Antimicrobial Activity of the Extract and Isolated Compounds from *Baccharis dracunculifolia* D. C. (Asteraceae)

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Baccharis dracunculifolia D.C. (Asteraceae) is the most important plant source of the Brazilian green propolis. Since propolis is known for its antimicrobial activity, the aim of this work was to evaluate the antimicrobial activities of *B. dracunculifolia* and some of its isolated compounds. The results showed that the leaves extract of *B. dracunculifolia* (BdE) presents antifungal and antibacterial activities, especially against *Candida krusei* and *Cryptococcus neoformans*, for which the BdE showed IC_{50} values of 65 μg mL^{-1} and 40 μg mL^{-1}, respectively. In comparison to the BdE, it was observed that the green propolis extract (GPE) showed better antimicrobial activity, displaying an IC_{50} value of 9 μg mL^{-1} against *C. krusei*. Also, a phytochemical study of the BdE was carried out, affording the isolation of ursolic acid (**1**), 2α-hydroxy-ursolic acid (**2**), isosakuranetin (**3**), aromadendrin-4′-methylether (**4**), baccharin (**5**), viscidone (**6**), hautriwaic acid lactone (**7**), and the clerodane diterpene **8**. This is the first time that the presence of compounds **1**, **2**, and **8** in *B. dracunculifolia* has been reported. Among the isolated compounds, **1** and **2** showed antibacterial activity against methicillin-resistant *Staphylococcus aureus*, displaying IC_{50} values of 5 μg mL^{-1} and 3 μg mL^{-1}, respectively. **3** was active against *C. neoformans*, showing an IC_{50} value of 15 μg mL^{-1} and a MIC value of 40 μg mL^{-1}, while compounds **4**–**8** were inactive against all tested microorganisms. The results showed that the BdE, similar to the GPE, displays antimicrobial activity, which may be related to the effect of several compounds present in the crude extract.

Key words: Baccharis dracunculifolia, Brazilian Green Propolis, Antimicrobial Activity

Introduction

The genus *Baccharis* (Asteraceae) is a strictly American genus with approximately 400 species (Verdi *et al*., 2005). Several *Baccharis* species are used in South American folk medicine as an antiseptic, anti-inflammatory, and to treat both gastric ulcers and skin sores (Feresin *et al*., 2003; Verdi *et al*., 2005). *Baccharis dracunculifolia* D. C., popularly known as "alecrim do campo" and "vassoura", is used in folk medicine as anti-inflammatory and for the treatment of gastrointestinal diseases (Da Silva Filho *et al*., 2004). Besides its use in traditional medicine, *B. dracunculifolia* has been described as the most important plant source of the South Eastern Brazilian propolis, which due to its colour is called green propolis (Bankova *et al*., 2000; Park *et al*., 2004).

Propolis is a natural resinous substance collected by honeybees (*Apis mellifera*) from buds and exudates of plants to be used as a protective barrier in the beehive that displays many biological activities, such as antibacterial, antifungal, antiviral (Kujumgiev *et al*., 1999; Leitão *et al*., 2004), anti-ulcer (Barros *et al*., 2007), anti-inflammatory (Reis *et al*., 2000), and antioxidant (Simões *et al*., 2004) activities. Currently, because of its biological activities, Brazilian green propolis is extensively used in foods and beverages, especially in Brazil and Japan, aiming to improve health and to prevent several diseases (Bankova *et al*., 2000; Park *et al*., 2004). The biological properties of propolis are a consequence of plant-derived products, since its composition is originated from plant exudates and substances secreted by the bee metabolism (Mar-

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cucci *et al.*, 2001; Simões *et al.*, 2004). Although much research has been implemented on propolis, its composition can be extraordinarily variable, because of this, creating a problem for its medical use and quality control (Bankova *et al.*, 2000; Leitão *et al.*, 2004; Missima *et al.*, 2007). Thus, the investigation of both the chemical and biological properties of propolis plant sources, such as *B. dracunculifolia*, is important not only for academic interest, but also for the chemical and biological standardization of propolis raw material (Missima *et al.*, 2007; Bankova *et al.*, 2000).

B. dracunculifolia and Brazilian green propolis have been reported to display similar anticariogenic (Leitão *et al.*, 2004), anti-ulcer (Lemos *et al.*, 2007), and immunomodulatory (Missima *et al.*, 2007) activities. However, it is still unknown whether *B. dracunculifolia* displays the same antimicrobial activity reported for Brazilian green propolis.

On the basis of the botanical origin of Brazilian green propolis and its antimicrobial activity, the aim of this work was to evaluate the *in vitro* antibacterial and antifungal activities of the crude extract and some isolated compounds from *B. dracunculifolia*, which have not yet been described. Additionally, the antibacterial and antifungal activities of *B. dracunculifolia* crude extract and its isolated compounds were compared to the antimicrobial activity of the Brazilian green propolis extract.

Materials and Methods

Plant and propolis material

Leaves of *Baccharis dracunculifolia* D. C. (Asteraceae) were collected in Cajuru (São Paulo State, Brazil), in November 2001. The plant material was authenticated by Jimi N. Nakagima (Federal University of Uberlândia, Uberlândia, MG, Brazil), and a voucher specimen (SPFR 06143) stored in the herbarium of the Biology Department of the University of São Paulo, campus of Ribeirão Preto, SP, Brazil.

Green propolis was produced and collected from *Apis mellifera* hives, in the same field and period of *B. dracunculifolia* material collection.

Preparation of green propolis and B. dracunculifolia extracts

The leaf rinsed extract of *B. dracunculifolia* was obtained by immersing the air-dried leaves (658 g) in dichloromethane for 30 s at room temperature, affording 35 g of leaf rinse crude extract (BdE) after removal of the solvent under vacuum below 40 °C.

The crude green propolis (3 g) was kept in a freezer for 24 h and powdered in a blender. The furnished powder was submitted to exhaustive maceration, followed by filtration, using ethanol/H_2O (7 : 3 v/v) at room temperature. The filtered extract was concentrated under vacuum to furnish 1.9 g of the crude hydroalcoholic green propolis extract (GPE).

HPLC analysis of the green propolis extract

The GPE was dissolved in methanol (5.0 mg/mL) and filtered through a 0.45 μm filter before injecting 15 μL onto a HPLC system. A gradient starting with 0.8% acetic acid, 0.3% ammonium acetate, 5.0% methanol/water and 25% acetonitrile, and finishing with 100% of acetonitrile, over 60 min (flow rate 1.0 mL/min), was used to separate the major compounds. Veratraldehyde was used as an internal standard. The phenolic compounds were identified by comparison with authentic standards previously obtained (Simões *et al.*, 2004; Da Silva Filho *et al.*, 2004; Missima *et al.*, 2007; Lemos *et al.*, 2007).

Drugs and chemicals

Dichloromethane and acetonitrile were from Acros Co. (NJ, USA); ethyl acetate, hexane, and methanol were from Mallinckordt Co. (Xalostoc, Mexico); dimethyl sulfoxide (DMSO) and veratraldehyde were from Sigma-Aldrich Co. (St. Louis, MO, USA); Sabouraud Dextrose broth and Mueller-Hinton broth were from Difco; Alamar Blue was from BioSource International; ethanol was bought from a local distillery and purified by distillation. All the other chemicals employed in this work were of analytical grade and were purchased locally.

General procedures

NMR spectra were recorded on a Bruker ARX 400 spectrometer. Vacuum-liquid chromatography (VLC) was carried out with Silica gel 60 H, 100–200 mesh ASTM (Merck Co., Darmstadt, Germany), in glass columns with 5–10 cm i. d. Column chromatography (CC) was carried out with Silica gel, 230–400 mesh (Merck). Analytical and semi-preparative chromatographic separations were

carried out on a Shimadzu (Kyoto, Japan) HPLC equipment: an SCL-10A*vp* controller, three LC-10AD pumps, an SPDM10A*vp* diode array detector and a Class-VP version 5.02 software controller, a Shimadzu UV-DAD detector (the channel was set at 281 nm), and Shimadzu columns (ODS column, 250×4.6 mm, 5 μm for analytical analysis; ODS, 250×20 mm, 15 μm for semi-preparative separations).

Isolation of the compounds

The crude BdE extract (35 g) was dissolved in methanol/H_2O (7:3) and submitted to sequential partition with hexane and dichloromethane, giving 2.6 g and 22.1 g of the crude fractions, respectively. The hexane fraction (BdE-h, 2.6 g) was chromatographed over silica gel using a VLC system and hexane/ethyl acetate mixtures in increasing proportions as eluent, giving four fractions. Fraction IV (0.62 g) was submitted to CC over silica gel, using hexane/ethyl acetate mixtures in increasing proportions as eluent, followed by preparative TLC (hexane/ethyl acetate 75:25), affording compounds **1** (80 mg), and **2** (15 mg). The dichloromethane fraction (BdE-d, 22.0 g) was chromatographed over silica gel using a VLC system and hexane/ethyl acetate mixtures in increasing proportions as eluent, giving six fractions. Fraction II (0.28 g) was washed with cold methanol to afford **1** (250 mg). Fractions III (2.5 g) and IV (6.5 g) were chromatographed over silica gel using a VLC system and hexane/ethyl acetate mixtures in increasing proportions as eluent. The resulting sub-fractions III.2 and IV.2 were submitted to semi-preparative reverse-phase HPLC purification using methanol/H_2O (75:25) as mobile phase. Fraction III.2 furnished compounds **3** (45 mg), **4** (40 mg), **5** (30 mg), and **6** (15 mg). Fraction IV.2 furnished compounds **7** (20 mg) and **8** (15 mg).

Antimicrobial assays

Activity against a panel of microorganisms was evaluated *in vitro*. All organisms were obtained from the American Type Culture Collection (Manassas, VA, USA) and include *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods (Muhammad *et al*., 2003). Susceptibility testing of *M. intracellulare* was done using the modified Alamar Blue procedure of Franzblau *et al*. (1998). Samples (dissolved in DMSO) were serially diluted by using 0.9% saline and transferred in duplicate to 96-well microplates. Microbial inocula were prepared after comparison of the absorbance (at 630 nm) of cell suspensions to the 0.5 McFarland standard and dilution of the suspensions in broth [Sabouraud Dextrose and cation-adjusted Mueller-Hinton broth (Difco) for the fungi and bacteria, respectively, and 5% Alamar Blue (BioSource International) in Middlebrook 7H9 broth with oleic acid-albumin-dextrose-catalase enrichment for *M. intracellulare*] to afford the recommended inocula. Microbial inocula were added to the samples to achieve a final volume of 200 μL and final sample concentrations starting with 200 μg mL^{-1} for crude extracts and 50 μg mL^{-1} for pure compounds. Growth, solvent, and media controls were included on each test plate. The plates were read either at 630 nm or at excitation and emission wavelengths of 544 and 590 nm (for *M. intracellulare*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies) prior to and after incubation. Percent growth was calculated and plotted with the concentration tested to afford the concentration that inhibited 50% of growth (IC_{50}). Minimum inhibitory concentration (MIC) was defined as the lowest test concentration that allows no detectable growth.

Results

Phytochemical study

The phytochemical study of the BdE led to the isolation of eight compounds. The chemical structures of all isolated compounds (Fig. 1) were established by UV-vis, ^{1}H and ^{13}C NMR data analysis, in comparison with the literature, as follows: ursolic acid (**1**) (Cunha *et al*., 2006a), 2α-hydroxyursolic acid (**2**) (Mahato and Kundu, 1994), isosakuranetin (**3**) (Bohlmann *et al*., 1981), aromadendrin-4′-methylether (**4**) (Banskota *et al*., 1998), baccharin (**5**) (Banskota *et al*., 1998), viscidone (**6**) (Le-Van and Thi, 1981), hautriwaic acid lactone (**7**) (Bohlmann *et al*., 1985), and the clerodane diterpene **8** (Saad *et al*., 1985).

HPLC analysis of the propolis extract

The HPLC analysis of the GPE (Fig. 2) allowed the identification of the following compounds: ar-

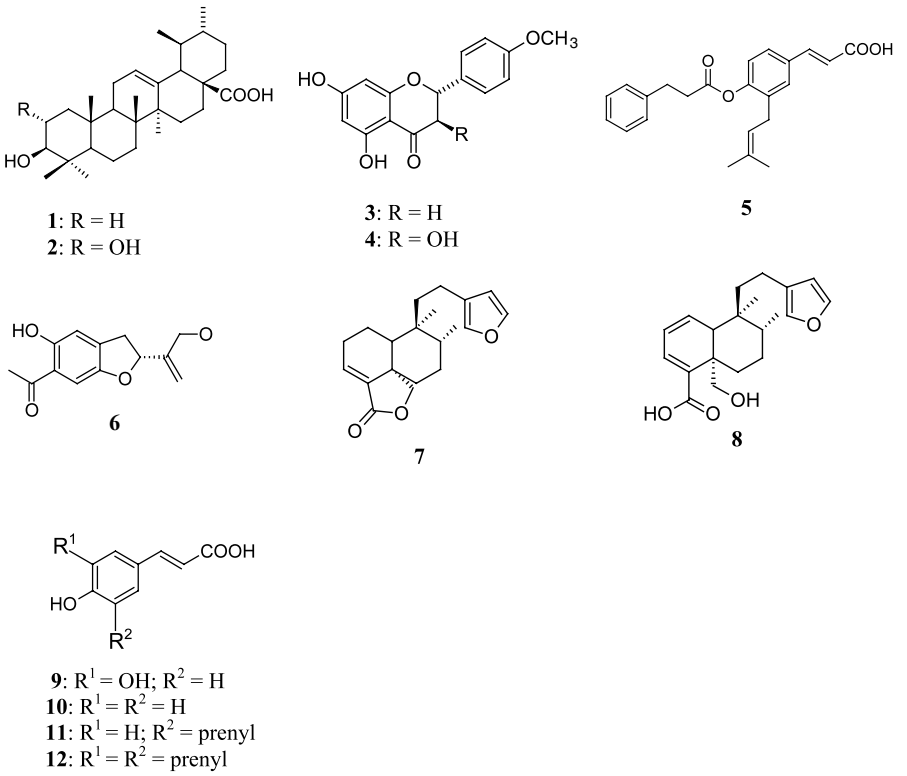

Fig. 1. Chemical structures of both isolated and identified compounds from BdE and GPE: ursolic acid (**1**); 2α-hydroxy-ursolic acid (**2**); isosakuranetin (**3**); aromadendrin-4′-methylether (**4**); baccharin (**5**); viscidone (**6**); hautriwaic acid lactone (**7**); clerodane diterpene (**8**); caffeic acid (**9**); *p*-coumaric acid (**10**); drupanin (**11**); artepillin C (**12**).

Table I. *In vitro* antifungal and antibacterial activities of crude extracts and isolated compounds from *B. dracunculifolia*.

	Antimicrobial activity IC_{50}[a]/MIC[b] [μg mL^{-1}]					
	C. albicans	*C. glabrata*	*C. krusei*	*C. neoformans*	MRSA[c]	*M. intracellulare*
Crude extracts						
BdE	150	100	65	40	100	–[d]
GPE	60	10	9	60	40	–[d]
Compounds						
1	–[d]	–[d]	–[d]	–[d]	5/10	15/20
2	–[d]	–[d]	–[d]	–[d]	3/7	–[d]
3	–[d]	–[d]	–[d]	15/40	–[d]	–[d]
4	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]
5	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]
6	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]
7	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]
8	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]
Amphotericin B	0.10/0.31	0.10/0.31	1.50/2.50	0.50/1.25	–	–
Ciprofloxacin	–	–	–	–	0.10/0.63	0.30/0.63

[a] 50% growth inhibitory concentration.
[b] Minimum inhibitory concentration.
[c] Methicillin-resistant *Staphylococcus aureus.*
[d] Not active at the highest tested concentration of 200 μg mL^{-1} for crude extracts and 50 μg mL^{-1} for pure compounds.

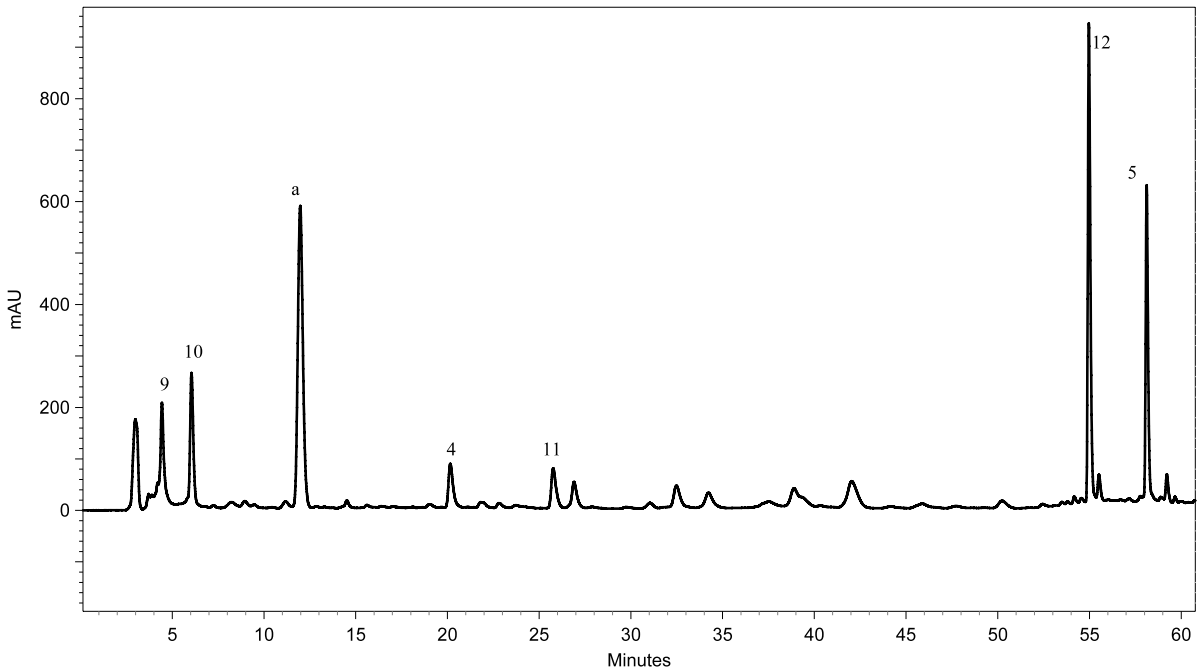

Fig. 2. HPLC profile of GPE. a, Internal standard (veratraldehyde); 4, aromadendrin-4′-methylether; 5, baccharin; 9, caffeic acid; 10, *p*-coumaric acid; 11, drupanin; 12, artepillin C.

omadendrin-4′-methylether (**4**), baccharin (**5**), caffeic acid (**9**), *p*-coumaric acid (**10**), drupanin (**11**), and artepillin C (**12**).

Antimicrobial activity

The antibacterial and antifungal activities are summarized in Table I. Both the BdE and GPE extracts displayed antifungal and antibacterial activities, especially against *C. krusei*, for which the GPE displayed an IC_{50} value of 9 μg mL^{-1}, while the BdE showed an IC_{50} value of 65 μg mL^{-1}. Among the isolated compounds, the triterpenes **1** and **2** showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), displaying IC_{50} values of 5 μg mL^{-1} and 3 μg mL^{-1}, respectively. **3** was active against *C. neoformans*, showing an IC_{50} value of 15 μg mL^{-1} and a MIC value of 40 μg mL^{-1}, while the related dihydroflavonoid **4** was inactive. Compounds **5**–**8** were inactive against all tested microorganisms in the antimicrobial assay.

Discussion

A great variety of chemical compounds have been identified from Brazilian propolis, such as flavonoids, diterpenes, and mainly prenylated *p*-coumaric acid derivatives, which are particularly found in green propolis and South American *Baccharis* species (Park *et al*., 2004; Kumazawa *et al*., 2003; Bankova *et al*., 2000). It is well known that the chemical composition of propolis can change depending on several factors, including the site of collection and the plant sources used in propolis production (Bankova *et al*., 2000). Such variations make it difficult to standardize the raw material and commercialize propolis products for medicinal purposes. For this reason, it is suggested that if *B. dracunculifolia* and Brazilian green propolis present comparable biological activities, *B. dracunculifolia* extracts could be successfully incorporated into pharmaceutical products to be used in foods and beverages.

Previous phytochemical studies of the aerial parts of *B. dracunculifolia* reported the isolation of *p*-coumaric acid derivatives, flavonoids, diterpenes, and triterpenes (Bohlmann *et al*., 1981; Nagatani *et al*., 2002; Da Silva Filho *et al*., 2004; Missima *et al*., 2007). Ursolic acid (**1**) and related pentacyclic triterpenes are compounds widely found in natural plants (Liu, 2005), and they possess several

biological activities, such as trypanocidal (Cunha *et al.*, 2006a), anti-inflammatory (Cunha *et al.*, 2006b), and antimutagenic (Resende *et al.*, 2006) activities. Pentacyclic triterpenes have been isolated from some Baccharis species (Verdi *et al.*, 2005), but neither ursolic acid (**1**) nor 2α-hydroxy-ursolic acid (**2**) were previously isolated from *B. dracunculifolia*. Also, both compounds **1** and **2** have not been previously reported from Brazilian green propolis. Regarding the antimicrobial assay, ursolic acid (**1**) and 2α-hydroxy-ursolic acid (**2**) showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Table I). Nevertheless, compounds **1** and **2** were inactive in the antifungal assay. According to the literature, **1** was identified as one of the active components in plants related to growth inhibition of Gram-negative and Gram-positive organisms (Liu, 2005).

Additionally, several flavonoids have been isolated from *Baccharis* species and propolis samples (Bohlmann *et al.*, 1985). Isosakuranetin (**3**) and aromadendrin-4′-methylether (**4**) are major flavonoids found in both green propolis and *B. dracunculifolia* (Park *et al.*, 2004). **3** was active only against *C. neoformans*, while **4** was inactive. Antimicrobial activity of flavonoids against different microorganisms has been reported (Cushnie and Lamb, 2005).

Moreover, baccharin (**5**) and viscidone (**6**) have frequently been isolated from *Baccharis* species (Bohlmann *et al.*, 1981, 1985) and propolis (Banskota *et al.*, 2001). However, it was observed that compounds **5** and **6** were inactive in the antimicrobial assay. The same antimicrobial profile may be observed for hautriwaic acid lactone (**7**) and the clerodane furanoditerpene **8**, which are known for their antifeedant activity (Cifuente *et al.*, 2002). Clerodane diterpenes have been reported from *Baccharis* species (Bohlmann *et al.*, 1981, 1985), but they are rare in propolis samples.

On the other hand, the HPLC analysis of the GPE showed as major compounds the prenylated *p*-coumaric acid derivatives baccharin (**5**) and artepillin C (**12**) (Fig. 2). Prenylated *p*-coumaric acid derivatives have been reported to possess significant antifungal and antibacterial activities (Marcucci *et al.*, 2001; Feresin *et al.*, 2003). Aga *et al.* (1994) suggested that the antibacterial activity of this class of compounds might be increased by the increasing number of prenyl residues attached. However, the mechanism of the antimicrobial activity of propolis is complex, and could be attributed to a synergism between phenolic and other compounds in the resin (Marcucci *et al.*, 2001).

In conclusion, the undertaken study provided biological evidence that *B. dracunculifolia*, like Brazilian green propolis, possesses antimicrobial activity. Since the BdE was more active towards most of the evaluated microorganisms than its isolated compounds, it is suggested that the antimicrobial activity of the BdE may be due to the effect of several compounds present in the crude extract. Finally, since *B. dracunculifolia* is the main botanical source of the Brazilian green propolis, further studies are in progress to disclose other important biological effects of this medicinal plant.

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