Evaluation of Antifungal Activity of *Piper solmsianum* C. DC. var. *solmsianum* (Piperaceae)

Marina Pereira De CAMPOS, a Valdir CECHINEL FILHO,a Rosi Zanoni Da SILVA, b Rosendo Augusto YUNES, b Susana ZACCHINO, c Sabina JUAREZ, c Rosana Cé BELLA CRUZ, a and Alexandre BELLA CRUZ.*a, b

Programa de Mestrado em Ciências Farmacêuticas/CCS and Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí (UNIVALI); 88302–202, Itajaí-SC, Brazil; a Departamento de Química, Universidad Federal de Santa Catarina (UFSC); 88040–900, Florianópolis-SC, Brazil; and b Farmacognosia, Faculdade de Farmácia y Bioquímica, Universidad Nacional de Rosario; Argentina. Received November 15, 2004; accepted April 14, 2005.

We have studied the crude methanolic extract (CME), some fractions (hexane, dichloromethane and ethyl acetate) and four pure compounds: eupomatenoid-3 (1), eupomatenoid-5 (2), conocarpan (3) and orientin (4), from *Piper solmsianum*, for possible antifungal activity against 12 pathogenic fungi. The minimal inhibitory concentration (MIC) was determined and the experiments showed that the CME exhibited antifungal action against all the dermatophytes tested, with MIC values of between 20 μg/ml to 60 μg/ml. Similar activity also was verified for the hexane, dichloromethane and ethyl acetate fractions. However, the starting material (CME), and all the fractions, did not exert inhibitory effect against hyaline hyphomycetes and were only discretely active against the zygomycetes and yeasts. Compounds 2, 3 and 4 also exhibited pronounced activity against all the dermatophytes tested (MIC<1 to 9 μg/ml) with potency as high as the standard antifungal drug (ketoconazole). Compound 3 also exhibited activity against all the yeasts tested. In conclusion, the antifungal activity of *P. solmsianum* seems to be related mainly to the presence of compounds 2, 3 (neolignans) and 4 (flavonoid), however it was verified that another active compound, as yet unidentified, exists in the plant.

Key words antifungal activity; *Piper solmsianum* var. *solmsianum*; neolignans; orientin

The incidence of opportunistic fungal infections in immunocompromised patients, such as those undergoing treatment with immunosuppressive drugs, intensive chemotherapy, AIDS patients and neonates, is increasing at an alarming rate. These mycoses are very difficult to eradicate, and present an enormous challenge for healthcare providers.

The diversity of plant species in Brazil, together with their known ethnopharmacological uses, creates enormous potential for finding new structures with antifungal properties.

The Piperaceae is a vast family of plants, which has been extensively used for medicinal purposes. It comprises about 10 genus and approximately 2000 species, most of them herbaceous, which are found mainly in tropical areas. *Piper* and *Peperomia*, the two greatest genera of the family, are well-represented in the Brazilian flora.

Within the Piperaceae family, the genus *Piper* has over 700 species, distributed throughout the tropical and subtropical regions of the world. Its phytochemistry has been object of extensive reviews.

With regard to the ethnopharmacological information, while the pungent and aromatic fruits of some species of *Piper* are used as spices, most of them find wide application in traditional systems of medicine as insecticides, antivirals, antimicrobials and particularly antifungals. These biological properties have been attributed to the presence of lignans and/or amides, such as alkyl or olefinic isobutylamides, flavonoids, kawa-lactones, butenolides and cyclohexane epoxides, among others.

Among the different species of *Piper* growing in Brazil, *Piper solmsianum* (syn. *P. leucatham* or *P. santosanum*) known popularly as “pariparoba”, is a shrub that measures 1—3 m. There are few reports on the chemical or biological studies of this plant. In contrast, phytochemical studies indicated the presence of aliphatic hydrocarbons, monoterprenes, sesquiterpenes, arylpropanoids and neolignans. Recently, we have demonstrated that the fractions and some pure compounds (lignans) from the leaves of this plant exhibit significant activity against Gram-positive bacteria.

The current study extends our previous work on the biological properties of *P. solmsianum* and describes the in vitro antifungal activity of extracts, fractions and pure compounds against a panel of opportunistic pathogenic fungi using the agar dilution method.

**MATERIALS AND METHODS**

**Plant Material** *Piper solmsianum* C. DC. var. *solmsianum* (Piperaceae) was collected in May, 2001, in Ponta Grossa, State of Paraná, Brazil, and identified by Dr. Elsie Franklin Guimarães (Rio de Janeiro Botanic Museum Herbarium). A voucher specimen was deposited in the same herbarium under the number RB 368597.

**Phytochemical Analyses** Air-dried leaves (1.36 kg) of *P. solmsianum* var. *solmsianum* were cut into small pieces and macerated at room temperature for one week in methanol. After filtration, the solvent was removed by rotary evaporation under reduced pressure, and given 269 g of crude methanolic extract. It was then suspended in water and successively partitioned with hexane, dichloromethane (DCM) and ethyl acetate (EtOAc), as previously described, afforded hexanic fraction (45.2 g), dichloromethane fraction (33.8 g) and ethyl acetate fraction (7.80 g).

A part of the hexanic fraction (3.3 g) was fractionated on silica gel column chromatography eluted with a gradient of hexane, hexane:EtOAc, EtOAc: methanol with increasing polarity and methanol. Fractions 8—15 (547.26 mg) were
rechromatographed as in previous cases, and eluted with hexane, hexane:EtOAc gradient and EtOAc, and given 64.5 mg of eupomatenoid-3 (1) and 375 mg of eupomatenoid-5 (2).

The DCM fraction (2.5 g) was similarly chromatographed and eluted with increasing amounts of hexane in EtOAc and EtOAc/methanol, yielding 686 mg of concarpan (3).

The EtOAc fraction (6.6 g) was fractionated on silica gel column chromatography eluted with chloroform/methanol gradient, and given several fractions. Fraction 20—34, which exhibited a positive result with FeCl₃ reagent, was eluted with a mixture of ethyl acetate/acetone/water (25:8:2) yielding 765 mg of a flavone identified as orientin (4), together with two flavonoids, as yet identified. The identification of the isolated compounds was performed by direct comparison with authentic samples previously described for this plant, and spectral data.³³

Microorganisms and Media To determine antimicrobial activity, microorganisms from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.) or clinical isolates provided by the Centro de Referencia Micología (C) (CEREMIC, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, (2000) Rosario, Argentina) and Control Lab (CL 35) (Control Lab, Rio de Janeiro, Brazil): Aspergillus flavus (ATCC 9170), Aspergillus fumigatus (ATCC 26934), Aspergillus niger (ATCC 9092), Rhizopus sp. (CL 35), Microsporum canis (C112), Microsporum gypseum (C115), Trichophyton mentagrophytes (ATCC 9972), Trichophyton rubrum (C137), Epidermophyton floccosum (C114), Cryptococcus neoformans (ATCC 32264), Candida albicans (ATCC 10231) and Candida tropicalis (ATCC 7349).

The yeasts used were cultivated on Sabouraud-dextrose agar (Merck, 5438) for 48 h at 37°C. Cell suspension in sterile distilled water was adjusted to give a final concentration of 1×10⁶ to 5×10⁶ yeast cells/ml, standardized with 0.5 on the McFarland scale (λ = 530 nm).

Filamentous fungi were maintained on Sabouraud-dextrose agar (Merck, 5438) and subcultured every 15 d to prevent pleomorphic transformations. The inocula were prepared by removing the sporulated fungi from the agar slant with a loop and suspending them in 10 ml of sterile water. The fungal suspensions were filtered through a sterile gauze to remove hypha. The resulting suspensions of conidia were vigorously vortexed and adjusted to 1.4×10⁵ CFU/ml by adding sterile distilled water and using a hemacytometer cell counting chamber.³³

Antifungal Assays The fungistatic activities of extract, fractions and isolated compounds were evaluated by the agar dilution method, using Sabouraud-dextrose agar.

Stock solutions of extracts, fractions or compounds in dimethylsulfoxide (DMSO) were diluted to give serial twofold dilutions which were added to each medium, resulting in concentrations ranging from 1000 to 1 µg/ml. The final concentration of DMSO in the assay did not exceed 2%. Inocula of 5 µl with the yeast cells or spore suspensions were added to Sabouraud-dextrose agar media. The antifungal agent ketoconazole (Sigma) was included in the assay as positive control. Drug-free solution was also used as a blank control. Tubes were incubated at 37°C for 24 to 72 h for yeasts and at 25°C for 5 to 15 d (up to 15 d for dermatophyte strains) according to the control fungus growth. MIC was defined as the lowest extract, fractions or compound concentration, showing no visible fungal growth after the incubation period. Fungal growth was analysed after an appropriate incubation period, specific to each fungi. Each assay was repeated three times.

RESULTS AND DISCUSSION

In a previous work, we reported the isolation of eupomatenoid-3 (1), eupomatenoid-5 (2), concarpan (3) and orientin (4) from Piper solmsianum³³ which were identified on the basis of their physical and spectral data (mp., IR, ¹H- and ¹³C-NMR). The compounds (1),²¹,²⁹ (2),³¹,²³,²⁹ and (3)²¹,²³,²⁹,³⁶ have been found in other Piper spp. In contrast, the flavonoid (4), was found only in P. solmsianum.³³

The total extract, fractions and isolated compounds from P. solmsianum var. solmsianum were evaluated against several opportunistic pathogenic fungi and the results are reported in Table 1. MIC = 1000 µg/ml for extracts and ≤250 µg/ml for pure compounds were considered active. The results showed that the crude methanolic extract

Table 1. Antifungal Activity of Extract, Fractions and Compounds of P. solmsianum var. solmsianum against Filamentous Fungi and Yeast, Expressed as Minimal Inhibitory Concentration (MIC)

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Filamentous fungi</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>CME</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Hexane Fr.</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>DCM Fr.</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>EtOAc Fr.</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>1</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>800</td>
</tr>
<tr>
<td>4</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Crude methanolic extract (CME); hexane fraction (Hexane Fr.); dichloromethane fraction (DCM Fr.); ethyl acetate fraction (EtOAc Fr.); eupomatenoid-3 (1); eupomatenoid-5 (2); concarpan (3); orientin (4); Aspergillus flavus (A. fla); Aspergillus fumigatus (A. fum.); Aspergillus niger (A. nig.); Rhizopus sp. (Rhyz. sp.); Microsporum canis (M. can.); Microsporum gypseum (M. gyp.); Trichophyton mentagrophytes (T. ment.); Trichophyton rubrum (T. rub.); Epidermophyton floccosum (E. floccosum); Cryptococcus neoformans (C. neo.); Candida albicans (C. alb.); Candida tropicalis (C. trop.).
(CME) exhibited pronounced antifungal activity against all the dermatophytes tested, with MIC values of 20 μg/ml for M. canis, M. gypseum, T. mentagrophytes and E. floccosum, and 60 μg/ml for T. rubrum. Although all the fractions displayed interesting activities against dermatophytes, the DCM fraction showed the best activity with MICs between 7 and 30 μg/ml similar to ketoconazole. However, the starting material (CME) and all the fractions did not exert inhibitory effect against hyaline hyphomycetes of the genus Aspergillus, and were only discretely active against the zigomycete Rhizopus sp. and the yeasts C. albicans and C. neoformans.

Compounds 2, 3 and 4 exhibited pronounced activity against all the dermatophytes tested (MIC≤9 μg/ml), with potency as high as the standard antifungal drug (ketoconazole), as can be observed in Table 1. Furthermore, these compounds showed good activity against C. neoformans, and compound 3 exhibited this activity against C. albicans. In contrast, compound 1 possesses the lowest antifungal activity against all the microorganisms tested. To the best of our knowledge, this study is the first to demonstrate antifungal activity for the flavonoid (4).

In a previous work Freixa and co-workers21 showed that eupomatenoid-6 and conocarpan exerted considerable activity against some dermatophytes and yeasts. They suggested that the absence of the methoxyl group at position 3 on the phenyl-propenyl-benzofuran structure and the saturation of the carbons 7 and 8 could play important roles in antimicrobial activity. In another study, (1) and three methylated derivatives proved not to be active against several bacteria tested.21 These compounds did not possess a phenolic hydroxyl group, suggesting that the phenolic hydroxyl present in the active structures is related to antifungal activity. Our results suggest that the presence of a hydroxyl group at position 4 of the phenyl-propenyl-benzofuran structure may also play an important role in the antimicrobial activity. Therefore, when this group is absent, the antifungal effects are lower (compare activity of compound 1 with those of compounds 2—4) (Fig. 1).

The most potent inhibitory effects were observed with (3) which showed MICs≤8 μg/ml against dermatophytes, this being the only compound able to inhibit members of the genus Aspergillus and Candida.

Mycotic infections are probably the most common among superficial infections in Brazil. As is well known, drugs used against dermatophytosis exhibit several side effects and have limited efficacy37,38 and there is a real need for new molecules with antifungal properties for the treatment of superficial mycoses.

Thus, the particular antifungal activity of P. solmsianum var. solmsianum, its neolignans 2 and 3 and its flavonoid 4 against dermatophytes open avenues for the development of new antifungal drugs which help in the treatment of obstinate superficial fungal infections.

Acknowledgements This work was supported by a grant from the Agencia de Promoción Científica y Tecnológica de Argentina (PICTR #260) to SZ. This work is part of the Iberoamerican Project PIBEAFUN X.7 (Search and development of new antifungal agents) of CYTED (Iberoamerican Program of Science and Technology for Development).

ABC and VCF thanks to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Ciência e Tecnologia de Santa Catarina (FUNCITEC) for financial support.

REFERENCES