SUPPLEMENTARY MATERIAL

GC×GC/qMS Analysis of *Campomanesia guazumifolia* (Cambess.) O. Berg Essential Oils and Their Antioxidant and Antimicrobial Activity

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Abstract: Brazilian biomes are recognized for their biodiversity and it is important for correct utilization and exploitation, to determine their chemical and biological value. This paper studies the essential oil of *Campomanesia guazumifolia* (Cambess.) O. Berg, an aromatic plant used in folk medicine. The chemical composition was investigated using GC×GC/qMS and the antioxidant and antimicrobial properties were also investigated. 68 compounds were identified and the major components (47%) were sesquiterpenes (bicyclogermacrene - 15%, globulol - 5% and spathulenol - 5%). Values of DPPH (IC_{50} of 26.1±0.5 µg/mL) and BCB (68.3±1.5%) indicated a significant antioxidant activity. The oil also showed a strong antimicrobial activity against *S. aureus* (MIC 15±0.1 µg/mL), *E. coli* (MIC 25±0.2 µg/mL) and *C. albicans* (MIC 5±0.1 µg/mL). The results represent the most detailed characterization of this essential oil reported today and this is the first study that has reported any biological activity of this essential oil.

EXPERIMENTAL SECTION

Material

Samples. Leaves of *Campomanesia guazumifolia* (Cambess.) O. Berg were collected from the wild in Dourados-MS (Brazil) in March 2012. The specie was identified and a voucher specimen was deposited at the *Herbarium* of the Universidade Federal da Grande Dourados. The register number was 4746.

Chemicals. Standard chemicals used: *n*-alkane (C_6-C_{30}), β-carotene, linoleic acid (≥98%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, quercetin (≥95%), butylated hydroxytoluene (BHT) (≥99%), were purchased from Sigma Aldrich (Saint Louis, MO, USA). Solutions of *n*-alkane (100 ppm) were prepared in *n*-hexane. Casein Soy, Sabouraud Broth and Polyoxyethylene Sorbitan Monopalmitate (Tween®-40) were purchased from Sigma Aldrich (Saint Louis, MO, USA), and anhydrous sodium sulfate p.a. grade from Merck (Darnstadt, Germany). Essential oil standards used: (-)-β-pinene (99%), α-terpineol (≥96%), limonene (97%), linalool (97%), α-pinene (98%), *(E)*-caryophyllene (≥98.5%), α-phellandrene (≥85%), α-humulene (≥96%), γ-terpinene (97%) and terpinolene (≥90%) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Standard solutions (1 ppm) were prepared by weight appropriate amounts of standard samples, dilution in hexane and stored at -4 °C. Methanol, chloroform and *n*-hexane (chromatographic grade) were obtained from JT Baker (Phillipsburg, NJ, USA).

Sample preparation

The essential oil was isolated from fresh leaves (300 g) by hydrodistillation using a Clevenger-type apparatus, according to the Brazilian Official Pharmacopoeia V (Brazilian official pharmacopoeia 2010). The essential oil was recovered, dried with anhydrous sodium sulfate, transferred to dark vials and, stored at -4 °C for further analysis. The plant essential oil (1000 mg) was diluted in *n*-hexane (1 mL) before gas chromatographic analysis. The experiments were run in triplicate.

GC×GC/qMS analysis

GC×GC/qMS analysis were carried out on a GC×GC/QP2010-Ultra qMS instrument (Shimadzu Corp., Kyoto, Japan). The system was provided with a loop-type

modulator (ZOEX Corp., TX, USA) cooled with liquid nitrogen and with the hot jet pulse time set at 500 ms (300 ºC) with modulation time of 5 s. The 1D column, the injection mode, carrier gas flow, mass spectrometry detector parameters, injector, interface, ion source and oven temperature were the same as above described for GC/qMS analysis giving a 65 Hz acquisition rate. The 2D column was a DB-17 (50% phenyl methylpolysiloxane, Agilent Technologies, CA, USA - 2.15 m × 0.18 mm × 0.18 µm). In order to calculate the linear-temperature-programmed retention indices (LTPRI) (von Mühlen & Marriott 2011) a linear C_6-C_{30} alkane mixture was analyzed using identical GC×GC/qMS conditions. Data were acquired by GCMS Solution software and processed using GC-Image software (2.2b1; ZOEX Corp., TX, USA). Compounds identification were based on chromatographic (retention times, LTPRI (Adams 2007) and standard compounds) and spectroscopic (mass spectra interpretation, comparison with NIST database, and standard compounds) data. An Identity Spectrum Match factor above 800 resulting from NIST and a LTPRI with a match window of ±10 were determined to be acceptable for tentatively identified compound. The relative amounts of individual compounds were calculated based on GC/qMS peak area and GC×GC/qMS peak volume without using correction factor.

Antioxidant activity analyses

DPPH: free radical scavenging assay. In order to estimate the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity, essential oil samples at different concentration ranges (1-100 µg mL^{-1}) were mixed in the freshly DPPH solution (0.1 mM in methanol, 3.0 mL) according to the method described in the literature (Gupta et al. 2011) with minor modifications. The mixture was shaken vigorously and left to stand at room temperature in the dark. After 30 min, absorbance (517 nm) was measured against a blank containing all reagents except the test samples, using a UV-VIS spectrophotometer (Femto, model 700 plus). BHT and ascorbic acid were used as positive control. The scavenging activity was calculated as follows:

% DPPH scavenging activity = $(A_0\text{-}A/A_0) \times 100$,

where: A_0 is the absorbance of the blank solution and A is the absorbance of the essential oil. The percentage of scavenging activity was plotted against the sample concentration to obtain effective concentration (IC_{50}) defined as the concentration of the

sample necessary to scavenge 50% of the DPPH radicals. Determination was performed in triplicate.

β-carotene bleaching (BCB) antioxidant activity. The antioxidant activity of the essential oil was measured on the basis of the β-carotene bleaching system according to the previously method reported in the literature (Jayaprakasha et al. 2001). Briefly, β-carotene was dissolved in chloroform (0.2 mg mL^{-1}) and an aliquot (1 mL) of this solution was mixed with linoleic acid (20 mg) and Tween-40® (200 mg). Subsequently, chloroform was removed under vacuum using a rotary evaporator (Fisatom, model 801) and then distilled water saturated with oxygen (50 mL) was slowly added with vigorous agitation to form an emulsion. Emulsion aliquots (5 mL) were mixed with the sample (0.2 mL). Control samples were prepared with methanol (0.2 mL) without sample. As soon as the emulsion was added to each tube, the absorbance (470 nm) was measured at against blank (zero time) using a UV-VIS spectrophotometer (Femto, model 700 plus). Tubes were placed in a water bath (50°C) and oxidation was monitored by absorbance measurements at 15 min intervals until the color of β-carotene in the control sample had disappeared (approximately 105 min). Butylated hydroxytoluene (BHT), ascorbic acid and quercetin were used as a reference. Antioxidant activity (AA) was calculated as percent inhibition relative to the control:

AA= [1- (Ai - At) / (A'i -A't)] x 100,

where Ai = absorbance of sample at zero time, At = absorbance of sample after incubation (105 min, 50°C), A'i= absorbance of control at zero time, and A't = absorbance of control after incubation (105 min, 50 °C). The analyses were performed in triplicate.

Antimicrobial activity

In order to evaluate the minimum inhibitory concentration (MIC) of the essential oil, gram-positive bacterium *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC 11103) and the yeast *Candida albicans* (ATCC10231), a micro-dilution assay was performed according to a method described in the literature (Hammer et al. 1998). The MIC was determined on 96 well culture plates by a micro dilution method using a microorganism suspension at a density of 10^5 CFU mL^{-1} with Casein Soy Broth incubated (24 hours, 37 ºC) for bacteria, and Sabouraud Broth incubated (72 hours at 25 °C) for yeasts. Serial dilutions were then made resulting in different concentrations (2 to

50 µg mL^{-1}). Proper blanks were assayed simultaneously and samples were tested in triplicate.

References

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Table 1: Identified compounds and their amounts in the *C. guazumifolia* essential oil.

Peak No.	Compound	GC×GC/qMS					Identification mode
		LTPRI[a]	LTPRI[b]	V (%)	Base Ion	Molecular Ion (Intensity)	
Alcohols							
1	4-methyl-Pentanol	830	815	0.29	56	102 (0.02)	MS[c], LTPRI
	TOTAL			**0.29**			
Monoterpenes							
2	α-Thujene	924	928	0.42	93	136 (6.85)	MS, LTPRI
3	α-Pinene	932	933	1.68	93	136 (5.64)	MS, LTPRI, SD[d]
4	Camphene	946	949	0.30	93	136 (7.83)	MS, LTPRI
5	Sabinene	969	973	0.38	93	136 (9.29)	MS, LTPRI
6	β-Pinene	974	976	0.33	93	136 (5.36)	MS, LTPRI, SD
7	β-Myrcene	988	990	0.90	93	136 (2.27)	MS, LTPRI
8	α-Phellandrene	1002	1005	2.55	93	136 (22.2)	MS, LTPRI, SD
9	Iso-Sylvestrene	1007	1010	0.31	93	136 (22.0)	MS, LTPRI
10	alpha-Terpinene	1014	1017	0.38	93	136 (38.8)	MS, LTPRI
11	o-Cymene	1026	1024	0.90	119	134 (31.5)	MS, LTPRI
12	Limonene	1024	1029	4.42	68	136 (18.6)	MS, LTPRI, SD
13	(Z)-β-Ocimene	1032	1038	0.54	93	136 (2.94)	MS, LTPRI
14	(E)-β-Ocimene	1044	1048	2.69	93	136 (6.13)	MS, LTPRI
15	γ-Terpinene	1054	1058	1.02	93	136 (25.3)	MS, LTPRI, SD
16	Terpinolene	1086	1088	0.40	93	136 (62.3)	MS, LTPRI, SD
	TOTAL			**17.2**			
Oxygenated Monoterpenes							
17	Linalool	1095	1100	0.48	71	154 (0.09)	MS, LTPRI, SD
18	Terpinen-4-ol	1174	1180	0.29	71	154 (18.7)	MS, LTPRI
19	α-Terpineol	1186	1194	0.32	59	154 (0.30)	MS, LTPRI, SD
	TOTAL			**1.09**			

Sesquiterpenes							
20	δ-Elemene	1335	1340	0.46	121	204 (7.51)	MS, LTPRI
21	α-Cubebene	1345	1353	0.35	161	204 (20.1)	MS, LTPRI
22	Cyclosativene	1369	1370	0.28	105	204 (17.4)	MS, LTPRI
23	Isoledene	1374	1376	0.53	105	204 (18.6)	MS, LTPRI
24	α-Copaene	1374	1379	1.17	119	204 (9.98)	MS, LTPRI
25	β-Elemene	1389	1396	1.29	81	204 (1.00)	MS, LTPRI
26	α-Gurjunene	1409	1413	1.14	105	204 (65.8)	MS, LTPRI
27	(E)-Caryophyllene	1417	1417	0.36	105	204 (27.3)	MS, LTPRI, SD
28	β-Duprezianene	1421	1424	2.08	91	204 (31.9)	MS, LTPRI
29	β-Gurjunene	1431	1434	0.99	161	204 (6.01)	MS, LTPRI
30	Aromadendrene	1439	1443	2.08	93	204 (28.8)	MS, LTPRI
31	α-Guaiene	1437	1447	0.92	105	204 (11.5)	MS, LTPRI
32	α-Humulene	1452	1458	1.74	93	204 (3.33)	MS, LTPRI, SD
33	Allo-aromadendrene	1458	1465	3.22	161	204 (57.8)	MS, LTPRI
34	γ-Gurjunene	1475	1477	0.72	105	204 (23.7)	MS, LTPRI
35	γ-Muurolene	1478	1481	0.97	161	204 (15.6)	MS, LTPRI
36	Germacrene D	1484	1486	4.44	161	204 (11.6)	MS, LTPRI
37	*cis*-Eudesma-6,11-diene	1489	1491	0.64	106	204 (22.3)	MS, LTPRI
38	α-Selinene	1498	1494	1.04	189	204 (6.79)	MS, LTPRI
39	*trans*-Muurola-4(14),5-diene	1493	1496	0.41	161	204 (50.1)	MS, LTPRI
40	Bicyclogermacrene	1500	1506	15.2	93	204 (13.7)	MS, LTPRI
41	δ-Amorphene	1511	1512	1.41	161	204 (44.1)	MS, LTPRI
42	β-Vatirenene	1527	1514	0.69	202	202 (100)	MS, LTPRI
43	γ-Cadinene	1513	1519	1.07	161	204 (15.4)	MS, LTPRI
44	7-*Epi*-α-selinene	1522	1521	0.54	161	204 (17.1)	MS, LTPRI
45	δ-Cadinene	1522	1528	2.05	161	204 (43.9)	MS, LTPRI
46	*trans*-Cadina-1,4-diene	1533	1537	0.35	119	204 (14.3)	MS, LTPRI
47	α-Cadinene	1537	1542	0.45	105	204 (8.14)	MS, LTPRI

48	α-Calacorene	1544	1550	0.43	157	200 (16.8)	MS, LTPRI
	TOTAL			**47.1**			
Oxygenated Sesquiterpenes							
49	Elemol	1545	1555	0.69	59	222 (0.04)	MS, LTPRI
50	Germacrene D-4-ol	1574	1560	0.63	81	222 (2.23)	MS, LTPRI
51	Epiglobulol	1564	1567	1.15	82	222 (2.99)	MS, LTPRI
52	Ledol	1569	1575	0.87	111	222 (2.09)	MS, LTPRI
53	Spathulenol	1577	1586	5.07	91	220 (3.44)	MS, LTPRI
54	Globulol	1590	1593	5.27	81	222 (2.12)	MS, LTPRI
55	Viridiflorol	1592	1600	3.63	109	222 (1.45)	MS, LTPRI
56	Guaiol	1600	1604	1.92	161	222 (2.64)	MS, LTPRI
57	Isoaromadendrene epoxide	1612	1617	0.75	55	220 (1.31)	MS, LTPRI
58	Cubenol	1645	1618	1.54	119	222 (3.33)	MS, LTPRI
59	1-epi-Cubenol	1627	1635	0.56	119	222 (0.09)	MS, LTPRI
60	γ-Eudesmol	1630	1639	1.16	161	222 (3.05)	MS, LTPRI
61	allo-Aromadendrene epoxide	1639	1645	1.84	119	220 (14.9)	MS, LTPRI
62	epi-α-Cadinol	1638	1648	2.46	161	222 (0.98)	MS, LTPRI
63	Torreyol	1644	1653	1.12	161	222 (0.10)	MS, LTPRI
64	α-Cadinol	1652	1662	3.77	95	222 (2.25)	MS, LTPRI
65	Aromadendrene oxide (2)	1678	1670	0.54	55	220 (5.96)	MS, LTPRI
66	Bulnesol	1670	1674	0.66	107	222 (2.39)	MS, LTPRI
67	8-Cedren-13-ol	1688	1686	0.39	91	220 (6.53)	MS, LTPRI
68	(2Z,6E)-Farnesol	1722	1727	0.33	69	222 (0.02)	MS, LTPRI
	TOTAL			**34.4**			

[a] LTPRI: literature values of linear-temperature-programmed retention index; [b] experimental values of linear-temperature-programmed retention index; [c] mass spectra; [d] standard compounds. Volume (%): percentage of volume of the compounds related to the total of identified peaks.