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Antimicrobial activity and chemical composition of *Brunfelsia uniflora* flower oleoresin extracted by supercritical carbon dioxide

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ABSTRACT. *Brunfelsia* genus is traditionally utilized in popular medicine due to its antibacterial and antifungal properties to name but a few. However, studies on the antimicrobial activity of *Brunfelsia uniflora* flower oleoresin have not been found yet. This study aimed to evaluate the chemical composition and antimicrobial activity of *B. uniflora* flower oleoresin obtained by supercritical carbon dioxide. Oleoresin from the plant dried flowers was obtained by carbon dioxide, and the chemical composition was analyzed by gas chromatographic-mass spectrometry. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of this oleoresin for seven bacteria and eight fungi

were determined using 96-well microtiter plates. The oleoresin MBC for *Bacillus cereus*, *Enterobacter cloacae*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Staphylococcus aureus* ranged from 0.01 to 0.08 mg/mL, whereas the controls streptomycin and ampicillin varied from 0.1 and 0.5 mg/mL. The oleoresin MFC for *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum* var. *cyclopium*, and *Trichoderma viride* varied from 0.01 to 0.08 mg/mL, whereas the controls bifonazole and ketoconazole ranged from 0.2 to 3.5 mg/mL. The oleoresin obtained by supercritical carbon dioxide presented bacteriostatic, bactericidal, fungistatic, and fungicidal activities that were higher than the positive controls streptomycin, ampicillin, bifonazole, and ketoconazole. The high antimicrobial activity was related to the high content of (*E*, *E*)-geranyllinalool that composes 21.0% of the oleoresin and a possible synergic action with fatty acid esters that made up 50.5% of the oleoresin. The oleoresin antimicrobial activity against common multiresistant bacteria in severe infectious processes as *P. aeruginosa* or against toxin-producing fungi such as *P. ochrochloron* or fungi that are difficult to control such as *T. viride* suggests the development of promising applications of this product in the food, farming, livestock, and pharmaceutical industry.

Key words: Minimal bacteriostatic concentration; (2*E*, 6*Z*)-farnesal; Minimal fungistatic concentration; (*E*)-nerolidol; (*E*, *E*)-geranyllinalool

INTRODUCTION

Brunfelsia genus comprises 50 species popularly known as lady-of-the-night, manacá, manacá-açu, jeretaca, cangambá, and Paraguayan jasmine. The species of this genus are appreciated for the exuberant effect of their chromatic variation and also for the fragrance of their flowers (Filipowicz and Renner, 2012). *Brunfelsia uniflora* (Pohl.) D. Don, synonym of *Brunfelsia hopeana* (Hook.) Benth, from the Solanaceae family, is native of the Brazilian Atlantic Forest, found in Amazon region as well as in several countries that are part of this biome such as Brazil, Bolivia, Peru, Ecuador, Colombia, and Venezuela. It is a shrub or small tree with simple leaves and purple or white individual or clustered flowers (Filipowicz and Renner, 2012).

This genus is traditionally utilized in popular medicine of American peoples, especially in the Amazon region due to its distinct and varied medicinal effects such as hallucinogenic, anti-inflammatory (Castioni and Kapetanidis, 1996; Filipowicz and Renner, 2012), antibacterial, and antifungal (Begum et al., 2007) effects. However, studies on antimicrobial activity of *B. uniflora* flower oleoresin extracted by supercritical carbon dioxide were not found.

Hydrodistillation is one of the most utilized extraction processes for essential oils of leaves; however, *B. uniflora* flowers are sensitive to heat and heating can result in losses of volatile compounds with reduced extraction yield (Bertrand et al., 2006). Extraction by supercritical carbon dioxide has the advantage of using low temperatures, and allows controlling extraction conditions such as temperature, pressure, and solvent flow. When utilizing carbon

dioxide as solvent, its advantage is the high extractive selectivity to obtain highly aggregated value compounds (Barros et al., 2014). Besides greater yield, the extraction by supercritical carbon dioxide is quick and does not leave organic solvent residues in the extract, which makes it an interesting technique to obtain plant extracts industrially (Mazutti et al., 2006).

Despite the use of new genomic tools, there has been a decrease in identification of new antibiotics and an increase in microorganism resistance to conventional antibiotics. The resistance to microorganisms is related to the overuse of antibiotics in livestock worldwide (Aarestrup, 2012). Common microorganisms such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Listeria monocytogenes* have become the main causes of hospitalizations and deaths in hospitals (Mead et al., 1999). Fungi of *Aspergillus* and *Penicillium* genera are also difficult to control in food preservation, increasing risks to human and animal health (Korsten, 2006). Therefore, the search for new alternatives to control microorganisms is necessary to produce and keep food safe, preserving human and animal health. Thus, this study aimed to evaluate the chemical composition and antimicrobial activity of *B. uniflora* flower oleoresin obtained by supercritical carbon dioxide.

MATERIAL AND METHODS

Biological material

Fresh flowers of *B. uniflora* were collected in the morning in the northern region of the State of Paraná, Brazil, at the coordinates 23°29.06'S 51°47.30'W and altitude of 670 m. Plant sample was identified and deposited in the Educational Herbarium of Paranaense University (HEUP) under the number 2855. The cultivation soil was evaluated for pH, macro- and micronutrients, and granulometry according to Camargo et al. (2009). The soil samples were collected at 20 cm depth from four different equidistant points 1 m away from the plant to obtain a compound sample (Gazim et al., 2007). This region has humid temperate climate (CFa) according to Köppen-Geiger classification, with hot humid summer, more intense winter and without dry season, and annual average temperature of 22°-23°C and monthly rainfall of 1400-1600 mm (Peel et al., 2007).

Extraction of *B. uniflora* flower oleoresin by supercritical carbon dioxide

The flowers were dried at room temperature (26°C) and ground in a cutting mill. *B. uniflora* flower oleoresin was obtained by supercritical carbon dioxide. For extraction, 10 g flowers was transferred to a bench extractor (White Martins S.A.; Quimis) and kept in contact with supercritical carbon dioxide at 100 bars, 30°C, for 60 min. After this time, the extraction occurred with constant flow of carbon dioxide (2 mL/min) for 3 h (Reverchon, 1997).

Gas chromatographic-mass spectrometry chemical identification

Gas chromatographic-mass spectrometric analysis (EI) was performed using an Agilent 5973 Network chromatograph coupled to an Agilent 5973 MSD spectrometer (Agilent Technologies, Santa Clara, CA, USA). For the separation, an Agilent 19091S-433 HP-5MS fused silica capillary column with 30 m x 0.25 mm ID and 0.25-μm film thickness was used. The temperature program - in the gas chromatography oven - was from 60° to 285°C at a rate

of 4.3°C/min. The carrier gas was helium; inlet pressure: 25 kPa; linear velocity: 1 mL/min at 210°C; injector temperature: 250°C; injection mode: splitless. Mass spectrometric scan conditions were source temperature of 200°C; interface temperature of 250°C; E energy of 70 eV; and mass scan range of 40-350 amu.

Antibacterial activity

The bacteria used for the antibacterial activity assay was *Bacillus cereus* Frankland and Frankland (clinical isolate), *Enterobacter cloacae* (Jordan) Hormaeche and Edwards (clinical isolate), *E. coli* (Migula) Castellani and Chalmers (ATCC 35218), *L. monocytogenes* (Murray et al.) Pirie (NCTC 7973), *P. aeruginosa* (Schroeter) Migula (ATCC 27853), *Salmonella enterica* subsp *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 13311), and *Staphylococcus aureus* subsp *aureus* Rosenbach (ATCC 6538). Microorganisms were obtained from the Mycological Laboratory, Institute for Biological Research 'Siniša Stanković', University of Belgrade, Serbia. The method used for the antibacterial activity was by microdilution (Hänel and Raether, 1988; Espinel-Ingroff, 2001). The bacterial suspension concentrations were of 1.0 x 10^5 CFU/mL adjusted with sterile saline solution. Inoculum was prepared daily and stored at 4°C until use. In order to verify the absence of contaminations, the inoculum was grown in solid medium to validate each inoculum. All experiments were in duplicate and repeated three times.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using 96-well microtiter plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/mL. Compounds to be investigated were dissolved in 5% DMSO solution containing 0.1% polysorbate-80 (v/v) (1 mg/mL) and added in Luria-Bertani (LB) broth medium (100 μL with bacterial inoculum (1.0 x 10^4 CFU/well) to achieve the wanted concentrations. The microplates were incubated at rotary shaker (160 rpm) for 24 h at 37°C. The lowest concentrations without visible growth at light microscope were defined as concentrations that completely inhibited bacterial growth or MIC. The MBCs were determined by serial subcultivation of 2 μL onto microtiter plates containing 100 μL broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate Manager 4.0 (Bio-Rad Laboratories) and compared with a blank and a positive control. Streptomycin (Sigma P 7794) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline). Solution of 5% DMSO was used as a negative control. All experiments were performed in duplicate and repeated three times.

Antifungal activity

For the antifungal bioassays, eight fungi were used: *Aspergillus fumigatus* Fresenius (ATCC 1022), *Aspergillus niger* van Tieghem (ATCC 6275), *Aspergillus ochraceus* Wilhelm (ATCC 12066), *Aspergillus versicolor* (Vuillemin) Tiraboschi (ATCC 11730), *Penicillium funiculosum* Thom (ATCC 8725), *Penicillium ochrochloron* Biourge (ATCC 9112), *Penicillium verrucosum* var. *cyclopium* (Westling) Samson, Stolk & Hadlok (food isolate), and *Trichoderma viride* Pers. (IAM 5061). Microorganisms were obtained from the Mycological Laboratory, Institute for Biological Research 'Siniša Stanković', University of

Belgrade, Serbia. The micromycetes were kept on malt extract agar and the cultures stored at 4°C and subcultured once a month (Booth, 1971). In order to investigate the antifungal activity of the compounds, a modified microdilution technique was used (Hänel and Raether, 1988; Espinel-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% polysorbate-80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 ml per well. The inocula were stored at 4°C for further use. Dilutions of inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of each inoculum. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in 5% DMSO solution containing 0.1% polysorbate-80 (v/v) (1 mg/mL) and added in broth malt extract medium with inoculum. The microplates were incubated at rotary shaker (160 rpm) for 72 h at 28°C. The lowest concentrations without visible growth at light microscope were defined as MIC. The minimal fungicidal concentration (MFC) was determined by serial subcultivation of 2 µL of tested compounds dissolved in medium and inoculated for 72 h onto microtiter plates containing 100 µL broth per well and with further incubation for 72 h at 28°C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Solution of 5% DMSO was used as a negative control. Commercial fungicides bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia) were used as positive controls (1-3500 mg/mL). All experiments were performed in duplicate and repeated three times.

Statistical analysis

All of the tests were carried out in triplicate. The results were expressed as mean values ± standard deviation, and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD test with $\alpha = 0.05$, to determine whether there is a statistically significant difference among the obtained results. The analysis was carried out by Statistical Package for the Social Sciences (SPSS) version 18.0.

RESULTS

The granulometric analysis of the soil showed 40.85% clay, 20.00% silt, and 39.15% sand, and the soil was classified as clayey according to Brasil (2008). High iron content (62.34 mg/dm^3) (Table 1) makes this soil red, highly fertile, and saturated for bases (V = 79.55%) and organic matter (OM = 35.60 g/dm^3), but with low phosphorus content (2.20 mg/dm^3).

Table 1. Analysis of pH, macro- and micronutrients of the soil utilized in the cultivation of *Brunfelsia uniflora*.

pH and macronutrients								
	(cmol/dm^3)						(mg/dm^3)	
pH ($CaCl_2$)	Al^{3+}	$H^+ + Al^{3+}$	Ca^{2+} Mg^{2+}	K^+	SB	CTC	P	C
6.22	0.0	3.42	13.00	0.31	13.31	16.73	2.20	20.65
Micronutrients (cmol/dm^3)								
V (%)	Ca		Mg	K	Ca/Mg	Ca/K	Mg/K	
79.55	8.75		4.25	0.31	2.06	28.44	13.81	

The chemical composition of *B. uniflora* flower oleoresin by supercritical carbon dioxide resulted in 50 compounds (Table 2). The predominant class was oxygenated diterpene

(*E*,*E*)-geranyllinalool (21.0%), followed by oxygenated sesquiterpenes (*E*)-nerolidol (3.9%) and (2*E*,6*Z*)-farnesal (0.5%). Also, a great amount of fatty acid esters was extracted (48.2%), making the presence of hydrocarbons (15.3%) evident, which is typical of oleoresin extraction technique (Table 2).

Table 2. Chemical composition of *Brunflesia uniflora* flower oleoresin extracted by supercritical carbon dioxide.

Peak	Compound	RI	Area (%)	Methods of identification
1	Camphor	1134	t	a.b.
2	n.i.	1382	0.4	a.b.
3	n.i.	1387	1.6	a.b.
4	(*E*)-nerolidol	1577	3.9	a.b.
5	Dodecyl acrylate	1694	0.2	a.b.
6	(2*E*,6*Z*)-farnesal	1723	0.5	a.b.
7	Hexadecanoic acid	1977	0.5	a.b.
8	(*E*,*E*)-geranyllinalool	2040	21.0	a.b.
9	n.i.	2461	0.1	a.b.
10	Tricosane	2305	0.1	a.b.
11	n.i.	2485	0.2	a.b.
12	Pentacosane	2501	0.4	a.b.
13	1,3-dihydroxy propan-2-yl hexadecanoate	2518	0.5	a.b.
14	n.i.	2638	0.1	a.b.
15	Eicosanoic acid, isoamyl ester	2654	0.2	a.b.
16	Phenyl palmitate	2681	0.3	a.b.
17	n.i.	2688	0.1	a.b.
18	l-tetracosanol	2701	0.8	a.b.
19	Heptacosane	2701	1.3	a.b.
20	Octadecanoic acid, 2,3-dihydroxypropyl ester	2724	0.8	a.b.
21	n.i.	2746	0.8	a.b.
22	Eicosanoic acid, hexyl ester	2809	0.2	a.b.
23	Octacosane	2801	0.3	a.b.
24	Hexacosyl acetate	2809	0.1	a.b.
25	Docosanoic acid, isoamyl ester	2844	2.3	a.b.
26	Nonacosane	2902	4.9	a.b.
27	n.i.	2916	0.3	a.b.
28	n.i.	2933	0.6	a.b.
29	Docosanoic acid hexyl ester	2966	6.2	a.b.
30	Eicosanoic acid phenylmethyl ester	2974	0.4	a.b.
31	Tetracosanoic acid isoamyl ester	3022	5.5	a.b.
32	Tricosanoic acid hexyl ester	3049	0.4	a.b.
33	Untriacontane	3100	4.8	a.b.
34	Docosanoic acid octyl ester	3129	1.2	a.b.
35	Tetracosanoic acid hexyl ester	3138	10.4	a.b.
36	Docosanoic acid phenylmethyl ester	3150	2.5	a.b.
37	Hexacosanoic acid isoamyl ester	3187	0.8	a.b.
38	Tetracosanoic acid 3-methylhexyl ester	3193	0.7	a.b.
39	Tricosanoic acid phenylmethyl ester	3224	1.7	a.b.
40	Tetracosanoic acid octyl ester	3231	0.6	a.b.
41	n.i.	3246	0.6	a.b.
42	Hexacosanoic acid hexyl ester	3288	2.3	a.b.
43	Tetracosanoic acid phenylmethyl ester	3295	4.6	a.b.
44	Tritriacontane	3305	3.5	a.b.
45	Octacosanoic acid octyl ester	3439	1.7	a.b.
46	Hexacosanoic acid hexyl ester	3445	3.1	a.b.
47	Pentacosanoic acid phenylmethyl ester	3486	0.5	a.b.
48	Octacosanoic acid octyl ester	3612	0.9	a.b.
49	Tricontanoic acid hexyl ester	3618	0.9	a.b.
50	Tricontanoic acid octyl ester	3839	0.3	a.b.
Total identified			96.1	-

a = identification based on retention index (RI); b = identification based on comparison of mass spectra; n.i. = unidentified compound; t = trace. Compound listed in order of elution from an HP-5MS column.

The bacteriostatic activity (MIC) for oleoresin varied from 0.005 to 0.04 mg/mL, whereas the positive controls streptomycin and ampicillin had MIC values ranging from 0.05

to 0.5 mg/mL. Thus, MIC values for *B. uniflora* oleoresin were in general effective and lower ($P \leq 0.05$) than the respective positive controls (Table 3). Oleoresin also presented greater bactericidal activity than the positive controls. MBC values ranged from 0.01 to 0.08 mg/mL for oleoresin, and from 0.1 to 0.5 mg/mL for the positive controls streptomycin and ampicillin (Table 3).

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Brunfelsia uniflora* oleoresin, streptomycin, and ampicillin.

Bacterium	*Brunfelsia uniflora* (mg/mL)		Streptomycin (mg/mL)		Ampicillin (mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	0.0050 ± 0.0003^{A}	0.0100 ± 0.0060^{a}	0.0500 ± 0.0300^{B}	0.1000 ± 0.0300^{b}	0.1000 ± 0.0100^{C}	0.1500 ± 0.0300^{b}
Enterobacter cloacae	0.0400 ± 0.0060^{A}	0.0400 ± 0.0020^{a}	0.5000 ± 0.0001^{C}	0.1000 ± 0.0600^{b}	0.3000 ± 0.0200^{B}	0.5000 ± 0.0900^{c}
Escherichia coli	0.0100 ± 0.0020^{A}	0.0200 ± 0.0020^{a}	0.1500 ± 0.0300^{B}	0.3000 ± 0.0300^{b}	0.1500 ± 0.0001^{B}	0.3000 ± 0.0300^{b}
Listeria monocytogenes	0.0050 ± 0.0003^{A}	0.0200 ± 0.0001^{a}	0.2500 ± 0.0600^{C}	0.5000 ± 0.0300^{c}	0.1000 ± 0.0300^{B}	0.1500 ± 0.0100^{b}
Pseudomonas aeruginosa	0.0400 ± 0.0001^{A}	0.0800 ± 0.0100^{a}	0.0500 ± 0.0100^{A}	0.1000 ± 0.0300^{b}	0.1000 ± 0.0600^{B}	0.2000 ± 0.0100^{c}
Salmonella enterica	0.0100 ± 0.0030^{A}	0.0400 ± 0.0001^{a}	0.0500 ± 0.0300^{B}	0.1000 ± 0.0001^{b}	0.1500 ± 0.0100^{C}	0.2000 ± 0.0001^{c}
Staphylococcus aureus	0.0100 ± 0.0001^{A}	0.0200 ± 0.0030^{a}	0.1250 ± 0.0300^{B}	0.2500 ± 0.0600^{c}	0.1000 ± 0.0300^{B}	0.1500 ± 0.0100^{b}

Different superscript capital letters and small case letters on the same column indicate statistical differences among the treatments according to Tukey's test ($P \leq 0.05$).

Moreover, *B. uniflora* oleoresin had action against *P. aeruginosa* (MIC of 0.0400 mg/mL and MBC of 0.0800 mg/mL). This is a common multiresistant bacterium in serious infectious processes in animals and humans (Magiorakos et al., 2012). Oleoresin action against *P. aeruginosa* was greater than the controls streptomycin (MIC of 0.0500 mg/mL and MBC of 0.1000 mg/mL) and ampicillin (MIC of 0.1000 mg/mL and MBC of 0.2000 mg/mL). It was also verified that Gram-positive bacteria were more sensitive to oleoresin than Gram-negative ones. For *B. cereus*, *S. aureus*, and *L. monocytogenes*, the MBC values were 0.0100, 0.0200, and 0.0200 mg/mL, respectively. For *E. coli*, *E. cloacae*, and *S. enterica*, however, the MBC values were greater, 0.0200, 0.0400, and 0.0400 mg/mL, respectively (Table 3).

The fungistatic activity (MIC) for oleoresin varied from 0.0012 to 0.04 mg/mL, but for the positive controls bifonazole and ketoconazole the MIC values varied from 0.1 to 2.5 mg/mL. Thus, the MIC values for *B. uniflora* oleoresin were smaller ($P \leq 0.05$) than the positive controls (Table 4). Oleoresin also showed greater fungicidal activity than the positive controls. MFC values ranged from 0.01 to 0.08 mg/mL for oleoresin and from 0.2 to 3.5 mg/mL for the positive controls bifonazole and ketoconazole (Table 4).

Table 4. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Brunfelsia uniflora* oleoresin, bifonazole, and ketoconazole.

Fungus	*Brunfelsia uniflora* (mg/mL)		Bifonazole (mg/mL)		Ketoconazole (mg/mL)	
	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	0.0200 ± 0.0060^{A}	0.0400 ± 0.0001^{a}	0.1500 ± 0.0300^{b}	0.2000 ± 0.0100^{b}	0.2000 ± 0.0300^{C}	0.5000 ± 0.0001^{c}
Aspergillus niger	$0.0400 \pm .0020^{A}$	0.0800 ± 0.0060^{a}	0.1000 ± 0.0300^{B}	0.2000 ± 0.0600^{b}	0.2000 ± 0.0300^{C}	0.3000 ± 0.0001^{c}
Aspergillus ochraceus	0.0400 ± 0.0010^{A}	0.0800 ± 0.0030^{a}	0.1500 ± 0.0100^{B}	0.2000 ± 0.0001^{b}	0.1500 ± 0.0100^{C}	0.2000 ± 0.0300^{b}
Aspergillus versicolor	0.0100 ± 0.0001^{A}	0.0400 ± 0.0030^{a}	0.1000 ± 0.0300^{B}	0.2000 ± 0.0001^{b}	0.2000 ± 0.0300^{C}	0.5000 ± 0.0100^{c}
Penicillium verrucosum	0.0400 ± 0.0030^{A}	0.0800 ± 0.0010^{a}	0.1500 ± 0.0600^{B}	0.2000 ± 0.0001^{b}	0.2000 ± 0.0600^{C}	0.5000 ± 0.0600^{c}
Penicillium ochrochloron	0.0012 ± 0.0001^{A}	0.0100 ± 0.0001^{a}	0.2000 ± 0.0001^{B}	0.2500 ± 0.0300^{b}	2.5000 ± 0.3300^{C}	3.5000 ± 0.0600^{c}
Penicillium funiculosum	0.0025 ± 0.0003^{A}	0.0500 ± 0.0030^{a}	0.2000 ± 0.0300^{B}	0.2500 ± 0.0200^{b}	0.2000 ± 0.0001^{B}	0.5000 ± 0.0100^{c}
Trichoderma viride	0.0200 ± 0.0001^{A}	0.0400 ± 0.0020^{a}	0.1500 ± 0.0001^{A}	0.2000 ± 0.0100^{b}	1.0000 ± 0.0100^{B}	1.0000 ± 0.0200^{c}

Different superscript capital letters and small case letters on the same column indicate statistical differences among the treatments according to Tukey's test ($P \leq 0.05$).

In general, the antifungal activity for oleoresin was greater than the controls bifonazole and ketoconazole (Table 4). It is noteworthy to point out the fungicidal activity against *P.*

ochrochloron and *T. viride*. For these fungi, the MFC values for oleoresin were 0.01 and 0.04 mg/mL, respectively, while they varied from 0.2500 to 0.2000 mg/mL for bifonazole and from 3.5000 to 1.0000 mg/mL for ketoconazole. Thus, the fungicidal action of oleoresin against *P. ochrochloron* was 25 and 350 times greater than bifonazole and ketoconazole, respectively. Against *T. viride* the oleoresin was 5 and 25 times more efficient than the respective positive controls. These results show that *B. uniflora* flower oleoresin has high activity against bacteria and fungi.

DISCUSSION

According to Morais (2009), abiotic factors such as temperature, rainfall, luminosity, and soil composition affect the chemical composition of plant secondary metabolites. Although *B. uniflora* cultivation soil is fertile, there was low phosphorus content (2.20 mg/dm^3) (Table 1). Prado et al. (2005) stated that phosphorus is a structural compound of nucleic acids, coenzymes, phosphoproteins, and phospholipids, and could interfere in the oleoresin chemical composition.

The chemical composition of *B. uniflora* flower oleoresin main compound was (*E*,*E*)-geranyllinalool (21.0%) (Table 2). The class of oxygenated diterpenes, mainly geranyllinalool, comprises floral aroma of plants that exhale smells (Falara et al., 2014). Geranyllinalool is utilized as flavoring in food, cosmetic, and perfume industry (Lapczynski et al., 2008). It is estimated that 100 kg of this compound is commercialized annually worldwide (Lapczynski et al., 2008). This compound has antispasmodic activity, is used against amenorrhea, rheumatism (Zrira et al., 2008), acne, arthritis, burning, ulcers, and is also used as expectorant and anti-thermal (Xie et al., 2013).

In the season of the year when there are high temperatures and heavy rainfall, *Brunfelsia* genus has great flower production (Filipowicz and Renner, 2012). Castioni and Kapetanidis (1996) obtained 0.07% essential oil yield from *Brunfelsia grandiflora* aerial part by steam distillation, obtaining more than 200 compounds. Studies carried out by our research group show that the yield of *B. uniflora* flower oleoresin obtained by supercritical carbon dioxide varies from 0.83 to 1.03%. However, according to Jorge (2016) the essential yield oil of *B. uniflora* by hydrodistillation is close to zero.

The amount of (*E*,*E*)-geranyllinalool of 21.0% obtained from *B. uniflora* flowers by supercritical carbon dioxide is greater than the ones found by hydrodistillation extraction of *Arnica montana* of 14.7% (Judžentienė and Būdienė, 2009), *Heliotropium stenophyllum* of 6.81% (Urzúa et al., 2013), *Exocarpium citri grandis* of 3.5% (Xie et al., 2013), but similar to *Ammi visnaga* of 22.7%, extracted by supercritical carbon dioxide (Zrira et al., 2008). Considering a greater extraction yield of supercritical carbon dioxide when compared to hydrodistillation and the high concentration of geranyllinalool in *B. uniflora* flower oleoresin, this is a good alternate source of this compound.

Oleoresin of *B. uniflora* is a potential source of geranyllinalool, but the concentration of chemical compounds of essential oils could vary according to flower stage (Bertrand et al., 2006), soil composition (Gazim et al., 2007), and extraction technique (Bertrand et al., 2006). Jorge (2016) reported that the pressure increase from 150 to 200 bars of supercritical carbon dioxide increases the extraction of geranyllinalool from 11.72 to 21.52% in oleoresin. Zrira et al. (2008) reported that extraction yield of *A. visnaga* oleoresin was greater (1.5%) by supercritical carbon dioxide when compared to hydrodistillation (0.4%). Moreover, the geranyllinalool percentage was 22.7% in oleoresin, and it was not identified in oils obtained

by other extraction methods. Besides a greater extraction yield, supercritical carbon dioxide preserves thermolabile compounds of the essential oil (Mazutti et al., 2006). This method also has extraction process variables such as pressure, extraction, temperature, fluid flow, extraction time, particle size, among others that could affect extraction yield of a single compound of the mixture such as geranyllinalool (Zorca et al., 2006).

The great antimicrobial potential of *B. uniflora* (Tables 3 and 4) against the evaluated microorganisms must probably be due to the presence of high concentration of geranyllinalool (21.0%) associated with high concentrations of fatty acid esters (50.5%). Zrira et al. (2008) reported that *A. visnaga* essential oil with 22.7% geranyllinalool showed inhibitory halos from 10 to 33 mm against *E. coli*, *S. aureus*, *Klebsiella pneumoniea*, *Proteus mirabilis*, *B. subtilus*, *Salmonella* sp, and *P. aeruginosa* (Feirouz and Salima, 2014). *A. montana* with 14.7% geranyllinalool showed from 10 to 18 mm of inhibitory halo against *P. aeruginosa*, which is resistant to most studied compounds (Lister et al., 2009). In our study, *B. uniflora* flower oleoresin presented MIC of 0.04 mg/mL and MBC of 0.08 mg/mL against *P. aeruginosa*. Therefore, *B. uniflora* oleoresin was highly efficient (MIC 0.04 mg/mL) against *P. aeruginosa*, a common multiresistant bacterium in severe infectious processes (Magiorakos et al., 2012).

The composition of *B. uniflora* oleoresin showed a variety of chemical compounds, having geranyllinalool as the main compound and more than 50% of fatty acid esters. Methyl esters present antimicrobial activity against different bacteria and fungi: *E. coli*, *P. aeruginosa*, *S. aureus*, *A. niger*, and *A. fumigatus* (Agoramoorthy et al., 2007; Chandrasekaran et al., 2008). Shen et al. (2012) verified that a series of 3-(2-furyl) acrylate monosaccharide esters and menthyloxycarbonyl monosaccharide esters synthesized in laboratory showed antifungal activity against *A. flavus*, *A. niger*, *A. fumigatus*, *Geotrichum candidum* and antibacterial activity against *B. subtilis*, *S. aureus*, *Staphylococcus epidermidis*, *E. coli*, and *P. aeruginosa*. Although the mechanism used by methyl esters to kill microorganisms is unknown, they might penetrate the peptideoglucan network of the cellular wall of these organisms (bacteria and fungi) and reach the cellular membrane causing its disintegration. Besides this action, esters make the transport of bioactive molecules easier and, thus, act sinergically for the bactericidal and fungicidal action (Karlová et al., 2010).

For fungi like *P. ochrochloron*, *B. uniflora* oleoresin showed efficiency with MIC of 0.0025 mg/mL. For Ristíc et al. (2000), *Phlomis fruticosa* essential oil was not effective for this fungus. Džamić et al. (2008) reported MIC of 25 µg/mL for *Salvia sclarea* essential oil. For *T.* viride, the obtained MIC in our study was 0.02 mg/mL. The MIC of essential oils for *T. viride* described in the literature is 0.6 mg/mL for *Lippia alba* (Glamočlija et al., 2011), 40 µL/mL for *L. nobilis*, 10 µL/mL for *Sassafras albidum* (Simić et al., 2004). Therefore, *B. uniflora* has great fungicidal action against *P. ochrochloron* and *T. viride.* Fungi from *Trichoderma* genus are the most common contaminants of mushroom cultivations and cause great economic losses worldwide (Castle et al., 1998; Glamočlija et al., 2011). Although commonly used synthetic fungicides are efficient and have low cost, studies suggest that they leave residual toxicity that may cause side effects, including carcinogenesis and teratogenesis (McCarroll et al., 2002; Glamočlija et al., 2011). Thus, *B. uniflora* flower oleoresin can be explored to the development of products to control fungi and bacteria.

CONCLUSIONS

Oleoresin of *B. uniflora* flower extracted by supercritical carbon dioxide presents

higher bacteriostatic, bactericidal, fungistatic, and fungicidal activity than the positive controls streptomycin, ampicillin, bifonazole, and ketoconazole. The high antimicrobial activity of the oleoresin is related to the higher content of (*E*,*E*)-geranyllinalool (21.0%) and possible synergic action with fatty acid esters (48.2%). The antimicrobial activity of *B. uniflora* oleoresin against common multiresistant bacteria in severe infectious processes such as *P. aeruginosa* or against toxin-producing fungi such as *P. ochrochloron* or fungi that are difficult to control as *T. viride* suggests the development of promising applications of this product in the food, farming, livestock, and pharmaceutical industry.

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