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Original Article

Phytochemistry and antimicrobial activity of *Campomanesia adamantium*

Q1 Stone Sá [a], Luíza T. Chaul [a], Virgínia F. Alves [b], Tatiana S. Fiuza [c], Leonice M.F. Tresvenzol [a], Boniek G. Vaz [d], Pedro H. Ferri [d], Leonardo L. Borges [e,f], José R. Paula [a,*]

[a] *Laboratório de Pesquisa em Produtos Naturais, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[b] *Laboratório de Microbiologia de Medicamentos e Alimentos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[c] *Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[d] *Instituto de Química, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[e] *Escola de Ciências Médicas, Farmacêuticas e Biomédicas, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brazil*
[f] *Campus Henrique Santillo, Universidade Estadual de Goiás, Anápolis, GO, Brazil*

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ABSTRACT

Campomanesia adamantium (Cambess.) O. Berg., Myrtaceae, is a plant popularly used for its anti-inflammatory, anti-diarrhoeal and urinary antiseptic activities. The aims of this study were to obtain the crude ethanolic extract and the hexane, dichloromethane, ethyl acetate, aqueous and concentrated aqueous tannin fractions from *C. adamantium* leaves, perform biomonitored fractionation to isolate and identify chemical compounds, study the chemical composition of the volatile oils of the leaves and flowers and test the antimicrobial activity of the ethanolic extract, fractions, isolated substances and volatile oils. Phytochemical screening and chromatographic and spectrometric techniques were used. Volatile oils were isolated by hydrodistillation in a Clevenger apparatus and analyzed by gas chromatography/mass spectrometry. The antimicrobial activity was tested by a broth microdilution test. The component stictane-3,22-diol was isolated and identified from the hexane fraction, while valoneic and gallic acid were isolated and identified from the concentrated aqueous tannin fraction. The major constituents of the volatile oils of the leaves were verbenene (13.91%), β-funebrene (12.05%) and limonene (10.32%), while those of the volatile oils of the flowers were sabinene (20.45%), limonene (19.33%), α-thujene (8.86%) and methyl salicylate (8.66%). Antibacterial activity was verified for the hexane fraction, while antifungal activity was observed for the aqueous fraction and concentrated aqueous tannin fraction and for vanoleic acid. These results may justify the popular use of *C. adamantium*.

Introduction

In Brazil, the Myrtaceae family comprises 23 genera and 976 species, of which fourteen genera and 211 species exist in the Cerrado (Sobral et al., 2010). The genus *Campomanesia* has 36 species, 31 of which are species of the Brazilian flora (Sobral et al., 2010; Govaerts et al., 2014).

Campomanesia adamantium (Cambess.) O. Berg is a native fruit species of the Cerrado biome, popularly known as "guabiroba-do-campo". This plant is a deciduous shrub ranging from 0.5 to 1.5 m in height (Lorenzi et al., 2008; Lima et al., 2011). The leaves of this plant have been used in the form of tea for their anti-inflammatory, anti-diarrhoeal, and urinary antiseptic activities and to treat stomach disorders (Piva, 2002; Lorenzi et al., 2008).

Terpenoids (Stefanello et al., 2008; Coutinho et al., 2008b, 2009), flavonoids (Ferreira et al., 2013) and chalcone derivatives (Pascoal et al., 2014) have been isolated and identified from *C. adamantium* leaves.

Several studies on *C. adamantium* have reported antioxidant activity of hexanic, chloroform and ethanolic extracts of the leaves and fruits of this plant (Vallilo et al., 2006; Ramos et al., 2008; Coutinho et al., 2008a, 2009; Alves et al., 2013). Antinociceptive and anti-inflammatory effects were observed with ethyl acetate fractions, aqueous fractions and flavonoids isolated from the hexanic fraction of the leaves (myricetin and myricitrin) (Ferreira et al., 2013); antimicrobial activity was observed with ethanolic extracts

* Corresponding author.
E-mail: jose_realino@ufg.br (J.R. Paula).

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of the leaves and fruits (Coutinho et al., 2009; Pavan et al., 2009; Cardoso et al., 2010a,b). Cardamonin isolated from ethanolic fractions of the leaves exhibited cytotoxicity in prostate cells (Pascoal et al., 2014).

There are no data in the literature about the antimicrobial activity of substances isolated from *C. adamantium* leaves. Due to the resistance of microorganisms to commonly used antimicrobials, the search for new substances with antimicrobial activity is necessary (Holetz et al., 2002; Canton and Onofre, 2010; Mulyaningsih et al., 2011).

The aims of this paper were to determine the chemical composition of volatile oils from the leaves and flowers of *C. adamantium*; evaluate the antimicrobial activity of these volatile oils and of ethanolic, hexanic, dichloromethane, ethyl acetate and aqueous extracts of the leaves against gram-positive and gram-negative bacteria and fungi; perform biomonitored fractioning of fractions that presented high antimicrobial activity; and isolate and identify substances and test their antimicrobial activity.

Material and methods

Plant material

Leaves and flowers of *Campomanesia adamantium* (Cambess.) O. Berg., Myrtaceae, were collected in Bela Vista, Goiás, Brazil (17° 02′ 01.1″ S; 48° 49′ 00.3″ W; at an altitude of 847 m). The plant material was identified by Prof. Dr. José Realino de Paula. A voucher specimen has been deposited at the Herbarium of Federal University of Goiás under the code number UFG-243832.

To obtain the crude ethanolic extract (CEE), the leaves were dried at 40 °C in a drying oven under forced ventilation. The plant material was ground in a Wiley knife mill. The powdered material was subjected to maceration using an ethanol:water solution (95:5, v/v) as the solvent mixture. A mechanical shaker was employed for 4 h to perform the maceration using a ratio of 1:4. The extract was filtered and evaporated under reduced pressure on a rotary evaporator at 40 °C (Ferri, 1996).

Fractionation of the CEE was conducted according to the methodology described by Ferri (1996). CEE was diluted in methanol:water (7:3, v/v), and successive liquid–liquid extractions were performed with hexane, dichloromethane and ethyl acetate. The solvents from each fraction were evaporated on a rotary evaporator under reduced pressure, and the aqueous fractions were lyophilized. Four fractions were obtained: hexanic (HF), dichloromethane (DF), ethyl acetate (AcF) and aqueous (AqF). The yields of the fractions were calculated according to the following equation:

$$\text{Yield}\ (\%) = \frac{(\text{fraction weight})}{\text{crude extract weight}} \times 100$$

ESI FT-ICR MS analysis

To identify the chemical compounds present in the CEE, HF, DF, AcF and AqF, an electrospray ionization source was used with Fourier-transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) at atmospheric pressure. Fractions were diluted in approximately 0.25 mg/ml in water:methanol (1:1) with 0.1% ammonium hydroxide for analysis in negative ion mode and 0.1% acetic acid for analysis in positive ion mode. The resulting solution was infused directly into the electrospray source (for ESI) at a flow rate of 5 ml/min. The mass spectrometer (model 9.4 T solariX, Bruker Daltonics, Bremen, Germany) was configured to operate in a range of *m/z* 150–2000. The general ESI analysis conditions were as follows: pressure, 3.0 bar; capillary voltage, 4.5 kV; and temperature of capillary ion transfer, 220 °C. ESI-FT-ICR MS spectra were acquired and processed using the Bruker Compass Data Analysis software (Bremen, Germany). MS data were processed, and the molecular formulas of the compounds were determined by measuring the *m/z* values. Structures for each compound were putatively assigned using the ChemSpider (www.chemspider.com) database.

Obtaining sub-fractions

HF (4 g) was fractionated by column chromatography (CC) with Vetec silica gel G60 0.05–0.2 mm (1:40) and eluted with hexane-ethyl acetate (5–100%), ethyl acetate-methanol (1:1) and methanol. Ninety 20-ml fractions were collected. After evaporation of the solvent, these fractions were grouped into eleven additional fractions (HF1–HF11) based on their chromatographic profiles by analytical thin layer chromatography (TLC) on silica gel G60 F254 (Vetec, Brazil) with mobile phases consisting of hexane-ethyl acetate mixtures (10–30%). The TLC analysis was based on the retention factors (R_f) of the spots observed under 254/365-nm light, visualized with vanillin/sulfuric acid solution. HF1 was analyzed by gas chromatography coupled with mass spectrometry (GC/MS). HF2 was sub-fractionated using silica gel CC (1:40) and eluted with hexane-dichloromethane (9:1 and 8:2), resulting in 33 fractions. These fractions were pooled into six fractions based on their chromatographic profiles (HF2/1 to HF2/6). The fractions HF2/3 and HF2/6 were analyzed by GC/MS, and fraction HF2/5 was analyzed by nuclear magnetic resonance spectroscopy (^{13}H and ^{13}C NMR). HF9 was sub-fractionated by silica gel CC (1:40) and eluted with hexane-ethyl acetate as the mobile phase (8:2 and 7:3), resulting in nineteen fractions, which were pooled into four fractions based on their chromatographic profiles (HF9/1 to HF9/6). The HF9/3 fraction was sub-fractionated by preparative thin layer chromatography (PTLC) and eluted with a mobile phase of hexane-ethyl acetate (7:3), resulting in three fractions: HF9/3/1, HF9/3/2, and HF9/3/3. The HF2 and HF9 samples were fractioned by silica gel CC (1:40), and the sub-fractions were pooled according to their chromatographic profiles. The new sub-fractions were further fractionated by CC, TLC and PTLC.

The results obtained from fractions HF1, HF2/3, HF2/6 and HF9/3/1/2/1 were analyzed by GC/MS.

Obtaining concentrated aqueous tannin fractions (CAqTF): extraction and purification of tannins

Powdered *C. adamantium* leaves (500 g) were subjected to extraction with acetone/water at a ratio of 1:1 on a mechanical shaker for 3 h. Subsequently, liquid–liquid extractions with ethyl ether and ethyl acetate were performed. Three fractions were obtained: ethyl ether and ethyl acetate fractions and a concentrated aqueous tannin fraction (CAqTF). The ethyl ether and ethyl acetate fractions were not used in the tests; only the CAqTF was used.

The CAqTF was lyophilized, and 12 g of this sample was subjected to CC using the polymeric vinyl gel Diaion® HP-20 (Sigma, USA) as an adsorbent (column size 28 × 4 cm). The eluents used were water, methanol:water (20–100%), and methanol. At the end of the elution, 88 20-ml fractions were obtained (AqF1–AqF88). These fractions were lyophilized and analyzed by TLC with a mobile phase consisting of acetone/toluene/formic acid (3:3:1) based on the retention factors (R_f) of the spots observed under UV light at 254/365 nm and by staining with $FeCl_3$/HCl solution (0.01 M). After analysing the TLC profile, fractions containing only one or two spots on the chromatographic plate after visualization were selected for analysis by high-performance liquid chromatography (HPLC). After HPLC analysis, the samples AqF80 and 88 exhibited peaks indicative of pure substances and were subjected to ^{1}H and ^{13}C NMR analysis.

Nuclear magnetic resonance (NMR) analysis was used to identify the individual substances in the HF and AqF. ^{1}H and

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