

Antifungal activity of drimane sesquiterpenes from *Drimys brasiliensis* using bioassay-guided fractionation

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ABSTRACT. Purpose. This study describes the antifungal effect of extracts and compounds isolated from *Drimys brasiliensis* acting against dermatophytes. **Methods.** The activities were evaluated by using the microbroth dilution method. **Results.** Bioassay-guided fractionation of the most active extract from the bark (CHCl₃) led to the isolation of the sesquiterpene drimanes polygodial, 1-β-(p-methoxycinnamoyl)-polygodial, drimanal and 1-β-(p-cumaroyloxy)-polygodial, which were selectively active against *Epidermophyton floccosum* and *Tricophyton rubrum*. **Conclusions.** The selective antifungal activity reported in this paper for drimanes isolated from *D. brasiliensis* opens the possibility that they could be helpful for the developing of new antifungal agents for treating the difficult to eradicate dermatomycoses produced by *E. floccosum*.

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INTRODUCTION

Due to the increasing number of immunocompromised individuals, fungal infections have increased in the last two decades, affecting millions of people worldwide (1). Among them, opportunistic systemic mycoses are associated with high rates of death (2) and skin fungal infections, although not life threatening, are very difficult to eradicate (3). They produce a variety of problems, such as Athletes' foot and nail infections, leading to the debilitation of the patients' quality of life, with the additional danger that they can spread to other areas of the body and to other individuals (4).

Although there appears to be many drugs for the treatment of superficial mycoses, there are in fact a limited number of efficacious antifungal drugs (5). They possess a series of limitations such as undesirable side effects or rapid development of resistance and, as a consequence, new antifungal agents are still needed to improve the treatment of superficial fungal infections (6,7).

In the course of our screening program for the detection of antifungal compounds, we found in preliminary assays that CHCl₃ extract of *Drimys brasiliensis* possesses antifungal activity. These effects were related to the presence of polygodial (**1**), the major compound present in plant of this genus (8,9), which has been reported as antifungal in previous studies (10,11).

In order to detect some new antifungal compounds with drimane skeleton in extracts of *Drimys brasiliensis*, we report here the bioassay-guided fractionation of chloroform and methanol extracts of different parts of the plant by using the microbroth dilution method.

MATERIAL AND METHODS

Plant material

Barks, stems and leaves of *Drimys brasiliensis* were collected in Rancho Queimado, state of Santa Catarina, Brazil, in December 1998. The plant was identified as *Drimys brasiliensis* Miers subsp. *sylvatica* (Saint Hilaire) Ehrendofer L Gott sb by Dr. Ademir Reis (Department of Botany, Universidade Federal de Santa Catarina) and a voucher specimen was deposited in the Barbosa Rodrigues Herbarium (Itajaí - SC), under number VC Filho 010.

Preparation of extracts

Barks of *Drimys brasiliensis* (1.85 Kg) were dried at 40°C for two days, powdered and successively extracted with chloroform and MeOH at room temperature for 10 days each. The extracts were concentrated under reduced pressure, giving residues of 30.2 g and 60.6 g, respectively. Stems (700 g) were processed in the same way as barks, giving 17.6 g and 14.8 g of CHCl₃ and MeOH extracts respectively. From 250 g of leaves were obtained similarly 24.5 g and 10.6 g of CHCl₃ and MeOH extracts respectively.

Isolation and identification of drimanes from CHCl₃ extract (barks)

Part of the bark extract (23 g) was subjected to column chromatography (Øi 4,5 cm), packed with silica gel 60-230 mesh (220 g), and eluted with hexane gradually enriched in ethyl acetate and ethanol. 75 fractions of 100 ml each were collected. Polygodial (**1**) was obtained from fractions 28-32, [elution solvent Hex-AcOEt (9:1)] and was further purified by repeated chromatographies, giving 3.1 g yield (0.22 % in relation to dry plant).

Fraction 48-49, eluted with hexane:ethyl acetate 6:4 (2.3 g), was submitted to column chromatography (Øi 3, 0 cm), packed with silica gel (46 g), eluted with hexane and gradually enriched with ethyl acetate and ethanol. 51 sub-fractions of 25 mL each were collected. Sub-fractions 14-18, eluted with hexane:ethyl acetate 7.5:2.5 (198.4 mg) were rechromatographed as indicated above, eluted with hexane:ethyl acetate 6:4, yielding 12 mg of 1-β-(p-methoxycinnamoyl)-polygodial (**2**) (8.5 X 10⁻⁴ % in relation to dry plant).

Fraction 50-54, eluted with hexane:ethyl acetate 3:7 (5.5 g), was submitted to column chromatography (Øi 2,5 cm), packed with silica gel (48 g), eluted with hexane and gradually enriched with ethyl acetate and ethanol. 72 sub-fractions of 25 mL each were collected. From the subfraction 16-25, eluted with hexane:ethyl acetate 6:4, 1-β-(p-cumaroyloxy)-polygodial was obtained (187 mg yield, 0.013 % in relation to dry plant). Finally, from sub-fraction 30-42, eluted with hexane:ethyl acetate 6:4, drimanol (**3**) was obtained (3.3 g yield, 0.23 % in relation to dry plant). All the compounds were identified on basis of their spectral data in

comparison with those of literature [8,9] and direct comparison with authentic samples.

Microorganisms and media

For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina were used: *Microsporum canis* C 112, *Epidermophyton floccosum* C 114, *Trichophyton rubrum* C 110, *T. mentagrophytes* ATCC 9972 and *Microsporum gypseum* C 115. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to 10⁵ cells/spores with colony forming units (CFU) /ml (12).

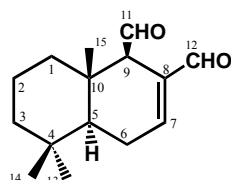
Antifungal susceptibility testing

Minimal Inhibitory Concentration (MIC) of each extract or compound was determined by using broth microdilution techniques according to the guidelines of the National Committee for Clinical Laboratory Standards for yeasts (M27-A2) (13) and for filamentous fungi (M 38 A) (14). MIC values were determined in RPMI 1640 (Sigma, St Louis, Mo, USA) buffered to pH 7.0 with MOPS. The starting inocula were 1x10⁵ to 5x10⁵ CFU/ml. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28-30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi.

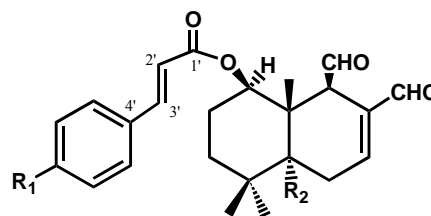
For the assay, stock solutions of extracts were two-fold diluted with RPMI 1000-1 µg/ml (final volume = 100 µl) and a final DMSO concentration ≤ 1%. A volume of 100 µl of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. Pure compounds were tested from 100 - 1 µg/ml. MIC was defined as the minimum inhibitory concentration of extract or pure compound which resulted in total inhibition of the fungal growth. ketoconazole, terbinafine and amphotericin B were used as positive controls.

Table 1. Minimum inhibitory concentration (MIC, µg/ml) of barks, stems and leaves of *Drimys brasiliensis* extracts against dermatophytes

	M.c	M. g	E. f.	T.r	T.m
CHCl ₃ barks	100	62.5	12.5	62.5	100
DB MeOH barks	500	1000	500	1000	1000
DBC CHCl ₃ stems	250	100	100	100	250
DBC MeOH Stems	>1000	>1000	>1000	>1000	>1000
DBF CHCl ₃ leaves	500	1000	500	500	1000
DBF MeOH leaves	>1000	>1000	>1000	>1000	>1000

Table 2. Minimum inhibitory concentration (MIC, µg/mL) of polygodial and some derivatives isolated from CHCl₃ extract of barks of *Drimys brasiliensis* against dermatophytes.

A



B

Nr	Compound	Ty	R ₁	R ₂	M.c.	M.g.	E. f.	T.r.	T.m.
1	Polygodial	A	-	-	>100	>100	3	25	>100
2	1-β-(p-methoxycinnamoyl) polygodial	B	OMe	H	100	50	25	25	62.5
3	Drimanial	B	OMe	OH	>100	>100	>100	>100	>100
4	1-β-(p-cumaroyloxy) polygodial	B	OH	H	62.5	50	25	25	25
	Ketoconazole				15	6.25	25	6.25	6.25
	Amphotericin B				50	12.5	0.3	12.5	12.5
	Terbinafine				0.01	0.006	0.004	0.003	0.006

RESULTS AND DISCUSSION

Results of the antifungal activities of chloroform and methanol extracts of bark, stems and leaves of *Drimys brasiliensis* are showed in Table 1.

CHCl₃ extracts of the three parts of the plant showed MIC values between 12.5 -1000 µg/ml against all dermatophytes tested. Chloroform extract of the bark was the most promising one, with MICs ranging from 12.5 to 100 µg/ml, and being *E. floccosum* the most sensitive species (MIC 12.5 µg/ml).

The bioassay-guided fractionation of CHCl₃ extract of barks led to the isolation of the antifungal drimane polygodial (**1**), 1-β-(methoxycinnamoyl)-

polygodial (**2**) and 1-β-(p-cumaroyloxy)-polygodial (**4**) together with the inactive drimanial (**3**) (table 2).

Compounds **1** and **2** have been previously isolated as antinociceptive from a highly related species *Drimys winteri* (8,9). In addition polygodial was previously isolated from *Polygonum hydropiper* (13), *Warburgia ugandensis* and *Warburgia stuhlmanii*, exhibiting insect antifeedant and antimicrobial activities (14,15) *Pseudowintera colorata* (16) and *Polygonum punctatum* (17) exhibit antimicrobial activities. Regarding the reported antifungal activity, polygodial previously showed potent antifungal activities against yeasts and filamentous

fungi being its activity due to the structural disruption of cell membranes (18, 19)

In this work we found that polygodial inhibit the growth of *Trichophyton rubrum* C 110 (see Microorganisms and Media above) with MIC 25 μ g/ml and *Epidermophyton floccosum* C 114 with MIC 3 μ g/ml. It was not active against the dermatophytes *Trichophyton mentagrophytes* ATCC 9972, *Microsporum canis* C112 and *Microsporum gypseum* C 115 up to 100 μ g/ml.

Previously reported antifungal activity of polygodial (20) showed lower MICs (0.78 and 3.13 mg/ml respectively) against the dermatophytes *T. rubrum* ATCC 28188 and *T. mentagrophytes* ATCC 28185 and 18748. No results were reported against the rest of dermatophytes *Epidermophyton floccosum*, *Microsporum canis* or *M. gypseum*. A comparison of the previous work and ours with respect to the activities of polygodial against *T. rubrum* and *T. mentagrophytes* may be meaningless due to the differences in methodology used.

Within the activity shown by drimane derivatives against the most sensitive fungus (*E. floccosum*), the following conclusions can be extracted a) polygodial showed the best activities with MIC = 3 μ g/ml; b) when a bulky substituent (*p*-methoxy or *p*-hydroxycinnamoyl) is present in position 1 of the polygodial, the activity diminishes 8 times [compare MIC of (1) (= 3 μ g/ml) with those of compounds (2) and (4) (= 25 μ g/ml each)]. This reduction seemed to be not dependent on the substituent R1 on the benzene ring of the cinnamoyl moiety; c) the presence of an OH in position 5 led to an inactive compound [compound (4), MIC > 100 μ g/ml].

Considering that the antifungal activity of drimane-type sesquiterpene dialdehydes was previously attributed to their α , β unsaturated aldehyde moiety (21), it is interesting to note that drimane 3, which possesses this structural feature, did not display any activity up to 100 μ g/ml.

Within the activity of cinnamoyl esters 2, 3 and 4, it is interesting to note that the change of a methoxyl by a hydroxyl group on the benzene ring (2 \rightarrow 4) does not modify the antifungal behavior of these compounds (MIC = 25 μ g/ml against *E. floccosum* for both compounds) while the presence of an hydroxyl group in the 5-position (2 \rightarrow 3) led to complete loss of activity.

CONCLUSIONS

Polygodial 1 together with three other cinnamoyl derivatives 2-4 were isolated from the chloroform extract of the bark of *Drymis brasiliensis*. Three of them showed interesting antifungal activities, this being the first report on the antifungal properties of cinnamoyl derivatives of polygodial.

Regarding antifungal activity of the isolated compounds, compound 1 strongly inhibits *E. floccosum* with MIC values of 3 μ g/ml and compounds 2 and 3 inhibit the same fungus at higher concentrations (25 μ g/ml), showing that the three compounds contribute to the antifungal activity observed in the whole bark. Although with a lesser incidence as the ethiological cause of dermatophytoses, *E. floccosum* produces arthroconidia, which survive for a longer time than other dermatophytes, therefore constituting an environmental source of contagion, sometimes leading to recurrent outbreaks of dermatophytosis in individuals and in institutions (6). Although it is desirable to develop compounds having a broad spectrum of activity, it is also important to keep in mind that the treatment of chronic different tineaes with the same broad spectrum antifungal agent leads sometimes to a high resistance to the available antifungal agents (4). Thus, one of the strategies for overcoming this problem is the treatment of fungal infections with the appropriate narrow spectrum agent when the ethiological agent is known (6).

Therefore, the selective antifungal activity reported in this paper for drimanes isolated from *D. brasiliensis* opens the possibility that polygodial (1) or its other drimane components (2) and (4) could be helpful for the developing of new antifungal agents for treating dermatomycoses produced by *E. floccosum*, usually very difficult to eradicate.

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