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Antifungal activity of the extracts and saponins from *Sapindus saponaria* L.

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ABSTRACT

Extracts from the dried pericarp of *Sapindus saponaria* L. (Sapindaceae) fruits were investigated for their antifungal activity against clinical isolates of yeasts *Candida albicans* and *C. non-albicans* from vaginal secretions of women with Vulvovaginal Candidiasis. Four clinical isolates of *C. albicans*, a single clinical isolated of each of the species *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and the strain of *C. albicans* ATCC 90028 were used. The hydroalcoholic extract was bioactivity-directed against a clinical isolate of *C. parapsilosis*, and showed strong activity. The n-BuOH extract and one fraction showed strong activity against all isolates tested. Further column-chromatography on silica gel separation of this fraction afforded two pure triterpene acetylated saponins: 3-O-(4-acetyl- β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-hederagenin (**1**) and 3-O-(3,4-di-acetyl- β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabynopyranosyl-hederagenin (**2**). The structures of the compounds were based on spectral data (¹H and ¹³C NMR, HSQC, HMBC and MS), and on with literature. The saponins isolated showed strong activity against *C. parapsilosis*.

Key words: Sapindaceae, *Sapindus saponaria*, saponins, antifungal activity, *Candida*.

INTRODUCTION

Species from Sapindaceae family are known for their traditional medicinal uses as a diuretic, stimulant, expectorant, natural surfactant, sedative, vermifuge and against stomachache and dermatitis in many parts of the world. Chemical investigations of this family have led to the isolation of saponins, diterpenes and flavonoids, among other secondary metabolites. Several saponins and acyclic sesquiterpene and diterpene oligoglycosides have been isolated as main secondary metabolites of

several Sapindaceae species used in traditional oriental medicine (Cavalcanti et al. 2001).

Sapindus saponaria L. (Sapindaceae), popularly known as 'sabão-de-soldado' and 'saboeiro', is a medium-sized deciduous tree occurring in the tropics, e.g., America and India, where the fruit is used as a soap and as a remedy against ulcers, scabies, joint pain, inflammations (Braga 1984, Abdel-Wahab and Selim 1985, Corrêa 1984, Albiero et al. 2002) and skin lesions caused by fungi (Murgu and Rodrigues-Filho 2006).

Abdel-Wahab and Selim (1985) detected the presence of carbohydrates, steroids and saponins in the leaves, stems, seeds and fruits of *S. saponaria* L. Flavo-

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noids were detected only in the stems and leaves. Tannins, essential oil and anthraquinones were detected only in the stems. β -sitosterol and α - and β -amyrin were found in the seeds; and rutin, luteolin and 4'-methoxyflavone in the seeds and leaves.

The acetylated saponin 3- β -O-[α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-glucopyranosyl] hederagenin was detected by Lemos et al. (1992) who observed antimicrobial activity against *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Cryptococcus neoformans*.

Ribeiro et al. (1995) isolated a monodesmosidic acetylated saponin and a mixture of two monodesmosidic saponins from a methanol extract of *S. saponaria* fruits. The compounds showed molluscicidal activity (LC₁₀₀/24h of 5-10 ppm) against the snail *Biomphalaria glabrata*.

Extracts of leaves and fruits of *Sapindus saponaria* were orally administered to rats, and the parameters of gastric secretion, evaluated after pylorus ligation, suggested that the fruits have an anti-gastric ulcer potential (Albiero et al. 2002).

C. albicans is an opportunistic pathogen that can cause local and systemic infections in predisposed persons, commonly affecting immunologically compromised patients and those undergoing prolonged antibiotic treatment. Yet, the information available on plants, particularly medicinal plants, active against this yeast has, until recently, not resulted in effective formulations for either human or animal use (Duarte et al. 2005). The other species, called non-*albicans*, are more difficult to eradicate (Jackson et al. 2005). It has become evident that there are differences in the susceptibility pattern to the antifungal drugs among different species, which is relevant to the clinical and epidemiological point of view (Richter et al. 2005).

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynecology, with a significant increase during the last year (Ziarrusta 2001). VVC may be treated with either topical or systemic antifungal agents. Fluconazole has been considered the drug of choice because it is well tolerated, has good oral bioavailability and is efficacious against most *Candida* spp. yeasts; however, it is expensive.

According to existing literature, the investigation of

natural products active against *Candida* spp. increased significantly in the last 10 years, and approximately 258 plant species, from 94 families were examined (Du et al. 2003).

In the present study, we describe the *in vitro* antifungal activity of extracts and subfractions from dried pericarps of the fruits of *S. saponaria*, as well as of the bioactivity-directed isolated saponins (1) and (2).

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

The NMR spectra were obtained in a VARIAN GEMINI 97.05T, using deuterated solvent, TMS as an internal standard and constant temperature of 298K. IR: film NaCl plates; ESI-MS were recorded on a Micro-mass Quattro LC, HRMS: CC: silica gel 60 (70-230 and 230-400 mesh); TLC: silica gel plates F₂₅₄ (0.25 mm thickness).

PLANT MATERIAL

The dried pericarps of *Sapindus saponaria* L. fruits were collected in February 2004 on the campus of the Universidade Estadual de Maringá. The plant was identified by the UEM Botany Department, and a voucher specimen (No. HUM 11710) is deposited in the UEM Herbarium, Paraná, Brazil.

ISOLATION OF THE CONSTITUENTS

The dried pericarps of *S. saponaria* fruits (450.0 g) were ground and extracted with EtOH:H₂O (9:1) at room temperature. The solvent was removed under *vacuum* at 40°C to yield an aqueous extract and a dark-brown residue. The aqueous extract from the crude hydroalcoholic extracts was lyophilized (50.1 g), and the residue from the crude extract was washed with EtOAc and subsequently with MeOH at room temperature. The evaporation in *vacuum* yielded 2.90 and 43.10 g for the EtOAc and MeOH residues, respectively. The MeOH extract was suspended in H₂O and then extracted with n-BuOH, which by evaporation gave a solid residue (28.9 g). The aqueous and n-BuOH extracts were assayed against *C. parapsilosis* by broth microdilution assay, to determine the minimal inhibitory concentrations (MIC) as described below.

The active n-BuOH extract (3.0 g) was fractionated by successive column chromatographies (CC). Development of the column, with mixtures of solvents of increased polarity, from $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ 90:9.5:0.5 v/v/v (50.0 mL) to $\text{MeOH}/\text{H}_2\text{O}$ 85:15 v/v (50.0 mL) afforded 14 principal fractions. The resulting fractions were assayed for antifungal activity (Table I). The active fraction F (148.0 mg) was chromatographed by CC on silica gel 60 (70-230 mesh) eluted with mixtures of solvents of increased polarity, from $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ 90:9.5:0.5 v/v/v (50.0 mL) to $\text{MeOH}/\text{H}_2\text{O}$ 85:15 v/v (50.0 mL) in order to yield 92 fractions and to obtain the following compounds: **1** (2.0 mg), **2** (2.1 mg). Identification of these compounds showed that they were 3-O-(4-acetyl- β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-hederagenin (**1**) and 3-O-(3,4-di-acetyl- β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabynopyranosyl-hederagenin (**2**). The chemical structures of compounds **1** and **2** are shown in Fig. 1. Structures were established with the use of spectroscopic methods (^1H and ^{13}C NMR, HSQC, HMBC and ESI/MS) and by comparing them with literature data (Ribeiro et al. 1995, Murgu and Rodrigues-Filho 2006). The hydroalcoholic extract was bioactivity-directed against a clinical isolate of *C. parapsilosis*. The n-BuOH extract and a subfraction were tested against *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. albicans* ATCC 90028. Saponins **1** and **2** were tested against a single clinical isolated of *C. parapsilosis*.

MICROORGANISMS USED AND GROWTH CONDITIONS

This research was conducted with 4 clinical isolates of *Candida albicans*, and one clinical isolate of each of the following species: *C. glabrata*, *C. parapsilosis* and *C. tropicalis* from vaginal secretions of women who were attended at the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá, Paraná, Brazil. The yeasts were identified according to classical methods (Larone 1995, Kurtzman and Fell 1998). For quality control, the isolate *Candida albicans* ATCC 90028 was included in each experiment. The yeasts were maintained in Sabouraud-dextrose agar "SDA" (DIFCO – USA) until use.

ANTIFUNGAL SUSCEPTIBILITY TESTING

The MICs of all the extracts, compounds and reference antifungal compounds were determined by the broth microdilution method. Fluconazole powder (Pfizer Inc., New York, NY, USA) was included as a control, and the test was performed according to the proposed NCCLS, M27-A standard guidelines (1997) but with some modifications for adaptation to natural compounds. The yeast suspension had its turbidity adjusted by spectrophotometer reading according to a 0.5 McFarland standard at 530 nm wavelength. Testing was performed in sterile, flat-bottom 96 well microliter plates. A volume of 100 μL of this adjusted inoculum suspension was dispensed in each well, resulting in inoculum size between 0.5 and 2.5×10^6 cells.mL $^{-1}$ tested against each drug concentration. The plates were incubated at 35°C for 72 h. A quality-control organism was included in each test, in order to check the accuracy of the drug dilutions and the reproducibility of the results (Padua et al. 2003). The MIC was defined as the first well with a significant reduction (approximately 80%) in growth, compared to that of the positive control. The MFC was defined as the lowest concentration yielding negative subcultures. Fluconazole powder (Pfizer Inc., New York, NY, USA) was included in the test control.

RESULTS AND DISCUSSION

The MIC results of the extracts, fractions and compounds tested are shown in Table I. The *in vitro* results were classified according to Aligiannis et al. (2001) and Duarte et al. (2005) who proposed a classification for plant materials, as follows: when the MIC is up to 0.5 mg mL $^{-1}$, the antifungal activity is considered strong. If the extracts or compounds display a MIC between 0.6 mg mL $^{-1}$ and 1.5 mg mL $^{-1}$ the antifungal activity is considered moderate; above 1.6 mg mL $^{-1}$ the antifungal activity is considered weak.

The crude hydroalcoholic extract obtained from dried pericarps of *S. saponaria* fruits were assayed for antifungal properties with microdilution-technique assays, against a clinical isolate of opportunistic pathogenic yeast from vaginal secretions (*C. parapsilosis*) showing a strong activity with MIC of 400.0 μg mL $^{-1}$. On the basis of this result, the crude hydroalcoholic extract was successively extracted with EtOAc and MeOH. The

TABLE I
Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)
of extracts, fractions and compounds obtained from dried pericarps *S. saponaria* fruits.

Tested material	MIC (MFC) $\mu\text{g mL}^{-1}$			
	<i>C. albicans</i> n = 4	<i>C. glabrata</i> n = 1	<i>C. parapsilosis</i> n = 1	<i>C. tropicalis</i> n = 1
Hydroalcoholic extract	n.d.	n.d.	400	n.d.
n-BuOH extract	300-600 (300–600)	600 (600)	300 (600)	300 (600)
Aqueous extract	n.d.	n.d.	1600	n.d.
Fraction A	n.d.	n.d.	250	n.d.
Fraction B	n.d.	n.d.	500	n.d.
Fraction C	n.d.	n.d.	500	n.d.
Fraction D	n.d.	n.d.	130	n.d.
Fraction E	n.d.	n.d.	70	n.d.
Fraction F	75–150 (75–150)	150 (150)	150 (150)	75 (75)
Subfraction F1	n.d.	n.d.	130	n.d.
Subfraction F2	n.d.	n.d.	250	n.d.
Subfraction F3	n.d.	n.d.	130	n.d.
Subfraction F4	n.d.	n.d.	130	n.d.
Saponin (1)	n.d.	n.d.	70	n.d.
Saponin (2)	n.d.	n.d.	250	n.d.
Fluconazole	0.5*	4	8	8

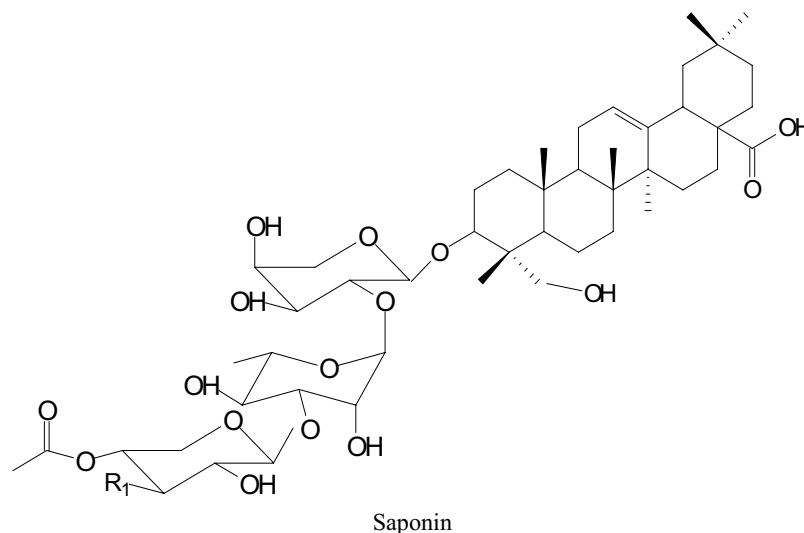
n.d. = not determined; *0.5 is the average value (Individual data are: 0.25, 0.5, 1.0, 0.25 $\mu\text{g mL}^{-1}$ respectively).

MeOH extract was further suspended in H_2O and n-BuOH. The n-BuOH extract showed activity against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. albicans* ATCC 90028. The results showed values of MIC from 300.0 to 600.0 $\mu\text{g mL}^{-1}$ against all the yeasts tested, strong activity against *C. albicans* and *C. tropicalis*, and moderate activity against *C. glabrata*, *C. parapsilosis* and *C. albicans* ATCC 90028. On the other hand, the aqueous extract was inactive with a MIC > 1600 $\mu\text{g mL}^{-1}$. n-BuOH extract (3.0 g) was subjected to column chromatography on silica gel to yielding some fractions. Fraction F showed significant anticandidal activity, with MIC from 75.0 to 150.0 $\mu\text{g mL}^{-1}$, and its activity against *C. tropicalis* was greater than against the other yeasts. Using activity-guided fractionation, antifungal saponins (**1**) and (**2**) were isolated from the Fraction F after separation by column chromatography on silica gel from the n-BuOH extract, with MIC 250.0 and 70.0 $\mu\text{g mL}^{-1}$ against *C. parapsilosis*, respectively. The

minimal fungicidal concentrations were twice the MIC for these organisms (Table I).

Our data, in agreement with an investigation of *Serjania salzmanniana* (Sapindaceae) for biologically active substances, led to isolation of two saponins: salzmanianoside A (3-O-[[β -D-glucopyranosyl-(1 \rightarrow 4)]-[α -L-rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl]gypsogenin) and salzmannianoside B (3-O-[[β -D-glucopyranosyl-(1 \rightarrow 4)]-[α -L-rhamnopyranosyl-(1 \rightarrow 3)]- α -rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl]hederagenin), both active against the human pathogenic fungi *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus* (Ekabo and Farnsworth 1996). The saponin structures and the MIC values reinforce the results of this study.

Studies on the antimicrobial properties detected in extracts of *S. mukurossi* and acetylated saponin of *S. saponaria*, showed that they were active against *Candida albicans* and *Cryptococcus neoformans* ATCC 32264,



Saponin 1	$R_1 = \text{OH}$
Saponin 2	$R_1 = \text{OAc}$

Fig. 1

as well as against the bacteria *Pseudomonas aeruginosa* ATCC 15422, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* (Lemos et al. 1992, Ahmad and Beg 2001).

Phytochemical analyses of several species of the genus *Sapindus* showed that they are rich in triterpenoid saponins possessing oleanolic acid and hederagenin as the genin (Murgu and Rodrigues-Filho 2006).

In recent years, triterpenoid saponins, with hederagenin or oleanolic acid as aglycone, have been found to possess antifungal activity against *C. glabrata*, *C. albicans*, *Trichosporon beigeli*, *Penicillium avelaneum* UC-4376, *Pyricularia oryzae*, *Cryptococcus neoformans*, *Coccidioides immitis* and *Saccharomyces cerevisiae*, as well as against the dermatophytes *Microsporum canis* and *Trichophyton mentagrophytes* (Du et al. 2003, Lee et al. 2001).

The saponins belong to the olean-type triterpenoid saponins, with C-28, C-30 dicarboxylic groups and olefinic double bond on C-12, which also showed antifungal activity against a panel of human-pathogenic opportunistic fungi (Escalante et al. 2002).

Despite advances in antifungal therapies, many

problems remain to be solved for most antifungal drugs available. Therefore, the azole drugs, especially fluconazole, are widely used to fight *C. albicans* infections. Not surprisingly, repeated fluconazole therapy for fungal infections in patients, such as vaginitis in women with *C. albicans* infections, has been associated with an increase in azole resistance, and the survey showed that 33.5% of clinical *C. albicans* isolates from women with vaginitis were resistant to fluconazole. In addition, several other *Candida* species, such as *C. krusei* and *C. tropicalis*, are inherently resistant to fluconazole. It is therefore very important to find antifungal drugs with novel chemical structures (Zhang et al. 2005).

In vitro studies have demonstrated that most *C. non-albicans* species from vaginal secretions are less sensitive than *C. albicans* to both topical and systemic antifungal drugs (Lynch et al. 1996). In the present study, the extract and subfractions of *S. saponaria* were more effective against *C. non-albicans*, (*C. tropicalis* and *C. parapsilosis*), suggesting that the dried pericarp of *S. saponaria* fruits is a potential candidate to supply phytotherapeutic inhibitors of *C. non-albicans* growth.

The findings revealed that the antifungal properties

of the extract of dried pericarp of *S. saponaria* fruits provide preliminary scientific validation for the traditional medicinal use of this plant, as a potential phytotherapeutic agent in certain fungal diseases and for the control of fungi in the environment. In addition, the extracts and fractions obtained from *S. saponaria* showed a fungicidal effect. This is very promising, considering that fluconazole, the medicine of choice in VVC treatment, exerts fungistatic activity. However, the extracts and active compound isolated from *S. saponaria* should be further studied in animal models in order to evaluate their *in vitro* efficacy and toxicity.

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RESUMO

Extratos do pericarpo de frutos de *Sapindus saponaria* L. (Sapindaceae) foram testados para a atividade antifúngica sobre isolados clínicos de leveduras de *Candida albicans* e *C. não-albicans* obtidos de secreção vaginal de mulheres com Candidíase Vulvovaginal. Foram avaliados quatro isolados clínicos de *C. albicans*, um de cada uma das espécies *C. glabrata*, *C. parapsilosis*, *C. tropicalis* e uma cepa referência de *C. albicans* ATCC 90028. O extrato hidroalcoólico foi biomonitorado contra um isolado clínico de *C. parapsilosis*, apresentando forte atividade. O extrato butanólico e uma fração apresentaram forte atividade contra todos os isolados testados. Posterior análise desta fração via cromatografia em sílica gel ($\text{CHCl}_3:\text{CH}_3\text{OH}$, 1:1, v/v) resultou no isolamento de duas saponinas triterpênicas puras mono e diacetiladas, 3-O-(4-O-acetil-O- β -D-xilopiranosil)-(1 \rightarrow 3)- α -L-ramnopiranosil-(1 \rightarrow 2)- α -L-arabinopiranosil-hederagenina (**1**) e 3-O-(3,4-di-O-acetil- β -D-xilopiranosil)-(1 \rightarrow 3)- α -L-ramnopiranosil-(1 \rightarrow 2)- α -L-rabinopiranosil-hederagenina (**2**) respectivamente. A elucidação estrutural das substâncias foi baseada em dados espectrais (RMN de ^1H e de ^{13}C , HSQC, HMBC, ESI/MS) e comparados com dados da literatura. As saponinas triterpênicas isoladas (**1**) e (**2**) apresentaram forte atividade contra *C. parapsilosis*.

Palavras-chave: Sapindaceae, *Sapindus saponaria*, saponinas, atividade antifúngica, *Candida*.

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