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Antimicrobial Activity of Ethanol Extract from Leaves of *Casearia sylvestris*

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Abstract

Casearia sylvestris Sw., also known as "guaçatonga," is a medicinal plant with broad use in South America. Among the popular applications attributed to this plant are anti-inflammatory, anticancer, antimicrobial, and antiulcer activities. Despite the broad popular use of this plant as a phytopharmaceutical agent, there are few studies about the antimicrobial potential of guaçatonga. In this work, we have studied the antimicrobial potential of an ethanol extract obtained from *C. sylvestris* leaves against three yeasts, two filamentous fungus, six Gram-negative bacteria, and two Gram-positive bacteria. Through two chromatographic steps using a Sephadex LH-20 column and RP-HPLC, we isolated and characterized two derived compounds of gallic acid: isobutyl gallate-3,5-dimethyl ether (IGDE) and methyl gallate-3,5-dimethyl ether (MGDE). Both compounds showed antimicrobial activity. IGDE was much more efficient than MGDE in inhibiting yeasts (*Candida albicans, Candida tropicalis*, and *Candida guilliermondii*) and Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*). This fact is probably associated with the higher hydrophobicity degree of IDGE compared with MGDE.

Keywords: Antibacterial, antifungical, antimicrobial, *Casearia sylvestris.*

Introduction

The Flacourtiaceae is a large family consisting of 89 genera and 1300 species found in tropical and temperate regions of the world. In South America, *Casearia sylvestris* Sw., popularly named "chá-de-bugre," "cafezinho-do-mato," and "guaçatonga," is a plant used in popular medicine against several diseases (Lorenzi & Matos, 2002).

The Brazilian Karajá Indians and Shipibo-Conibo Indians of Peru prepare a bark maceration to treat diarrhea. Other Indian tribes in Brazil mash the roots or seeds of Guaçatonga to treat wounds and topical leprosy. The plant is also a popular herbal medication employed in Bolivian herbal medicine, in which it is considered to be analgesic, antacid, anti-inflammatory, anticancer, and antimicrobial. Nowadays, *C. sylvestris* is used as an active principle in pomades against Herpes and is also being commercialized in plant mixtures used in the elaboration of teas and infusions with phytotherapeutic purposes (Alves, 2000; Basile et al., 1990; Beutler et al., 2000; Chiappeta, 1983; Mans et al., 2000; Menezes et al., 2004; Morita et al., 1991; Mors et al., 2000; Raslan et al., 2002; Simões et al., 2001).

Concerning the popular use of *C. sylvestris,* we verified that few studies have been performed at a deeper level regarding its antimicrobial potential. The majority of guaçatonga studies are restricted to its antileishmanial, trypanocidal, larvicidal, gastric antiulcer, anti-inflammatory, genotoxicity, and antioxidant activities (Basile et al., 1990; De Mesquita et al., 2005; Esteves et al., 2005; Maistro et al., 2004; Rodrigues et al., 2006). However, Mosaddik et al. (2004) showed that the leaves of other genres of *Casearia* plants (*C. costulata* Jessup., *C. grewiifolia* Benth., *C. grayi* Jessup, and *C. multinervosa* CT White & Sleumer) can inhibit *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* growth.

In this work, we studied the antimicrobial potential of ethanol extracts from leaves of *C. sylvestris*, as we isolated,

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purified, and characterized two compounds of this material that showed antibacterial and antifungicidal activity.

Materials and Methods

Plant material and alcohol extract

The fresh leaves of *C*. sylvestris were collected in the city of Campinas, S.P., Brazil, in March 2005. The material was identified by the botanists from the herbarium of the Biological Institute of Unicamp, where a voucher was deposited under the number UEC 118743. The ethanol extract was produced by using 500 g of fresh leaves that were washed and stirred with 1000 mL of ethanol (analytical grade) in a blender for 15 min at room temperature and then filtered using analytical filter paper. The alcohol extract was centrifuged at 30,000× *g* for 15 min, and the supernatant was concentrated to semisolid paste using a rotavapor. This paste was lyophilized to yield 5.6 g of a light-green powder that was stored at −20°C (Weniger, 1991).

Sephadex LH-20 column chromatography

A 1.3 g portion of powder obtained from ethanol extract was dissolved in 10 mL of HPLC-grade methanol and applied to a column (1.5-cm diameter and 77-cm height) packed with Sephadex LH-20 (particle size 25–100 mm; Sigma Chemical Co., Nepean, ON, Canada) and eluted with methanol. Methanol fractions (8 mL each) were collected in test tubes placed in an LKB Bromma 2112 Redirac fraction collector (Pharmacia, Uppsala, Sweden) and their absorbances read at 280 nm. Eluates were then pooled into fractions I–VIII. Solvent was evaporated under vacuum at 40°C. Dried fractions were stored in tinted glass bottles at −20°C until used.

Analytical and preparative reverse phase high-performance liquid chromatography

Fractions eluted from Sephadex LH-20 column chromatography were subjected to a second chromatography process using reverse phase high-performance liquid chromatography (RP-HPLC). A Shimadzu HPLC system (Kyoto, Japan) was used for analytical and preparative HPLC of isolated compounds. Conditions for preparative HPLC were as follows: Hilber prepacked column RT (10 × 250 mm) with Lichrosorb RP-18 (Merck, Darmstadt, Germany); water:acetonitrile:methanol:acetic acid (79.5:18:2:0.5, v/v/v/v) as the mobile phase; flow rate of 3 mL/min; UV-Vis spectrophotometric detector adjusted at 280 nm. Each peak eluted was collected, the solvent was evaporated under vacuum at 40°C, and finally was stored at − 20°C until used.

Microorganisms

The microorganisms are characterized and stocked in the microbiology laboratory of the Biology Institute of the State University of Campinas (UNICAMP). The microorganisms were three yeasts (*Candida albicans, Candida tropicalis*, and *Candida guilliermondii*), two filamentous fungi (*Aspergillus flavus* and *Aspergillus niger*), six Gram-negative bacteria (*Escherichia coli*, *Salmonella enteritidis, Shigella sonnei, Pseudomonas aeruginosa, Serratia marcescens* and *Klebsiella pneumoniae*), and two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*), obtained from clinical isolates of several materials from the Clinical Hospital of the State University of Campinas (UNICAMP, Campinas, Brazil).

Proton and carbon nuclear magnetic resonance spectroscopy

The compounds selected from RP-HPLC were characterized by proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR)s. NMR spectra were recorded using a General Electric 300-NB spectrometer (General Electric, Palo Alto, CA, USA). ^{1}H (at 300 MHz), (1H) correlation spectroscopy (COSY, at 300 MHz), and ^{13}C (at 75.5 MHz) NMR data were collected at room temperature in deuterated methanol (CD_3OD). Chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) as an internal standard.

Antimicrobial tests

The microorganisms were grown in appropriately fortifed agar poured in bioassay plates. Mueller-Hinton agar (DIFCO, Detroit, MI, USA) was prepared for the bacteria. RPMI 1640 standard cell culture (Sigma Chemicals, St. Louis, MO, USA) buffered with a 0.165 M MOPS buffer and fortified with 20 g of glucose per liter of 1.5% agar was used for the fungi (Hoffman & Pfaller, 2001).

The test solutions were obtained from fractions (or isolated compounds) dissolved in aqueous solution of DMSO (dimethyl sulfoxide) 1% (final concentration of 40 mg/mL). The dilutions for MIC (minimum inhibitory concentration) determinations were serially done (two-fold) up to a concentration of 4.9 μg/mL. Test solution (100 μL) was added to the 6.2-mm-diameter wells in agar (Barry, 1986), as well as the positive and negative controls. The positive controls were prepared using antibiotics solutions (cloramphenicol and streptomycin for bacteria and miconazol for fungi) in a 1 mg/mL concentration. The solvent was used as the negative control (1% aqueous DMSO).

The activity of fractions from the ethanol extract (and isolated compounds) of *C. sylvestris* leaves was determined by inhibition zone (IZ) measurement of fungi or bacteria growth caused by the test solution. The absence of IZs (visual analysis) indicated the last dilution allowing microbial growth, thus it was considered the MIC (NCCLS, 1990). Bacteria were added directly to the cooling agar (at about 55°C), and spores for the fungus and yeast were applied as a lawn just prior to coring and filling the wells. Plates were stored for both bacteria and fungi screening for 48 h in an

incubator at 37°C. Then, measurements of inhibition zones were done.

Results and Discussion

The results of chromatography using Sephadex LH-20 column are shown in Figure 1. There were eight peaks with only peak III demonstrating activity against the microorganisms tested. For selection of peaks with antimicrobial activity, only *Escherichia coli* and *Candida albicans* were used.

The fractions corresponding with peak III were pooled and lyophilized. This material was dissolved in methanol and subjected to RP-HPLC chromatography. The chromatographic profile obtained is shown in Figure 2. Peaks 2 and 3 were selected because of antimicrobial activity against *Escherichia coli* and *Candida albicans*, and structures were solved by NMR.

The compounds correspondenting with peaks 2 and 3 were characterized by ^{1}H and ^{13}C NMR spectroscopy. We identified two compounds derived of gallic acid (3,4,5-trihidroxybenzoic acid). The compound corresponding with peak II was identified as isobutyl gallate-3,5-dimethyl ether (IGDE) (Fig. 3), and the compound corresponding to peak III was identified as methyl gallate-3,5-dimethyl ether (MGDE) (Fig. 3). The spectroscopic data a are: as follows: IGDE^{1}H NMR: δ ppm 7.02 (2H, s, H-2 and H-6), 3.58 (6H, s, H-2′ and H-3′), 2.80 (2H, d, H-4′), 2.20–2.05 (1H, multi, H-5′), 0.98 (6H, s, H-6′ and H-7′); ^{13}C NMR: δ ppm C 188.02 (C1′), 148.02 (C4), 126.42 (C2 and C6), 124.28 (C3 and C5), 124.02 (C1), 50.01 (C4′), 25.18 (C5′), 22.94 (C6′ and C7′), 19.88 (C2′ and C3′); and MGDE^{1}:H NMR: δ ppm 6.92 (2H, s, H-2 and H-6), 3.64 (6H, s, H-2′ and H-3′), 2.60 (3H, d, H-4′); ^{13}C NMR: δ ppm C 186.44 (C1′), 150.61 (C4), 134.28 (C1), 128.28 (C2 and C6), 124.48 (C3 and C5), 30.80 (C4′), 24.28 (C2′and C3′).

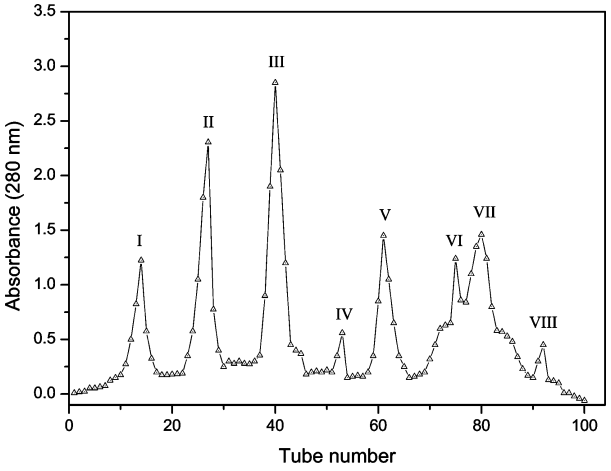

Figure 1. Chromatographic profile of elution of ethanol extract from leaves of *C. sylvestris* using Sephadex LH-20 column. The peak III showed antimicrobial activity against *Escherichia coli* and *Candida albicans.*

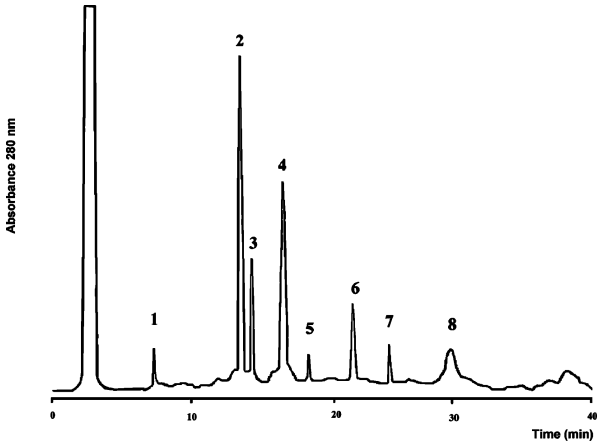

Figure 2. The chromatographic profile of fractions elution, corresponding with peak III (see Fig. 1) using RP-HPLC. Peaks 2 and 3 showed activity against *Escherichia coli* and *Candida albicans*.

The complete antimicrobial assays using either IDGE or MGDE compounds are shown in Table 1. Among the microorganisms tested, the Gram-positive bacteria. *E. faecalis* and *S. aureus* were the most sensitive microorganisms either to IGDE (MIC 9.8 μg/mL for both) or MGDE (MIC 39.0 μg/mL for both). The Gram-negative bacteria, *E. coli, S. enteritidis*, and *P. aeruginosa*, showed just some degree of sensibility to IGDE and MGDE (see Table 1). The *C. albicans, C. tropicalis*, and *C. guilliermondii* yeasts were significantly sensitive for IGDE (MIC 78.0, 156.0, and 156.0 μg/mL, respectively) but not to MGDE. No filamentous fungi tested (*A. flavus* and *A. niger*) was sensitive to IGDE or MGDE.

According to our results, the Gram-positive bacteria and yeasts are more sensitive to the most hydrophobic

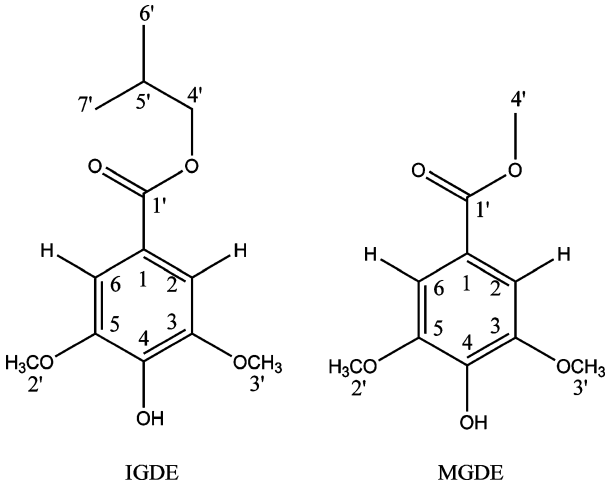

Figure 3. Structures of isobutyl gallate-3,5-dimethyl ether (IGDE) and methyl gallate-3,5-dimethyl ether (MGDE).

Table 1. The minimal inhibitory concentration[a] of IGDE and MGDE (in μg/mL) against bacteria and fungi.

Microorganism	IGDE MIC (μg/mL)	MGDE MIC (μg/mL)
Gram-negative bacteria		
Escherichia coli	156.0[b]	312.0
Salmonella enteritidis	156.0	156.0
Shigella sonnei	–[c]	–
Pseudomonas aeruginosa	312.0	624.0
Serratia marcescens	–	–
Klebsiella pneumoniae	–	–
Gram positive bacteria		
Enterococcus faecalis	9.8	39.0
Staphylococcus aureus	9.8	39.0
Yeast		
Candida albicans	78.0	–
Candida tropicalis	156.0	–
Candida guilliermondii	156.0	–
Filamentous fungi		
Aspergillus flavus	–	–
Aspergillus niger	–	–

[a] Method for determination of MIC of IGDE and MGDE from leaves of *C. sylvestris*: agar diffusion.
[b] Last concentration that caused inhibition in bacterial growth.
[c] (–) Presence bacterial growth = inhibitory activity negative.

compound (IGDE) relative to the less hydrophobic compound (MGDE). Similar to our results, Shibata et al. (2005) showed that *S. aureus* is sensative to alkyl gallates, and higher hydrophobicity of the tested compound leads to greater inhibition of bacterial growth. Shibata et al. (2005) verified that the presence of alkyl gallates synergistically increases the sensitivity of *S. aureus* to β-lactam antibiotics. Also, Kubo et al. (2002) showed that *S. cerevisiae* is sensitive to alkyl gallate derivatives with bigger hydrophobicity index.

We have verified that MGDE was in a general way less efficient than IGDE for inhibiting microbial growth. Probably this fact is related to the low degree of hydrophobicity of MGDE, which is chemically much more similar to the gallic acid than IGDE. In fact, Chanwitheesuk et al. (2007) verified that gallic acid inhibits neither *C. albicans* nor *Aspergillus* spp. growth. Furthermore, these authors verified that Gram-negative bacteria have low sensitivity to gallic acid, but Gram-positive bacteria are very sensitive to the same compound.

Finally, we can conclude that *C. sylvestris* presents a good action potential against microorganisms, mainly against Gram-positive bacteria and yeasts. A part of this antimicrobial activity is associated with two compounds derived from gallic acid, IGDE and MGDE.

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