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Short Communication

Antifungal activity of extracts from *Piper aduncum* leaves prepared by different solvents and extraction techniques against dermatophytes *Trichophyton rubrum* and *Trichophyton interdigitale*

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Abstract

The effects of different solvents and extraction techniques upon the phytochemical profile and anti-*Trichophyton* activity of extracts from *Piper aduncum* leaves were evaluated. Extract done by maceration method with ethanol has higher content of sesquiterpenes and antifungal activity. This extract may be useful as an alternative treatment for dermatophytosis.

Key words: antifungal activity, *Piper aduncum* L., dermatophytes, *Trichophyton*, extraction techniques.

Dermatophytes are a group of closely related fungi that affect keratinous tissue (skin, hair, and nails) of humans and other vertebrates, causing superficial infections, dermatophytosis, commonly known as ringworm or tinea. They belong to three anamorphic (asexual or imperfect) genera, *Epidermophyton*, *Microsporum* and *Trichophyto*n (Weitzman and Summerbell, 1995). In recent years the number of infections caused by these fungi has increased considerably and *Trichophyton rubrum* has been the most common dermatophyte since the fifties of last century, accounting for 80-90% of the strains, followed by *T. interdigitale* (Seebacher *et al.*, 2008).

The drugs used against dermatophytosis exhibit several side effects, have limited efficacy, and are very expensive (Gupta and Cooper, 2008; Kyle and Dahl, 2004). Such considerations have led to the search for alternative treatment methods, which have included plant extracts or plant-derived compounds based on the knowledge that plants have their own defense against fungal pathogens (Gurgel *et al.*, 2005).

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Piper aduncum L. (*Piperaceae*) is a tropical shrub widespread in South and Central America, growing naturally in the Amazon and in the Atlantic Forests of Brazil. Its extracts have displayed a broad range of antifungal activity and phytochemical studies have described the isolation of metabolites with antifungal effects (Baldoqui *et al.*, 1999; Lago *et al.*, 2004; Lentz *et al.*, 1998; Okunade *et al.*, 1997). Given these properties and the fact that currently no studies have adequately assessed the anti-*Trichophyton* activity of the *P. aduncum*, we aimed to study this plant for its effect against *T. rubrum* and *T. interdigitale*. Additionally, in view of the fact that solvent and extraction technique are fundamental parameters influencing secondary metabolites extraction, the effects of different solvents (ethanol and n-hexane) and extraction techniques (maceration, ultrasonic, decoction and soxhlet) were determined on the phytochemical profile and anti-*Trichophyton* activity of extracts from *P. aduncum* leaves.

Leaves of *P. aduncum* (adult plants) were collected in the region of Governador Valadares city, state of Minas Gerais, Brazil. The plant was identified by one of us (B.G.B.) and a voucher specimen was deposited at the Faculdade de Ciências Biológicas e da Saúde, Universidade Vale do Rio Doce, under the number 423.

The powder of the air-dried leaves (15 g) was extracted with either 80% ethanol or n-hexane (150 mL) by different extraction techniques: (i) maceration for a week at room temperature; (ii) Soxhlet apparatus for 4 h at boiling temperature; (iii) decoction for 6 h at boiling temperature and (iv) ultrasonic extraction into the ultrasonic bath (40 kHz, QUIMIS) for 4 h at 20°C. After filtration, the resulting solution was concentrated to dryness under reduced pressure using a rotary evaporator at a temperature lower than 40 °C.

The *Trichophyton* strains employed in this study were either acquired from the American Type Culture Collection (ATCC, Rockville, MD) or were clinical isolates from the Laboratório de Micologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. The reference strains were *T. rubrum* (ATCC 28189) and *T. interdigitale* (ATCC 9533).

The antifungal activity was determined by broth microdilution assay following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi M38-A (CLSI, 2002), with modifications, as described previously (Santos and Hamdan, 2005). Test concentrations for extracts ranged from 0.005 to 5 mg/mL. Fluconazole (Pfizer) was used as a standard antifungal agent at concentration ranging from 0.125 to 64 μg/mL. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract that completely inhibited visible growth of microorganisms in the microdilution wells and the minimum fungicidal concentration (MFC) was determined by sub-culturing the negative wells on potato dextrose agar (PDA) plate and was the lowest concentration that yielded negative sub-cultures.

Extracts were analyzed by Gas Chromatography/Mass Spectrometry (GC-MS) using Solid Phase Micro Extraction (SPME) analysis mode, as described previously (Siqueira *et al.*, 2007).

The susceptibility profiles of *T. rubrum* (ATCC 28189) and *T. interdigitale* (ATCC 9533) for each evaluated extract showed nearly identical results (data not shown). Considering only the MIC values, both ethanol and hexane extracts obtained by different extraction methods showed the same result (0.31 mg/mL), except ethanol extract yielded by ultrasonic technique that displayed MIC of 0.62 mg/mL. MFC values were either the same or twice higher than those of the corresponding MIC, ranging from 0.31 mg/mL to 1.25 mg/mL. The maximum MFC was obtained with the ethanol extract yielded by ultrasonic extraction. Because the strongest lethal activity to the two reference species was achieved to ethanol extract obtained to maceration (0.31 mg/mL), we also assessed its activity against eight clinical isolates of *Trichophyton* strains. As shown in Table 1, the MICs of the fluconazole-resistant strains were identical to those of sensitive strains (0.31 mg/mL), and the MFC values ranged from 0.31 mg/mL to 0.62 mg/mL.

The antifungal activity of members from *Piperaceae* species is well known. This plant family is among the most tested and has the highest number of positive results for the antifungal activity in a screening program of Latin American medicinal plants (Fenner *et al.*, 2006). Specifically about *P. aduncum*, several pharmacological effects have been attributed to it, including antifungal activity. However, its anti-dermatophyte activity has not been adequately evaluated yet. At the best of our knowledge, it is the first time that quantitative MIC and MFC values were deter-

Table 1 - Antifungal activity of fluconazole and maceration-ethanol extract from *Piper aduncum* leaves against clinical isolates of *Trichophyton* strains.

Clinical strains	Fluconazole	Extract	
	MIC (μg/mL)[a]	MIC (mg/mL)[a]	MFC (mg/mL)[a]
T. rubrum (I)[b]	> 64.0	0.31	0.62
T. rubrum (II)	32.0	0.31	0.62
T. rubrum (III)[b]	> 64.0	0.31	0.31
T. interdigitale (I)[b]	> 64.0	0.31	0.31
T. interdigitale (II)[b]	> 64.0	0.31	0.31
T. interdigitale (III)[b]	> 64.0	0.31	0.31
T. interdigitale (IV)	32.0	0.31	0.62
T. interdigitale (V)	16.0	0.31	0.31

[a]Results are representative of at least two independent experiments, each performed in triplicate.
[b]Fluconazole-resistant strains (MICs ≥ 64 μg/mL).

mined to extracts from *P. aduncum* leaves against *Trichophiton* species. Lentz *et al.* (1998) showed that of the 92 extracts from Honduran medicinal plants subjected to antifungal screens, ethanol extract (prepared by percolation) of *P. aduncum* displayed the broadest range of antifungal activity, including against *T. interdigitale*. However, as in this study the agar well tests were employed, neither MIC nor MFC values were determined.

In the present investigation all extracts evaluated showed a marked fungicidal effect. In many situations, fungicidal drugs are often preferred over drugs with fungistatic activity, since fungi recur more often when fungistatic, rather than fungicidal, drugs have been used. Therefore, considering that the relapse of infection remains a problem, particularly with tinea pedis/unguium, fungicidal activity is desirable for treatment of dermatophytic fungal infections (Kyle and Dahl, 2004).

The chemical composition of extracts was analyzed by GC-MS technique. The major constituents, compounds whose concentration was higher than 3% in at least one of the extracts, and their relative concentrations are given in Table 2. Extracts are predominantly composed of sesquiterpenes (cariofilene, α-calacorene, γ-elemene, Cis- γ-cadinene, germacrene D, linalool oxide, nerolidol, β-elemene, δ-cadinene, α-cariofilene). Monoterpene (linalool) and fatty alcohol (falcarinol) were also characterized as main components.

Navickiene *et al.* (2006) analyzed the essential oil obtained by hydrodistillation of *P. aduncum* leaves, occurring in Brazil, by GC-MS and found that it was active against *Cladosporium cladosporioides* and *C. sphaerospermum*. The analysis revealed a high amount of monoterpenes (45.2%), among which linalool occurred as the most abundant (31.7%), but the sesquiterpenes were predominant (52%). Our data confirm the predominance of sesquiterpenes and monoterpenes among the volatile compounds of *P. aduncum* leaves, but the chromatographic profiles were not the same in both studies. This discrepancy could be due to many factors, including different soil type and climate conditions, time of collection of plant materials and extraction techniques. One obvious reason for this discrepancy is that the samples were different (essential oil from fresh leaves *vs.* ethanol or hexane extracts from air-dried leaves). Another aspect that has to be taken into account is that previous researches have shown that phytochemical composition of *P. aduncum* can differ widely when it grows at different geographical location. Vila *et al.* (2005) reported high sesquiterpene contents in Panama samples, whilst Bolivian samples were composed predominantly of monoterpenes. Additionally, the phenylpropanoid dillapiol has been detected as major constituent in the Amazonian species (Maia *et al.*, 1998) but not in specimens occurring in southern region of Brazil (Navickiene *et al.*, 2006), as used in this study, or in samples from Panama and Bolivia (Vila *et al.*, 2005).

Regarding different procedures for the preparation of crude extracts, ultrasonic technique with ethanol was less efficient than both maceration and decoction in extracting the major volatile compounds (Table 2). The reduced content of terpenes observed in the extract prepared by ultrasonic technique can be related, at least in part, to its lower anti-*Trichophyton* activity, since these compounds are known for their antifungal activities (Paduch *et al.*, 2007).

Table 2 - Major compounds identified in the extracts from *Piper aduncum* leaves prepared by different solvents and extraction methods.

Compounds	Concentration (%)				
	Ethanol			Hexane	
	Maceration	Ultrasonic	Decoction	Maceration	Decoction
Caryophyllene	6.78	7.56	5.63	6.06	5.51
α-calacorene	-	5.41	-	-	-
γ-elemene	14.48	-	13.49	13.27	14.78
Cis- γ-cadinene	17.16	-	15.39	-	-
Falcarinol	-	6.06	-	-	-
Germacrene D	17.16	-	15.39	17.84	18.93
Linalool	2.44	8.0	1.89	0.74	0.56
Linalool oxide	0.55	4.34	-	-	-
Nerolidol	13.41	18.45	15.24	15.26	15.78
β-elemene	3.12	-	2.68	3.57	3.83
δ-cadinene	6.01	-	5.73	3.51	4.51
α-caryophyllene	7.49	8.25	6.06	6.65	6.26
Total	88.6	58.07	81.5	66.9	70.16

- Not detected.

Among the identified compounds, germacrene D and δ-cadinene, which were previously reported to have antifungal activity (Cheng *et al.*, 2005; Sahin *et al.*, 2004), were absent in ultrasonic-ethanol extract, but occurred in appreciable amounts in all other extracts. Indeed, a plant extract is a complex chemical mixture, whose biological activity relies upon synergistic effects between their constituents (Cos *et al.*, 2006). Thus, it is difficult to attribute its activity to a single or particular constituent. Additionally, phytochemical studies of *P. aduncum* have reported the isolation of other metabolites with antifungal activity such as chromenes, dihydrochalcones and prenylated benzoic acids (Baldoqui *et al.*, 1999; Lago *et al.*, 2004).

In conclusion, our findings demonstrated that extracts from *P. aduncum* leaves have strong fungicidal activity against *Trichophyton* species, including those exhibiting resistance to fluconazole. This good antifungal performance suggests that *P. aduncum* extracts may be useful as an alternative treatment for dermatophytosis caused by *Trichophyton* species. In addition, the maceration method using ethanol is a suitable way to prepare these bioactive extracts. Our data reinforce the importance of choosing the correct extraction scheme to ensure that active constituents are maintained during the extract preparation process.

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