J. Braz. Chem. Soc., Vol. 16, No. 6A, 1130-1133, 2005.
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0103 - 5053 $6.00+0.00

Article

Antifungal Activity of the Extracts and Neolignans from *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck

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Extratos de folhas de *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck (Piperaceae), uma planta medicinal utilizada no Brasil para tratar doenças infecciosas, foram testados para a atividade antifúngica sobre as leveduras *Candida albicans*, *C. krusei*, *C. parapsilosis* e *C. tropicalis*. O extrato em acetato de etila apresentou boa atividade contra *C. albicans* com uma CIM de 125 μg mL^{-1}, moderada atividade contra *C. krusei* e *C. parapsilosis* (CIM de 500 μg mL^{-1}) e foi inativo contra *C. tropicalis* (CMI> 1000 μg mL^{-1}). Com base nestes resultados, o extrato em acetato de etila foi fracionado em nove frações em cromatografia de coluna de sílica gel. As frações hexano e clorofórmio mostraram variados níveis de atividade antifúngica contra as leveduras testadas. Posterior separação da fração hexânica em cromatografia de coluna resultou nas substâncias puras eupomatenóide-6, eupomatenóide-5, eupomatenóide-3 e conocarpano. A elucidação estrutural das substâncias foi baseada em dados espectrais (RMN de ^{1}H e de ^{13}C, HSQC, HMBC, gNOE, IV e EM). O conocarpano foi a única substância isolada, com atividade contra as leveduras. As propriedades antifúngicas do extrato de *P. regnellii* demonstraram preliminar validação científica do uso da planta na medicina popular.

Piper regnellii (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck (Piperaceae) is a medicinal plant traditionally used in Brazil to treat infectious diseases. The extracts obtained from the leaves of *P. regnellii* were investigated for their antifungal activities against the yeasts *Candida albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. The EtOAc extract presented a significant activity against *Candida albicans* with MIC at 125 μg mL^{-1}, and a moderate activity against both *C. krusei* and *C. parapsilosis* with MIC at 500 μg mL^{-1}. *Candida tropicalis* was not inhibited by this extract at concentrations as high as 1000 μg mL^{-1}. Based on these findings, the EtOAc extract was fractionated by silica gel column chromatography into nine fractions. The hexane and $CHCl_3$ fractions showed varied levels of antifungal activity against all test yeasts. Further column chromatography separation of the hexane fraction afforded the pure compounds eupomatenoid-6, eupomatenoid-5, eupomatenoid-3 and conocarpan. The structure of the compounds was based on spectral data (^{1}H and ^{13}C NMR, HSQC, HMBC, gNOE, IR and MS). Conocarpan was the only active compound on the yeasts. The antifungal property of *P. regnellii* extract provides preliminary scientific validation for the traditional medicinal use of this plant.

Keywords: *Piper regnellii*, neolignans, antifungal activity

Introduction

Piper regnellii (Miq) C. DC. var. *pallescens* (C. DC.) Yunck (Piperaceae) is an herbaceous plant popularly known as "pariparoba" and is distributed in tropical and subtropical regions of the world.[1] Leaf and root are used as crude extracts, infusions or plasters to treat wounds, reduction of swellings and skin irritations.[2-4] In a screening of Brazilian medicinal plants, we have reported the antimicrobial activity of the hydroethanolic extract of the leaves of *P. regnellii* (Miq) C. DC against the bacteria *Staphylococcus aureus* and *Bacillus subtilis* and against the yeasts *Candida krusei* and *Candida tropicalis*.[5]

The search for active constituents from different *Piper* species has been intensified in recent years, with the finding that several species have been shown to have a number of biological activities.[6] Phytochemical study of *P. regnellii* roots has shown the accumulation of several

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phenylpropanoids and dihydrobenzofuran neolignans including (+)-conocarpan as major compound. Conocarpan, a dihydrobenzofuran neolignan, has been isolated from several Piperaceae species.[7,8] This compound displays a variety of biological activities including anti-PAF,[9] antifungal[10,11] and insecticidal activity.[7,12]

In the present study we describe the *in vitro* antifungal activity of ethanolic extract and fractions from leaves of *Piper regnellii* as well as of the bioactivity-directed isolation of eupomatenoid-6, eupomatenoid-5, eupomatenoid-3 and conocarpan.

Experimental

General experiments procedures

The NMR spectra were obtained in a BRUKER ARX400 (9.4 T) and VARIAN GEMINI 300 (7.05T), using deuterated solvent, TMS as internal standard and constant temperature of 298K. IR: film NaCl plates; ES-MS were recorded on a Micromass Quattro LC, HRMS: Autospec-Micromass EBE and EI-MS on a GC/MS-SHIMADZU QP 2000 A. CC: silica gel 60 (70-230 and 230-400 mesh); TLC: silica gel plates F_{254} (0.25 mm thickness).

Plant material

The leaves of *Piper regnellii* (Miq.) C. CD. var. *pallescens* (C. DC.) Yunck were collected in August 2001 in Horto of Medicinal Plants "Prof. Irenice Silva" in the Campus of Universidade Estadual de Maringá. The plant material was identified by Marilia Borgo from the Botanical Department of Universidade Federal do Paraná, and a voucher specimen (n. HUM 8392) is deposited at the Herbarium of Universidade Estadual de Maringá, Paraná, Brazil.

Isolation of the constituents

Dried leaves (200 g) of *P. regnellii* were ground and extracted with EtOH:H_2O (9:1) at room temperature. The solvent was removed under vacuum at 40 °C to give an aqueous extract and a dark green residue. The aqueous extract from the crude hydroalcoholic extracts was lyophilized (13.9 g) and the residue from crude extract was washed with EtOAc. The organic solvent was removed to give the EtOAc extract (15.3 g). The aqueous and EtOAc extracts were assayed against *S. aureus* by bioautography and broth microdilution assay to determine the MICs as described below.

The active EtOAc extract (10.7 g) was submitted to vacuum column chromatography (silica gel 150 g) and eluted with hexane (1000 mL), $CHCl_3$ (1400 mL), $CHCl_3$/EtOAc 19:1, v/v (1000 mL), $CHCl_3$/EtOAc 9:1, v/v (700 mL), $CHCl_3$/EtOAc 1:1, v/v (500 mL), EtOAc (500 mL), acetone (700 mL), MeOH (1400 mL) and MeOH/H_2O 9:1, v/v (1800 mL). The resulting fractions were assayed for antifungal activity (Table 1). The active hexane fraction (2.4 g) was chromatographed by column chromatography on silica gel 60 (70-230 mesh) eluted with hexane, hexane/$CHCl_3$ (49:1, 19:1, 9:1 and 1:1, v/v), $CHCl_3$, EtOAc, acetone and MeOH in order to yield 72 fractions and to obtain the following compounds: **1** (98.5 mg), **2** (82.9 mg) and **3** (55.1 mg). Fractions 40-44 (328 mg) were chromatographed by CC in silica gel 60 (230-400 mesh) with hexane:CH_2Cl_2:EtOAc (12:7:1, v/v/v) to afford compound **4** (181.3 mg). The compounds were identified as eupomatenoid-6 (**1**), eupomatenoid-5 (**2**), eupomatenoid-3 (**3**) and conocarpan (**4**), respectively. Structures were established with the use of spectroscopic methods (UV, EI-MS, ^{1}H NMR, ^{13}C NMR, H-H COSY, gNOE, HETCOR, HMBC and by comparing them with literature data.[7,13,14] Compounds **1**- **4** were tested against *Candida albicans*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*.

Microorganisms used and growth conditions

A single clinical isolated of each species (*Candida albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*), obtained from vaginal mucosa, was selected to testing. The yeasts were maintained in agar Sabouraud-dextrose (Merck).

Antifungal susceptibility testing

The minimal inhibitory concentrations (MICs) of all the extracts, compounds and reference antifungal compounds were determined by microdilution techniques in Sabouraud broth (Merck) for yeasts.[15] Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard (10^4 colony-forming units [CFU] mL^{-1}) and diluted to 1:10 for the broth microdilution procedure. Microtiter trays were incubated at 37 °C and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of compounds at which the microorganism tested does not demonstrate visible growth. MBC was defined as the lowest concentration yielding negative subcultures or only one colony. Nystatin (Sigma Chemical Co., St.Louis, MO, USA) was included in the test as control.

Results and Discussion

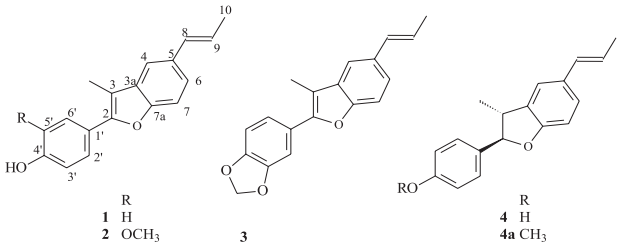
The results of the antifungal activities of extracts, fractions and compounds appear in Table 1. The *in vitro*

results were classified as follow: when the MIC is equal or smaller than 100 μg mL^{-1}, the antifungal activity was considered significant. If the extracts or compounds displayed a MIC from 100 to 500 μg mL^{-1} the antifungal activity was considered moderate; from 500 to 1000 μg mL^{-1} the antifungal activity was considered weak; over 1000 μg mL^{-1} the extracts were considered inactive.

The EtOAc extract obtained from leaves of *P. regnellii* presented a moderate activity against *C. albicans* with MIC of 125 μg mL^{-1}, a weak activity on both *C. krusei and C. parapsilosis* with MIC of 500 μg mL^{-1}, and was inactive against *C. tropicalis* at a concentration >1000 μg mL^{-1}. On the other hand, the aqueous extract was inactive against all yeasts tested (MIC > 1000 μg mL^{-1}) (Table 1).

On the basis of these findings, the EtOAc extract was fractionated on silica gel into nine fractions. The hexane and $CHCl_3$ fractions showed varied levels of antifungal activity against all yeasts. The hexane fraction showed significant anticandidal activity with a MIC from 62.5 to 125 μg mL^{-1}. $CHCl_3$ fraction was less active than hexane fraction with a MIC from 31.2 to 500 μg mL^{-1}. The $CHCl_3$/EtOAc, EtOAc, acetone, MeOH, and MeOH/H_2O fractions showed no activity against the organisms tested. The minimal fungicidal concentrations were within twofold dilution of the MIC for these organisms.

Further separation of the hexane fraction by column chromatography afforded the pure compounds eupomatenoid-6 (**1**), eupomatenoid-5 (**2**), eupomatenoid-3 (**3**) and conocarpan (**4**). The pure compound conocarpan (**4**) was the only active compound on the yeasts at concentrations of 6.3 to 12.5 μg mL^{-1} (Table 1). Conocarpan was methylated to afford *O*-methylconocarpan (**4a**), which was inactive against all the yeasts (data not shown). It is suggested that the antifungal activity of conocarpan can be related with the presence of the phenolic hydroxyl.



Under the conditions employed in the present study, **1-3** compounds were also inactive against all yeasts tested. The higher activity of conocarpan could also possibly be attributed to the absence of a double bond at C-2, since the derivatives **1-3** were inactive.

Studies on the antimicrobial properties of conocarpan have shown that it was active against *C. albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*, as well as against the dermatophytes *Microsporum gypseum* and *Tricophyton mentagophytes*.[16]

In recent years, a number of plants of *Piper* species have been found to possess antifungal activity (*Candida albicans, Cryptococcus neoformans, Saccharomyces cerevisae, Cladosporium sphaerospermum, C. cladosporiodes, Microsporum gypseum* and *Tricophyton*

Table 1. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of extracts, fractions and compounds obtained from leaves of *P. regnellii*

Tested material	MIC (MFC) / (μg mL^{-1})			
	C. albicans	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
Extracts				
Aqueous	inactive[a]	inactive	inactive	inactive
EtOAc	125(250)	500(1000)	500(1000)	inactive
Fractions obtained from EtOAc extract				
Hexane	62.5(62.5)	125(250)	125(250)	125(250)
$CHCl_3$	62.5(62.5)	500(1000)	31.2(62.5)	125(250)
$CHCl_3$:EtOAc (19:1)	>100	>100	>100	>100
$CHCl_3$:EtOAc (9:1)	>100	>100	>100	>100
$CHCl_3$:EtOAc (1:1)	>100	>100	>100	>100
EtOAc	>100	>100	>100	>100
Acetone	>100	>100	>100	>100
MeOH	>100	>100	>100	>100
MeOH:H_2O (9:1)	>100	>100	>100	>100
Compounds isolated from hexane fraction				
Eupomatenoid-6 (**1**)	>100	>100	>100	>100
Eupomatenoid-5 (**2**)	>100	>100	>100	>100
Eupomatenoid-3 (**3**)	>100	>100	>100	>100
conocarpan (**4**)	6.3(6.3)	12.5(25)	25(50)	6.3(12.5)
Positive control (Nystatin)	1.0(n.d.)	4.0(n.d.)	8.0(n.d.)	8.0(n.d.)

[a] Samples with MIC>1000 μg mL^{-1} were considered inactive. n.d. not determined.

mentagrophytes) which has allowed the isolation of the active principles followed by their characterization as benzofuran neolignans in leaves of *P. fulvescens*[16], benzoic acid derivatives in leaves of *P. dilatatum*,[17] and pyrrolidine and pyperidine amides in leaves and stems of *P. hispidum,*[18,19] seeds and leaves of *P. tuberculatum*[19,20] and of the leaves of *P. arboreum.*[20]

The findings revealed that the antifungal properties of *P. regnellii* extract provide preliminary scientific validation for the traditional medicinal use of this plant. However, the extracts and active compound isolated from *P. regnellii* should be further studied in animal models in order to evaluate the *in vitro* efficacy and toxicity.

Acknowledgments

To Marinete Martinez Vicentim for help in the experiments. This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Capacitação de Aperfeiçoamento de Pessoal de Nível Superior, Capes, and Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá.

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Received: March 8, 2005
Published on the web: September 15, 2005