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Antimicrobial Activity of the Crude Extracts and Fractions of Three *Baccharis* Species

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Abstract

The antimicrobial activities of crude extracts and fractions from three *Baccharis* L. species were tested against *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8738), *Pseudomonas aeruginosa* (ATCC 9027), and *Candida albicans* (ATCC 10231) using the microdilution plate method. The results showed that the crude extract from female *B. burchellii* Baker had moderate activity against *S. aureus* (minimum inhibitory concentration [MIC], 0.9 $mg.mL^{-1}$). Among the fractions obtained from this extract, the dichloromethane fraction showed the highest activity against *S. aureus* (MIC, 0.4 $mg.mL^{-1}$). The ethyl acetate fractions from female *B. burchellii* (MIC, 0.6 $mg.mL^{-1}$ and 1.2 $mg.mL^{-1}$, respectively) and *B. aracatubaensis* Malag (MIC, 1.1 $mg.mL^{-1}$ for both) were moderately effective against *S. aureus* and *P. aeruginosa*. The extracts from *B. organensis* Baker showed no significant activity against any organism tested. None of the extracts from *Baccharis* species showed any activity against *C. albicans*. In addition, the chemical investigation of the dichloromethane and ethyl acetate fractions from female *B. burchellii* was carried out, resulting in the identification of *trans*-ferulic acid, ethyl caffeoate, naringenin, and 7-hydroxy-benzaldehyde compounds. These phenolic compounds were found in other species of *Baccharis* and have been shown to possess antimicrobial activity. The results obtained in this work with respect to *B. burchellii* indicate that this species is a promising source of compounds with antimicrobial activities.

Keywords: Asteraceae; Baccharis; Antimicrobial; Phenolic compounds

Introduction

The *Baccharis* L. genus consists of about 500 species distributed exclusively in the Americas, found in the southern United States to southern Argentina and Chile [1,2]. There are about 178 described species in Brazil, mainly located in the southeastern and southern regions [3]. Species of this genus are well known for their use in folk medicine, especially in South America. These plants are used for the treatment of various diseases such as ulcers, gastritis, inflammation, diabetes, and skin infections [4-6]. Numerous biological activities have been attributed to essential oils, extracts, and compounds isolated from the *Baccharis* genus [7-9]. Campos et al. noted that several species of this genus have shown anti-inflammatory, anti-diabetic, anti-ulcer, or anti-microbial activities. However, there are very few reports on the antimicrobial activities of the genus *Baccharis* [10]. In this context, the aim of this study was to evaluate the antimicrobial activities of crude extracts (male and female specimens) and fractions (female specimens) from *Baccharis organensis* Baker, *Baccharis burchellii* Baker, and *Baccharis aracatubaensis* Malag, as well as to perform a chemical analysis of fractions obtained from female *B. burchellii*.

Materials and Methods

Chemicals and reagents

Dimethyl sulfoxide (DMSO), methanol (MeOH), ethyl acetate (EtOAc), and dichloromethane (CH_2Cl_2) were purchased from Tedia (Fairfield, OH, USA). Deuterium solvents ($CDCl_3$, DMSO-d_6, and CD_3OD) (≥ 99.9% D) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Mass spectrometry (MS)-grade methanol and acetonitrile (ACN) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Distilled and deionized water was obtained using a Millipore system (Millipore Milli-RO plus, MA, USA). Tryptic soy broth (TSB), Mueller-Hinton broth (MHB), sabouraud dextrose broth (SDB), tryptic soy agar (TSA), sabouraud agar, chloramphenicol, 2,3,5-triphenyltetrazolium chloride (TTC), and ketoconazole were purchased from Millipore-Sigma (Darmstadt, Germany).

Chemical analysis

NMR data were acquired at 303 K in $CDCl_3$ for all compounds by using a Bruker AVANCE 600 NMR spectrometer operating at 14.1 Tesla, and ^1H and ^{13}C spectra were recorded at 600.13 and 150.61 MHz, respectively. The spectrometer was equipped with a 5-mm quadrinuclear inverse detection probe with z-gradient. One-bond and long-range ^1H-^{13}C correlations from the HSQC and HMBC NMR experiments were obtained with average coupling constants $^1J_{(H,C)}$ and $^{LR}J_{(H,C)}$ optimized for 140 and 8 Hz, respectively. The ^1H and ^{13}C NMR chemical shifts are given in ppm relative to the tetramethylsilane (TMS) signal as the internal reference, and the coupling constants (J) in Hz. Low-resolution electrospray ionization mass spectrometry (LRESIMS) experiments were performed on a Thermo LTQ XL Ion Trap, equipped with an ESI source. Silica gel 60 (70-230 mesh) and sephadex LH-20 (25-100 μm) were used for column chromatography (CC), and precoated silica gel plates (60 F_{254} Merck, 0.2 mm, aluminum) were used for analytical thin layer chromatography (TLC). Gel plates were sprayed with p-anisaldehyde and heated, followed by exposure to $UV_{254/366}$ light for visualization of compounds.

Plant material collection

Botanical materials of male and female specimens of *Baccharis* were collected separately and randomly along a transect within the same population in November 2013 in the "Morro do Canal", Municipality

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of Piraquara, Paraná State, Brazil. The *B. aracatubaensis* (leaves) and *B. organensis* (leaves) samples were collected at [25º30'52-48'' S/48º59'10-41'' O] and [25º30'52-39'' S/48º59'10-78'' O], respectively, at an elevation of 1200-1300 m. The *B. burchellii* (cladodes) samples were collected in the proximity of one river in [25º31'11-54'' S/49º00'21-17'' O] at an elevation of 906 m. The species were identified by Osmar dos Santos Ribas, Dr. Gustavo Heiden, and Dr. Angelo Alberto Schneider. The voucher specimens were deposited in the Botanical Museum of Curitiba (MBM), under the registration numbers: (MBM-286268/MBM-286267), (MBM-386275/MBM-386266), and (MBM-386257/MBM-386256), respectively.

The access to the botanical material was authorized and licensed by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Conselho de Gestão do Patrimônio Genético (CGEN/MMA) and registered as Nº 010304/2013-4.

Collection and purification of the extracts

The air-dried botanical materials from the male and female specimens of *B. aracatubaensis* (0.986 kg and 1.0 kg, respectively), *B. burchellii* (1.1 kg and 1.2 kg, respectively), and *B. organensis* (0.490 kg and 0.560 kg, respectively) were extracted successively with a solution of ethanol:water (90:10, v/v), at room temperature. The solvent was removed from the extracts under reduced pressure to obtain the crude extracts Ba-M (129.1 g) and Ba-F (121.6 g), Bb-M (211.4 g) and Bb-F (230.4 g), and Bo-M (158.2 g) and Bo-F (184.7 g), respectively. The crude extracts from all the three female species were defatted with n-hexane; then, the crude extracts Bb-F and Bo-F were subjected to liquid-liquid partitioning with the solvents: CH_2Cl_2 (3 × 500 mL) to yield Bb-D (6.1 g) and Bo-D (14.5 g) fractions; EtOAc (3 × 500 mL) to yield Bb-Ae (26.7 g) and Bo-Ae (9.6 g) fractions; and remaining aqueous to yield Bb-Aq (30.5 g) and Bo-Aq (18.9 g) fractions, respectively. The crude extract of *B. aracatubaensis* was subjected to liquid-liquid partitioning with the solvents EtOAc (3 × 500 mL) to yield Ba-Ae (6.2 g) and remaining aqueous residue to yield Ba-Aq (26.2 g) fractions.

Part of the Bb-Ae fraction (4.5 g) was subjected to silica gel CC and was eluted with increasing concentrations of CH_2Cl_2 in n-hexane (100:0 to 10:90, v/v), followed by EtOAc in CH_2Cl_2 (100:0 to 30:70, v/v), and MeOH in EtOAc (100:0 to 70:30, v/v), affording 181 sub-fractions (30 mL each) that were pooled into 10 groups according to TLC analysis. Groups 2 (48.8 mg), 5 (131.8 mg), and 6 (171.9 mg) resulted in compounds 1, 2, and 3, respectively. Part of the Bb-D fraction (3.2 g) was subjected to silica gel CC and was eluted with increasing concentrations of CH_2Cl_2 in n-hexane (100:0 to 10:90, v/v), followed by EtOAc in CH_2Cl_2 (100:0 to 30:70, v/v), and MeOH in EtOAc (100:0 to 70:30, v/v), affording 66 sub-fractions (30 mL each) that were pooled into 6 groups according to TLC analysis. Group 2 (639.5 mg) resulted in compounds **2** and **4**, and from of group 3 (191.3 mg) resulted in compounds **2** and **3**.

Trans-Ferulic acid (1): $C_{10}H_{10}O_4$ - ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.03 (1H, *d, J* = 1.9 Hz, H-2), 7.07 (1H, *dd, J* = 1.9, 8.2 Hz, H-6), 6.91 (1H, *d, J* = 8.2 Hz, H-5), 6.28 (1H, *d, J* = 15.9 Hz, H-8), 7.60 (1H, *d, J* = 15.9 Hz, H-7), 3.92 (3H, *s,* OCH_3); ^{13}C-NMR (600 MHz, $CDCl_3$) δ 167.1 (C-9), 148.9 (C-3), 146.7 (C-4), 127.4 (C-1), 123.0 (C-6), 114.7 (C-5), 115.8 (C-8), 109.1 (C-2), 144.6 (C-7), 56.2 (OCH_3); LRESIMS *m/z* 195 $[M+H]^+$, 177 $[M - H_2O]^+$ (100%).

Caffeate ethyl (2): $C_{11}H_{12}O_4$ - ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.87 (1H, *d, J* = 2.1 Hz, H-2), 7.00 (1H, *dd, J* = 2.1, 8.1 Hz, H-6), 7.07 (1H, *d, J* = 8.1 Hz, H-5), 6.25 (1H, *d, J* = 15.9 Hz, H-8), 7.56 (1H, *d, J* = 15.9 Hz, H-7), 4.25 (2H, *q, J* = 7.1 Hz, H-12), 1.33 (3H, *t, J* = 7.1 Hz, H-11). ^{13}C NMR (600 MHz, $CDCl_3$) δ 167.4 (C-9), 146.3 (C-3), 144.9 (C-4), 127.4 (C-1), 122.4 (C-6), 114.6 (C-5), 144.4 (C-8), 116.2 (C-7), 115.6 (C-2), 14.1 (C-12), 60.7 (C-11); LRESIMS *m/z* 207 $[M - H]^-$, 179 $[M - C_2H_5]^-$ (100%).

Naringenin (3): $C_{15}H_{12}O_5$ - ^{1}H NMR (600 MHz, $CDCl_3$) δ 12.03 (1H, *s*, H-5), 7.32 (2H, *d, J* = 8.5, H-2' H-6'), 6.88 (2H, *d, J* = 8.5, H-5' H-3'), 5.98 (1H, *d*, *J* = 2.1, H-6), 6.00 (1H, *d*, *J* = 2.1, H-8), 5.35 (1H, *dd*, *J* = 13.1, 3.0, H-2), 3.08 (1H, *dd*, *J* = 17.2, 13.1, H-3α), 2.78 (1H, *dd*, *J* = 17.2, 3.0, H-3β); ^{13}C-NMR 196.0 (C-4), 163.8 (C-5), 103.4 (C-9), 156.0 (C-4'), 127.9 (C-2' C-6'), 115.1 (C3' C-5'), 179.8 (C-10), 95.4 (C-6), 96.9 (C-8), 79.0 (C-2), 43.4 (C-3); LRESIMS *m/z*: 271 $[M - H]^-$, 177 $[M - C_6H_6O]^-$ (24%), 151 $[M - C_7H_6O_2]^-$ (100%).

7-Hidroxy-benzaldehyde (4): $C_7H_6O_2$ - ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.76 (2H, *d, J* = 8.5, H-2, H-6), 6.93 (2H, *d, J* = 8.5, H-3, H-5), 9.76 (1H, *s*). ^{13}C NMR (600 MHz, DMSO-d_6) 115.8 (C-3, C-5), 128.3 (C-1), 132.0 (C-2, C-6), 163.2 (C-4), 191.0 (CHO). LRESIMS *m/z*: 121 $[M - H]^-$, 106 $[M - H_2O]^-$ (24%), 93 $[M - CHO]^-$ (100%), 77 $[M - H_2O - CHO]^-$ (22%).

Antimicrobial assay: The antimicrobial assays of the crude extracts (male and female specimens) and fractions (female specimens) from *Baccharis organensis*, *B. burchellii*, and *B. aracatubaensis* were performed using Clinical and Laboratory Standards Institute (CSLI) microdilution method [11]. The samples were tested against *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8738), *Pseudomonas aeruginosa* (ATCC 9027), and *Candida albicans* (ATCC 10231). The microbial suspensions used for inoculation were prepared at 10^5 CFU (colony forming unit/ml) by diluting fresh cultures at McFarland 0.5 density. Positive controls used were 100 $\mu g.mL^{-1}$ chloramphenicol for bacteria and 500 $\mu g.mL^{-1}$ ketoconazole for yeast. The crude extracts and the CH_2Cl_2 fractions were solubilized in 20% MeOH and 5% DMSO, and the EtOAc and aqueous fractions were solubilized in water.

In this assay, the crude extracts were used at concentrations between 0.78 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$, and the fractions were used at concentrations between 0.39 $\mu g.mL^{-1}$ and 50 $\mu g.mL^{-1}$. In each well of the microplate, was added 100 µL MHB for bacterial strains or 100 µL of SDB for yeast strain. In the first well, was added 100 µL of extracts or essential oils, and then performed serial dilutions (1:1, v/v), followed by addition of 10 µL of the inoculum into each well, and incubated at 35 °C for 20 h. After the incubation period, was added 20 µL of 0.125% TTC solution to all wells of the plates, followed by two hours of incubation. The absorbance was measured using a spectrophotometer at 540 nm. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the extract showing no visible bacterial growth after the incubation period.

Results and Discussion

Identification of the compounds

The chromatographic fractionation was achieved only for the fractions from female *B. burchellii* (Bb-Ae and Bb-D), resulting in the identification of the four compounds by LRESIMS and 1D and 2D NMR and upon comparison with previous literature. In the analysis of the Bb-Ae fraction from group 2 was identified, *trans*-ferulic acid (**1**) [12]; from group 5 resulted in isolation of caffeoate ethyl (**2**) [13]; from group 6, a mixture of caffeoate ethyl (**2**) and naringinin (**3**) [14]. In the analysis of the Bb-D fraction was identified from group 2, a mixture of caffeoate ethyl (**2**) and 7-hydroxy-benzaldehyde (**4**) [15]; from group 3 was identified caffeoate ethyl (**2**) and naringinin (**3**).

All compounds have been identified for the first time from the female cladodes of *B. burchellii* and are commonly found in this genus.

Antimicrobial activity

The antimicrobial activities of the samples from *B. organensis*, *B. burchellii*, and *B. aracatubaensis* were evaluated according to the microdilution method described by CLSI [11]. According to the results summarized in Table 1, the crude extract from female *B. burchellii* showed a MIC of 0.9 mg.mL^{-1} against *S. aureus,* which was the highest activity among all the crude extracts analyzed. This extract was fractionated and the dichloromethane fraction (Bb-D) showed the highest antimicrobial activity against *S. aureus*, with a MIC of 0.4 mg.mL^{-1} [16]. Caffeoate ethyl, naringenin, and 7-hydroxy-benzaldehyde compounds were identified upon chemical analysis of this fraction. Furthermore, the ethyl acetate fractions from *B. burchellii* (Bb-Ae) and *B. aracatubaensis* showed moderate activity, with MIC values ranging between 0.6 and 1.2 mg.mL^{-1}. Ethyl caffeate, naringenin, and *trans*-Ferulic acid compounds were identified in the Bb-Ae fraction. Campos et al. had reported that the extracts and/or fractions from this genus are constituted mainly of phenolic compounds such as flavonoids, phenolic acids, and terpenes, and these possess antimicrobial activity [10], corroborating with the results obtained in this work. According to a survey conducted by Coppo and Marchese, the antibacterial activity of polyphenols can be attributed mainly to flavonols, flavones, isoflavones, flavanones, and flavan-3-ol [17]. Among the compounds tested against *S. aureus*, naringenin demonstrated strong antimicrobial activity [18-20]. Rangel observed antimicrobial activity of the extracts from *Baccharis nitida* against *S. aureus* strains [21]. Other compound classes, such as diterpenes, identified from *B. dracunculifolia*, *B. grisebachii*, *B. trimera*, *B. incarum*, and *B. dentata*, also showed activity against *S. aureus* strains [22-26].

In tests conducted using samples from *Baccharis* against *Pseudomonas aeruginosa*, MIC between 1.1 and 26.5 mg.mL^{-1} was obtained (Table 1). The fractions that showed moderate activities were Ba-Ae (MIC, 1.1 mg.mL^{-1}) and Bb-Ae (MIC, 1.2 mg.mL^{-1}), which were from *B. aracatubaensis* and *B. burchellii*, respectively [16]. Previous studies have reported antimicrobial activities against *P. aeruginosa* in other species of *Baccharis* such as *B. dracunculifolia*, *B. articulata* [27,28], and *B. nitida* [21]. In the plant kingdom, phenolic compounds are involved in the plant defense; and since they are synthesized in response to microbial infections [29], they can also be effective antimicrobials against a wide variety of microorganisms [30,31].

Conclusion

The crude extracts and fractions from *Baccharis aracatubaensis*, *B. burchellii*, and *B. organensis* showed significant antibacterial activity against tested strains. The highest activity against *S. aureus* was exhibited by the dichloromethane fraction from female *B. burchellii*, and moderate activity was observed in the crude extract from its male specimens and the ethyl acetate fraction from its female specimens. The ethyl acetate fractions from female *B. burchellii* and *B. aracatubaensis* showed moderate activity against *P. aeruginosa*. Extracts from *B. organensis* showed no significant activity against any organism tested. Neither the extracts nor fractions from *B. organensis*, *B. aracatubaensis*, or *B. burchellii* showed antifungal activity. Phenolic derivate compounds identified in the dichloromethane and ethyl acetate fractions from *B. burchellii* were the *trans*-ferulic, ethyl caffeate, naringinin, and 7-hydroxy-benzaldehyde compounds. These phenolic compounds were found in other species of the *Baccharis* and have been shown to possess antimicrobial activity. In this context, the results obtained in this work, with respect to *B. burchellii*, indicate that this species is a promising source of compounds with antimicrobial activity.

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Species	Extract and Fraction	Antimicrobial Activity (MIC mg.mL^{-1})			
		S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*
Baccharis aracatubaensis	Ba-M	8.0	16.0	8.0	---
	Ba-F	9.8	19.7	19.7	---
	Ba-Ae	1.1	4.5	1.1	---
	Ba-Aq	13.2	26.5	26.5	---
Baccharis burchellii	Bb-M	4.0	14.8	3.7	---
	Bb-F	0.9	8.1	4.0	---
	Bb-D	0.4	2.9	2.9	---
	Bb-Ae	0.6	4.9	1.2	---
	Bb-Aq	3.2	26.0	6.5	---
Baccharis organensis	Bo-M	3.6	14.4	14.4	---
	Bo-F	4.0	16.0	16.0	---
	Bo-D	2.6	10.4	5.2	---
	Bo-Ae	5.6	11.7	5.6	---
	Bo-Aq	6.6	26.4	26.4	---

Ba-M and Ba-F: crude extract from *B. aracatubaensis* male and female, respectively; Ba-Ae and Ba-Aq: fractions ethyl acetate and aqueous from *B. aracatubaensis* female, respectively; Bb-M and Bb-F: crude extract from *B. burchellii* male and female, respectively; Bb-D, Bb-Ae and Bb-Aq: fractions dichloromethane, ethyl acetate and aqueous from *B. burchellii* female, respectively; Bo-M and Bo-F: crude extract from *B. organensis* male and female, respectively; Bo-D, Bo-Ae and Bo-Aq: fractions dichloromethane, ethyl acetate and aqueous from *B. organensis* female, respectively; ---: Not activity; Positive control for antifungal activity: ketoconazole (500 µg.mL^{-1}); Positive control for antibacterial activity: chloramphenicol (100 µg.mL^{-1}); Negative control: Methanol/DMSO/H_2O (20:5:75, v/v) or H_2O.

Table 1: Antimicrobial activity of crude extracts and fractions from *B. aracatubaensis*, *B. burchellii* and *B. organensis*.

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