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Antifungal activity of fractions and two pure compounds of flowers from *Wedelia paludosa* (*Acmela brasiliensis*) (Asteraceae)

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Received January 28, 2003, accepted March 2, 2003

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Pharmazie 58: 567–569 (2003)

Wedelia paludosa (Acmela brasiliensis) (Asteraceae), a traditionally used native Brazilian medicinal plant, showed antifungal activity against dermatophytes in dilution tests. The hexane, dichloromethane and butanol fractions displayed activity against *Epidermophyton floccosum, Trichophyton rubrum* and *Trichophyton mentagrophytes,* with minimal inhibitory concentrations between 250 and 1000 μg/mL. Two pure compounds, identified as kaurenoic acid (**1**) and luteolin (**2**), also showed activity against these dermatophytes.

1. Introduction

Superficial fungal infections, although rarely life threatening, have debilitating effects on a person's quality of life, sometimes limiting their daily activities or affecting their relationships since they disfigure skin conditions and can be transmitted to other individuals by direct contact. They are produced by a group of fungi that characteristically infect the keratinized areas of the body, mainly *Trichophyton* spp., *Microsporum* spp. and *Epidermophyton* spp. [1, 2].

A major concern in treatment of mycoses is the limited number of efficaceous antifungal drugs [3]. Many of those have undesirable side effects and antifungal drug resistance is becoming a major problem in certain populations [4], especially those infected with HIV. As a consequence, there is an urgent need for new generations of antifungal agents [5–7].

One of the most promising sources of new antifungal compounds are plants used in traditional medicine to treat infected wounds, athletes's foot or respiratory disorders. This approach is particularly interesting in Brazil, which possesses a very rich biodiversity because of its tropical climate and a long tradition in ethnomedicine [8, 9].

Another strategy for finding new antifungal compounds is the comparative design [10]. In this approach, plants being effective in microorganisms other than fungi, could be helpful in finding new antifungal compounds since they could act with similar mechanisms of action.

Both approaches converge to *Wedelia paludosa*, recently reclassified as *Acmela brasiliensis* (Asteraceae). It is a native medicinal plant which grows in several regions of Brazil, being known as margaridão, pingo de ouro, pseudoarnica or vedelia. The flowers and the whole plant are used in folk medicine against several disorders, including infections of the respiratory tract, inflammation, and affections in general [11–13]. Regarding their chemical composition, previous reports have shown the presence of several types of constituents such as kaurenoic acids [14, 15], terpenes [16, 17], eudesmanolide lactones [18] and flavonoids [12].

Since we have previously found that the hydroalcoholic extract of flowers of *W. paludosa (A. brasiliensis)* possesses antibacterial effects [11] and some diterpenes kauranes isolated from it exhibited tripanosomicid, antiparasitic and anti-HIV activities [19], we have decided to study its antifungal properties by testing it against different opportunistic pathogenic fungi with the agar dilution method.

2. Investigations, results and discussion

A methanolic extract of *Wedelia paludosa* was fractionated into hexane, dichloromethane, ethyl acetate, and butanol according to reported methods [20]. Concentrations up to 1000 μg/mL and 250 μg/mL for fractions and pure compounds, respectively, were incorporated into growth media according to reported procedures [21]. When fractions and pure compounds showed MICs $\leq$ 1000 μg/mL or 250 μg/mL respectively, they were considered active.

None of the extracts tested possessed activity against the yeasts *Candida albicans*, *C. tropicalis*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*, or against the filamentous fungi *A. fumigatus*, *A. flavus*, *A. niger* or *Microsporum gypseum* (Table). However, all fractions were active at least against one of the following dermatophytes: *Epidermophyton floccosum*, *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum canis* (Table), with MICs ranging from 250 to 1000 μg/mL.

The fact that *Wedelia paludosa* shows activity only against dermatophytes make it interesting for further invertigatiens. It is interesting to note that among dermatophytes, it selectively inhibits *Epidermophyton floccosum* and mainly *Trichophyton* spp. *E. floccosum* is a fungus that produces arthroconidia which survive for a longer time than those

Table: Minimal inhibitory concentrations (MICs in μg/mL) of different fractions and two pure compounds, kaurenoic acid (1) and luteolin (2), of flowers from *Wedelia paludosa* (*Acmela brasiliensis*)

Material tested	Ca	Ct	Cn	Sc	Afu	Afl	An	Mc	Mg	Ef	Tr	Tm
HEX	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000	>1000	500
DCM	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000	>1000	1000	1000	250
EA	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000
BuOH	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000	>1000	1000	250	500
1	>250	>250	>250	>250	>250	>250	>250	>250	>250	50	100	100
2	>250	>250	>250	>250	>250	>250	>250	>250	>250	250	>250	125

Candida albicans (Ca); *Candida tropicalis* (Ct); *Cryptococcus neoformans*.(Cn); *Saccharomyces cerevisiae* (Sc); *Aspergillus fumigatus* (Afu); *Aspergillus flavus* (Afl); *Aspergillus niger* (An); *Microsporum canis (Mc)*; *Microsporum gypseum* (*Mg*); *Epidermophyton floccosum* (Ef); *Trichophyton rubrum* (Tr); *Trichophyton metagrophytes* (Tm). HEX = hexane fraction; DCM = dichloromethane fraction; EA = ethyl acetate fraction; BuOH = butanol fraction; (**1**) = kaurenoic acid; (**2**) = luteolin.

of other dermatophytes, therefore constituting an environmental source of contamination, which could lead to recurrent outbreaks of dermatophytosis in individuals and in institutions [22]. In turn, *Trichophyton*. spp. are responsible for 80–93% of chronic or recurrent dermatophyte infections [22].

The bioassay-guided fractionation of the most active fractions, led to the isolation of (−)-kaur-16-en-19-oic acid (kaurenoic acid) (**1**) and luteolin (**2**) from the hexane and dichloromethane fractions, respectively.

Kaurenoic acid (**1**) was the most active compound showing the best activities against *Epidermophyton floccosum* (MIC = 50 μg/mL) followed by *Trichophyton rubrum* (MIC = 100 μg/mL) and *T. mentagrophytes* (MIC = 100 μg/mL). This tetracyclic diterpene has previously demonstrated selective antibacterial activity against Gram-positive bacteria, being bacteriolytic for *Bacillus cereus* [23, 24]. Besides its activity, it is interesting to note that compound **1** showed very low or no citoxicity when tested against human tumor cell lines [25].

Luteolin **(2)**, isolated from the dichloromethane fraction, showed MIC values of 125 μg/mL against *T. mentagrophytes* and 250 μg/mL against *E. floccosum*, being inactive against the other fungi tested. This compound has previously demonstrated antibacterial properties mainly against Gram-negative bacteria (*Enterobacter cloacae*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*) [26]. It also exhibited inhibitory activity against methicillin-resistant *S. aureus* (MRSA) [27] and against oral cariogenic *Streptococci* and periodontopathic *Porphyromonas gingivalis* [28].

In conclusion, our results together with those previously reported, indicate that the flowers of *W. paludosa* (*A. brasiliensis*) actually possess antifungal activities against dermatophytes, validating the popular use of this part of the plant in traditional medicine for the treatment of infectious dermal diseases. In contrast, *W. paludosa* would not exert activity against systemic mycoses and in these infections its use should be discouraged. Compound **2** and mainly the non-toxic compound **1** appear to be the most active constituents detected in this plant. Their selectivity against two genera of dermatophytes is particularly promising. According to Di Domenico [29] it is important to keep in mind that although compounds having a broad spectrum of action are usually searched, the fact that fungi represent one of the most diverse collection of microorganisms in the biosphere, narrow spectrum agents are intensely needed too.

3. Experimental

3.1. Plant material

Flowers of *Wedelia paludosa* (*Acmela brasiliensis*) (Asteraceae) were collected at "Lagoa da Conceição", in Florianopolis city, State of Santa Catarina, Brazil, in November 1998, and identified by Dr. Ademir Reis (Barbosa Rodrigues Herbarium [BRH, Itajaí (SC)]. A voucher specimen (V. C. Filho 002) has been deposited at the BRH.

3.2. Phytochemical analysis

Air-dried leaves (630 g) were powdered and extracted with methanol (5 L) at room temperature for approximately ten days. After solvent removal, the extract was concentrated under reduced pressure and successively partitioned with *n*-hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol (BuOH) [20] respectively, to give the following yields for each fraction: *n*-hexane (6.8 g), DCM (10.6 g), EA (8.5 g) and *n*-BuOH (5.5 g). The *n*-hexane and DCM fractions showed the most suitable phytochemical profile and antifungal activity and were selected for phytochemical evaluation. The *n*-hexane fraction (6.0 g) was chromatographed using a silica gel column eluted with a mixture of *n*-hexane: EA of increasing polarity. Elution with 9 : 1 v/v gave a white solid (0.11 g), which was identified as kaurenoic acid (**1**) by direct comparison with an authentic sample. The dichloromethane fraction (5.0 g) was chromatographed as in the previous case, to give a yellow solid (0.04 g), identified as luteolin (**2**) by direct comparison with an authentic sample.

3.3. Evaluation of biological activity

3.3.1. Microorganisms and media

The following microorganisms used for the fungistatic evaluation were purchased from the American Type Culture Collection (ATCC, Rockville, MD) or were clinical isolates provided by Centro de Referencia Micológica (C, CEREMIC, Facultad de Ciencias Bioquimicas y Farmacéuticas, Suipacha, Rosario, Argentina): *Candida albicans* (ATCC10231); *Cryptococcus neoformans* (ATCC32264); *Saccharomyces cerevisiae* (ATCC9763); *Aspergillus fumigatus* (ATCC26934), *Aspergillus flavus* (ATCC9170); *Aspergillus niger* (ATCC9092); *Candida tropicalis* (C113). The microorganisms used were cultivated on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C. The assays were carried out using the agar dilution method. Cell suspension in sterile distilled water was adjusted to give a final concentration of 10^6 viable yeast cells/mL, standardized with number 0.5 on the McFarland scale and with 90% T (530 nm). The dermatophytes *Epidermophyton floccosum* (C114); *Trichophyton rubrum* (C137); *Microsporum canis* (C112); *Microsporum gypseum* (C115) and *Trichophyton metagrophytes* (ATCC9972) were maintained on Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to previously reported procedures [30] and adjusted to 10^6 spores with colony forming ability/mL.

3.3.2. Antifungal assays

The fungistatic activities of different extracts were evaluated by the agar dilution method, using Sabouraud-chloramphenicol agar. The assay was carried out in the macro dilution plates [31, 32].

Stock solutions of extracts or compounds in dimethylsulfoxide (DMSO) were diluted to give serial twofold dilutions that were added to each medium, resulting in concentrations ranging from 1000 to 7.8 μg/mL. The final concentration of DMSO in the assay did not exceed 2%. Inoculums of 5 μL having the yeast cells or spore suspensions were added to Sabouraud-chloramphenicol media. The antifungal agent ketoconazole (Janssen Pharmaceutical) was included in the assay as positive control. Drug-free solution was also used as a blank control. Plates were incubated at 30 °C for 24, 48 or 72 h (according to the control fungus growth) up to 15 days for dermatophyte strains. MIC was defined as the lowest extract or compounds concentration, showing no visible fungal growth after the incubation period.

Acknowledgements: This research is part of the Iberoamerican project X.7 PIBEAFUN (Search and development of natural antifungal agents and analogues) of X.A (Iberoamerican Network on Medicinal Natural Products)

which belongs to the Iberoamerican Program of Science and Technology for the Development (CYTED-Subprogram X). SAZ is gratefully to the Agencia de Promociones Cientificas y Tecnológicas de la Argentina (PICT 06-06454) and to OEA (Project Profit of the Regional Flora), for financial support. VCF and RAY thank to CNPq (Brazil) for financial support.

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