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Article

Analysis of the Chemical Composition of the Essential Oils Extracted from *Lippia lacunosa* Mart. & Schauer and *Lippia rotundifolia* Cham. (Verbenaceae) by Gas Chromatography and Gas Chromatography-Mass Spectrometry

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Lippia lacunosa e *L. rotundifolia* são duas espécies brasileiras que formam um complexo de difícil delimitação taxonômica. A composição química do óleo essencial das folhas e flores dessas plantas foi investigada por cromatografia com fase gasosa (CG) e por cromatografia com fase gasosa acoplada à espectrometria de massas (CG-EM). Principais constituintes dos óleos essenciais de *L. lacunosa* (flores e folhas): mirceno (14,7% e 11,9%), mircenona (45,2% e 64,2%), *Z*-ocimenona (5,7% e 5,2%), e *E*-ocimenona (14,7% e 4,1%), respectivamente; *L. rotundifolia* (flores e folhas): α-pineno (8,7% e 1,8%), mirceno (5,1% e 3,6%), limoneno (26,0% e 7,9 %), *cis*-pinocanfona (4,5% e 3,1%) e mirtenal (22,3% e 16,7%), respectivamente. Os óleos essenciais de *L. lacunosa* apresentaram um forte e agradável aroma de manga, que foi relacionado à presença de mirceno e mircenona. Diferenças fundamentais na composição química de seus óleos essenciais podem representar uma poderosa ferramenta na classificação botânica das espécies.

Lippia lacunosa and *L. rotundifolia* (Verbenaceae) are two Brazilian species of complex taxonomic delimitation. The composition of the essential oils from leaves and flowers of these plants was investigated by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) analysis. The major components of the essential oils of flowers and leaves of *L. lacunosa* were: myrcene (14.7% and 11.9%), myrcenone (45.2% and 64.2%), *Z*-ocimenone (5.7% and 5.2%), and *E*-ocimenone (14.7% and 4.1%), respectively; whereas in *L. rotundifolia* (flowers and leaves) were α-pinene (8.7% and 1.8%), myrcene (5.1% and 3.6%), limonene (26.0% and 7.9 %), *cis*-pinocamphone (4.5% and 3.1%) and myrtenal (22.3% and 16.7%), respectively. The essential oils from *L. lacunosa* exhibited a strong and pleasant mango aroma, which was related to the presence of myrcene and myrcenone. The marked differences in the chemical composition of their essential oils may represent a powerful tool for the botanical classification.

Keywords: *Lippia lacunosa*, *Lippia rotundifolia*, myrcene, myrcenone, (*E*)-ocimenone, limonene, myrtenal, GC, GC-MS

Introduction

The genus *Lippia* (Verbenaceae) comprises about 200 species occurring mainly in Central and South America, as also in some areas of Tropical Africa.[1] One of the main diversity centers of the genus *Lippia* is located at the "Cadeia do Espinhaço" Mountains, in the State of Minas Gerais, Brazil.[2] There are some taxonomical controversies on the number of *Lippia* species. Moldenke[3] compared the descriptions given by various authors to this genus, and presumably all the authors

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included *Acantholippia*, *Aloysia* and *Phyla* in their concept of *Lippia*.[3,4] Some species, like *L. alba* and *L. graveolens* are largely used in folk medicine and culinary.[1] As part of our continuing study on *Lippia* species occurring in Brazil, *L. lacunosa* Mart. & Schauer and *L. rotundifolia* Cham. were selected for investigation. Included in the Section Corymbosae, they form a complex of very difficult taxonomic delimitation, and were considered as synonyms in many herbarium samples.[5] They both possess a developed underground system, coriaceous leaves, and corymb inflorescences with pink flowers. They are strongly aromatic due to a dense layer of glandular hairs. Distinction between them is very difficult and is only accomplished by analyses of the leaf (foliar limbus) base morphology and of the floral bracts.[5] However, pollen analysis showed relevant differences[6] and further taxonomic studies led to the classification of these plants as two different species. In this way, chemical studies might contribute as an extra tool to reinforce this delimitation.

The aim of this work was to investigate the chemical composition of the essential oils obtained from flowers and leaves of *L. lacunosa* and *L. rotundifolia*. To the best of our knowledge, no previous studies concerning their chemistry have been published.

Experimental

Plant material

Fresh leaves and flowers of *L. lacunosa* and *L. rotundifolia,* cultivated, from original clones[4] originary from Diamantina (MG-Brazil), were collected at the campus of the Federal University of Juiz de Fora, Juiz de Fora, Brazil, (22°46'48.6"S, 43°22'24.5"W) in August, 2005. The plants were authenticated by Dr. Fatima Regina Gonçalves Salimena, and voucher specimens were deposited at the Herbarium of the Departamento de Botânica, Universidade Federal de Juiz de Fora (CESJ 41.691 and CESJ 31.376, for *L. lacunosa,* and *L. rotundifolia*, respectively).

Essential oil extraction

The essential oils from fresh leaves and flowers of *L. lacunosa* and *L. rotundifolia* were obtained separately by hydrodistillation in a Clevenger-type apparatus for 2 h. The oils were dried over anhydrous sodium sulphate and stored in sealed vials at low temperature.

GC and GC-MS analyses

Gas chromatographic analyses were performed using a HP 5890 series II gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) detector and a HP-5 (5% phenyl/95% dimethylpolysiloxane) fused silica capillary column (30m × 0.25mm; film thickness 0.25μm). Hydrogen was the carrier gas (1.0 mL min^{-1}). The injector temperature was kept at 250 °C and the oven temperature program was from 60° to 240 °C at a rate of 3 °C min^{-1}. Detector (FID) was operated at 280 °C. Pure oils (0.03 μL) were injected in split mode (100:1).

The GC-MS analyses were performed in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Palo Alto, CA), equipped with a HP5-MS capillary column (30m × 0.25mm × 0.25μm), operating in electronic ionization mode at 70eV, with transfer line maintained at 260 °C, while quadrupole and ion source temperature were held at 150 °C and 230 °C, respectively. Helium (1.0 mL min^{-1}) was used as carrier gas. Oven temperature program, injector temperature and split rate were the same as stated for GC analyses.

A standard solution of *n*-alkanes (C_7-C_{26}) was used to obtain the retention indices.[7] Individual volatile components were identified by comparison of their mass spectra (MS) and retention indices (RI) with those reported in literature[8] and also to the Wiley Registry of Mass Spectral Data, 6th Edition (Wiley Interscience, New York).

Isolation of myrcenone from L. lacunosa essential oil

Approximately 0.7 mL of *L. lacunosa* essential oil was submitted to silica gel column chromatography eluted with a mixture of hexane-ethyl acetate (98:2). Seventy five fractions of 1 mL were obtained. Fractions 11-21 afforded 2 mg of pure myrcene as green fluorescent oil. Fractions 59-61 were pooled together to afford 30 mg of pure myrcenone (99% purity by GC). The identity of the myrcenone was confirmed by ^{1}H and ^{13}C NMR data measurement on a Bruker Avance DRX400 (Karlsruhe, Germany) at 25 °C, operating at 400.13 MHz for ^{1}H and 100.61MHz for ^{13}C NMR spectra were recorded in $CDCl_3$ using TMS as internal standard. Data were in accordance with those in the literature.[9] ^{1}H NMR ($CDCl_3$): 1.96 (s, C$\underline{H}_3$), 2.22 (s, C$\underline{H}_3$), 3.37 (s, C$\underline{H}_2$), 5.18 (d, 11, 1H, CH=C$\underline{H}_2$), 5.19 (sl, 1H, C=C$\underline{H}_2$), 5.25 (d, 17.5, 1H, CH=C$\underline{H}_2$), 5.32 (sl, 1H, C=C$\underline{H}_2$), 6.24 (sl, C=C$\underline{H}$), 6.53 (dd, 17.5, 11, C$\underline{H}$=CH_2); ^{13}C-NMR ($CDCl_3$): 198.41 (C=O), 156.84 (CH_2=$\underline{C}$), 141.03 (CH_2=$\underline{C}$), 138.58 (C=$\underline{C}$H), 122.81 (C=$\underline{C}$H), 120.24 ($\underline{C}H_2$=C), 115.31 ($\underline{C}H_2$=C), 48.38 (CH2), 28.10 (CH_3), 21.16 (CH_3).

Direct olfactive analysis of L. lacunosa essential oil (leaves)

Direct olfactive analysis was carried out on May 2007 with a new sample of the essential oil obtained from leaves

of *Lippia lacunosa* collected on May 14th, 2007, from the same *specimen* at the Campus of the Federal University of Juiz de Fora. Flowers were not available by this time of the year. GC-MS analysis of this oil, in the same conditions described above, revealed the same chemical composition reported here for that one collected in August 2005. Direct olfactive analyses were performed by a panel composed of three trained analysts from the perfumery company "MANE do Brasil", located at Estrada do Guerenguê, 1.421, CEP 22.713-000, Jacarepaguá, Rio de Janeiro, RJ, Brazil.

Results and Discussion

The flowers (fluorescent greenish-yellow oil) and leaves (fluorescent light green oil) of *L. lacunosa* yielded 0.38% and 0.44% of oil, respectively, while the distillation of *L. rotundifolia* yielded 0.33% and 0.01% of oil, respectively for flowers (light yellow oil) and leaves (light yellow oil). The main compounds (with relative % peak area above 0.1) identified in the essential oils of *L. lacunosa* and *L. rotundifolia* are listed in Table 1. The essential oils from leaves and flowers of *L. lacunosa* and from the flowers of *L. rotundifolia* showed a high content of monoterpenes (over 80%) and very low contents of sesquiterpenes.

In the oils of *L. lacunosa* flowers and leaves, 67 and 57 compounds were detected, respectively. Twenty six compounds were identified in the essential oils from both flowers and leaves of *L. lacunosa*, of which the major components were myrcene (flowers: 14.7, leaves: 11.9%), myrcenone (flowers: 45.2%, leaves: 64.2%), *(Z)*-ocimenone (flowers: 5.7%, leaves: 5.2%), and *(E)*-ocimenone (flowers: 14.7%, leaves: 4.1%), respectively. In the oils of *L. rotundifolia* 54 (flowers) and 108 (leaves) compounds were detected, while 58 different compounds were identified in both oils. Among them, the most abundant were limonene (flowers: 26.0, leaves: 7.9%) and myrtenal (flowers: 22.3%, leaves: 16.7%), respectively.

The oils from *L. rotundifolia* contain a variety of mono- and sesquiterpenes in amounts below 1%. In the oil from the leaves 51 identified compounds accounted for 88% of the oil, whereas in that obtained from the flowers 41 identified compounds accounted for 97.8% of the oil. A difference between the essential oils from the two plants concerns their yields: *L. rotundiflolia* leaves essential oil yield (0.01%) is much lower than that of the flowers (0.33%).

The major component of *L. lacunosa* essential oils, myrcenone, has been described as an important constituent of other *Lippia* species: *L. adoensis*[10] (0.8 to 14.9% in wild plants; 0.06 to 0.8% from cultivated plants), *L. alba*[11] (38 to 50%), *L. multiflora*[12] (54.6%), *L. juneliana*[13] (17.2%, in plants growing wild), *L. javanica*[14] (myrcenone, and *(E)*- and *(Z)*-ocimenone were the major constituents) and *L. asperifolia*[15] (ocimene (80%), and a mixture of ketones: myrcenone, ocimenone, etc, referred by the author as "tagetenones"); and *Aloysia*[16] (*A. triphylla,* 36.5%, cultivated) essential oils, as well as in *Hyptis mutabilis*[17] (14.8%,) oil, a genus belonging to the Lamiaceae, which is a family taxonomically very close to the Verbenaceae. In this way, the oil from the leaves of *L. lacunosa* was submitted to column chromatography, to afford pure myrcenone (99% purity by GC), as a yellow fluorescent oil. Its identity was confirmed by comparison of its NMR data with those in literature.[9] By this procedure, myrcene was also separated in its pure form as a green fluorescent oil.

The essential oils of *L. lacunosa* flowers and leaves exhibited a strong and pleasant mango aroma, the former one being sweeter than the latter, which resembled more the aroma of unripe mangos. That difference may, in part, be due to the myrcene/myrcenone ratio, since both single substances, separated by column chromatography, exhibited mango aroma. In view of these findings we decided to have the essential oil from the leaves evaluated by a panel, involving three analysts from the perfumery company "MANE do Brasil". As a result, the identified notes described in the panel were: tea/fruity (mango) as head notes; fruity/green as heart notes; and vegetal-like as base note. This result corroborates our initial findings, and it is interesting to note that the heart and base notes (green and "vegetal-like") described in the panel are in agreement with our perception of unripe mango aroma (green). A survey on the literature about volatile substances related to the aroma of mango fruits revealed that, in some mango varieties, myrcene plays an important role. Andrade *et al.*[18] studied the volatiles of 15 Brazilian mango fruit varieties, extracted by simultaneous distillation-extraction followed by GC-MS analysis. They could distinguish between three aroma groups, one of which was rich in myrcene (30.3-57.1%). Also, in another work by MacLeod and Pieris,[19] myrcene and (*Z*)-β-ocimene were demonstrated to be characteristic of the aroma of two varieties from India and Sri Lanka. Lopes *et al.,*[20] analysing the aroma of commercial Brazilian mangos by High Resolution Gas Chromatography-Olfatometry-Aroma Extract Dilution Analysis-Mass Spectrometry (HRGC-O-AEDA-MS) reported that myrcene is the main constituent of the varieties *Carlota* and *Coração de Boi*, and that it contributes to the "unripe" (green) flavour of the fruit.

The presence of dihydrotagetone, tagetone, as well as *(Z)*-and *(E)*-ocimenone in the essential oils of flowers and leaves of *L. lacunosa* are also noteworthy, as they are the main constituents of Tagetes oil,[21] a yellow to dark orange liquid with a strong aromatic fruity odor, obtained by stem distillation of *Tagetes minuta* L.

Table 1. Chemical composition (relative % peak area) of the essential oils from *Lippia lacunosa* and *L. rotundifolia* (flowers and leaves) obtained by GC and GC-MS on a HP-5 column

Constituents	RI[a]	*Lippia lacunosa*			*Lippia rotundifolia*		Identification Method[b]
		Flowers	Leaves[d]	Leaves[e]	Flowers	Leaves	
tricyclene	926	---	---	---	0.1	---	1,2
A-thujene	930	---	---	---	1.4	0.6	1,2
α-pinene	938	---	---	---	8.7	1.8	1,2
camphene	953	---	---	---	0.8	0.3	1,2
thuja-2,4(10)-diene	958	---	---	---	0.1	---	1,2
sabinene	977	---	0.1	---	2.2	0.9	1,2
β-pinene	980	---	---	---	9.5	3.0	1,2
1-octen-3-ol	981	0.3	---	---	---	---	1,2
myrcene	992	14.7	11.9	15.2	5.1	3.6	1,2
N.I.[c]	999	---	---	0.7	---	---	
α-phellandrene	1007	---	---	---	0.1	0.1	1,2
α-terpinene	1019	---	---	---	0.3	0.2	1,2
para-cymene	1028	---	---	---	0.7	1.3	1,2
limonene	1032	1.8	0.8	1.3	26.0	7.9	1,2
1,8-cineole	1035	---	---	---	1.4	0.4	1,2
(Z)-β-ocimene	1040	---	---	---	0.8	0.3	1,2
(E)-β-ocimene	1051	---	---	---	0.2	2.1	1,2
dihydrotagetone	1056	0.4	0.4	1.3	---	---	1,2
γ-terpinene	1062	---	---	---	2.9	2.9	1,2
cis-sabinene hydrate	1071	---	---	---	0.1	---	1,2
N.I.[c]	1088	---	0.1	---	---	---	
para-mentha-2,4(8) diene	1088	---	---	---	0.2	0.2	1,2
N.I.[c]	1101	1.0	1.0	---	0.4	0.8	1,2
N.I.[c]	1114	---	---	0.1	---	---	
trans-thujone	1120	---	---	---	0.1	0.3	1,2
N.I.[c]	1125	---	---	---	0.1	---	1,2
α-campholenal	1129	---	---	---	0.1	0.1	1,2
N.I.[c]	1133	---	---	0.1	---	---	
trans-pinocarveol	1142	---	---	---	1.5	0.5	1,2
ipsdienol	1147	---	---	1.0	---	---	1,2
myrcenone	1153	45.2	64.2	35.0	---	---	1,2,3
(Z)-tagetone	1157	0.5	0.5	---	---	---	1,2
N.I.[c]	1158	---	---	0.7	---	---	
N.I.[c]	1167	0.7	0.2	---	---	---	1,2
trans-pinocamphone	1164	---	---	---	0.1	---	1,2
pinocarvone	1166	---	---	---	3.0	0.1	1,2
borneol	1169	---	---	---	0.3	2.4	1,2
cis-pinocamphone	1177	---	---	---	4.5	3.1	1,2
terpinen-4-ol	1180	---	---	---	1.1	0.7	1,2
N.I.[c]	1189	0.1	0.1	0.6	---	---	
α–terpineol	1192	0.5	0.4	0.6	0.3	0.2	1,2
myrtenal	1197	---	---	---	22.3	16.7	1,2
N.I.[c]	1197	0.1	0.1	0.7	---	---	
N.I.[c]	1208	0.4	0.1	---	---	---	1,2
N.I.[c]	1209	---	---	0.4	---	---	
(Z)-ocimenone	1234	5.7	5.2	6.1	---	---	1,2
(E)-ocimenone	1243	14.7	4.1	13.4	---	---	1,2
N.I.[c]	1248	---	0.1	0.3	---	---	
N.I.[c]	1256	---	---	---	0.2	---	1,2

Table 1. continuation

Constituents	RI[a]	*Lippia lacunosa*			*Lippia rotundifolia*		Identification Method[b]
		Flowers	Leaves[d]	Leaves[e]	Flowers	Leaves	
piperitone	1258	---	---	---	0.2	---	1,2
N.I.[c]	1269	---	---	0.9	---	---	
thymol	1293	0.5	0.2	0.1	---	---	1,2
trans–sabinyl acetate	1294	---	---	---	t	0.3	1,2
myrtenyl acetate	1328	---	---	---	1.2	2.1	1,2
δ-elemene	1340	---	---	---	---	0.3	1,2
neryl acetate	1368	---	---	0.5	---	0.2	1,2
α-copaene	1378	---	---	---	0.1	0.7	1,2
geranyl acetate	1386	0.5	0.5	---	---	---	1,2
β-bourbonene	1386	---	---	---	---	0.9	1,2
β-elemene	1393	---	---	---	0.3	10.9	1,2
(E)-caryophyllene	1421	---	0.7	2.7	0.9	7.9	1,2
α-humulene	1456	0.4	0.1	0.4	0.2	1.5	1,2
allo-aromadendrene	1463	---	---	---	---	0.2	1,2
trans–cadina-1(6),4-diene	1475	---	---	---	---	0.2	1,2
γ-gurjunene	1478	---	---	---	---	0.1	1,2
germacrene D	1480	---	---	0.8	---	---	1,2
γ-muurolene	1482	0.3	0.1	---	0.3	2.2	1,2
ar-curcumene	1484	---	---	---	0.2	1.0	1,2
β-selinene	1487	---	---	---	---	0.3	1,2
muurola-4(14),5-diene	1492	---	---	---	---	0.5	1,2
α-muurolene	1501	---	---	---	---	0.2	1,2
germacrene A	1506	---	---	---	---	1.9	1,2
γ-cadinene	1516	---	---	---	---	0.7	1,2
δ-cadinene	1525	---	0.8	---	---	0.9	1,2
germacrene B	1559	---	---	---	---	0.3	1,2
caryophyllene oxide	1584	0.4	1.1	0.7	---	2.5	1,2
epi-α-cadinol and/or *epi*-α-muurolol	1643	---	0.2	---	---	0.6	1,2
α-muurulol	1649	---	---	---	---	0.7	1,2
Monoterpenes		84.5	88.3	74.5	95.6	52.5	
Total Identified Compounds		85.9	91.3	79.7	97.3	87.0	

[a]RI - retention index obtained using an HP-5 column, t = trace (below 0.05%). [b]Identification Methods: 1-Retention indices; 2-Wiley library; 3-^{1}H and ^{13}C NMR. [c]N.I.-not identified. [d]Essential oil extracted in August 2005. [e]Essential oil extracted in May 2007.

The aroma of the essential oils from *L. rotundifolia* (flowers and leaves) is somewhat different from those of *L. lacunosa*. The essential oil from the flowers has a strong and pleasant floral scent, less pronounced in the essential oil from the leaves. Myrtenal, the second major constituent of the flowers oil, and the major one on the leaves oil, is also found in eucalyptus, mint, pepper and cumin seeds essential oils. It is used in perfumery, its odour being described as spicy, with the following odour descriptions: sweet cinnamon, tonka, terpene, camphor and jam,[22] as well as an insecticide[23] (from *Myrtus communis* L.). This substance is also used by the European grapevine moth (*Lobesia botrana*) in its chemical communication system.[24]

Conclusions

Although *L. lacunosa* and *L. rotundifolia* are considered as synonyms in many herbarium samples, there are marked differences in the composition of their essential oils. The olfactive description of the volatile constituents of both the investigated oils could be included in the herbarium tags of the species, as "floral" for *L. rotundifolia*, and "mango aroma", for *L. lacunosa*, being of great help for the differentiation of the species and further botanical classification.

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Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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