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Antioxidant and antifungal activities of extracts and condensed tannins from *Stryphnodendron obovatum* Benth.

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The antioxidant activity of stem-bark extracts from Stryphnodendron obovatum *Benth., including fractions and isolated compounds, was evaluated by DPPH in thin-layer chromatography. All the fractions and isolated compounds showed antioxidant activity. Antifungal activity was determined by the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against the yeasts* Candida albicans, Candida parapsilosis, Candida krusei *and* Candida tropicalis. *All extracts (CE, EtOAc and FW), subfractions (F1-F12) and the compounds I, II and III were inactive against the yeasts. Against* C. parapsilosis *and* C. albicans, *fractions F13-15 and F20 showed moderate antifungal activity, and fractions F16-19 and F21-22 showed good activity. Chemical isolation of the ethyl-acetate fraction resulted in the identification of three compounds: epigallocatechin, gallocatechin and epigallocatechin-(4β→8)-gallocatechin.*

Uniterms
- *Stryphnodendron obovatum*
- Leguminosae
- Antioxidant activity
- Antifungal activity
- Condensed tannins

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INTRODUCTION

The genus *Stryphnodendron* Mart., family Leguminosae, includes about 48 species, all native to central savannas of Brazil, including *Stryphnodendron obovatum* Benth. (Cronquist, 1988). The stem bark of several species of *Stryphnodendron,* which contains about 20% tannins, is used by the local population for wound healing and treatment of leukorrhea and diarrhoea (Santos and Mello, 2004). Tannin-rich plants are used in folk medicine because of their antimicrobial properties, and act as scavengers of free radicals (Santos and Mello, 2004).

The search for compounds with antimicrobial activity is urgent and indispensable for the treatment of infectious diseases caused by microorganisms resistant to traditional antimicrobial drugs (Irobi *et al.,* 1994). For this reason, Toledo (2002) and Lopes *et al.* (2003) evaluated the antimicrobial activity of crude extracts and fractions isolated from two other species of *Stryphnodendron* [*S. adstringens* (Mart.) Coville and *S. polyphyllum* Mart.]; both species showed good activity. Simeray *et al*. (1982) and Foo *et al*. (2000) also reported antimicrobial activity of tannins present in different species.

The anticarcinogenic and antimutagenic potentials of tannins may be related to their antioxidative properties important in preventing cellular oxidative damage, including lipid peroxidation (Haslam, 1998). Okamura *et al.*

(1993), Bors *et al.* (2000), Demirezer *et al.* (2001) and Velázquez *et al*. (2003) demonstrated the antioxidant activity of extracts and tannins isolated from red wine, *Eucalyptus rostrata* Schltdl., *Camellia sinensis* (L.) Kuntze, *Rumex patientia* L., *Aristolochia giberi* Hook, *Schinus weinmannifolia* Engler, and *Piper fulvescens* DC.

The aim of the present investigation was to evaluate the antioxidant and antifungal activities of extracts, fractions and compounds isolated from *S. obovatum*. This is the first time that chemical and biological activities have been evaluated in this species of plant.

MATERIAL AND METHODS

Plant material

The stem bark of *Stryphnodendron obovatum* Benth was collected in the city of Assis, state of São Paulo (22°35'20.8"S; 50°24'18.7"W; 546 m altitude) in February 2001. The plant was identified by Prof. Dr. Cássia Mônica Sakuragui. A voucher specimen is deposited in the Herbarium of the Biology Department, State University of Maringá, Brazil (HUM 8137).

Extraction and isolation

The stem bark (700 g) was submitted to turbo-extraction with acetone-water (7:3 v/v) for 30 min, yielding a crude extract (CE). The CE (100 g) was partitioned with a mixture of ethyl-acetate/water (1:1, 12 times), resulting in aqueous (FW, 70.5 g) and ethyl-acetate (EtOAc, 17.9 g) fractions. A 10 g subsample of the EtOAc was subjected to column chromatography on Sephadex®LH-20 with an eluent gradient between EtOH and water (1:1) and EtOH (100%), to yield 22 subfractions (F1 to F22). Subfractions F8 and F10 were rechromatographed under the same conditions, yielding 3 compounds which were identified by NMR (Varian Mercury Plus BB, 300 MHz) (^{1}H, ^{1}H/^{1}H COSY), and mass spectroscopy (ESI-MS, Quattro LCZ Micromass, Manchester, UK) and polarimetry (Perkin Elmer Polarimeter 241). The compounds were identified as epigallocatechin (I) (31.8 mg) and gallocatechin (II) (17.1 mg), by comparison with data in the literature (Mello *et al*., 1996).

Compound III (13.9 mg), after derivatisation, showed the following data of ^{1}H RMN ($CDCl_3$): the MS supplied to the pseudo-molecular ion [M+H+]+ in *m/z* 611.2 (19%) and the peak base in *m/z* 305.0 (100%).

Antioxidant Activity

Antioxidant activity was determined by thin-layer chromatograph DPPH (1,1-diphenyl-2-picrylhydrazyl) radical at 0.2% in MeOH direct by TLC, as recommended by Cuendt *et al.* (1997) and Hostettmann *et al.* (2003). Solutions of the crude extract, the ethyl-acetate fraction, and all the subfractions were all tested at a concentration of 100 µg/ml. The compounds isolated were tested at a concentration of 10 µg/ml. The reference drugs astilbin, gallic acid, quercetin and rutin (Sigma Chemical Co., St. Louis, Missouri, USA) were prepared at a concentration of 10 µg/ml.

Antifungal Activity

Antifungal activity of the crude extract, fractions, all subfractions, and isolated compounds was evaluated by the method of microdilution in broth (NCCLS, 2002), determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). The yeasts used were strains of *Candida albicans* (ATCC 573), and *Candida parapsilosis, Candida tropicalis* and *Candida krusei* obtained from clinical samples. Nystatin (Sigma) was used as the reference drug.

RESULTS AND DISCUSSION

The crude extract (CE) partitioned with ethyl-acetate/water resulted in the fractions ethyl-acetate (EtOAc) and aqueous (FW). The EtOAc fraction was separated on Sephadex® LH-20 column in 22 subfractions (F1-F22).

Because of the high tannin concentration in the bark of *S. obovatum* (13 - 19%) in the bark of *S. obovatum* (Sanches *et al*., 2002) and in view of literature data indicating that tannins may have antioxidant activity because of their capacity to scavenge radicals (Santos and Mello, 2004), the CE, the fractions (EtOAc and FW), and the chromatographic subfractions were analysed by their ability to scavenge DPPH$^{\cdot}$. The results demonstrated the capacity of the CE, the EtOAc and FW fractions and the subfractions (F1-F12) to reduce the DPPH radical. In particular, the subfractions F5-F12, at concentrations of 100 µg/ml, showed high antioxidant activity compared to quercetin, rutin, gallic acid and astilbin controls (Fig. 1). The subfractions F13-F22 were not analysed.

These results have been confirmed by other investigators. Okamura *et al.* (1993) demonstrated that tannins present in *Eucalyptus* sp. have potential antioxidant activity. Bors *et al*. (2000) demonstrated the antioxidant activity of proanthocyanidins and hydrolysable

tannins in red wine and green tea. Hatano *et al*. (2002) studied the inhibitory effect of NADP-dependent lipid peroxidation, besides the effect of inhibiting the autoxidation of linoleic acid; these effects were attributed to the scavenging activity of radicals, based on results from cacao polyphenols.

The crude extract (CE), the fractions (EtOAc and FW) and the subfractions (F1-F22) were also analysed by their antifungal activity against *C. albicans*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*. The results show values of MIC and MFC >1,000 µg/ml for CE, fractions (EtOAc, FW) and subfractions (F1-F12) against all the yeasts tested. On the other hand, the subfractions (F13-F22) showed good or moderate antifungal activity against *C. albicans* and *C. parapsilosis* but no activity against *C. krusei* and *C. tropicalis*. Against *C. albicans* and *C. parapsilosis*, the subfractions F13-F22 showed a MIC of 31.5 µg/ml to 125 µg/ml. The fungicidal action was moderate for subfractions F13-F18 (125-250 µg/ml) and for the subfractions F21 and F22 (125 µg/ml) against *C. albicans*. Whereas against *C. parapsilosis*, only as subfractions F21 and F22 (125 µg/ml) showed moderate fungicidal activity, in accordance with the proposal of Holetz *et al*. (2002) (Table 1).

Laks (1987) studied the antifungal activity of flavonoids derived from a thiolysis reaction with alkyl thiol resulting in epicatechin-4-alkylsulphides. This compound showed a MIC greater than 500 ppm for fungal and gram-negative bacteria. Extracts from the bark of *Bridelia ferruginea* Benth. were tested at a concentration of 5 µg/ml against *Staphylococcus aureus, C. albicans*, *Staphylococcus epidermidis, Escherichia coli* and *Streptococcus lactis*. For *Proteus vulgaris, Proteus mirabilis, Streptococcus pyogenes and Klebsiella* sp. the inhibition zone was measured at between 4 and 20 mm, whereas the control antibiotic chloramphenicol produced zones between 15 and 36 mm. Preliminary phytochemical analysis of plant extracts indicated the presence of phenols and tannins (Irobi *et al.*, 1994). Gülçin *et al*. (2003) studied antioxidant and antimicrobial activity of extracts from the seeds of *Pimpinella anisum* L. in nine species of bacteria and one species of fungus, and observed strong activity against *S. aureus*. The extracts showed strong antioxidant activity, reducing potential, radical DPPH and oxidate anion scavenging, hydrogen peroxide scavenging, and metal chelating activity, compared with several standards such as BHA, BHT and tocopherol. Chattopadhyay *et al.* (2001) evaluated the antimicrobial activity of a crude extract from leaves of *Alstonia macrophylla* Wall, against *S. aureus*, *Staphylococcus saprophyticus, Steptococcus faecalis, E. coli, P. mirabilis, Trichophyton rubrum, Trichophyton mentagrophytes* var. *mentagrophytes* and *Microsporum gypseum*, which showed MIC 64-1,000 µg/ml and 32-128 µg/ml for dermatophytes. However, these extracts were inactive against other microorganisms. Studies with *S. adstringens* (Mart.) Coville by Toledo (2002) demonstrated that subfractions of the EtOAc fraction of the stem bark showed antibacterial activity against gram-positive (*S. aureus* and *Bacillus subtilis*) and gram-negative (*Pseudomonas aeruginosa* and *E. coli*) bacteria; one subfraction (F3#12) showed the best activity against *P. aeruginosa*. Lopes *et al*. (2003), in studies with *Stryphnodendron polyphyllum* Mart., observed activity against *S. aureus* in fractions 15 and 9, which showed a MIC of 125 µg/ml and a minimum bactericidal concentration of 125-250 µg/ml.

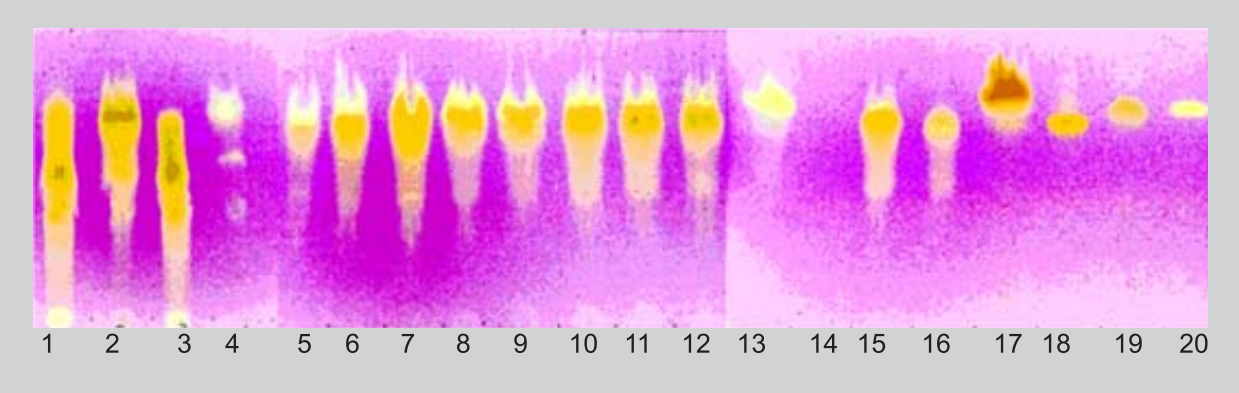

FIGURE 1 - Antioxidant activity of crude extract, fractions, subfractions and isolated compounds from *Stryphnodendron obovatum* on DPPH radical. 1. CE, 2. EtOAc, 3. FW, 4. F2, 5. F3, 6. F5-7, 7. F8, 8. F9, 9. F10, 10. F11, 11. F12, 12. Compound I, 13. Compound II, 14. not sampled, 15. Compound III, 16. F11.3, 17. quercetin, 18. rutin, 19. gallic acid, 20. astilbin

TABLE 1 - Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of crude extract, fractions, subfractions and compounds isolated from *S. obovatum* against yeasts

	MIC (MFC) µg/ml			
	C. albicans	***C. parapsilosis***	***C. krusei***	***C. tropicalis***
Crude extract	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)
Water fraction	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)
Ethyl-acetate fraction	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)
F1 to F12	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)	>1000 (>1000)
F13	125 (250)	125 (1000)	>1000 (>1000)	>1000 (>1000)
F14	125 (250)	125 (500)	>1000 (>1000)	>1000 (>1000)
F15	125 (125)	62.5 (500)	>1000 (>1000)	>1000 (>1000)
F16	62.5 (125)	62.5 (1000)	>1000 (>1000)	>1000 (>1000)
F17	62.5 (125)	31.5 (1000)	>1000 (>1000)	>1000 (>1000)
F18	62.5 (125)	31.5 (500)	>1000 (>1000)	>1000 (>1000)
F19	62.5 (1000)	62.5 (500)	>1000 (>1000)	>1000 (>1000)
F20	125 (500)	125 (500)	>1000 (>1000)	>1000 (>1000)
F21	62.5 (125)	62.5 (125)	>1000 (>1000)	>1000 (>1000)
F22	62.5 (125)	62.5 (125)	>1000 (>1000)	>1000 (>1000)
Compound I	>100 (>100)	>100 (>100)	>100 (>100)	>100 (>100)
Compound II	>100 (>100)	>100 (>100)	>100 (>100)	>100 (>100)
Compound III	>100 (>100)	>100 (>100)	>100 (>100)	>100 (>100)
Nystatin	1 (-)	8 (-)	4 (-)	8 (-)

Condensed tannins are usually isolated in a Sephadex® LH-20 column, mainly with alcoholic solvents, to separate first the monomeric, and next the di-, tri-, and oligomeric flavan-3-ols (Thompson *et al.,* 1972). The present study demonstrated the presence of fungicidal activity against *C. albicans* and *C. parapsilosis,* in subfractions F13 to F22. These subfractions may be compounds of higher molecular weight than subfractions F1 to F12, which could account for the greater activity of the former. Greater activity of high-molecular-weight compounds was observed previously by Holetz (2003) in a trypanosomatid protozoan, using CE and EtOAc subfractions from *S. adstringens* (Mart.) Coville.

The antifungal activity of the subfractions F13-F22 on *C. albicans* and *C, parapsilosis* may result from the properties of bark tannins, such as: extracellular enzyme inhibition, deprivation of substratum, inhibition of oxidative phosphorylation, as well as mechanisms that involve deprivation of iron (Scalbert, 1991).

The subfractions (F8 and F10) obtained from EtOAc fraction chromatography on Sephadex® LH-20 column were rechromatographed under the same conditions. The compounds I, II, and III were isolated and identified as epigallocatechin, gallocatechin, and epigallocatechin-(4β→8)-gallocatechin, respectively, using ^{1}H NMR, ^{1}H/^{1}H COSY, and mass spectroscopy and polarimetry, and comparing their data with the literature (Fig. 2). Although these substances have previously been isolated from *Psidium guajava* L. (Myrtaceae) (Tanaka *et al*., 1992), *Cistus incanus* L. (Cistaceae) (Petereit, 1992) and *S. adstringens* (Mart.) Coville (Mello *et al.*, 1996), this is the first time that they have been isolated from *S. obovatum*. These compounds, at concentration of 10 µg/ml, showed capacity of scavenging the DPPH radical, indicating antioxidant activity. On the other hand, the compounds I, II and III showed no antifungal activity as observed for subfractions F8 and F10.

CONCLUSIONS

From the mixture of compounds from the EtOAc, the following compoundswere isolated and identifued epigallocatechin, gallocatechin, and epigallocatechin-(4β→8)-gallocatechin using ^{1}H RMN, ^{1}H/^{1}H COSY, mass spectroscopy and polarimetry, and comparing their data with the literature. The results demonstrated that the extracts, fractions and subfractions all possessed antioxidant activity. In particular, fractions F5-F12 showed high activity compared to the quercetin, rutin, gallic acid and astilbin controls. The present study demonstrated the presence of fungicidal activity against *C. albicans* and *C. parapsilosis* in subfractions F13 to F22. These

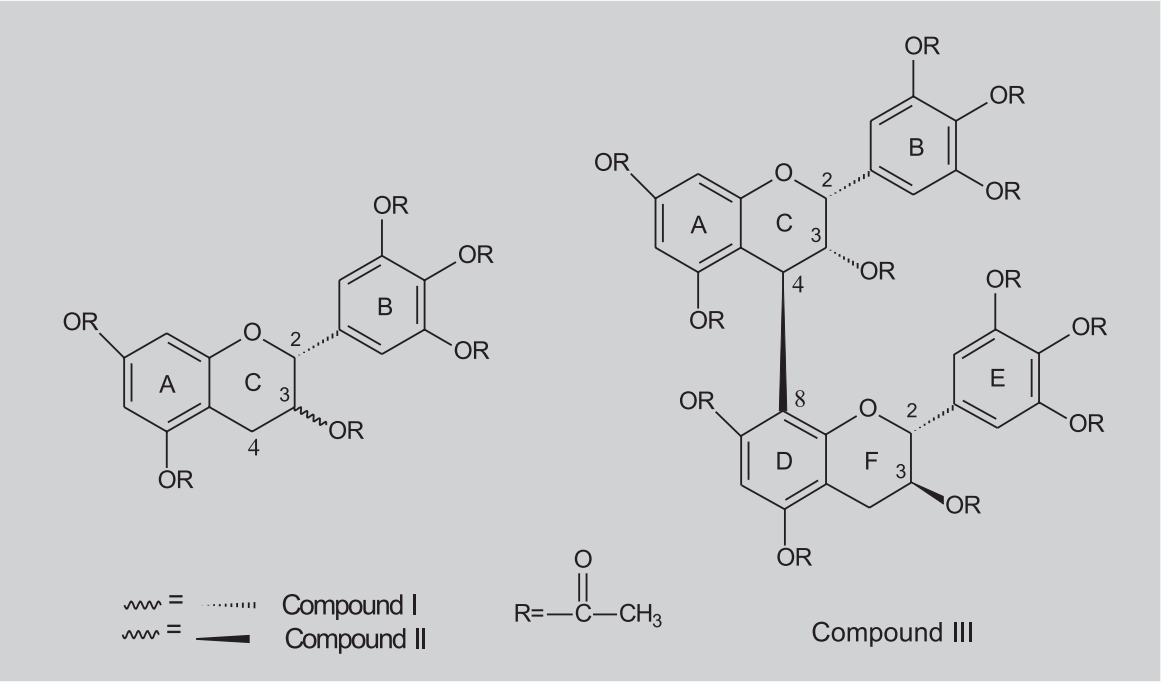

FIGURE 2 - Chemical structure at the isolated compounds

subfractions may be compounds of higher molecular weight than subfractions F1 to F12, which could account for the greater activity of the former.

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RESUMO

Atividades antioxidante e antifúngica de extratos e taninos condensados de *Stryphnodendron obovatum* Benth.

Atividade antioxidante de extrato, frações, subfrações e substâncias isoladas das cascas de Stryphnodendron obovatum *Benth. foi avaliada através da redução do radical 1,1-difenil-2-picrilhidrazila (método DDPH˙) em cromatografia em camada delgada. O extrato bruto (CE, acetona:água), as frações acetato de etila (EtOAc) e aquosa (FW), as subfrações (F1-F12) e as substâncias isoladas I, II e III apresentaram a capacidade de reduzir o radical DDPH˙. A atividade antifúngica foi determinada pela concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) frente às amostras de leveduras* Candida albicans, Candida parapsilosis, Candida krusei *e* Candida tropicalis. *O extrato bruto (CE), as frações (EtOAC e FW), e os compostos isolados I, II e III, como também as subfrações cromatográficas (F1-F12) foram inativos frente a todas as leveduras testadas. Por outro lado, as subfrações cromatográficas F13-15 e F20 apresentaram atividade antifúngica moderada. Já as subfrações F16-19 e F21-22 mostraram boa atividade antifúngica frente às cepas de* C. albicans *e* C. parapsilosis. *As substâncias I, II, e III, isoladas da fração EtOAc por cromatografia e recromatografia em coluna de Sephadex® LH-20, foram identificadas como sendo os monômeros de flavan-3-ol, epigalocatequina e galocatequina, e um dímero, epigalocatequina-(4β→8)-galocatequina, respectivamente.*

UNITERMOS: Stryphnodendron obovatum. *Leguminosae. Atividade antioxidante. Atividade antifúngica. Taninos condensados.*

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