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HPLC/DAD/ESI-MS Analysis of Non-volatile Constituents of Three Brazilian Chemotypes of *Lippia alba* (Mill.) N. E. Brown

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Aqueous preparations and ethanolic extracts of three Brazilian chemotypes of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) were investigated for the chemical variability of their non volatile constituents by HPLC/DAD/ESI-MS analysis. The main class of compounds in all the extracts investigated was phenylpropanoids, mainly verbascoside, followed by the flavonoids tricin-7-*O*-diglucuronide (present in *Lippia alba* chemotypes 2 and 3), luteolin-7-*O*-glucuronide (present in *L. alba* chemotype 1) and mono-and di-*O*-glucuronic derivatives of apigenin and tricin. Four iridoids, geniposidic acid, theveside, 8-*epi*-loganin and mussaenoside were also identified.

Keywords: *Lippia alba*, Verbenaceae, Brazilian chemotypes, flavonoid glycosides, phenylpropanoid glycosides, iridoids.

Lippia alba (Mill.) N. E. Brown (Verbenaceae), is a very common herb in Brazil, where is popularly known as 'Erva-cidreira'. It occurs in all regions of the country as a spontaneous or cultivated plant. In Brazilian folk medicine infusions and decoctions of its leaves are traditionally used as a sedative and for gastrointestinal disorders. Ethanolic preparations are also popularly used for fever, coughs and asthma [1,2].

There are approximately eighteen chemotypes of *L. alba*, mainly based on the composition of their essential oil. Brazilian chemotypes have been classified by Matos and coworkers according to the percentage of citral (chemotype 1), carvone (chemotype 2) and linalool (chemotype 3). However, the last is hardly found in Brazil as a spontaneous plant [3,4].

Several studies have been carried out regarding the characterization of volatile compounds, but to the best of our knowledge nothing has been reported yet concerning the distribution of non-volatile constituents by chemotypes, which would be useful for pharmacological purposes. Since the chemical variability of the three Brazilian chemotypes seems to be important for both the volatile and non-volatile constituents, the present study aimed to investigate the polar extracts of these chemotypes in order to establish differences between them.

Infusions, decoctions and ethanolic extracts of the leaves of three chemotypes of *L. alba* were investigated according to their traditional uses [4,5].

The samples were submitted to HPLC/DAD/ESI-MS analyses in order to obtain a complete characterization of these preparations. In Fig. 1 the HPLC/DAD profiles of the infusions of the three chemotypes of *L. alba* at different wavelengths are presented: 240 nm (monitoring of iridoids), 330 nm (monitoring of phenylpropanoids) and 350 nm (monitoring of flavonoids).

Results are shown in Table 1, where the identified constituents in the extracts of the three chemotypes are presented.

Table 1: Identified compounds in the three extracts of the *Lippia alba* compared by chemotypes.

Cpds	LA1I	LA2I	LA3I	LA1D	LA2D	LA3D	LA1E	LA2E	LA3E
1	+	+	+	+	+	+	-	-	-
2	+	+	+	+	+	+	-	-	-
3	+	+	+	+	+	+	-	-	-
4	t	+	+	t	+	t	-	-	-
5	t	t	t	-	-	-	-	-	-
6	-	t	t	t	+	t	-	-	-
7	t	+	+	t	+	+	-	-	-
8	+	t	t	t	t	t	-	-	-
9	+	-	-	t	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	-
13	+	+	+	t	t	t	-	-	-

Cpds: compounds; **LA**: *Lippia alba*; **1**, **2**, **3**: chemotypes: 1 (citral), 2 (carvone), 3 (linalool);
I: infusion; **D**: decoction; **E**: ethanolic extract.
Iridoid compounds: (**1**) theveside; (**2**) geniposidic acid; (**3**) 8-*epi*-loganin; (**4**) mussaenoside; ***Flavonoid derivatives***: (**5**) apigenin-7-*O*-glucuronide; (**6**) apigenin-7-*O*-diglucuronide; (**7**) tricin-7-*O*-diglucuronide; (**8**) tricin-7-*O*-glucuronide: (**9**) luteolin-7-*O*-glucuronide; ***Phenylpropanoid derivatives***: (**10**) calceolarioside E; (**11**) verbascoside; (**12**) isoverbascoside; (**13**) β-OH-acteoside diastereisomers; **t**: traces; **+** presence of the compound; **-**: absence of compound.

The constituents of the extracts from these chemotypes belong mainly to three classes of compounds: iridoids, flavonoids and phenyl-propanoids. This finding is in good agreement with literature data [5]. Four iridoids, theveside (**1**), geniposidic acid (**2**), 8-*epi*-loganin (**3**) and mussaenoside (**4**), were detected in infusions and decoctions of all chemotypes studied, in accord with literature data [6-8].

Concerning the phenylpropanoid content of the samples, four phenylpropanoids were detected, namely, calceolarioside E (**10**), verbascoside (**11**), isoverbascoside (**12**) (isobaric isomer of verbascoside), and a pair of diastereoisomeric forms of β-OH-acteoside (**13**). Compound **13** was detected in infusions and decoctions, but not in the ethanolic extracts. Phenylpropanoids **10**-**12** have been previously reported in the literature for *L. alba* whereas β-OH-acteoside was detected for the first

Table 2: Positive and negative MS fragmentation and Uv-vis absorption data of the compounds detected in the three chemotypes of *Lippia alba*.

Compound	t_R (min)	UV-vis Λ_{max} (MeOH)	eV	MW	ESI-MS$^+$ *m/z* (rel. intensity, %)	ESI-MS$^-$ *m/z* (rel. intensity, %)
Theveside (**1**)	3.8	236	80	390	413 (100) $[M+Na^+]^+$; 429 (20) $[M+K^+]^+$	389 (100) $[M-H]^-$; 227 (20) $[Aglycone-H]^-$
8-*epi*-loganin (**2**)	4.4	238	80	390	413 (72) $[M+Na^+]^+$; 429 (24) $[M+K^+]^+$	389 (100) $[M-H]^-$; 227 (14) $[Aglycone-H]^-$
Geniposidic acid (**3**)	4.8	234	80	374	397 (100) $[M+Na^+]^+$; 413 (25) $[M+K^+]^+$; 787 (5) $[M+K^+]^+$	373 (73) $[M-H]^-$; 211 (30) $[Aglycone-H]^-$
Mussaenoside (**4**)	12.6	238	80	390	413 (50) $[M+Na^+]^+$; 429 (36) $[M+K^+]^+$; 229 (20) $[Aglycone+H]^+$	389 (100) $[M-H]^-$; 227 (18) $[Aglycone-H]^-$
Apigenin 7-*O*-glucuronide (**5**)	25.1	342	180	446	447 (100) $[M+H]^+$; 271 (12) $[Aglycone+H]^+$	445 (35) $[M-H]^-$; 285 (100) $[Aglycone-H]^-$
Apigenin 7-*O*- diglucuronide (**6**)	18.4	350	180	622	623 (100) $[M+H]^+$	621 (45) $[M-H]^-$; 269 (38) $[Aglycone-H]^-$; 351 (100) $[M-H-Aglycone]^-$ or $[(2x\ glucuronic\ acid)-H]^-$
Tricin 7-*O*-diglucuronide (**7**)	19.4	350	180	682	683 (100) $[M+H]^+$	681 (78) $[M-H]^-$; 329 (12) $[Aglycone-H]^-$; 351 (100) $[(2x\ glucuronic\ acid)-H]^-$
Tricin 7-*O*-glucuronide (**8**)	25.6	348	180	506	507 (100) $[M+H]^+$; 331 (10) $[Aglycone]^+$	505 (100) $[M-Hx]^-$; 329 (12) $[Aglycone-H]^-$; 351 (100) $[(2\ glucuronic\ acid)-H]^-$
Luteolin 7-*O*-glucuronide (**9**)	21.8	350	180	462	463 (100) $[M+H]^+$	461 (35) $[M-H]^-$; 285 (100) $[Aglycone-H]^-$
Calceolarioside E (**10**)	21.2	330	180	610	-	609 (100) $[M-H]^-$; 447 (20) $[M-H-162]^-$
Verbascoside (**11**)	22.3	330	180	624	-	623 (100) $[M-H]^-$; 461 (9) $[M-H-caffeic\ acid]^-$; 161 (20) $[caffeic-\ acid-H_2O-H]^-$
Isoverbascoside (**12**)	23.9	330	180	624	-	623 (100) $[M-H]^-$; 461 (9) $[M-H-caffeic\ acid]^-$; 161 (20) $[caffeic-\ acid-H_2O-H]^-$
β-OH-acteoside diastereoisomers (**13**)	16.7/17.3	330	180	640	-	639 (100) $[M-H]^-$; 621 (30) $[M-H-H_2O]^-$; 459 (16) $[M-H-caffeic\ acid-\ H_2O]^-$; 179 (20) $[caffeic\ acid-H]^-$

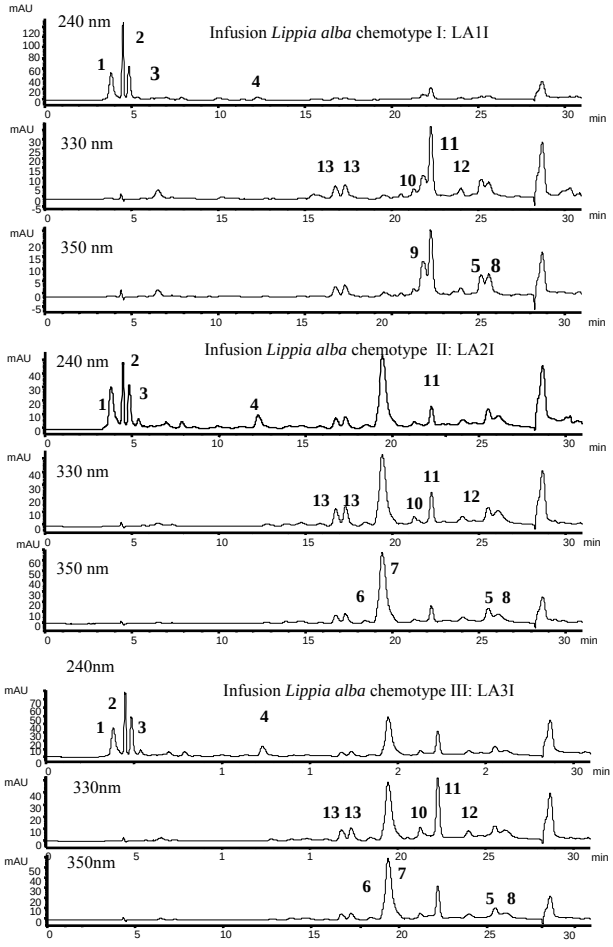

Figure 1: Chromatographic profiles at 240, 330 and 350 nm of a representative samples (infusions) of the three chemotypes of *Lippia alba* considered. Compound **7** was only detected in chemotypes 2 and 3, and compound **9** in chemotype 1.

time in this plant. Its presence was confirmed by, comparing UV, MS and retention time data with those reported previously for this compound [9,10].

Finally, five flavonoids, namely apigenin-7-*O*-monoglucuronide (**5**), apigenin-7-*O*-diglucuronide (**5**); tricin-7-*O*-monoglucuronide (**8**) tricin-7-*O*-diglucuronide (**7**) and luteolin-7-*O*-glucuronide (**9**), were detected, in accordance with literature data [11,12].

As shown in Table 1, and as expected, the main class of compounds of *L. alba* was phenylpropanoids, represented mainly by verbascoside (**11**), followed by the flavonoids tricin-7-*O*-diglucuronide (**7**) and luteolin-7-*O*-glucuronide (**9**). From the results described above, the flavonoids **7** and **9** could be used as markers to distinguish the three Brazilian chemotypes of *L. alba,* since **7** seems to be present only in chemotypes 2 and 3, and compound **9**, only in chemotype 1.

In addition, a pair of diastereoisomeric forms of β-OH-acteoside (**13**) was detected. These are an analogue of verbascoside with a hydroxyl group in the β position: 3,4-dihydroxyl-phenyl-ethanol moiety.

Table 2 reports the UV-vis absorptions and MS fragmentation profiles of all the compounds detected in *L. alba* infusions, decoctions and ethanolic extracts.

To the best of our knowledge, this is the first time that β-OH-verbascoside (**13**) has been found in *L. alba*, and tricin-7-*O*-diglucuronide (**7**) in the *Lippia* genus.

For each chemotype, only small differences were observed among the three preparations. However, differences were detected between the chemotypes, especially regarding the flavonoids (Figure 1). These differences should be taken into consideration when pharmacological studies are carried out.

Experimental

Chemicals: All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were purchased from Merck (Darmstadt, Germany). Formic acid (85 %) was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA). A 0.45 mm PTFE membrane filter was purchased from Waters Co. (Milford, MA).

Plant material: Dried leaves of three cultivated chemotypes of *L. alba* (Mill.) N. E. Brown, were collected in 2008 from the Herbarium CESJ (Federal University of Juiz de Fora), MG, Brazil.

Herbal preparations: *Infusions*: Dried powered leaves of each chemotype of *L. alba* (1 g) were extracted with 20 mL of boiling water. The mixture was cooled for 20 min and then filtered. *Decoctions*: Dried powered leaves of three chemotypes of the plant (1 g) were put in 20 mL of water and both were boiled for 2 min before filtration. *Ethanolic extracts*: Dried powered leaves of each chemotype of *L. alba* (1 g) were extracted three times successively with EtOH for 24 h each time. All preparations were lyophilized and then freeze-dried. For HPLC-DAD-MS analysis, the samples were obtained by dissolving and filtering the solid residues (1 mg exactly weighed) in 1 mL of MeOH.

General experimental procedures: HPLC/DAD/ESI-MS analysis was performed on a HP 1100 L instrument with DAD and managed by a HP 9000 workstation interfaced with a HP 1100 MSD API-USA. The column used was a Varian Polaris TM C18-E (250 x 4.6 mm i.d., 5 µm maintained at 26°C. Eluents were H_2O adjusted to pH 3.2 with formic acid (A), and acetonitrile (B). A multi-step linear gradient was applied from 87% A to 85% in 10 min; in 10 min to 75% B and a plateau for 3 min; 2 min to 95% CH_3CN and a final plateau for 3 min. Total time analysis was 28 min, and equilibration time 10 min. Flow rate: 0.8 mL min $^{-1}$. Oven temperature: 26°C. UV-vis spectra were registered between 220-500 nm and the chromatographic profiles were registered at 240, 330 and 350 nm. Mass spectrometry conditions were optimized in order to achieve sensitive values: negative and positive ionisation mode, scan spectra from *m/z* 100 to 800, gas temperature: 350°C, nitrogen flow rate: 10L min^{-1}, nebulizer pressure 30 psi, quadrupole temperature: 30°C, capillary voltage: 3500 V. Applied fragmentors range: 80-180 V.

Identification of constituents was carried out by HPLC/DAD/ESI-MS analysis. UV-vis and mass spectra of the peaks were compared with those of authentic standards, previously isolated compounds and literature data.

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