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Chemical and antifungal investigations of six *Lippia* species (Verbenaceae) from Brazil

Cristiano Soleo Funari [a], Fernanda Patrícia Gullo [b], Assunta Napolitano [d], Renato Lajarim Carneiro [c], Maria José Soares Mendes-Giannini [b], Ana Marisa Fusco-Almeida [b], Sonia Piacente [d], Cosimo Pizza [d], Dulce Helena Siqueira Silva [a,*]

[a] NuBBE, Institute of Chemistry, São Paulo State University, Araraquara, CP 355, CEP 14801-970, SP, Brazil
[b] Faculty of Pharmaceutical Sciences, São Paulo State University, Rodovia Araraquara-Jaú, km 1, 14801-902 Araraquara, SP, Brazil
[c] Federal University of São Carlos, Department of Chemistry, 13565-905 São Carlos, SP, Brazil
[d] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano, SA, Italy

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ABSTRACT

The *Lippia* genus is used in ethnobotany as food, beverages, seasoning and antiseptic remedies, among others. The chemical compositions of fifteen extracts of six *Lippia* species were investigated comparatively by HPLC–PDA. To avoid data replication of previous works on this genus, *Lippia lupulina* Cham. root ethanol extract was selected for isolation procedures based on Principal Component Analyses (PCA) of such data. Seven compounds previously unreported in this genus were isolated from this extract (a triterpene, two furanonaphtoquinones, a furanochromone, an isoflavone, a stilbene and an iridoid). The activities of extracts, fractions and pure compounds towards *Candida albicans, Candida krusei, Candida parapsilosis* and *Cryptococcus neoformans* were investigated. Two fractions from the extract of *Lippia salviaefolia* leaves showed marked inhibition of fungal growth, in addition to verbascoside and asebogenin, which showed MICs lower than 15.6 μg/ml and may be promising leads for the development of new antifungal agents, especially against *C. neoformans*.

1. Introduction

The *Lippia* genus comprises approximately 200 species distributed throughout the Central and South Americas as well as in tropical Africa, and it is estimated that Brazil hosts 70–75% of the known species (Arthur, Joubert, De Beer, Malherbe, & Witthuhn, 2011). It is used in ethnobotany worldwide as food, beverages, seasoning and remedies (Pascual, Slowing, Carretero, Sánchez, & Villar, 2001). Some *Lippia* species have antiseptic and healing properties, among other uses (Lorenzi & Matos, 2002; Pascual et al., 2001). Infusions of leaves and flowers of *Lippia lupulina* Cham. from the *Cerrado* biome have been employed by local people from Minas Gerais State (Southeastern Brazil) to threat mouth and throat infections (Rodrigues & Carvalho, 2001), but only one report of the chemistry of its essential oil is available in the literature (Zoghbi, Andrade, Silva, & Maia, 2002). Leaf and stem ethanol extracts of *Lippia salviaefolia* Cham. contain flavonoids and phenylpropanoids, including aromadendrin (**12**) and phloretin (**13**). The formal counteracted oxidative stress in human embryonic kidney HEK-293 cells and the latter inhibited human melanoma M14 cancer cell growth and induced concentration dependent apoptosis (Funari et al., 2011).

Lippia sidoides Cham., also investigated in the present work, has been widely used in Northeastern Brazil as a general use antiseptic (Lemos et al., 2007). Dried and milled leaves, flowers and fruits of this plant have been used as a substitute for *Thymus vulgaris* in spice mixtures for pizzas and meats (Lorenzi & Matos, 2002). Recently, this species was included in the Brazilian Health Ministry priority list of 71 species for phytoceutical product development (Ministério da Saúde & DAF/SCTIE/MS, 2009) due to its reported antiseptic properties.

Immunocompromised patients, such as HIV-infected individuals, transplant recipients and cancer patients, are especially vulnerable and die mainly due to opportunistic invasive fungal infections (IFIs) (Chandrasekar, 2010). The most common causative agents of these infections are *Candida* spp., *Aspergillus* species, and *Cryptococcus neoformans* (Kriengkauykiat, Ito, & Dadwal, 2011).

Amphotericin B and fluconazole are the drugs of choice for treatment of cryptococcosis. However, some recent isolates have shown resistance to fluconazole. In addition, amphotericin B has high toxicity and therefore its use should be limited (Mdod et al., 2011). Polyenes, azoles and echinocandins are now the main classes of antifungal drugs available to control these infections, but

* Corresponding author. Tel.: +55 19 33016659; fax: +55 19 33016692.
E-mail address: dhsilva@iq.unesp.br (D.H.S. Silva).

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with the changing spectrum of pathogens and their increasing resistance to these antifungal agents, together with possible side effects produced by current therapies, the development of new antifungal scaffolds is critical (Chandrasekar, 2010).

The important role of natural products in the development of new antimicrobial agents (antibacterial, antifungal, antiviral and antiparasitic), entities or therapies is well documented (Newman & Cragg, 2007). In this study, the chemical composition of fifteen ethanol extracts of six different *Lippia* species from Brazil were compared by high performance liquid chromatography coupled to a photodiode array detector (HPLC–PDA). To avoid replication procedures, the root extract of *L. lupulina* Cham. (EERLlup) was selected for fractionation and isolation. Isolated compounds were subsequently tested against opportunistic human pathogenic fungal strains (e.g., *Candidaparapsilosis, Candida krusei, Candida albicans* and *C. neoformans*). In addition, extracts, fractions and compounds previously extracted from leaves and stems of *L. salviaefolia* Cham., which presented chromatographic profiles and chemical compositions similar to *L. sidoides* Cham., were also investigated for their antifungal properties.

2. Experimental

2.1. General information

One- and two-dimensional Nuclear Magnetic Resonance (NMR) experiments were performed on a Bruker DRX-600 spectrometer at 14.1 T or on a Varian INOVA 500 and Bruker DRX-500 spectrometers at 11.7 T. Isolated compounds were analyzed by electrospray ionization ion trap mass spectrometry (ESI-ITMS^n) using a Thermo-Finnigan Spectra System HPLC coupled to an LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The mass spectra were acquired in negative and positive modes. The ESI source parameters were a capillary voltage of −4.0 V, a spray voltage of 5 kV and a tube lens offset of 20 V in negative mode. In positive mode, the spectra were acquired with a capillary voltage of 23 V, a spray voltage of 5 kV and a tube lens offset of 50 V. In both ionization modes, the capillary temperature was 280 °C. Data were acquired in MS scanning mode.

Semi-preparative HPLC–UV analyses were carried out on an Agilent 1100 series instrument equipped with a G-1312 binary pump, a G-1328A rheodyne injector and a G-1365B multiple wavelength detector. Preparative HPLC–UV analyses were carried out using a Varian Prep-Star 400 system. Column chromatography (CC) separations were performed over silica gel (0.035–0.070 mm, Acros Organics, USA) or Sephadex LH-20 (Pharmacia Biotech, Sweden). Analytical TLC analyses were performed on silica gel w/ UV plates (Sigma–Aldrich, USA). Spots on TLC plates were visualized under UV light and by spraying anisaldehyde–H_2SO_4 reagent followed by heating at 120 °C (Wagner & Bladt, 1996).

Specific rotation measurements were performed at λ 289 nm in a Jasco P-1020 polarimeter (Japan) with a cylindrical glass cell (10 mm I.D. × 10 mm, model CG1-10, Jasco, Japan).

2.2. Plant material

Aerial parts of *L. salviaefolia* Cham. and *Lippia velutina* were collected in Mogi-Guaçu (São Paulo-Brazil) in 2006 (voucher specimens n° Lima 90 and n° Brumati TI73, respectively) and identified by Dr. Maria Inês Cordeiro of the Herbarium Maria Eneida P. Kaufmann – Instituto Botânico de São Paulo, São Paulo, Brazil. Aerial parts of *Lippia balansae* Briq. and *Lippia lasiocalycina* Cham. were collected in Santa Cruz do Rio Pardo and Pratânia (São Paulo), respectively, in 2008 (voucher specimens n° FEA 402 and n° FEA 3556, respectively) and identified by Dr. Giselda Durigan of the Herbarium Coleção Botânica da Floresta Estadual de Assis, São Paulo, Brazil. Aerial parts and roots of *L. lupulina* Cham. and *L. sidoides* Cham. were collected in Iaras state (São Paulo) in 2009 (voucher specimens n° FEA 3638 and n° FEA 3639, respectively) and also identified by Dr. Giselda Durigan.

2.3. Extraction

Plant materials were dried in an oven with air circulation at 45 °C and then ground in a knife mill. Each extraction was performed in ethanol at a ratio of 7:2 (v/w) in three steps, each 24 h long at room temperature. The solutions were concentrated at 40 °C to give extracts of the flowers (EEFLb), leaves (EELLb) and stems (EESLb) from *L. balansae* Briq.; leaves and stems combined (EELSLlas) from *L. lasiocalycina* Cham.; leaves (EELLsid), stems (EESLsid) and roots (EERLsid) from *L. sidoides* Cham.; flowers (EEFLlup), leaves (EELLlup), roots (EERLlup) and stems (EESLlup) from *L. lupulina* Cham.; and leaves (EELLv) and stems (EESLv) from *L. velutina*. In addition, *L. salviaefolia* Cham. leaves and stems were previously extracted and concentrated following the same procedure described above to obtain EELLsal and EESLsal, respectively (Funari et al., 2011).

2.4. Dereplication by HPLC–PDA

Each extract (10 mg) was dissolved in MeOH (1 ml) and filtered through a PTFE membrane (0.20 μm, Sartorius AG, Germany). These solutions (15 μl) were analyzed in a Shimadzu HPLC equipped with a degasser (DGU-20A$_3$), two pumps (LC 20AT), an auto-sampler (SIL-20A), a photodiode array detector (SPD-M20A) and an oven (CTO-20A). Separation was achieved on two coupled C18 columns (Phenomenex Onyx Monolithic, 100 × 4.6 mm coupled to a Phenomenex Synergi Hydro-RP, 250 × 4.6 mm, 4 μm particle size) using H_2O (solvent A) and MeOH (solvent B). The elution was carried out at 1 ml/min using the following gradient: 30–50% B (0–25 min) and 50–100% B (25–70 min). Detection was achieved at 254 nm, and the compounds were identified by comparison of their retention times and UV spectra with the following reference compounds isolated previously from *Lippia salviaefolia* Cham.: (2*S*)- and (2*R*)-3′,4′,5,6-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**1a**/**1b**), (2*S*)- and (2*R*)-3′,4′,5,8-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**2a**/**2b**), (2*S*)- and (2*R*)-eriodictyol 7-*O*-β-D-glucopyranoside (**3a**/**3b**), forsythoside B (**4**), 6-hydroxyluteolin-7-*O*-β-glucoside (**5**), verbascoside (**6**), aromadendrin (**7**), naringenin (**8**), phloretin (**9**), asebogenin (**10**) and sakuranetin (**11**) (Funari et al., 2011).

2.5. Multivariate curve resolution and principal component analyses

Principal component analyses (PCA) were not performed directly on chromatograms but instead using the individual contribution of each peak/substance found by Multivariate Curve Resolution–Alternating Least Squares (MCR–ALS) in four regions of the chromatograms. Each HPLC–PAD analysis gave rise to a $\mathbf{X}_{txw}$ matrix with t retention times and w wavenumbers; thus, each retention time dataset was a UV–vis spectrum (or a sum of spectra) for a single substance. MCR–ALS decomposes this matrix into two matrices, so that:

$$\mathbf{X}_{txw} = \mathbf{C}^{*}_{txn}\mathbf{S}^{t}_{wxn} + \mathbf{E}_{txw}$$

where **C** is a matrix that contains the relative concentration of n substances at the t retention times, **S** is a matrix containing the pure spectrum of the n substances and **E** is the matrix of errors or lack of fit. If the relative concentration of a substance in **C** is summed from all t retention times, a relative concentration or area for this substance is obtained from that chromatogram. If matrix **X** is built using more than one chromatogram, MCR–ALS can avoid problems

from variation in the retention times because it uses both retention time and UV–vis spectrum data to identify each substance in all of the chromatograms. Thus, MCR–ALS finds the contribution of every substance presenting the same UV spectrum (over a short time interval where peak shift can occur) in each sample. Thus, PCA is performed on a matrix which contains the samples (or chromatograms) in the rows and the relative concentrations of each substance in each sample, in the columns. All chemometric procedures were performed in a Matlab 2011a (Mathworks, Inc., Natick, MA, USA) environment. MCR–ALS was downloaded from http://www.mcrals.info/. PCA was accomplished using routines developed in our laboratory. The shifting of chromatographic bands was circumvented using the area of the peaks, taking each peak area as one variable for each sample.

2.6. Isolation procedures

The crude ethanol extract of *L. lupulina* roots (EERLLup, 9.5 g) was dissolved in MeOH–H_2O 8:2 (v/v) (330 ml) and extracted with Hexane (4 × 150 ml) to give fraction FHex1 (1.6 g) and a precipitate (6.9 g). H_2O was added to the hydromethanolic phase up to 45:55 (v/v) and it was extracted with EtOAc (3 × 250 ml) to give fraction FAc1 (0.6 g). The hydromethanolic phase was then concentrated, diluted in H_2O (500 ml) and extracted with *n*-BuOH (3 × 170 ml) to give fractions FBu1 (0.4 g) and FAq1 (1.2 g). All fractions were then concentrated at 40 °C. FHex1 (0.6 g) was submitted to medium pressure liquid chromatography (MPLC) over silica gel (101.5 g; 39.5 × 2.6 cm i.d.) and eluted with Hex–$CHCl_3$ in a linear gradient (20–100% B in 170 min., 17 ml/min) to afford compounds **12** (335.3 mg) and **13** (8.0 mg). FAc1 (0.6 g) was submitted to liquid chromatography at atmospheric pressure (LC) over silica gel (25.0 g; 8.0 × 2.6 cm) and eluted with Hex–EtOAc 1:0, 1:1 and 0:1 (v/v) to give subfractions B1–B3 (75 ml each). Purification of B2 (20.0 mg, 20 injections) by HPLC–UV was performed on a C18 column (Waters Symmetry, 300 × 7.8 mm) with H_2O–MeOH (1:1 v/v), at 3 ml/min and UV detection at 220 and 254 nm to yield compounds **14** (3.4 mg, t_R = 13.4 min.), **15** (0.3 mg, t_R = 16.9 min.) and **16** (5.0 mg, t_R = 17.5 min.). Fraction FBu1 (1.0 g) was chromatographed by size exclusion chromatography (SEC) on a Sephadex LH 20 column (180.0 × 3.0 cm) eluted with MeOH (3.0 l) to give compounds **17** (18.5 mg), **18** (13.5 mg) and subfraction C1. The latter (40 mg, 8 injections) was purified by HPLC–UV on a semi-preparative C18 column (Phenomenex Synergi Hydro-RP, 250 × 21.2 mm) with H_2O–MeOH (7:3 v/v) at 12.0 ml/min and UV detection at 230 nm to afford **19** (5.0 mg, t_R = 35.1 min.). EELLsal and EESLsal were previously submitted to liquid–liquid extraction to give FHex2, FAc2, FBu2 and FAq2, and FHex3, FAc3, FBu3 and FAq3, respectively (Funari et al., 2011).

2.7. Antimicrobial susceptibility testing

This study evaluated the antifungal activity of crude extracts and fractions of *Lippia* against the pathogenic yeasts *Candida parapsilosis* (ATCC 22019), *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258) and *Cryptococcus neoformans* (90012) from the mycology collection of the Clinical Mycology Laboratory, Department of Clinical Analyses, School of Pharmaceutical Sciences, UNESP, Araraquara. The minimum inhibitory concentrations (MICs) were determined according to the microdilution method described by Rodriguez-Tudela, Barchiesi and the Subcommittee on Antifungal Susceptibility Testing (2008) using 96-well plates with serial dilutions of stock solutions of natural compounds in DMSO into RPMI-1640 culture medium. The concentrations tested ranged from 250 to 0.48 μg/ml (Scorzoni et al., 2007).

Inoculum was prepared in RPMI-1640 without sodium bicarbonate supplemented with L-glutamine and 2% glucose and buffered with 0.165 M MOPS at pH 7.0. Yeast suspensions were prepared to a final concentration of 1.0×10^4 CFU/ml in RPMI-1640 and 100 μl was added to each well. The plates were incubated in a shaker at 37 °C and 150 rpm for 24 (*Candida* species) or 48 h (*C. neoformans*). Amphotericin B and fluconazole were used as positive controls. MICs were read at 490 nm using a plate reader after visualization with Alamar Blue. The interpretation of the results was performed according to Scorzoni et al. (2007). MICs below 75 μg/ml were regarded as strong antifungal activity, whereas MICs between 75 and 150 μg/ml indicated moderate activity and MICs from 150 to 250 μg/ml indicated low activity. No antifungal activity was associated to MICs greater than 250 μg/ml.

3. Results and discussion

3.1. Dereplication by HPLC–PDA

In our continuous effort to investigate species of the genus *Lippia* native to Brazil, 15 ethanol extracts of six different *Lippia* species were analyzed by HPLC–PDA. The method was developed for the ethanol extracts of *L. salviaefolia* Cham. leaves (EELLsal) and stems (EESLsal) because the compounds **1**–**11** (Fig. 1) were previously isolated from them (Funari et al., 2011). After following the usual steps for HPLC method development employing a C18 silica-based packed column (Snyder, Kirkland, & Glajch, 1997), a C18 monolithic column was coupled in series prior to it, improving separation without exceeding the backpressure allowed by our HPLC–PDA system (250 bar). Analyses of all extracts and reference compounds were performed using this method and the presence of **1**–**11** was determined in each extract based on retention times and UV spectra. The results are summarized in Table 1.

Ethanol extracts from *L. balansae* (EELLb), *L. velutina* (EELLv) and *L. sidoides* (EELLsid) leaves and from *L. balansae* (EEFLb) flowers exhibited similar chromatographic profiles to the observed for the extract from *L. salviaefolia* leaves (EELLsal). The unusual interconverting flavanone glucosides **1a**/**1b** and **2a**/**2b** were detected in all of them. Their partial interconversions *via* a common chalcone intermediate were previously proposed (Funari et al., 2011). This intermediate could be achieved by means of an acid-catalyzed keto-enolic tautomerization with C ring opening and C2–C3 double bond formation. The subsequent Michael-type nucleophilic attack of the 6′-hydroxyl or the 2′-hydroxyl on the α,β-unsaturated ketone might lead to compounds **1a**/**1b** or **2a**/**2b**, respectively (Supplementary data). Flavanone glucosides **3a/3b**, flavone glucoside **5**, flavanones **8** and **11** and dihydrochalcones **9** and **10** were detected in these extracts (Table 1). Different chromatographic profiles were observed for the ethanol extracts of *L. lasiocalycina* leaves and stems combined (EELSLlas) as well as from *L. lupulina* leaves and flowers (EELLlup and EEFLlup, respectively), in which no flavonoids were detected. Phenylpropanoids forsythoside B (**4**) and verbascoside (**6**) were detected in the former two extracts, while only **6** was detected in the latter.

Regarding the chromatographic profiles of extracts from stems, *L. balansae* (EESLb) and *L. velutina* (EESLv) were similar to *L. salviaefolia* (EESLsal), while *L. sidoides* extract (EESLsid) had only partial similarity with the latter. Dissimilar profiles were observed for the extracts from stems of *L. lupulina* (EESLlup) and from leaves plus stems of *L. lasiocalycina* (EELSLlas). No flavonoids (reference compounds) were detected in EESLlup and EELSLlas, but phenylpropanoids **4** and **6** were found as major compounds in these extracts. In addition, the two extracts of roots investigated in this study, *L. sidoides* (EERLsid) and *L. lupulina* (EERLlup), showed different chromatographic profiles, but phenetyl glucosides **4** and **6** were detected in both (Table 1). Three chromatograms representing very

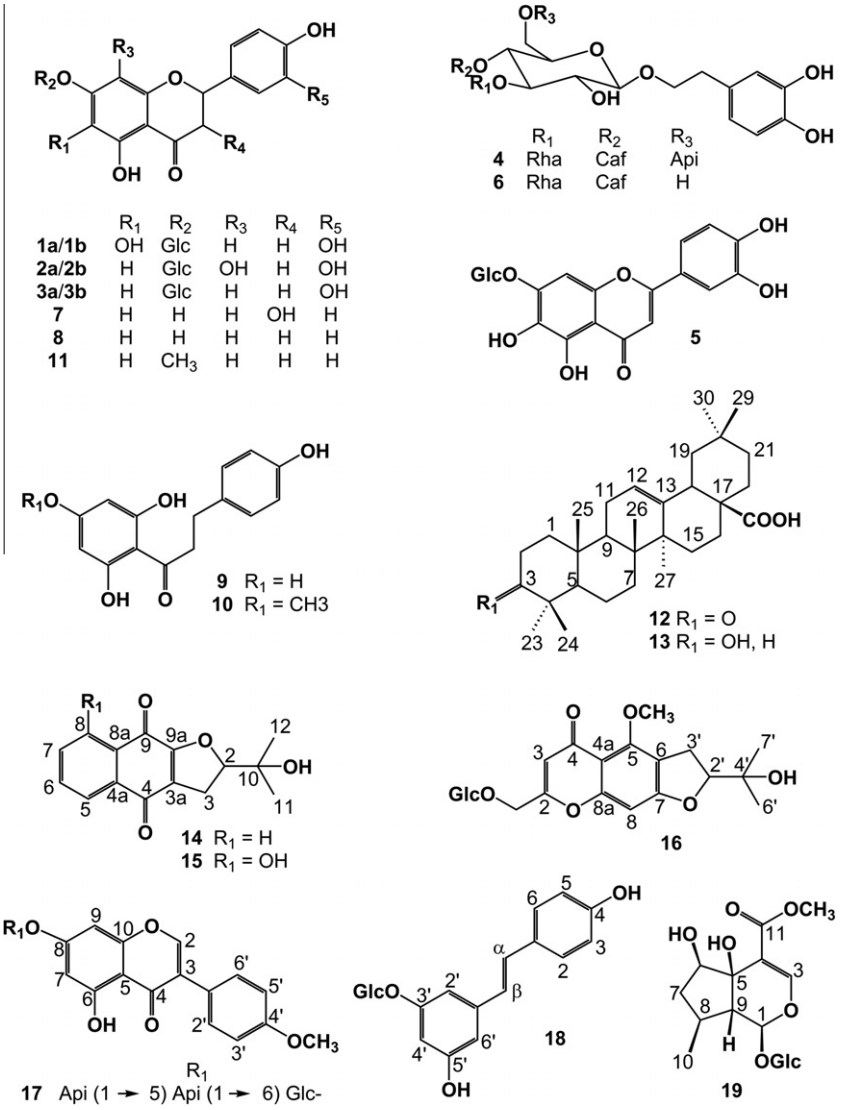

Fig. 1. Compounds employed as chemical markers during dereplication studies by HPLC–PDA (**1**–**11**) and compounds isolated from *L. lupulina* Cham. root extract (EERLlup) (**12**–**19**). Glucopyranosyl, rhamnopyranosyl, apiofuranosyl and caffeoyl are indicated as Glc-, Rha-, Api- and Caf-, respectively.

dissimilar HPLC–PDA profiles relative to the reference extract EELLsal are shown in Fig. 2.

To improve our dereplication and to support the selection of a new extract for further study, a cluster analysis was performed on HPLC–PDA data acquired from the fifteen extracts under analysis. The shifting of chromatographic peaks is the main problem when PCA is performed on a chromatographic dataset. This problem comes from the lack of bilinearity among the chromatographic profiles of each sample (Carneiro, Braga, Bottoli, & Poppi, 2007). As a result, the model identifies more than one principal component for one chromatographic profile, leading to a misinterpretation of the cluster formation. This problem can be solved by peak alignment or, as employed in this work, by using each peak area as one variable for each sample. MCR–ALS is a deconvolution method employed to find chromatographic peaks with identical UV spectra among all the chromatograms (on small intervals of the chromatograms where peak shifting might occur). This method then provides a relative area for identical peaks and was performed on the chromatograms of the extracts separated in four small regions to avoid interpretation mistakes. These mistakes could occur with any compounds that have similar UV spectra but large differences in retention time. This procedure was helpful in determining the area of peaks for the same compound but with different retention times across the samples. PCA was then performed on the relative concentrations of the compounds found by MCR–ALS in the extracts and combined to give a general overview of the cluster separations (Fig. 3).

As shown in Fig. 3, EELLb, EELLsid and EELLv composed a clear cluster with the reference extract EELLsal (**C**, **F**, **G** and **E** in Fig. 3, respectively), corroborating our visual analyses. EEFLb was very similar to the reference extract EELLsal (**A** and **E** in Fig. 3, respectively), with samples EESLsal and EESLb (**M** and **K** in Fig. 3, respectively) having less similarity. Despite the qualitative similarities among the chromatographic profiles of EESLb, EESLsal and EESLv (**K**, **M** and **O** in Fig. 3, respectively), the latter was far from the first two in PCA. This observation might be explained by the relative intensities among peaks containing variations from one sample to another. In this case, the PCA again indicated dissimilarities.

It should be noted that EERLlup (**I** in Fig. 3) was out of any cluster, corroborating the visual observations described above. In addition to showing a dissimilar profile when compared with the previously studied extracts EELLsal and EESLsal (**E** and **M** in Fig. 3), EERLlup presented intense peaks at retention times (t_R) greater than 35 min., which could not be identified from the reference compounds

Table 1
Occurrence of reference compounds in fifteen extracts of six *Lippia* spp.

Compound	t_R (min)	λ_{max} (nm)	*L. salviaefolia*		*L. balansae*			*L. velutina*		L sidoides			*L. lasiocalycina*	*L. lupulina*		
			Leaves	Stems	Leaves	Stems	Flowers	Leaves	Stems	Leaves	Stems	Roots	Leaves + stems	Leaves	Stems	Flowers	Roots
(2*S*)- and (2*R*)-3′,4′,5,6-Tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**1a/1b**)	13.7	286; 329	+	+	+	+	+	+	+	+							
(2*S*)- and (2*R*)-3′,4′,5,8-Tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**2a/2b**)	15.8	286; 329	+	+	+	+	+	+		+							
(2*S*)- and (2*R*)-Eriodictyol 7-*O*-β-D-glucopyranoside (**3a/3b**)	19.4	283; 328	+		+		+	+		+							
Forsythoside B (**4**)	19.6	291; 332		+		+			+		+	+	+	+	+		+
6-Hydroxyluteolin-7-*O*-β-glucoside (**5**)	21.1	281; 346	+		+		+	+		+							
Verbascoside (**6**)	21.1	289; 332		+		+			+		+	+	+	+	+	+	+
Aromadendrin (**7**)	21.5	296; 330	+		+		+	+		+	+						
Naringenin (**8**)	38.7	289; 326	+	+	+	+	+	+	+	+	+						
Phloretin (**9**)	39	286; 326	+	+	+	+	+	+	+	+							
Asebogenin (**10**)	48.7	285; 335	+	+	+	+	+	+		+							
Sakuranetin (**11**)	50	287; 335	+	+	+	+	+	+	+	+	+						

available (Fig. 2). These results led to the selection of this extract for fractionation and isolation procedures to identify unreported non-volatile compounds in *Lippia* genus.

3.2. Isolation and characterization of the root extract of *L. lupulina*

The ethanol extract of roots of *L. lupulina* (EERLlup), a species native to the *Cerrado* biome (São Paulo State, Brazil), was selected for phytochemical investigation (see Subsection 3.1). It exhibited a variety of secondary metabolite classes (Fig. 1). Its partition hexane fraction (FHex1) was chromatographed using MPLC and gave the triterpenes oleanonic (**12**) and oleanolic acids (**13**) (Mahato & Kundo, 1994). From the ethyl acetate fraction (FAc1), the furanonaphtoquinones stenocarpoquinone (**14**) (Schmeda-Hirschmann & Papastergiou, 2003) and avicequinone E (**15**) (Williams et al., 2006) were isolated by LC and HPLC–DAD, in addition to the furanochromone prim-*O*-glycosylcimifugin (**16**) (Sasaki, Taguchi, Endo, & Yosioka, 1982). The *n*-butanol fraction (FBu1) was submitted to SEC to afford the isoflavone triglycoside biochanin A (7-*O*-β-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**17**) (da Silva, Velozo, & Parente, 2000), the stilbene glycoside piceid (**18**) (Lu, Berthod, Hu, Ma, & Pan, 2009), and a subfraction which was further purified by HPLC–DAD to afford the iridoid glycoside β-dihydrohastatoside (**19**) (Teborg & Junior, 1991). Their identification was carried out by 1D and 2D NMR and ESI-MS experiments and compared with the literature.

Compound 12 was isolated as a white solid. $[\alpha]_D^{25}$ + 72.7 (*c* 0.49, MeOH). ESI-ITMS *m/z* 455 $[M+H]^+$ (calc. for $C_{30}H_{46}O_3$ + H). ^{13}C NMR spectral data (11.7 T, $CDCl_3$, TMS, δ ppm) *δ*: 39.0 (C-1), 34.0 (C-2), 218.6 (C-3), 47.3 (C-4), 55.1 (C-5), 19.5 (C-6), 32.1 (C-7), 39.1 (C-8), 45.8 (C-9), 36.7 (C-10), 23.4 (C-11), 122.0 (C-12), 143.7 (C-13), 41.7 (C-14), 27.5 (C-15), 22.8 (C-16), 46.4 (C-17), 41.0 (C-18), 45.8 (C-19), 30.5 (C-20), 33.7 (C-21), 32.3 (C-22), 26.3 (C-23), 21.3 (C-24), 14.9 (C-25), 16.7 (C-26), 25.7 (C-27), 182.3 (C-28), 32.9 (C-29) and 23.4 (C-30).

Compound 13 was isolated as a white solid. ESI-ITMS *m/z* 495 $[M+K]^+$ (calc. for $C_{30}H_{48}O_3$ + K). ^{13}C NMR spectral data (11.7 T, $CDCl_3$, TMS, δ ppm) *δ*: 38.4 (C-1), 27.2 (C-2), 79.0 (C-3), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 32.6 (C-7), 39.3 (C-8), 47.6 (C-9), 37.1 (C-10), 22.9 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.7 (C-15), 23.4 (C-16), 46.5 (C-17), 41.0 (C-18), 45.9 (C-19), 30.7 (C-20), 33.8 (C-21), 32.4 (C-22), 28.1 (C-23), 15.5 (C-24), 15.3 (C-25), 17.1 (C-26), 25.9 (C-27), 183.0 (C-28), 33.0 (C-29) and 23.6 (C-30).

Compound 14 was isolated as a yellow solid. $[\alpha]_D^{25}$ + 42.3 (*c* 0.17, MeOH). On line UV spectrum: λ_{max} at 248, 254, 292 and 345 nm. ESI-ITMS *m/z* 281 $[M+Na]^+$ (calc. for $C_{15}H_{14}O_4$ + Na). ^1H NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 4.90 (*dd*, *J* = 9.2 and 10.5 Hz, H-2), 3.16 (*dd*, *J* = 10.5 and 16.9 Hz, H-3), 3.20 (*dd*, *J* = 9.2 and 16.9 Hz, H-3), 7.06 (*dd*, *J* = 1.5 and 7.5 Hz, H-5), 7.81 (*ddd*, *J* = 1.5, 7.5 and 8.0 Hz, H-6), 7.77 (*ddd*, *J* = 1.5, 7.5 and 8.0 Hz, H-7), 8.09 (*dd*, *J* = 1.5 and 7.5, H-8), 1.28 (*s*, H-11), 1.38 (*s*, H-12); ^{13}C NMR spectral data extracted from HMBC and HSQC experiments (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 93.1 (C-2), 28.7 (C-3), 125.4 (C-3a), 183.3 (C-4), 134.1 (C-4a), 126.4 (C-5), 134.8 (C-6), 133.7 (C-7), 126.5 (C-8), 132.8 (C-8a), 178.6 (C-9), 161.7 (C-9a), 72.0 (C-10), 25.3 (C-11) and 25.3 (C-12).

Compound 15 was isolated as a yellow solid. ESI-ITMS *m/z* 275 $[M+H]^+$ (calc. for $C_{15}O_5H_{14}$ + H). ^1H NMR spectral data (14.1 T, CD_3OD, TMS, δ ppm) *δ*: 4.91 (*m*, H-2), 3.17 (*m*, H-3), 7.60 (*d*, *J* = 7.7 Hz, H-5), 7.70 (*dd*, *J* = 7.7 and 8.0 Hz, H-6), 7.25 (*d*, *J* = 8.0 Hz, H-7), 1.29 (*s*, H-11), 1.40 (*s*, H-12); ^{13}C NMR spectral data extracted from HMBC and HSQC experiments (14.1 T, CD_3OD, TMS, δ ppm) *δ*: 93.1 (C-2), 28.6 (C-3), 126.2 (C-3a), 182.6 (C-4), 134.6 (C-4a), 118.9 (C-5), 137.6 (C-6), 124.5 (C-7), 162.7 (C-8),

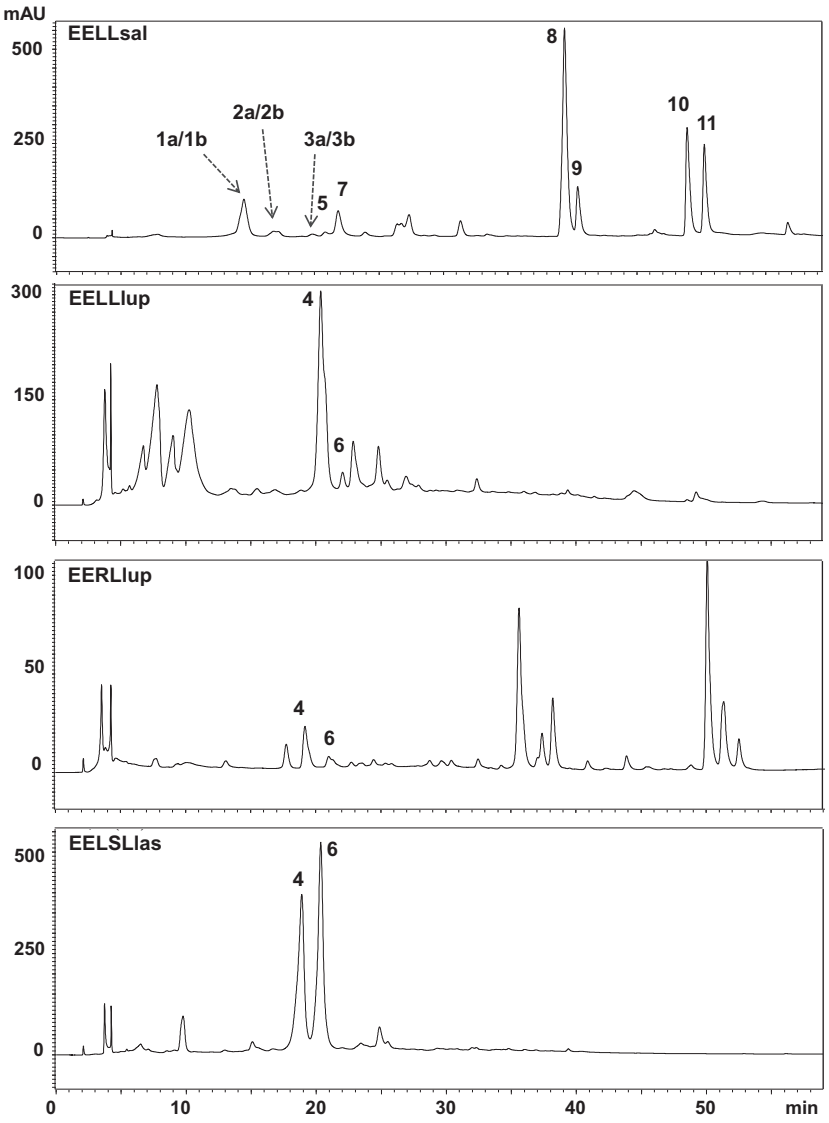

Fig. 2. Selected representative HPLC–PDA chromatograms of the ethanol extracts investigated: leaves of *L. salviaefolia* Cham. (EELLsal) and *L. lupulina* Cham. (EELLlup), roots of *L. lupulina* Cham. (EERLlup) and leaves plus stems of *L. lasiocalycina* Cham (EELSLas). Identified peaks: (2*S*)- and (2*R*)-3′,4′,5,6-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**1a**/**b**), (2*S*)- and (2*R*)-3′,4′,5,8-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**2a**/**b**), (2*S*)- and (2*R*)-eriodictyol 7-*O*-β-D-glucopyranoside (**3a**/**b**), forsythoside B (**4**), 6-hydroxyluteolin-7-*O*-β-glucoside (**5**), verbascoside (**6**), aromadendrin (**7**), naringenin (**8**), phloretin (**9**), asebogenin (**10**) and sakuranetin (**11**).

115.8 (C-8a), 162.0 (C-9a), 72.0 (C-10), 25.0 (C-11) and 25.0 (C-12).

Compound 16 was isolated as a redish solid: ESI-ITMS *m/z* 491 $[M+Na]^+$ (calc. for $C_{22}O_{11}H_{28}$ + Na). ^{1}H NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 6.41 (*s*, H-3), 6.67 (*s*, H-8), 4.64 (*d*, *J* = 14.7 Hz, 2-CH_2-), 4.79 (*d*, *J* = 14.7 Hz, 2-CH_2-), 4.79 (*m*, H-2′), 3.37 (*m*, H-3′), 1.27 (*s*, 4′-CH_3), 1.33 (*s*, -4′-CH_3), 4.45 (*d*, *J* = 7.5 Hz, H-1″), 3.27–3.40 (*m*, H-1″, H-3″, H-4″ and H-5″), 3.67 (*m*, H-6″) and 3.90 (*m*, H-6″); ^{13}C NMR spectral data extracted from HMBC and HSQC experiments (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 164.6 (C-2), 110.5 (C-3), 176.5 (C-4), 112.1 (C-4a), 156.7 (C-5), 118.2 (C-6), 166.6 (C-7), 94.1 (C-8), 160.8 (C-8a), 66.9 (2-CH_2-), 92.2 (C-2′), 28.6 (C-3′), 71.9 (C-4′), 25.3 (4′-CH_3), 103.9 (C-1″), 74.6 (C-2″), 77.7 (C-3″), 71.3 (C-4″), 78.0 (C-5″) and 62.5 (C-6″).

Compound 17 was isolated as a brown solid. $[\alpha]_D^{25}$ − 76.0 (*c* 0.1, MeOH). Online UV spectrum: λ_{max} at 259 and 324 nm. ESI-ITMS *m/z* 733 $[M+Na]^+$ (calc. for $C_{32}O_{18}H_{38}$ + Na). ^{1}H NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 8.20 (*s*, H-2), 6.56 (*d*, *J* = 2.3 Hz, H-6), 6.73 (*d*, *J* = 2.3 Hz, H-8), 7.52 (*d*, *J* = 8.8 Hz, H-2′), 7.01 (*d*, *J* = 8.8 Hz, H-3′), 7.01 (*d*, *J* = 8.8 Hz, H-5′), 7.52 (*d*, *J* = 8.8 Hz, H-6′), 3.85 (*s*, 4′-OCH_3), 5.02 (*d*, *J* = 7.0 Hz, H-1″), 3.49–3.54 (*m*, H-2″, H-3″), 3.38 (*t*, *J* = 9.2 Hz, H-4″), 3.72–3.74 (*m*, H-5″), 3.65 (*dd*, *J* = 7.0 and 10.9 Hz, H-6″a), 4.09 (*br d*, *J* = 10 Hz, H-6″b), 5.01 (*d*, *J* = 2.5 Hz, H-1‴), 4.00 (*d*, *J* = 2.5 Hz, H-2‴), 3.81 (*d*, *J* = 9.8 Hz, H-4‴a), 4.08 (*d*, *J* = 9.8 Hz, H-4‴b), 3.57 (*d*, *J* = 9.8 Hz, H-5‴a), 3.81 (*d*, *J* = 9.8 Hz, H-5‴b), 4.99 (*d*, *J* = 2.7 Hz, H-1⁗), 3.95 (*d*, *J* = 2.7 Hz, H-2⁗), 3.78 (*d*, *J* = 9.8 Hz, H-4⁗a), 3.98 (*d*, *J* = 9.8 Hz, H-4⁗b), 3.59 (*m*, H-5⁗). ^{13}C NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 155.7 (C-2), 124.5 (C-3), 182.4 (C-4), 163.9 (C-5), 100.7 (C-6), 164.2 (C-7), 95.4 (C-8), 159.1 (C-9), 107.9 (C-10), 124.1 (C-1′), 130.8 (C-2′, C-6′), 114.6 (C-3′, C-5′), 161.1 (C-4′), 55.5 (4′-OCH_3), 101.3 (C-1″), 74.1 (C-2″), 77.6 (C-3″), 71.3 (C-4″), 76.9 (C-5″), 68.8 (C-6″), 110.2 (C-1‴), 78.3 (C-2‴), 79.6 (C-3‴), 74.9 (C-4‴), 71.5 (C-5‴), 110.2 (C-1⁗), 77.4 (C-2⁗), 80.5 (C-3⁗), 74.8 (C-4⁗) and 65.2 (C-5⁗).

Compound 18 was isolated as a brown solid. $[\alpha]_D^{25}$ − 26.2 (*c* 0.32, MeOH). Online UV spectrum: λ_{max} at 215, 306 and 318 nm. ESI-ITMS *m/z* 429 $[M+K]^+$ (calc. for $C_{20}O_{22}H_8$ + K). ^{1}H NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 7.37 (*d*, *J* = 9.0 Hz, H-2, H-6),

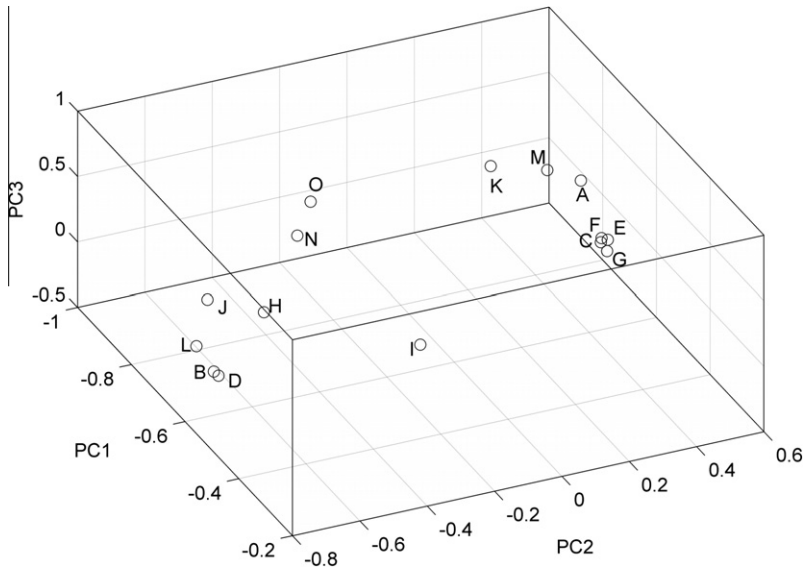

Fig. 3. Projection of the samples in the score space, evidencing that extract EERLlup (**I**) was outside of the clusters. The scores were calculated by PCA from the relative concentrations of compounds present in the samples obtained by MCR–ALS. Ethanol extracts: EEFLb (**A**), EEFLLup (**B**), EELLb (**C**), EELLlup (**D**), EELLsal (**E**), EELLsid (**F**), EELLv (**G**), EELSLas (**H**), EERLlup (**I**), EERLsid (**J**), EESLb (**K**), EESLlup (**L**), EESLsal (**M**), EESLsid (**N**) and EESLv (**O**).

6.78 (*d*, *J* = 9.0 Hz, H-3, H-5), 7.03 (*d*, *J* = 16.5 Hz, H-α), 6.85 (*d*, *J* = 16.5 Hz, H-β), 6.80 (*t*, *J* = 2.0 Hz, H-2′), 6.46 (*t*, *J* = 2.0 Hz, H-4′), 6.66 (*t*, *J* = 2 Hz, H-6′), 4.91 (*d*, *J* = 7.5 Hz, H-1″), 3.43–3.50 (*m*, H-2″, H-3″ and H-5″), 3.39 (t, *J* = 9.1 Hz, H-4″), 3.72 (*dd*, *J* = 5.9 and 12.1 Hz, H-6″a) and 3.96 (*dd*, *J* = 2.3 and 12.1 Hz, H-6″b). ^{13}C NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 130.3 (C-1), 128.9 (C-2, C-6), 116.5 (C-3, C-5), 158.5 (C-4), 130–4 (C-α), 126.7 (C-β), 141.5 (C-1′), 107.1 (C-2′), 160.5 (C-3′), 104.2 (C-4′), 159.6 (C-5′), 108.4 (C-6′), 102.5 (C-1″), 75.0 (C-2″), 78.1 (C-3″), 71.6 (C-4″), 78.3 (C-5″) and 62.6 (C-6″).

Compound 19 was isolated as a colourless solid. Online UV spectrum: λ_{max} at 231 nm. ^{1}H NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 5.59 (*d*, *J* = 2.2 Hz, H-1), 7.41 (*s*, H-3), 4.03 (*t*, *J* = 6.2 Hz, H-6), 1.21 (*ddd*, *J* = 6.2, 9.0 and 13.2 Hz, H-7a), 2.0 (*ddd*, *J* = 6.2, 7.8 and 13.2 Hz, H-7b), 1.50 (*m*, H-8), 1.94 (*dd*, *J* = 2.2 and 10.3 Hz, H-9), 1.04 (*d*, *J* = 6.6 Hz, H-10), 3.62 (*s*, 11-OCH_3), 4.49 (*d*, *J* = 7.9 Hz, H-1′), 3.10 (*dd*, *J* = 7.9 and 9.2 Hz, H-2′), 3.18–3.30 (*m*, H-3′, H-5′), 3.15–3.24 (*m*, H-4′), 3.57 (*dd*, *J* = 5.7 and 12.0 Hz, H-6′ a), 3.80 (*dd*, *J* = 2.0 and 12.0 Hz, H-6′ b). ^{13}C NMR spectral data (11.7 T, CD_3OD, TMS, δ ppm) *δ*: 95.7 (C-1), 154.0 (C-3), 112.7 (C-4), 72.8 (C-5), 76.8 (C-6), 40.4 (C-7), 31.5 (C-8), 55.7 (C-9), 19.7 (C-10), 167.9 (C-11), 51.5 (11-OCH_3), 100.0 (C-1′), 74.3 (C-2′), 77.4 (C-3′), 71.6 (C-4′), 78.1 (C-5′) and 62.5 (C-6′).

Compounds **12**–**19** (Fig. 1) are reported here for the first time in *L. lupulina* Cham. Oleanolic acid (**13**) was previously isolated from *Lippia triphylla* by Ono et al. (2008), while oleanonic acid (**12**) had not been isolated in the *Lippia* genus. Both **12** and **13** showed insecticidal activity against *Sitophilus oryzae* (L.) (Pungitore, García, Gianello, Sosa, & Tonn, 2005) and toxicity towards *M. tuberculosis* H37Rv (Caldwell, Franzblau, Suarez, & Timmermann, 2000). Compounds **14**–**19** have not been reported previously in the Verbenaceae family. Avicequinone E (**15**) was active against human ovarian cancer cells line A2780 with an IC_{50} of 8.8 μM, while stenocarpoquinone (**14**) presented an IC_{50} of 50 μM in the same test (Williams et al., 2006). No reports of the biological activity of biochanin A-7-*O*-β-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**17**) were found in the literature. Piceid (**18**) is abundant in nature and in food, such as grapes, cocoa and peanuts, and some of its biological activity has been reported, mainly its strong antioxidant activity (Counet, Callemien, & Collin, 2006). No reports on the biological activity of β-dihydrohastatoside (**19**) were found in the literature, but iridoid glycosides are known to have a wide range of activities (Tundis, Loizzo, Menichini, Statti, & Menichini, 2008).

3.3. Antifungal assay

The clinical importance of systemic mycosis has increased rapidly in recent years, mainly due to the increasing incidence of AIDS and immunocompromised or severely ill patients. Some *Lippia* species have been employed in ethnomedicine as antimicrobial agents (Lorenzi & Matos, 2002; Pascual et al., 2001), such as *L. lupulina* Cham. (Rodrigues & de Carvalho, 2001) and *L. sidoides* Cham. (Lemos et al., 2007). In this study, the influence of EERLlup, EELLsal and EESLsal, together with their partition fractions, was investigated against opportunistic human yeast pathogens (*C. albicans, C. krusei, C. parapsilosis* and *C. neoformans*). Table 2 summarizes the results obtained for these samples.

EELLsal was the most active among the crude extracts, with MICs of 125 μg/ml for *C. albicans, C. krusei* and *C. parapsilosis*, and 62.5 μg/ml for *C. neoformans* (Table 2). According to Scorzoni et al. (2007), EELLsal presents moderate activities against the *Candida* strains used and strong activity against *C. neoformans*. EESLsal, which presented MICs of 250 μg/ml for *Candida* strains and 125 μg/ml for *C. neoformans*, was considered moderately active against all yeasts tested. The MICs for EERLlup were outside the maximum concentration tested (250 μg/ml). EELLsal and EELLsid showed similar chromatographic profiles and chemical compositions (Table 1). Because *L. sidoides* Cham. is largely used in ethnopharmacology as a general antiseptic (Lorenzi & Matos, 2002), such similarities might be associated with the antifungal activities observed for EELLsal (Table 2).

Among the partition fractions of the three crude extracts, FBu1 and FAq1 from EERLlup and FAc2 from EELLsal showed stronger activities than their original crude extracts for all tested strains (Table 2). FBu1 presented stronger activity against *C. albicans*, *C. krusei* and *C. neoformans*, with MICs of 62.5, 15.6 and 31.2 μg/ml, respectively, and moderate activity against *C. parapsilosis* (MIC of 125 μg/ml). FAq1 presented stronger activities against *C. krusei* and *C. neoformans* with MICs of 62.5 μg/ml for both strains, and

Table 2
Antifungal activity of the ethanol extracts of leaves (EELLsal) and stems (EESLsal) of *L. salviaefolia*, roots of *L. lupulina* (EERLlup) and of their partition fractions (MICs in μg/ml).[a]

Extract/ fraction	*Candida albicans*	*Candida krusei*	*Candida parapsilosis*	*Cryptococcus neoformans*
EERLlup	>250	>250	>250	>250
FHex1	>250	>250	>250	>250
FAc1	125	125	>250	125
FBu1	**62.5**	**15.6**	125	**31.2**
FAq1	125	**62.5**	250	**62.5**
EELLsal	125	125	125	62.5
FHex2	>250	>250	>250	**62.5**
FAc2	**62.5**	**31.2**	62.5	**31.2**
FBu2	125	**62.5**	125	**31.2**
FAq2	250	125	250	**62.5**
EESLsal	250	250	250	125
FHex3	250	250	>250	250
FAc3	125	125	>250	125
FBu3	250	125	>250	250
FAq3	>250	>250	>250	>250

[a] Best MIC values are boldfaced.

moderate activities towards *C. albicans* and *C. parapsilosis* (MICs of 125 and 250 μg/ml, respectively). FAc2 showed strong activity against all tested strains, with MICs of 62.5 μg/ml towards *C. albicans* and *C. parapsilosis*, and 31.2 μg/ml towards *C. krusei* and *C. neoformans*. Thus, the purified compounds used for antifungal activity evaluation were selected from the most active fractions FBu1 (**17** and **18**) and FAc2 (**7**–**11**). Additionally, **4** and **6**, the interconverting isomers **1a**/**1b** and **2a**/**2b** from FBu3, and **12** from FHex1 were also assayed. Table 3 shows the antifungal activity measured for these compounds.

Sakuranetin (**11**) and oleanonic acid (**12**) did not show any activity at the maximum concentration assayed (250 μg/ml). On the other hand, eight pure compounds (**4**, **6**–**10** and **17**–**18**) and a mixture of the interconverting isomers (**1a**, **1b**, **2a** and **2b**) inhibited at least one strain with MIC ⩽ 250 μg/ml. Verbascoside (**6**), a phenylpropanoid isolated from the ethanol extract of stems of *L. salviaefolia* (EESLsal) (Funari et al., 2011), showed the strongest activity with an MIC of 25.0 μmol/l (or 15.6 μg/ml) against *C. neoformans*, which was approximately 6 times less active than the positive control amphotericin B (MIC of 4.3 μmol/l). This strain was the most susceptible because **4**, **6**, **10** and **17** as well as the mixture of isomers **1a**, **1b**, **2a** and **2b** showed MICs < 100 μmol/l. Biochanin A 7-*O*-β-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**17**) showed MIC of 88.0 μmol/l towards *C. krusei*, which was close to that observed for amphotericin B (MIC of 69.3 μmol/l). This isoflavone triglycoside presented similar inhibitory activity against *C. neoformans* (MIC of 88.0 μmol/l) and only twofold lower activity towards *C. albicans* and *C. parapsilosis* (MICs of 176 μmol/l) (Table 3). The presence of isoflavone glucoside **17** in EERLlup might be associated with the well-known antifungal activity of isoflavones and their wide occurrence in plant roots.

It should be noted that compounds **4**, **6**–**10** and the mixture of interconverting isomers **1a**/**1b**/**2a**/**2b**, which were active against one or more strains, were detected in the extracts of *L. sidoides* Cham. leaves and stems (Table 1). Infusions or ethanol extracts and tinctures prepared with aerial parts of this species have been widely used in Brazil as a general antiseptic (Lorenzi & Matos, 2002). Recently, this species was included in the Brazilian Health Ministry's priority list of species for phytotherapeutic product development (Ministério da Saúde, 2009).

Several compounds derived from various species of *Lippia* have been studied for their antioxidant and antimicrobial activities and for their use as food seasonings. It has been shown that *L. pseudothea* presented an MIC of 625 mg/ml towards *C. albicans*, and *L. sidoides* showed an MIC of 625 mg/ml for both *C. albicans* and *C. neoformans*. The technique used to determine these antifungal activities was bio-autography and indicated that the antioxidant activity of the extracts was due to coumarins and flavonoids. Terpenoids and the same flavonoids were associated to the observed antimicrobial properties (Fabri, Nogueira, Moreira, Bouzada, & Scio, 2011). Our results confirmed the antimicrobial potential of *Lippia* spp. in addition to revealing their effective inhibition of major human fungal pathogens. This is the first report on *L. salviaefolia* and *L. lupulina* producing compounds with potential antifungal activity against *C. krusei* and *C. neoformans*.

Given the poor arsenal of antifungal drugs and the problems regarding toxicity and increased fungal resistance to the usual therapies, the treatment of human mycoses is not always effective. For this reason, there is a growing interest in finding novel, effective antifungal drugs. Considering the immense Brazilian biodiversity, exploring plants for novel antifungal compounds should be a priority.

In addition to having increased the chemical knowledge on thirteen extracts from five species of the *Lippia* genus by comparison with phytochemical markers, HPLC–PDA combined with statistics led us to the selection of the ethanol extract of roots of *L. lupulina*

Table 3
Antifungal activity of compounds isolated from ethanol extracts of roots from *L. lupulina* (EERLlup) (**12**, **17** and **18**) and of leaves (EELLsal) and stems (EESLlsal) from *L. salviaefolia* (**1**–**11**) (MIC in μg/ml and μmol/l)[a].

Compound/strain	*Candida albicans*		*Candida krusei*		*Candida parapsilosis*		*Cryptococcus neoformans*	
	(μg/ml)	(μmol/l)	(μg/ml)	(μmol/l)	(μg/ml)	(μmol/l)	(μg/ml)	(μmol/l)
Isomeric mixture (**1**/**2**)[b]	**62.5**	**134.1**	**62.5**	**134.1**	**62.5**	**134.1**	**31.2**	**66.9**
Forsythoside B (**4**)	125	165.3	250	330.7	125	165.3	**62.5**	**82.7**
Verbascoside (**6**)	125	200.3	125	200.3	125	200.3	**15.6**	**25.0**
Aromadendrin (**7**)	250	868.0	250	868.0	125	434.0	125	434.0
Naringenin (**8**)	250	919.1	250	919.1	>250	>919.1	125	459.6
Phloretin (**9**)	250	912.4	250	912.4	125	456.2	**62.5**	**228.1**
Asebogenin (**10**)	>250	>868.0	250	868.0	>250	>868.0	**15.6**	**54.2**
Sakuranetin (**11**)	>250	>874.1	>250	>874.1	>250	>874.1	>250	>874.1
Oleanonic acid (**12**)	>250	>550.7	>250	>550.7	>250	>550.7	>250	>550.7
Biochanin A triglycoside (**17**)[c]	125	176.0	**62.5**	**88.0**	125	176.0	**62.5**	**88.0**
Piceid (**18**)	125	320.5	125	320.5	125	320.5	125	320.5
Amphotericin B	2.0	2.2	64	69.3	8.0	8.6	4.0	4.3
Fluconazole	2.0	6.5	2.0	6.5	1.0	3.3	0.1	0.2

[a] Best MIC values are boldfaced.
[b] (2*S*)- and (2*R*)-3′,4′,5,6-Tetrahydroxyflavanone-7-*O*-β-glucopyranoside and (2*S*)- and (2*R*)-3′,4′,5,8-tetrahydroxyflavanone-7-*O*-β-glucopyranoside.
[c] Biochanin A 7-*O*-β-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside.

for further phytochemical studies. These studies resulted in the isolation of eight compounds, including seven previously unreported in the *Lippia* genus. The reported strategy was effective at avoiding replication of time-consuming isolation procedures, which might lead to compounds previously isolated from *L. salviaefolia* leaves and stems (Funari et al., 2011). Furthermore, the ethyl acetate and *n*-butanol fractions from the ethanol extract of *L. salviaefolia* leaves (FAc2 and FBu2, respectively) showed strong inhibition of fungal growth, along with verbascoside (**6**) and asebogenin (**10**), previously isolated from *L. salviaefolia*. Therefore, these natural products might be considered promising prototypes for the development of new antifungal agents, especially against *C. neoformans*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.06.077.

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