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Short Report

Composition and Antimicrobial Activity of the Essential Oil from *Aloysia sellowii*

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Os óleos essenciais obtidos das partes aéreas de *Aloysia sellowii,* coletadas em duas diferentes localidades (A e B) do sul do Brasil, foram analisadas por CG, CG-EM, e através de cromatografia gasosa com fases quirais (CGC). Dos quarenta e oito componentes identificados, representando cerca de 93 e 91% dos óleos das regiões A e B, respectivamente, 1,8-cineol, β-pineno, sabineno e β-(*Z*)-santalol foram os principais componentes. Um "screening" preliminar das duas amostras de óleos de *A. sellowii* mostrou atividade frente a *Artemia salina* (LC50 = 2,85 μg mL^{-1}), além de exibirem atividade antimicrobiana frente a bactérias Gram-positivas e Gram-negativas e frente a leveduras (MIC 1,7-16 mg mL^{-1}).

The essential oils obtained from aerial parts of *Aloysia sellowii* from two different collection locations Southern Brazil (A and B), were analyzed by GC, GC/MS, and chiral phase gas chromatography (CPGC). It was identified 48 compounds representing *ca* 93% and 91% of the oils of samples A and B, respectively. Besides them 1,8-Cineole, β-pinene, sabinene, and β-(*Z*)-santalol were the major components. In a preliminary biological screening the samples of essential oils of *A. sellowii* displayed strong activity in the brine shrimp lethality assay (LC50 = 2.85 μg mL^{-1}) as well as antimicrobial activity against Gram-positive and Gram-negative microorganisms and two yeasts (MIC 1.7-16 mg mL^{-1}).

Keywords: *Aloysia sellowii,* essential oil, enantiomeric composition, chiral phase Gas Chromatography

Introduction

Aloysia (Verbenaceae) is a genus that comprises about 30 species widespread all over the Americas, mainly in the South and Central America countries.[1] *Aloysia sellowii* (Briq.) Moldenke [= *Lippia affinis* (Briq.)],[2] known as "garupá", "cidrozinho" or "erva de sepultura" is a large aromatic shrub up to 2-4 meters in height, found throughout the greater part of Southern of Brazil (Rio Grande do Sul), Uruguay, Paraguay and Argentina.[3, 4] In Rio Grande do Sul aerial part of this plant is traditionally used by local people as diuretic, stomach ache agent and as a remedy for colds, influenza, and for the treatment of respiratory disorders.[3, 4] There are few studies published about the chemical composition of the essential oils of this genus in spite of their interesting aromas. *A. gratissima*[5] and *A. triphylla*[2, 6] are the most widely studies species.

In our continuing research on the essential oils of Rio Grande do Sul aromatic plants,[7, 8] we have investigated the chemical composition of the essential oil of *A. sellowii*, its antimicrobial activity and the biological screening by brine shrimp lethality assay, which is considered an efficient tool for preliminary evaluation of the pharmacological activity.[9] In addition, we report on the enantiomeric distributions of the chiral monoterpene constituents in *A. sellowii.*

Results and Discussion

The leaf oils of *A. sellowi* were obtained by hydrodistillation in 1.2-1.4 % yield. Its constituents were identified by GC-MS using an electron-impact ionization technique and by co-chromatography with authentic samples**.** Twenty-eight compounds, making up 92.75 % of the oil from Sample A (Santa Maria), and thirty-four compounds, making up 91.04 % of the oil from Sample B (Livramento), were identified. Both oils displayed some qualitative and quantitative differences (see Table 1). The major constituent of the sample A was 1,8-cineole (43.30 %), which is present in only 1.16% in sample B. The oil

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Table 1. Percentage composition of the essential oils of *Aloysia sellowii*

	Compound[a]	% in samples		Kovats Indices		Identification[d]
		A	B	Apolar[b]	Polar[c]	
01	α-Thujene	0.26	0.35	931	1007	RI, GC-MS, Co
02	α-Pinene	5.18	1.87	939	1016	RI, GC-MS, Co
03	Camphene	0.33	-	953	1060	RI, GC-MS
04	β-Pinene	12.60	14.58	980	1114	RI, GC-MS, Co
05	Sabinene	-	12.62	976	1065	RI, GC-MS, Co
06	Myrcene	1.31	2.32	991	1160	RI, GC-MS, Co
07	Carene	0.32	-	1001	1189	RI, GC-MS
08	Limonene	1.27	1.13	1028	1193	RI, GC-MS, Co
09	1,8-Cineole	43.30	1.16	1033	1200	RI, GC-MS, Co
10	Linalool	0.72	0.58	1095	1562	RI, GC-MS, Co
11	Undecane	0.71	0.66	1100	1530	RI, GC-MS, Co
12	*cis*-β-Dihydroterpineol	-	1.08	1138	-	RI, GC-MS
13	Isoborneol	-	6.55	1156	1492	RI, GC-MS, Co
14	Pinocampheol	-	3.15	1167	1519	RI, GC-MS
15	Terpinen-4-ol	1.83	3.03	1177	1602	RI, GC-MS, Co
16	Isomentol	-	0.53	1178	-	RI, GC-MS, Co
17	*cis*-Dihydrocarvone	-	0.25	1194	-	RI, GC-MS, Co
18	*trans*-Dihydrocarvone	-	0.36	1198	-	RI, GC-MS, Co
19	Carvacrol ethyl ether	-	1.03	1294	-	RI, GC-MS
20	Carvacrol	-	5.25	1297	1572	RI, GC-MS, Co
21	β-Bourbonene	0.22	-	1384	-	RI, GC-MS, Co
22	Vanillin	0.25	-	1392	-	RI, GC-MS, Co
23	*cis-threo*-Davanafuran	1.28	1.77	1414	1569	RI, GC-MS, Co
24	ethyl-Vanillin	0.40	0.27	1452	-	RI, GC-MS
25	β-Farnesene (E)	-	0.34	1457	-	RI, GC-MS, Co
26	γ-Muurolene	2.95	0.83	1477	1639	RI, GC-MS, Co
27	γ-Curcumene	-	0.78	1480	-	RI, GC-MS
28	Cubenol	3.07	6.90	1492	1633	RI, GC-MS
29	α-Bisabolene (Z)	0.60	-	1504	-	RI, GC-MS, Co
30	*cis*-Calamenene	0.44	-	1521	-	RI, GC-MS
31	Thymohydroquinone	2.75	-	1552	1794	RI, GC-MS
32	Caryophyllene oxide	-	3.30	1574	1987	RI, GC-MS, Co
33	Turmerol	0.78	-	1577	-	RI, GC-MS
34	Spathulenol	3.47	-	1579	2164	RI, GC-MS, Co
35	Globulol	0.69	-	1582	-	RI, GC-MS, Co
36	Viridiflorol	-	0.36	1591	-	RI, GC-MS
37	Guaiol	4.91	-	1595	2077	RI, GC-MS, Co
38	Cedrol	-	0.31	1597	2170	RI, GC-MS, Co
39	Isobornyl isobutyrate	-	0.32	1602	-	RI, GC-MS
40	*epi*-Cedrol	-	0.40	1608	-	RI, GC-MS, Co
41	α-Acorenol	0.44	-	1630	2207	RI, GC-MS, Co
42	6-hydroxy-Isobornyl Isobutyrate	-	0.58	1638	-	RI, GC-MS
43	β-Eudesmol	0.32	-	1647	2240	RI, GC-MS, Co
44	Isoamyl geranate	-	1.34	1650	1707	RI, GC-MS
45	β-Bisabolol	2.02	2.62	1665	2189	RI, GC-MS, Co
46	*epi*-α-Bisabolol	0.33	-	1688	2235	RI, GC-MS, Co
47	β-Santalol (Z)	-	12.88	1703	2004	RI, GC-MS, Co
48	β-Santalol acetate (Z)	-	1.54	1823	2378	RI, GC-MS
	Total	92.75	91.04			

[a]Compounds listed in order of elution from a SE-54 column; [b]Kovats Indices determined on apolar SE-54 column (50-250 °C; 4 °C min^{-1}); [c]Kovats Indices determined on polar PEG-20M column (50-250 °C; 4 °C min^{-1}); [d]Identification: RI, Kovats index by Adams RP[17], GC-MS, gas chromatography-mass spectroscopy; Co, co-injection of authentic material.

of sample B was richer in sesquiterpenoids than the oil of sample A, and was characterized by a high content of sabinene (12.62%) and β-santalol (Z) (12.88%), which are not present in sample A. The enantiomeric composition of the chiral monoterpene constituents were analyzed on a dual column system,[10] using two 25 m fused silica columns coated with modified cyclodextrins (2,6-Me-3-Pe-β-CD and 6-Me-2,3-Pe-β-CD) as the chiral stationary phases. Of the chiral monoterpenes identified in the oils of *A. sellowi*, the main constituents were: (-)-

sabinene, (+)- β-pinene, (+)- α-pinene, and (+)-terpinen-4-ol (see Table 2).

The essential oils were screened for biological activity using the brine shrimps lethality test.[11] Samples A and B displayed high toxicity (LC50 = 2.85 μg mL^{-1}) in the brine shrimp bioassay. Antimicrobial activity of samples A and B was determined by the broth micro dilution method,[12,13] to determine the minimum inhibitory concentration (MIC) and the minimum lethal concentration (MLC). Table 3 shows that sample B exhibited a more potent antifungal activity against *S. cerevisiae* than sample A, probably due to the presence of carvacrol in sample B (> 5 %).[14] Yet for antibacterial activity, our results indicate that *S. epidermidis* (MIC = 1.7 mg mL^{-1}, samples A and B) was found to be the most sensitive while *S. aureus, M. luteus*, *K. pneumoniae, E. coli* and *S. setubal* were the most resistant bacteria (MIC = 6.7-20 mg mL^{-1}). A chemical-composition and antibacterial activity comparison in our results (Table 3) demonstrates that the compounds present in greatest proportions as 1,8-cineole (> 40 % in sample A and only 1.16 % in sample B)[15] are not necessarily responsible for the greatest share of total activity. Thus, the activity of the less abundant components as carvacrol, terpinen-4-ol, linalool, β-pinene, R (+)-limonene, myrcene[16] should be considered or at least the activity could be attributed to a synergistic effect among components. Although the MICs and the MLCs results varied between test organisms, in most cases the MIC and MLC are equivalent, indicating an antimicrobial action of the oils. To our knowledge, the antimicrobial activity of *A. sellowii* essential oils is reported here for the first time.

Table 2. Enantiomeric distribuitions (%) of monoterpenes in the *A. sellowii* essential oils determined by co-injection with authentic material

Compound	Sample A (*ee*)		Sample B (*ee*)		Kovats Indices	
	(+) %[a]	(-) %[a]	(+) %[a]	(-) %[a]	(+)	(-)
Thujene	100	-	100	-	920[c]	-
α-Pinene	98	02	98	02	969	964[b]
β-Pinene	92	08	89	11	980	973[c]
Sabinene	-	-	-	100	-	985[c]
Limonene	89	11	93	07	1067	1061[c]
Linalool	67	33	89	11	1255	1250[b]
Terpinen-4-ol	78	22	92	08	1321	1330[c]

[a] Percentage of enantiomeric excess of the chiral monoterpenes analyzed on a dual column system with chiral stationary phase (2,6-Me-3-Pe-β-CD and 6-Me-2,3-Pe-β-CD); Kovats Indices determined on: [b] 6-Me-2,3-Pe-β-CD and [c] 2,6-Me-3-Pe-β-CD.

Table 3. Antibacterial activity of the oils of *A. sellowii* (minimal inhibitory concentration (MIC) and the minimal letal concentration (MLC) in mg mL^{-1})

Organisms[a]	Sample A[b]		Sample B[b]		Chloramph.[b]	Nistatine[b]
	MIC	MLC	MIC	MLC	MIC	MIC
S. aureus	6.7	16.0	16.0	16.0	2.50 x 10^{-2}	-
S. epidermidis	1.7	1.7	1.7	3.4	6.25 x 10^{-3}	-
M. luteus	16.0	16.0	6.7	6.7	6.25 x 10^{-3}	-
K. pneumoniae	6.7	6.7	6.7	>20.0	1.25 x 10^{-2}	-
E. coli	8.0	>20.0	6.7	16.0	1.25 x 10^{-2}	-
S. setubal	6.7	16.0	16.0	16.0	1.25 x 10^{-3}	-
C.albicans	16.0	>20.0	16.0	16.0	-	8.12 x 10^{-3}
S. cerevisiae	4.0	8.0	2.0	4.0	-	4.06 x 10^{-3}

[a] ATCC (American Type Culture Collection); [b] Mean of 3 replicates in mg mL^{-1}.

Experimental

Plant material

The leaves of about 6-10 individuals of a wild population of *Aloysia sellowii* (to give a representative sample) were collected in two localities of Rio Grande do Sul (Santa Maria and Livramento), in three successive years at flowering time (October- November 2001-2003, one collection/year). Specimens were identified by Prof. Gilberto Zanetti (Dept. of Biology, University of Santa Maria, RS, Brazil). Voucher specimens (HDFI 166-170) were deposited at the Herbarium of the University of Santa Maria, RS, Brazil.

Essential oil isolation and chemical analysis

Fresh plant leaves were subjected to hydro distillation for 4h using a modified Clevenger-type apparatus, followed by exhaustive extraction of the distillate with diethyl ether. After removal of the solvent, the yield of the crude oils was 1.4 % and 1.2 % (m/v) for sample A and B, respectively. The physical properties for sample A and B were: d 25: 0.92; ηd 25: 1.48, $[\alpha]_D^{25}$: + 9.54 (in Hexane, c =0.22) and d 25: 0.89; ηd 25: 1.50, $[\alpha]_D^{25}$: -17.9 (in hexane, c = 0.18), respectively. The essential oils were submitted to GC analysis in a Varian 3800 Gas Chromatograph equipped with a capillary fused silica column (25 m x 0.25 mm; film thickness 0.2 μm) coated with SE-54 (The GC conditions used were: carrier gas H_2 (1 mL min^{-1}); injector split/splitless 220 ^{o}C; FID Detector 280 °C; column temperature 50 ^{o}C to 250 ^{o}C at 4 ^{o}C min^{-1}. GC-MS analyses were performed on a HP 5973-6890 GC-MSD system operating in the EI mode at 70 eV, equipped with a HP-5 crosslinked capillary column (30 m x 0.25 mm; film thickness 0.2 μm). The temperature of the column and the injector were the same as those from GC. The identification of the components of the oils was

based on comparison of the retention times and Kovats Indices ($Ir_i = 100.n + 100.\Delta n.\ tr_i - tr_n / tr_m - tr_n$) [17] on both columns and mass spectra with those of NBS/NIST Library[18] and those described by Adams.[19] The chiral monoterpene constituents of *A. sellowii* oils were identified by peak enrichment in enantioselective capillary GC with two fused capillary columns, 25 m x 0.25 mm, film thickness 0.2 μm, coated with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin and octakis-(6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin, each diluted with the polysiloxane OV-1701 (1:1), using a Varian 3800, equipped with a flame ionization detector (FID), and hydrogen as the carrier gas. All runs were performed with a temperature program from 35 °C for 15 min and 35 °C to180 °C at 3 °C min^{-1}.

Toxicity assays

The preliminary biological evaluation of the essential oils of *A. sellowii* was determined using the brine shrimp lethality test according to Meyer *et al.*[11] with some modifications.[9] The brine shrimp eggs were hatched in artificial sea water (3.8 g sea salt per liter of water) and, after hatching (2 days old), the shrimp larvae were used for experimental bioassay. The oil was dissolved in DMSO and them added to the saline solution containing 3% of tween 20. Emulsification was assisted by ultrasound and the dilutions of this stock solution were made with saline solution containing tween 20. Then, shrimp larvae were placed in multi-welled culture plate and test solution was added to each well (in triplicate). Saline solution with 3% tween 20 and DMSO was used as a negative control (LC50>1000 μg mL^{-1}), while potassium dichromate (LC50= 20 μg mL^{-1}) and cinchonine (LC50= 100 μg mL^{-1}) were used as standard positive controls.[9] Survivors were counted after 24h and the LC50 values were determined in μg mL^{-1}, using the Finney probit analysis[20] obtained values with 95% confidence intervals.

Antibacterial assays

The antibacterial activities of the oils of samples A and B were assayed using the broth micro dilution method. A collection of eight microorganisms were used, including three Gram-positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, three Gram-negative bacteria: *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella setubal*, and two yeasts: *Saccharomyces cerevisiae* and *Candida albicans*. Standard strains of microorganisms were obtained from American Type Culture Collection (ATCC), and standard antibiotics chloramphenicol and nistatine were used in order to control the sensitivity of the microbial test.[21] The minimal inhibitory concentration (MIC) was determined on 96 well culture plates by a micro dilution method using a microorganism suspension at a density of 105 CFU mL^{-1} with Casein Soy Broth incubated for 24 hours at 37 °C for bacteria, and Sabouraud Broth incubated for 72 hours at 25 °C for yeasts. The cultures that did not present growth were used to inoculate plates of solid medium (Muller Hinton Agar and Sabouraud Agar) in order to determine the minimal letal concentration (MLC). Proper blanks were assayed simultaneously and samples were tested in triplicate. Technical data have been described previously.[12, 13]

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