JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 933–939

Received October 23, 2008
Accepted April 07, 2009
DOI 10.1211/jpp/61.07.0013
ISSN 0022-3573

Research Paper

The role of polar phytocomplexes on anticonvulsant effects of leaf extracts of *Lippia alba* (Mill.) N.E. Brown chemotypes

Antônio C. Neto[a], Joaquim C. Netto[b], Paulo S. Pereira[a], Ana M.S. Pereira[a], Sílvia H. Taleb-Contini[a], Suzelei C. França[a], Márcia O.M. Marques[c] and René O. Beleboni[a]

[a]Unidade de Biotecnologia, Universidade de Ribeirão Preto, [b]Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo and [c]Instituto Agronômico de Campinas, Campinas, São Paulo, Brasil

Abstract

Objectives The purpose of the present work was to characterize the pharmacological profile of different *L. alba* chemotypes and to correlate the obtained data to the presence of chemical constituents detected by phytochemical analysis.

Methods Essential oils from each *L. alba* chemotype (LP1–LP7) were characterized by gas chromatography–mass spectrometry (GC-MS) and extracted non-volatile compounds were analysed by HPLC and GC-MS. The anticonvulsant actions of the extracted compounds were studied in pentylenetetrazole-induced clonic seizures in mice and their effect on motor coordination was studied using the rota-rod test in rats. The synaptosomes and synaptic membranes of the rats were examined for the influence of LP3 chemotype extract on GABA uptake and binding experiments.

Key findings Behavioural parameters encompassed by the pentylenetetrazole test indicated that 80% ethanolic extracts of LP1, LP3 and LP6 *L. alba* chemotypes were more effective as anticonvulsant agents. Neurochemical assays using synaptosomes and synaptic membranes showed that *L. alba* LP3 chemotype 80% ethanolic extract inhibited GABA uptake and GABA binding in a dose-dependent manner. HPLC analysis showed that LP1, LP3 and LP6 80% ethanolic extracts presented a similar profile of constituents, differing from those seen in LP2, LP4, LP5 and LP7 80% ethanolic extracts, which exhibited no anticonvulsant effect. GC-MS analysis indicated the occurrence of phenylpropanoids in methanolic fractions obtained from LP1, LP3 and LP6 80% ethanolic extracts and also the accumulation of inositol and flavonoids in hydroalcoholic fractions.

Conclusions Our results suggest that the anticonvulsant properties shown by *L. alba* might be correlated to the presence of a complex of non-volatile substances (phenylpropanoids, flavonoids and/or inositols), and also to the volatile terpenoids (β-myrcene, citral, limonene and carvone), which have been previously validated as anticonvulsants.

Keywords anticonvulsants; *Lippia alba* (Mill.) N.E. Brown chemotypes; GABA; polar complexes

Introduction

Lippia alba (Mill.) N.E. Brown (Verbenaceae) (syn. *Lippia geniculata* HBK or *Lantana alba* Mill.) is a shrub, about 0.5–1.0 m high, commonly found in Brazil and popularly known as '*cidreira*' or '*falsa melissa*' due to its sensorial properties.[1] The genus *Lippia* encompasses several species mainly from the Americas and Africa. Geographic distribution, environmental and plant physiological conditions may contribute to the great morphological and chemical diversity of *L. alba* chemotypes, probably accounting for their different pharmacological properties.[2,3]

A decoction or infusion of *L. alba* leaves is commonly used in Brazilian folk medicine as a sedative and for treatment of gastrointestinal disorders, among other uses. The actions of *L. alba* on the central nervous system (CNS), such as sedative and anticonvulsant effects, have been mainly attributed to the volatile constituents found in essential oil, such as β-myrcene, citral, limonene and carvone.[4,5] Despite the presence of volatile compounds in

Correspondence: Prof. Dr René Oliveira Beleboni, Unidade de Biotecnologia, Universidade de Ribeirão Preto, Av. Costabile Romano, 2201, Ribeirânia, 14096-380, Ribeirão Preto, SP, Brasil.
E-mail: rbeleboni@unaerp.br; reneusp@yahoo.com

the infusions or decoctions not being completely ruled out, the presence of non-volatile constituents should be taken into account for a better characterization of CNS activities of *L. alba*, as previously reported by Zetola *et al*.[1]

The purpose of this work was to characterize the pharmacological profile of different *L. alba* chemotypes and to correlate the obtained data to the presence of chemical constituents detected by phytochemical analysis. Moreover, considering that the knowledge of the molecular mechanisms involving *L. alba* CNS activity is still in a preliminary stage, we investigated the possible mode of anticonvulsant action of a selected *L. alba* chemotype (LP3) on GABA neurotransmission.

Materials and Methods

Chemicals and reagents

4-Amino-*n*-[2,3-[^{3}H]-butyric acid ([^{3}H]-GABA) was purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK) and the ScintVerse liquid scintillation fluid from Fisher Scientific (Pittsburgh, USA). Diazepam and pentylenetetrazole (PTZ) were from Sigma (St Louis, USA). Reagents *N, O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Aldrich-Sigma (Milwaukee, USA), while apigenin, luteolin and naringenin were from Fluka Riedel-deHaen (Sigma-Aldrich Kft, Budapest, Hungary). Solvents of analytical grade were from Synth (São Paulo, Brazil) and all other reagents from Merck (Darmstadt, Germany) or Reagen (Rio de Janeiro, Brazil).

Animals

Swiss albino male mice, 18–25 g, and Wistar albino male rats, 150–250 g, bred at the ANILAB Company (Paulínia, Brazil) and at the University of São Paulo (Ribeirão Preto, Brazil), respectively, were housed in standard cages under a 12-h light–dark cycle at room temperature with free access to rat platelet food and water. All animal experimental procedures followed the guidelines established by the Guide for the Care and Use of Laboratory Animals/Brazilian Society for Neuroscience and Behavior. This work received the approval of the Ethics Committee at the Universidade de Ribeirão Preto-UNAERP (Official Number: 137/08).

L. alba (Mill.) N.E. Brown cultivation

One hundred individual specimens of different *L. alba* (Mill.) N.E. Brown chemotypes (LP1–LP7) were collected in São Paulo State, Brazil, in September 2005 and cultivated up to February 2006, according to an agronomic protocol designed to provide equal conditions of plant development. Voucher specimens (1304–1310) were deposited at the Herbarium of Ribeirão Preto University, São Paulo, Brazil.

Collection of *L. alba* leaves, essential oil extraction and ethanol extract preparation

Leaves from each *L. alba* chemotype, collected in February 2006, were dried and pulverized. To obtain essential oils, *L. alba* dry material (20 g) was added to 0.25 L of distilled water and extracted for 2 h using a Clevenger type equipment. Crude ethanol extracts were prepared using 100 g of dried and pulverized leaves macerated with 80% ethanol for 72 h (25°C). After filtration, the organic solvent was concentrated. The dried extract was dissolved in Milli Q water before use in the behavioural and neurochemical assays.

Phytochemical analysis

Volatile compound analysis

Essential oils obtained from each *L. alba* chemotype (LP1–LP7) were characterized in a Shimadzu QP-5000 gas chromatograph coupled to a mass spectrometer, with a DB-5 (30 m × 0.25 mm × 0.25 μm) capillary column, under the following conditions: helium as carrier gas at 1.0 ml/min; injector split at 240°C (split ratio: 1/20); detector at 230°C; electron impact ionization at 70 eV; and oven temperature programmed from 60°C up to 240°C at 3°C/min. Volatile constituents were characterized individually, by comparison of mass spectra with software Nist 62 lib. and by calculation of Kovats retention index.[6,7]

Non-volatile compound analysis

Hydroalcoholic and methanolic fractions were prepared as follows. Dry powdered 80% ethanolic extracts (LP1–LP7) were separately dissolved in 20% methanol (0.01 g/ml). Samples were filtered (0.45 μm;, Millex HN nylon; Millipore, Billerica, USA) and the supernatant applied into a C18 SPE cartridge (Supelclean LC-18 SPE; Supelco, Bellefonte, USA). To obtain the hydroalcoholic fractions, the cartridge was eluted with 6 ml 20% (v/v) methanolic solution on an SPE manifold (Supelclean, Visiprep; Supelco) at a flow rate of 2 ml/min. After this procedure, the cartridge was dried under vacuum and then 6 ml of pure methanol was passed through the cartridge, producing the methanolic fractions. Both types of fraction were dried under nitrogen and analysed by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS).

The HPLC analysis was carried out on a Shimadzu LC10AD*vp* system equipped with Supelco LC18 column (Supelcosil RP-18, 250 × 4.6 mm i.d.), coupled to a diode array detector, monitored at 210–340 nm. A gradient system of orthophosphoric acid 0.03% (A) and acetonitrile (B) was used. The gradient programme consisted of six periods: (1) 0–20 min, 7–17% B; (2) 20–30 min, 17% B; (3) 30–45 min, 17–25% B; (4) 40–60 min, 25–40% B; (5) 60–65 min, 40–10% B; and (6) 65–70 min, 10% B. The flow-rate and the sample injection volume were 1.0 ml/min and 20 μl (10 mg/ml), respectively.

The GC-MS analysis of all hydroalcoholic and methanolic fractions was performed on a Varian Saturn 3900 GC/2100MS/injector CP8410. The GC was fitted with a VF-5ms (WCOT fused silica) capillary column (30 m × 0.25 mm i.d.). Volumes (1 μl; 10 mg/ml) of sample solutions in chloroform were injected into the capillary column. Helium at 1 ml/min was used as the carrier gas and the injector temperature was 250°C. The column was programmed from 80°C (2 min) to 290°C (30 min) at 10°C/min. The MS was operated in the scan mode (m/z 50–600 amu). EI spectra were obtained at 70 eV.

Hydrolysis of samples and derivatization procedures were carried out as follows. Dried fractions obtained from the SPE cartridge extraction were introduced into a vial containing 4 ml 2 M HCl solution. Hydrolyses were performed for 90 min, at 80°C. After cooling, fractions were extracted with 10 ml of ethyl acetate and the organic solvent was evaporated. Following this procedure, ethyl acetate residues were trimethylsilylated by adding 50 μl of pyridine and 100 μl of silylating agent containing BSTFA + TMCS (1000 : 10, v/v). The vial was vortex mixed and heated at 80°C for 90 min. After cooling, 50 μl of chloroform was added to the derivatized solutions and the trimethylsilyl (TMS) derivatives were submitted to GC-MS analysis.

Behavioural assays

Anticonvulsant activity

All *L. alba* chemotype 80% ethanolic extracts (LP1–LP7) were evaluated for anticonvulsant activity against pentylenetetrazole (PTZ)-induced clonic seizures according to the procedure described by Velisek *et al.*[8] Male Swiss mice allocated to control (n = 7) and experimental (n = 7) groups were treated with vehicle (saline 100 μl, i.p), diazepam (1 mg/g, kg, i.p) or with each *L. alba* chemotype extract (300 mg/kg) 60 min before the injection of convulsant agent PTZ (80 mg/kg, i.p). The mice were observed during 30 min, inclusive for the latency time for clonic convulsions registration. Crisis duration and the number of mice that presented clonic convulsions as well as the percentage of animal mortality were recorded. Also, the crisis intensity or severity was denoted, based on the score as follows: 0, absence of convulsive behaviour; 1, myoclonia; 2, clonic seizures without postural reflex impairment; 3, clonic seizures with postural reflex impairment; 4, seizures with hind paw extension; and 5, tonic extension and animal death. Statistical significance was assessed using analysis of variance followed by Dunnett's test ($P < 0.05$).

Rota-rod test

The motor coordination performance of rats treated with saline (100 μl, i.p.), diazepam (10 mg/kg, i.p.) and LP1–LP7 extracts (300 mg/kg, i.p.) was measured using a rota-rod apparatus (Acceler Rota-rod, Jones & Roberts, for rats 7750; Ugo Basile, Comerio, Italy) at a rotating speed of 8 rev/min, 60 min after treatments. The time of each rat remaining on the bar and the number of falls per group were registered for 2 min. Statistical significance was assessed using analysis of variance followed by Dunnett's test ($P < 0.05$).

Neurochemical assay

Preparation of synaptosomes and synaptic membranes

Rat cerebral cortices were rapidly removed and homogenized in ice-cold 0.32 M sucrose using a Potter-Elhvejen, Labo Stirrer LS-50 Yamato type equipment. The sample was centrifuged for 10 min at 1700g (4°C) and the supernatant centrifuged for 20 min at 21 200g (4°C). The pellet was resuspended in Krebs-phosphate buffer (in mM: NaCl 124; KCl 5; KH_2PO_4 1.2; $CaCl_2$ 0.75; $MgSO_4$ 1.2; Na_2HPO_4 20; glucose 10; pH 7.4), referred to as synaptosome P2 fraction and used in the GABA uptake assay. The protein content was determined according to Lowry *et al.*[9] as modified by Hartree.[10]

For synaptic membrane preparation, the synaptosome P2 fraction was homogenized in 5 mM Tris-HCl buffer (pH 7.4) and centrifuged for 5 min at 3000g (4°C). The pellet was carefully washed in 50 mM Tris-HCl buffer (pH 7.4) and finally suspended in the same buffer. The synaptic membranes had their protein content assessed as described above, centrifuged (3000g/5 min/4°C) and stored at −20°C for at least 24 h before use in the GABA binding assay.

GABA uptake and binding assays

Uptake and binding assays were respectively started by adding 5 nM or 10 nM radio-labelled [^{3}H]-GABA to synaptosomal suspensions (0.1 mg of protein/ml) or synaptic membranes (0.5–1.0 mg of protein/ml) in the presence or absence of increasing concentrations (0.001235–0.1 mg/ml) of LP3 *L. alba* chemotype 80% ethanolic extract in a final volume completed with 50 mM Tris-HCl buffer (0.3 ml for binding assay) or Krebs-phosphate buffer (0.5 ml for uptake assay). Samples were incubated for 5 and 30 min at 25°C for GABA uptake and GABA binding assays, respectively.

All reactions were carried out in triplicate and stopped by centrifugation (3000g, for 3 min at 4°C). Pellets were washed twice with ice-cold distilled water and homogenized in 10% trichloroacetic acid for the GABA uptake experiment, or in 50 mM Tris-HCl buffer for the GABA binding assay, and then centrifuged at 3000g, for 3 min at 4°C for the uptake experiments. Samples of supernatants or whole tube content, in the case of uptake and binding experiments, respectively, were transferred to scintillation vials containing 5 ml of the biodegradable scintillation fluid ScintiVerse. Radioactivity was quantified in a scintillation counter (Beckman, LS-6800) with a counting efficiency of 35–40% for [^{3}H]. Non-specific GABA uptake and GABA binding were respectively estimated in parallel probes with nipecotic acid (6 mM, final concentration) or non-radiolabelled GABA (1 mM, final concentration). These values were subtracted from those of total GABA uptake or GABA binding. Results were expressed as % of inhibition of GABA uptake or GABA binding in relation to control. Statistical significance was assessed using analysis of variance followed by Dunnett's test ($P < 0.05$).

Results

Phytochemical analysis

Table 1 shows results of the phytochemical analysis of the essential oils from *L. alba* chemotypes performed by GC-MS. Each of the chemotypes presented a great variety of volatile constituents. Myrcene was particularly found in essential oils of *L. alba* chemotypes LP2 (4.95%), LP3 (15.42%), LP4 (52.03%), LP6 (14.07%) and LP7 (1.75%), while limonene was found in chemotypes LP4 (1.43%), LP5 (17.54%) and LP7 (16.62%) and carvone in LP1 (5.53%) and LP5 (78.46%). Z-citral was abundant in LP2 (36.69%), LP3 (33.49%), LP6 (35.98%) and LP7 (38.32%). Linalool was found only in LP1 *L. alba* chemotype essential oil (77.95%).

HPLC analysis of LP1, LP3 and LP6 80% ethanolic extracts presented a similar profile of constituents, while this

Table 1 Volatile compounds (%) found in *L. alba* chemotypes essential oils (LP1–LP7)

Oil compound	IK	IK[a]	LP1	LP2	LP3	LP4	LP5	LP6	LP7
α-Pinene	932	926	–	–	–	5.62	–	–	–
Sabinene	970	967	0.77	–	–	4.25	–	–	0.92
6-Methyl-5-hepten-2-one	979	985	–	trace	1.06	–	–	2.13	3.29
Myrcene	988	991	–	4.95	15.42	52.03	–	14.07	1.75
Limonene	1026	1031	–	trace	–	1.43	17.54	–	16.62
1,8-Cineole	1027	1033	8.80	–	–	–	–	–	–
Linalool	1098	1101	77.95	–	–	–	–	–	–
Trans-sabinol	1140	1140	–	–	–	7.80	–	–	–
Neo-iso-dihydro carveol	1225	1226	–	1.36	–	–	–	2.13	3.29
Myrtenyl acetate	1235	1235	–	24.27	23.4	–	–	25.58	29.13
Carvone	1244	1242	5.53	–	–	–	78.46	–	–
Geraniol	1250	1255	–	1.53	–	–	–	–	0.95
Geranial (*Z*-citral)	1265	1270	–	36.69	33.49	–	–	35.98	38.32
Geranyl acetate	1379	1383	–	–	–	–	–	–	0.52
β-Bourbonene Transcaryophyllene	1383	1384	–	–	–	–	–	–	0.44
Transcaryophyllene	1419	1418	2.13	11.00	14.17	7.07	–	12.14	–
α-Guaiene	1438	1439	–	–	–	5.32	–	–	–
α-Humulene	1452	1454	–	–	–	2.39	–	–	–
Germagrene D	1480	1480	–	2.13	1.77	3.65	1.94	–	4.69
α-(*E*-*E*)-Farnesene	1504	1508	–	–	–	4.41	–	–	–
Cubebol	1514	1514	–	–	–	–	–	–	0.35
Caryophyllene oxide	1580	1581	–	5.71	3.99	–	–	4.30	–
Total %			95.18	87.64	94.12	85.17	97.94	94.20	96.79

IK, Experimental Kovats index; LP1–LP7, *Lippia alba* chemotypes; –, not found. [a]IK taken from Adams[7].

profile was different from those seen in LP2, LP4, LP5 and LP7 80% ethanolic extract (data not shown).

GC-MS analysis of the methanolic and hydroalcoholic fractions from *L. alba* 80% ethanolic extracts allowed the detection of different components and their identification (Figures 1a, 2a). The identity of phenylpropanoids from methanolic fractions (Figures 1b–1d) and inositol from hydroalcoholic fractions (Figure 2b) obtained from LP1, LP3 and LP6 chemotype 80% ethanolic extracts were verified by GC-EI/MS data compared with those in the National Institute of Standard and Technology (NIST; Washington, USA) mass library. Those classes of compounds were not found in LP2, LP4, LP5 and LP7 extracts, which showed no anticonvulsant action.

The trimethylsilylated flavonoid derivatives of synthetic apigenin, naringenin and luteolin were individually analysed by GC-EI-MS under the same conditions described above (data not shown). The retention times obtained and the fragmentation patterns showed in EI/MS spectra obtained from hydroalcoholic fractions (Figure 2c, 2d) confirmed the presence of that class of compound in LP1, LP3 and LP6 chemotypes, but not in LP2, LP4, LP5 and LP7 80% ethanolic extracts.

Anticonvulsant activity and effect on motor coordination of *L. alba* chemotypes 80% ethanolic extracts

Table 2 shows the different pharmacological profiles of the seven *L. alba* chemotype 80% ethanolic extracts (LP1–LP7) when measured by the PTZ seizure test in mice. Extracts from all the *L. alba* chemotypes were tested at the same dose (300 mg/kg).

The control group treated with saline presented the highest percentage of mice not protected against seizures induced by PTZ (76.20%), while all mice treated with diazepam (1 mg/kg) were protected. Only the mice treated with 80% ethanolic extracts from LP1, LP3 and LP6 *L. alba* chemotypes were significantly protected against seizures. It was found that 23.81% (LP1 and LP6) and 28.57% (LP3) of mice exhibited seizures, which meant that the treated mice developed seizures at an incidence of about 2- to 3-fold lower than mice in the saline control group.

Unlike diazepam, no experimental group (LP1–LP7) was effective in decreasing the latency time for convulsion. However, treatment with extracts of *L. alba* chemotypes LP1 (14.03 ± 2.40 s), LP3 (9.22 ± 4.0 s) and LP6 (15.00 ± 7.0 s) was effective in decreasing seizure duration in relation to the saline control group (20.86 ± 6.28 s), while treatment with LP2, LP4, LP5 and LP7 extract was not effective.

Regarding the degree of seizure severity/intensity, only those groups treated with diazepam (1.3 ± 0.14) or with *L. alba* chemotype extracts of LP1 (3.1 ± 1.15) and LP3 (3.2 ± 1.07) presented significant decreased scores when compared with the saline group (4.5 ± 0.16).

The percentage of animal mortality was significantly lower in the groups treated with diazepam (0.0%) and *L. alba* chemotypes extracts of LP1 (23.81%), LP3 (28.57%) and LP6 (23.81%) when compared with the control group treated with saline (76.20%).

Considering the behavioral parameters encompassed by the PTZ test, extracts of chemotypes LP2, LP4, LP5 and LP7 showed no anticonvulsant activity.

Unlike diazepam (10 mg/kg, i.p.), when compared with the saline group, LP1–LP7 80% ethanolic extracts did not

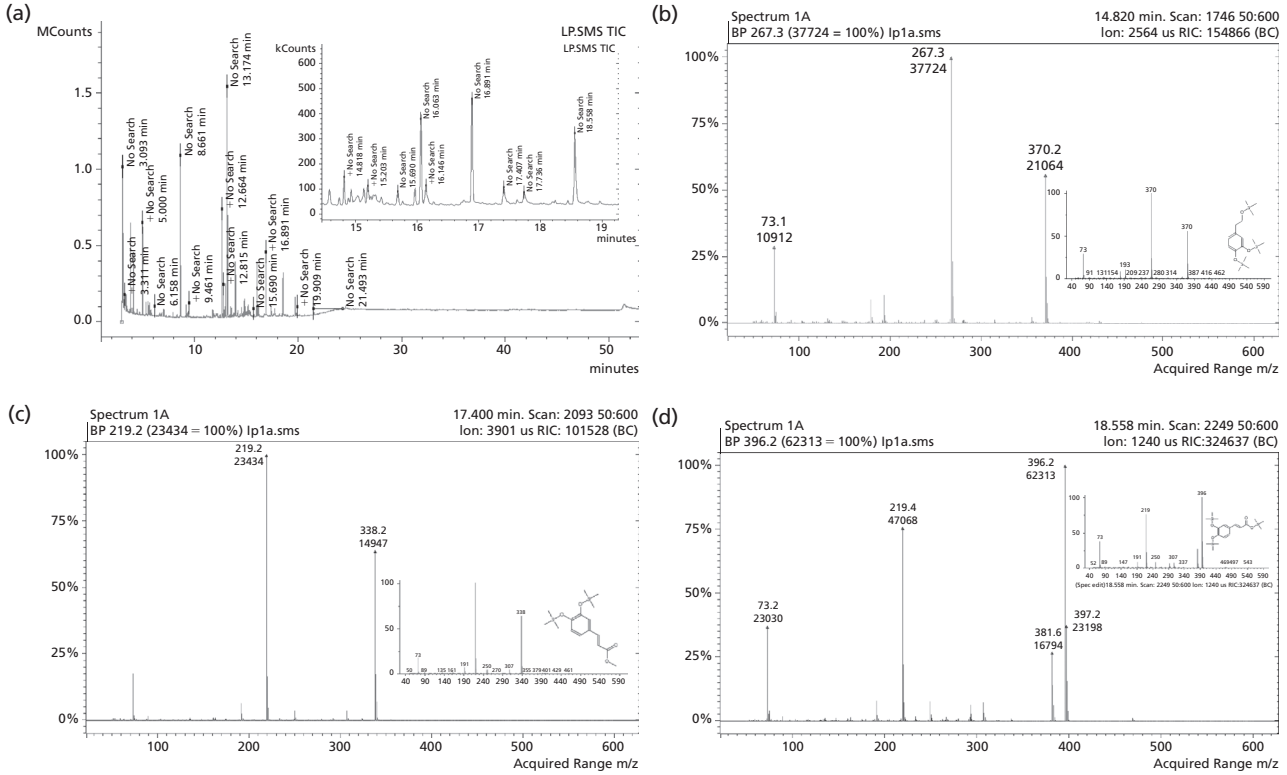

Figure 1 Representative GC chromatogram of methanolic fractions from LP1, LP3 and LP6 chemotypes (a). Mass spectra of phenylpropanoid class compounds (b–d).

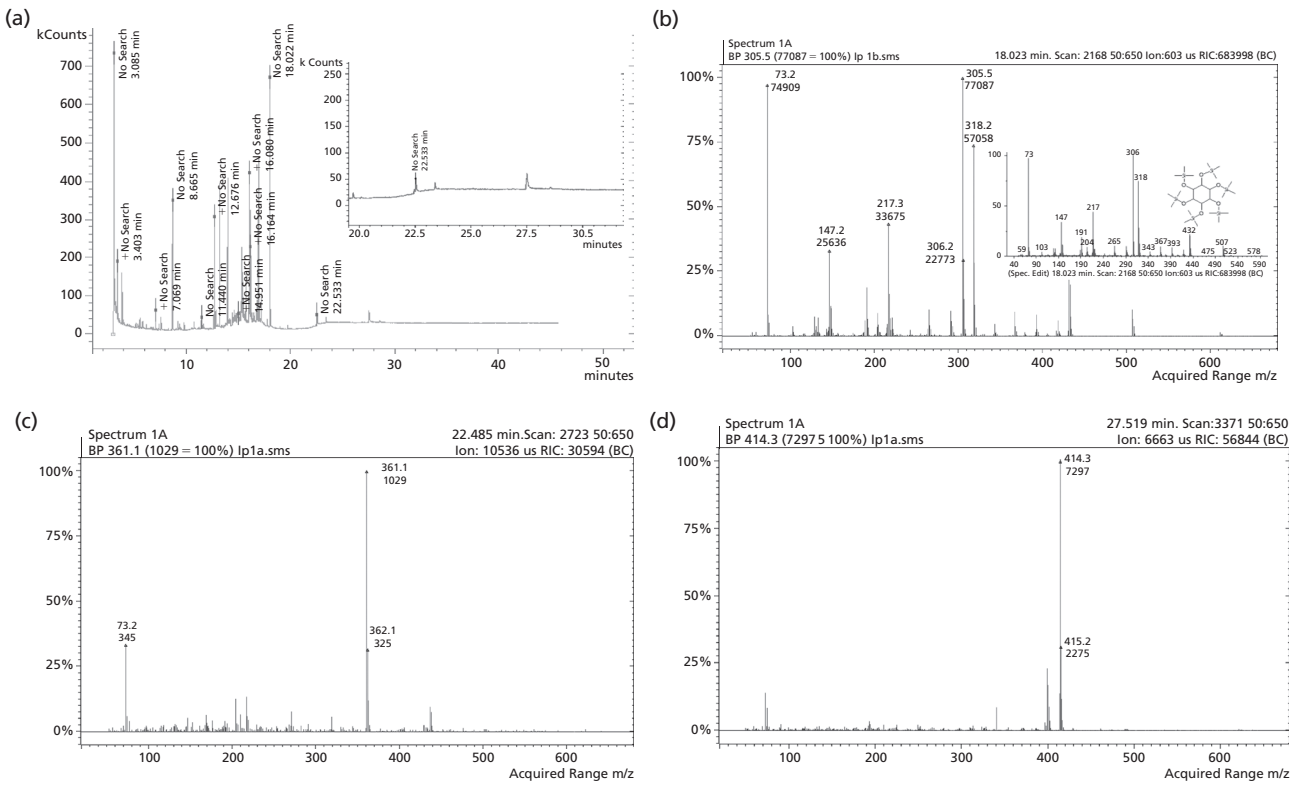

Figure 2 Representative GC chromatogram of hydroalcoholic fractions from LP1, LP3 and LP6 chemotypes (a). Mass spectra of inositol (b) and flavonoidic compounds (c, d).

Table 2 Pharmacological profile of *L. alba* chemotypes (LP1–LP7; 300 mg/kg) when assessed against pentylenetetrazole seizure test in mice

Sample	Score	Animal mortality (%)	Latency (min)	First seizure duration (s)	Seizure protection (%)
Saline	4.5 ± 0.16	76.20 ± 8.25	6.26 ± 2.31	20.86 ± 6.28	23.81 ± 8.24
Diazepam	1.3 ± 0.14**	0.00*	–	–	100.00*
LP1	3.1 ± 1.15**	23.81 ± 21.82*	4.90 ± 5.71	14.03 ± 2.40*	76.19 ± 21.82*
LP2	3.8 ± 0.50	42.86 ± 28.57	8.70 ± 2.86	23.36 ± 11.26	57.14 ± 28.57
LP3	3.2 ± 1.07**	28.57 ± 24.74*	5.49 ± 4.89	9.22 ± 4.0*	71.43 ± 24.74*
LP4	4.0 ± 0.54	42.86 ± 28.57	7.71 ± 1.97	23.50 ± 6.42	57.14 ± 28.57
LP5	4.0 ± 0.43	38.10 ± 8.25	9.82 ± 5.58	23.70 ± 9.30	61.90 ± 8.25
LP6	3.4 ± 0.72	23.81 ± 8.25*	6.25 ± 5.51	15.00 ± 7.0*	76.19 ± 8.24*
LP7	3.9 ± 0.65	47.62 ± 21.82	13.29 ± 6.35	21.50 ± 6.11	52.38 ± 21.82

*$P < 0.05$, **$P < 0.01$, compared with saline group (analysis of variance followed by Dunnett's test).

cause alterations in rat motor coordination at the same dose used in the PTZ test (300 mg/kg, i.p.) (data not shown).

GABA uptake and GABA binding assays

Table 3 shows the effect of increasing concentrations of LP3 *L. alba* chemotype 80% ethanolic extract on [^{3}H]-GABA uptake and [^{3}H]-GABA binding in rat cerebral cortex synaptosome P2 fraction and synaptic membrane. In both cases, the inhibitory effect promoted by the LP3 extract was in a dose-dependent manner. The maximum inhibition induced by LP3 ethanolic extract for uptake and binding assays was 50.31% and 58.40, respectively, at a dose of 0.1 mg/ml.

Discussion

In this work, leaves from seven chemotypes, LP1–LP7, proved to be distinguished by their essential oil composition, were used as source of 80% ethanolic extracts used in the PTZ test, and, in the case of the LP3 chemotype, for investigations at a molecular level on GABA neurotransmission in rats.

The PTZ seizure test indicated a variable anticonvulsant efficacy among different *L. alba* chemotypes. Ethanolic extracts of the *L. alba* chemotypes were not efficient enough to improve all behavioural parameters measured by the PTZ test, at least to the same extent observed for the group treated with diazepam. LP1, LP3 and LP6 *L. alba* 80% ethanolic extracts were particularly efficient in protecting the mice against seizures, and in decreasing seizure duration, degree of crisis severity and animal mortality. Considering all behavioural parameters measured by the PTZ test, the LP1, LP3 and LP6 *L. alba* chemotypes could be considered the most pharmacologically effective, since chemotypes LP2, LP4, LP5 and LP7 demonstrated no anticonvulsant activity.

Table 3 Dose–response inhibitory effect of increasing concentrations of LP3 *L. alba* chemotype extract on [^{3}H]-GABA uptake and [^{3}H]-GABA binding using synaptosome P2 fraction and synaptic membranes from rat cerebral cortex, respectively

Treatment	^{3}[H]-GABA uptake inhibition (%)	^{3}[H]-GABA binding inhibition (%)
Control	0.00 ± 6.11	0.00 ± 15.61
LP3 (0.001235 mg/ml)	12.44 ± 6.74	26.02 ± 7.24**
LP3 (0.0037 mg/ml)	18.39 ± 8.38	32.09 ± 4.70**
LP3 (0.011 mg/ml)	24.46 ± 5.65*	48.53 ± 3.77**
LP3 (0.033 mg/ml)	38.16 ± 7.61**	56.98 ± 4.41**
LP3 (0.1 mg/ml)	50.31 ± 5.99**	58.40 ± 12.71**

Data were obtained from three independent experiments and performed in triplicate. *$P < 0.05$, **$P < 0.01$, compared with saline control group (analysis of variance followed by Dunnett's test).

Comparative chromatographic analysis by HPLC of assayed hydroalcoholic and methanolic fractions demonstrated that LP1, LP3 and LP6 presented a similar profile of constituents, differing from those outlined for LP2, LP4, LP5 and LP7. In addition, UV spectra data for LP1, LP3 and LP6 hydroalcoholic fractions allowed the detection of flavonoidic substances (λ_{max}: 240 nm; 340 nm), not detected in LP2, LP4, LP5 and LP7 extracts, and also other classes of compounds.

GC-MS analysis confirmed the presence of flavonoids and detected the accumulation of inositol in hydroalcoholic fractions of LP1, LP3 and LP6. Our findings concerning the flavonoids reinforce those described by Hennebelle *et al.*,[11] who reported the presence of flavonoid glycosides in *L. alba* polar extract. Zetola *et al.*[1] also attributed the sedative and myorelaxant actions of *L. alba* to the presence of flavonoids in its leaf extracts. Flavonoids, such as apigenin and chrysin, have been reported as partial agonists of benzodiazepine receptors.[12–15] Despite exceptions, an increase in the inhibition transmission, particularly mediated by GABA, could lead to sedative and anticonvulsant effects.[16,17] Additionally, the sedative and sleep-enhancing properties of flavonoid glycosides from *Valeriana* and *Polygonum* species were reported by Fernandez *et al.*[18] and Datta *et al.*,[19] respectively. Fernandez *et al.*[18] verified that aglycones tested were inactive and reinforced the importance of the sugar moieties in flavonoidic compounds for the CNS depressant action. To the best of our knowledge, inositol has never been reported in *L. alba* extracts. Moon *et al.*[20] have shown that inositol (±)-1,2:4,5-di-*O*-isopropylidene-3, 6-di-*O*-(2-propylpentanoyl)-myo-inositol presents a better anticonvulsant action than valproic acid, but through different molecular mechanisms.

Data obtained from GC-MS analysis of LP1, LP3 and LP6 methanolic fractions demonstrated that chemotypes accumulate phenylpropanoid derivatives of benzoic and caffeic acids. No flavonoids were detected in the methanolic fractions. Henebelle *et al.*[11] demonstrated that six out of

eleven polyphenols in the polar extract of *L. alba* were phenylpropanoids, among them acteoside, a phenylpropanoid glycoside with antioxidant, anti-inflammatory, anti-nephritic, anti-hepatotoxic[21] and neuroprotective properties.[22] Moreover, it is known that phenylpropanoids derived from caffeic acid exhibit neuroprotective effects.[23,24]

Regarding the anticonvulsant molecular mechanism, the 80% ethanolic extract of *L. alba* LP3 chemotype, one of the most effective anticonvulsants evaluated and also that obtained in a larger amount, inhibited GABA uptake and GABA binding in a dose-dependent manner. These data suggest that the presence of non-volatile compound(s), probably phenylpropanoids, flavonoids and inositols, might function as GABA agonist(s) or GABA uptake inhibitor(s) leading to an enhanced and sustained GABA inhibitory activity, which could play a role in *L. alba* anticonvulsant molecular mechanism. As has been broadly acknowledged, drugs that enhance GABA-mediated inhibitory transmission, and subsequently affect neuronal repetitive firing, are of relevance in the development of new phytomedicines or innovative medicines based on natural products useful as anticonvulsant agents.[17]

Conclusions

Overall results suggest that the anticonvulsant properties shown by the extracts of *L. alba* are related to the presence of a complex of non-volatile substances, among them phenylpropanoids, flavonoids and inositols, in addition to those volatile substances (β-myrcene, citral and limonene) whose anticonvulsant actions have been previously established.[5] This effect is probably a result of their action on GABA neurotransmission. Moreover, the non-volatile compounds could be of relevance as phytochemical markers for future purification of LP1, LP3 and LP6 80% ethanolic extracts and for the identification of *L. alba* chemotypes with anticonvulsant actions in addition to information obtained by essential oil analysis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

The authors are grateful to CAPES and FAPESP for financial support.

Acknowledgements

The authors are grateful to Valeria Melo and Rosane França for technical assistance.

References

1. Zetola M *et al*. CNS activities of liquid and spray-dried extracts from *Lippia alba* – Verbenaceae (Brazilian false melissa). *J Ethnopharmacol* 2002; 82: 207–215.
2. Matos FJA. As ervas cidreiras do nordeste do Brasil. Estudo de três quimiotipos de *Lippia alba* (Mill). N.E.Br-Verbenaceae. *Rev Bras Farmácia* 1996; 77: 137–141.
3. Zoghbi MGB *et al*. Essential oils of *Lippia alba* (Mill.) N. E. Br growing wild in the Brazilian Amazon. *Flav Frag J* 1998; 13: 47–48.
4. Vale TG *et al*. Behavioral effects of essential oils from *Lippia alba* (Mill.) N.E. Brown chemotypes. *J Ethnopharmacol* 1999; 67: 127–133.
5. Viana GS *et al*. Anticonvulsant activity of essential oil and active principles from chemotypes of *Lippia alba* (Mill.) N.E. Brown. *Biol Pharm Bull* 2000; 23: 1314–1317.
6. Mclafferty FW, Stauffer D. *The Wiley/NBS Registry of Mass Spectral Data*. New York: John Wiley & Sons, 1989.
7. Adams RP. *Identification of Essential Oil Components by Gas Chromatography Mass Spectroscopy*. Illinois: Allured Publishing Corporation, 1995.
8. Velisek L *et al*. Excitatory aminoacid antagonist and pentylenotetrazol-induced seizures during ontogenesis II: the effects of MK-801. *Psychopharmacology* 1991; 104: 510–514.
9. Lowry OH *et al*. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
10. Hartree EF. Determination of protein: a modification of the Lowry method gives a linear photometric response. *Anal Biochem* 1972; 48: 422–477.
11. Hennebelle T *et al*. Phenolics and iridoids of *Lippia alba*. *Nat Prod Commun* 2006; 1: 727–730.
12. Viola H *et al*. Isolation of pharmacologically active benzodiazepine receptor ligands from *Tilia tomentosa* (Tiliaceae). *J Ethnopharmacol* 1994; 44: 47–53.
13. Viola H *et al*. Apigenin, a component of *Matricaria recutita* flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. *Planta Med* 1995; 61: 213–216.
14. Viola H *et al*. Sedative and hypnotic properties of *Salvia guaranitica* St. Hil, and of its active principle, cirsiliol. *Phytomedicine* 1997; 4: 47–52.
15. Avallone R *et al*. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. *Biochem Pharmacol* 2001; 59: 1387–1394.
16. Beleboni RO *et al*. Pharmacological and biochemical aspects of GABAergic neurotransmission: pathological and neuropsychobiological relationships. *Cell Mol Neurobiol* 2004; 240: 707–728.
17. Bernásková K *et al*. GABA uptake blocker NNC-771 exhibits marked anticonvulsant action in two cortical epileptic models in immature rats. *Epilepsia* 1999; 40: 1184–1189.
18. Fernández S *et al*. Sedative and sleep-enhancing properties of linarin, a flavonoid isolated from *Valeriana officinalis*. *Pharmacol Biochem Behav* 2004; 77: 399–404.
19. Datta BK *et al*. Analgesic, antiinflammatory and CNS depressant activities of sesquiterpenes and a flavonoid glycoside from *Polygonum viscosum*. *Pharmazie* 2004; 59: 222–225.
20. Moon SC *et al*. Crystal structure and anticonvulsant activity of (±)-1,2:4,5-di-O-isopropylidene-3,6-di-O-(2-propylpentanoyl)-myo-inositol. *Carbohydrate Res* 2007; 342: 1456–1461.
21. Lee KY *et al*. Acteoside of *Callicarpa dichotoma* attenuates scopolamine-induced memory impairments. *Biol Pharm Bull* 2006; 29: 71–74.
22. Kyung AK *et al*. Acteoside and its aglycones protect primary cultures of rat cortical cells from glutamate-induced excitotoxicity. *Life Sci* 2006; 79: 709–716.
23. Nakajima Y *et al*. Water extract of própolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. *Life Sci* 2007; 80: 370–377.
24. Taleb-Contini SH *et al*. Neuropharmacological effects in mice of *Lychnophora* species (Vernonieae, Asteraceae) and anticonvulsant activity of 4,5-di-O-[E]-caffeoylquinic acid isolated from the stem of *L. rupestris* and *L. staavioides*. *Basic Clin Pharmacol Toxicol* 2008; 102: 281–286.