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Full Length Research Paper

Antimicrobial activity of *Piper arboreum* and *Piper tuberculatum* (Piperaceae) against opportunistic yeasts

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In the scope of our ongoing research on bioactive agents from natural sources, 24 extracts and fractions obtained from *Piper arboreum* Aub. and *Piper tuberculatum* Jacq. (Piperaceae) were screened for antifungal activity by using broth microdilution method. The current investigation reveals that *P. arboreum* extracts and fractions were more effective against *Candida krusei* and *Candida parapsilosis* than *Cryptococcus neoformans*. The growth of *Candida albicans* was weakly affected by all the tested extracts and fractions. The strongest effects were observed for hexane and ethyl acetate fractions from leaves of *P. arboreum*, with MIC values (in µg/ml) of 15.6 and 31.2 µg/ml against *C. krusei*, respectively. Additionally, phytochemical investigation of the hexane fraction of *P. arboreum* leaves furnished 3 pyrrolidine amides; piperyline, 4,5-dihydropiperyline and tetrahydropiperyline, which could be responsible, at least in part for the observed antifungal activity. The most active compound, tetrahydropiperyline, displayed MIC values of 15.6 µg/ml against *C. krusei*, *C. parapsilosis* and *C. neoformans*.

Key words: Antifungal, antimicrobial, *Piper arboreum, Piper tuberculatum,* Piperaceae, *Candida, Cryptococcus neoformans.*

INTRODUCTION

Piperaceae have been extensively studied as a source of bioactive compounds (Parmar et al., 1997; Alecio et al., 1998; Kato and Furlan, 2007 Regasini et al., 2009). Phytochemical investigations of *Piper* genus have led to the isolation of typical classes of secondary metabolites such as amides, terpenes, benzoic acid derivatives and hydroquinones in addition to lignans, neolignans, flavornoids and a few alkaloids (Lago et al., 2004; Navickiene et al., 2000; Navickiene et al., 2006, Regasini et al., 2008).

As part of our research aiming to discover potent antimicrobial compounds in Piperaceae species, we have previously described the occurrence of trypanocidal chromenes in *Piper aduncum, Piper gaudichaudianum* and amides in *Piper tuberculatum* (Batista-Júnior et al., 2008; Cotinguiba et al., 2009). Additionally, hydroquinones and flavanones from leaves of *P. crassinervium* have been reported as well (Lopes et al., 2008). In this context, we have screened various plants of *Piper* genus collected in São Paulo state (Brazil). *Piper arboreum* Aub. and *P. tuberculatum* Jacq. were chosen for biological and chemical investigation and to our knowledge there are no previous reports on antifungal effects of these species on opportunistic yeasts (Silva et al., 2002).

In some Afro-Brazilian traditional communities, a decoction of *P. arboreum*, popularly known as “pau-de-Angola” and “alecrim-de-Angola” has been largely used against venereal diseases and infections of the urinary throat (Agra et al., 2007). On the other hand, *P. tuber-*

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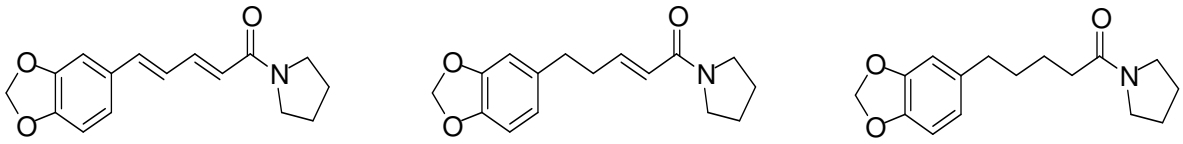

Piperyline 4,5-dihydropiperyline tetrahydropiperyline

Figure 1. Structures of piperyline, 4,5-dihydropiperyline and tetrahydropiperyline. 3 piperamides isolated from hexane fraction of *Piper arboreum* leaves.

culatum (vernacular names: “pimenta darta” and “pimenta longa”) has been used as soporific and antidote for snake bite (Felipe et al., 2007).

Thus, the aim of the current investigation was to screen the antimicrobial activity of extracts and fractions of green fruits, branches, leaves, and compounds from *P. arboreum* and *P. tuberculatum* against *Candida albicans*, *Candida krusei*, *Candida parapsilosis* and *Cryptococcus neoformans*, using broth microdilution test.

MATERIALS AND METHODS

Plant material

Specimens of *P. arboreum* and *P. tuberculatum* were cultivated from seeds under greenhouse conditions at the Institute of Chemistry, São Paulo state university, Araraquara-SP, Brazil. Plant material was collected in May of 2006 and identified by Dr. Guillermo E. D. Paredes (Universidad Pedro Ruiz Gallo, Lamba-yeque, Peru). The vouchers specimens Kato-163 and Cordeiro-1936 were deposited at the herbarium of the Institute of Biosciences, São Paulo University, São Paulo-SP, Brazil.

Extraction

Shade-dried and powdered plant material (leaves, green fruits or branches) of *P. arboreum* and *P. tuberculatum* (30.0 g) were extracted with ethanol (5 x 350 ml), for 3 weeks at room temperature. After filtering, the solvent was evaporated under reduced pressure to yield a thick syrup, which was dispersed in methanol: water (4:1) and then successively partitioned with hexane and ethyl acetate. Samples of the ethanol extracts and the hexane, ethyl acetate and lyophilized hydromethanol fractions were tested for potential antifungal activity.

Isolation and identification of piperamides (piperyline, 4,5-dihydropiperyline and tetrahydropiperyline)

The hexane fraction of the leaves of *P. arboreum* (880 mg) was subjected to column chromatography with silica gel (18 x 3.3 cm i.d.) and eluted with hexane : ethyl acetate (4:1). 25 fractions (10 ml) were collected and checked by TLC on silica gel F254 plates developed with hexane : ethyl acetate (6:4) and revealed with Dragendorff reagent. Fractions 10 - 13 (520 mg) were purified by preparative TLC [hexane : dichloromethane : acetone : acetic acid (6:3:1:0.1), 4 elution] to yield piperyline (310 mg), 4,5-dihydropiperyline (135 mg) and tetrahydropiperyline (4.5 mg). The molecular structures of these compounds (Figure 1) were identified by comparison with literature data, mainly ^{1}H and ^{13}C NMR δ values (Alecio et al., 1998; Navickiene et al., 2000: Silva et al., 2002).

Microorganisms and growth conditions

The test organisms included *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. neoformans* ATCC 90012. The microorganisms were originally obtained from the Department of Clinical and Toxicological Analysis of 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 minor modifications (Rodriguez-Tudela et al., 1996). 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 prepared in DMSO and to each well of a 96 well U-bottomed culture plate was 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 (piperyline, 4,5-dihydropiperyline and tetrahydropiperyline) 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 was used as positive control, exhibiting a MIC value ranged from 2.0 to 0.06 µg/ml for the *Candida* spp. and *C. neoformans*.

The MIC was calculated as the minimal concentration of the test sample, which shows complete inhibition of each fungi strain. For the extracts and fractions, the MIC was defined as the lowest concentration able to inhibit any visible fungal growth. Results were visually and spectrophotometrically analyzed. For extracts and fractions showing a MIC lower than 100 µg/ml, the antifungal activity was considered potent; from 100 to 500 µg/ml, the anti-microbial activity was moderate; from 500 to 1000 µg/ml, the anti-microbial activity was weak; over 1000 µg/ml the extract was considered not active (Holetz et al., 2002).

Minimum fungicidal concentration (MFC)

All tested samples in the MIC study, whether showing or not any microbial growth, 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

Table 1. *In vitro* antifungal activity (Minimum Inhibitory Concentation (MIC in µg/ml) of extracts, fractions *Piper arboreum* and *Piper tuberculatum* and piperamides piperyline, 4,5-dihydropiperyline and tetrahydropiperyline.

Plant part	Extract or fraction tested	*P. arboreum*				*P. tuberculatum*			
		Ca	Ck	Cp	Cn	Ca	Ck	Cp	Cn
Green fruits									
	Ethanol	250	125	250	250	>1000	>1000	>1000	>1000
	Hexane	250	62.5	125	125	>1000	250	>1000	250
	Ethyl acetate	>1000	250	250	>1000	>1000	>1000	>1000	>1000
	Hydromethanol	>1000	> 1000	>1000	>1000	>1000	>1000	>1000	>1000
Leaves									
	Ethanol	250	62.5	125	125	>1000	250	250	>1000
	Hexane	250	15.6	62.5	125	>1000	125	250	250
	Ethyl acetate	>1000	31.2	62.5	250	>1000	>1000	>1000	>1000
	Hydromethanol	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Branches									
	Ethanol	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
	Hexane	>1000	250	>1000	250	>1000	250	>1000	250
	Ethyl acetate	>1000	> 1000	>1000	>1000	>1000	>1000	>1000	>1000
	Hydromethanol	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Compounds									
piperyline		250	31.2	31.2	125	—	—	—	—
4,5-dihydropiperyline		125	31.2	15.6	31.2	—	—	—	—
tetrahydropiperyline		125	15.6	15.6	15.6	—	—	—	—
amphotericin B[a]		2.00	2.00	1.00	0.06	—	—	—	—
fluconazole[a]		2.00	62.5	8.00	4.00	—	—	—	—

Ca = *Candida albicans*, Ck = *Candida krusei*, Cp = *Candida parapsilosis*, Cn = *Cryptococcus neoformans*, a = positive controls.

plate after the period of incubation. The whole series of tests was performed in triplicate.

RESULTS AND DISCUSSION

The clinical relevance of fungal infections increased enormously due to the increasing of the immunocompromised host in the second half of the 20th century including infected HIV, transplant recipients and patients with neoplasm (Clark and Hajjeh, 2002; Hage et al., 2002). The crude mortality from opportunistic fungal diseases still exceeds 50% in most human studies and has been reported to be as high as 95% in bone marrow transplant recipients infected with *Aspergillus* sp. (Romani, 2004). The commonly used antifungal drugs, such polyene macrolides and azoles are toxic or limited in their spectrum and efficiency (Wakabayashi et al., 1998; Helmerhorst et al., 1999). For these reasons there is need for new molecules, particularly those from plant extracts, which can serve as lead compounds for further development in antifungal chemotherapy.

Natural products have long been used as templates for the development of new antimicrobial compounds, which may be useful against fungal diseases, such as papulacandins, lipopetides isolated from *Papularia sphaerosperma*, which were employed in the design and development of echinocandins (Denning, 2002). In this context, the screening of plant extracts has been a valid strategy being exploited to discover antifungal agents (Aliero and Afolayan, 2006; Kilani et al., 2007; Akerele et al., 2008; Akinpelu et al., 2008; Makut et al., 2008; Masoko et al., 2008; Adegoke et al., 2009).

In this work, 24 extracts and fractions of *P. arboreum* and *P. tuberculatum*, as well as 3 piperamides (piperyline, 4,5-dihydropiperyline and tetrahydropiperyline) were tested at concentrations ranging from 1000 to 0.48 µg/ml against 4 opportunistic yeasts (*C. albicans*, *C. krusei*, *C. parapsilosis* and *C. neoformans*). These results were summarized in Table 1.

In general, ethanol extracts (crude extracts) obtained from leaves exhibited stronger antifungal activity than did those from green fruits and branches. Hexane fractions were more effective than ethyl acetate and hydromethanol fractions, indicating that the potential fungitoxic compounds were in the low-polarity fractions. The hexane and ethyl acetate fractions of *P. arboreum* leaves exhibited the best activity against *C. krusei*, with values of MIC (µg/ml) of 15.6 and 31.2, respectively. On the other hand, these fractions exhibited potent anti-*Candida parapsilosis* activity, which MIC values of 62.5 µg/ml. All the extracts and fractions present weak activity against

C. albicans and *C. neoformans*, except for the hexane fractions of green fruits and leaves of *P. arboreum*, which exhibited moderate inhibition (MIC = 125 µg/ml) on *C. neoformans*.

In view of the results presented by hexane fraction obtained from leaves of *P. arboreum*, it was selected for phytochemical study, leading to isolation of 3 pyrrolidine amides (piperyline, 4,5-dihydropiperyline and tetrahydropiperyline). Piperyline showed moderate antifungal activity against *C. krusei* and *C. parapsilosis*, exhibiting MIC (in µg/ml) values of 62.5 and 31.2, respectively. The hydrogenated analogues of piperyline (amides 4,5-dihydropiperyline and tetrahydropiperyline) were also evaluated, exhibiting better potential antifungal than piperyline. Tetrahydropiperyline displayed potent activity on *C. krusei*, *C. parapsilosis* and *C. neoformans*, with value of MIC of 15.6 µg/ml. Altogether, these data indicates a clear positive correlation between potent antifungal effect and reduction of double bounds in intermediate chain of piperamides.

Moreover, amides piperyline, 4,5-dihydropiperyline and tetrahydropiperyline showed potent anti-*Candida krusei* activity, which was significant data, because *C. krusei* has natural resistance against the commercial drug fluconazole (Rex et al., 1995), suggesting its potential application for treating of *C. krusei* infections and, considering possible diverse mechanism of action from those of azole drugs.

Additionally, minimal fungicidal concentration (MFC) of all extracts, fractions and compounds piperyline, 4,5-dihydropiperyline and tetrahydropiperyline were also evaluated against the 4 fungi and showed MFC values higher than 1000 µg/ml, indicating a fungistatic behavior.

It may be concluded from the study that *P. arboreum* has potential antimicrobial activity based on toxic effect against four opportunistic yeasts. Furthermore, hexane fraction of the leaves of *P. arboreum* could be an important source of promising antifungal compounds, useful for developing of novel bioactive agents. Three known pyrrolidine alkylamides have been isolated from *P. arboreum* leaves, which could be responsible, at least in part for the observed antifungal effect. In view of these findings, further chemical and pharmacological investigations to identify others secondary metabolites and to evaluate the potential of these *Piper* species as an antimicrobial *in vivo* are recommended.

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