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Chemical composition, seasonal variation and a new sesquiterpene alcohol from the essential oil of *Lippia integrifolia*

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ABSTRACT: Chemical investigations on the essential oil of *Lippia integrifolia* performed by column chromatography, HPLC, GC–MS, ^{1}H- and ^{13}C-NMR spectroscopy led to the identification of 78 components. A new sesquiterpene alcohol, *trans*-africanan-1α-ol (4), was characterized as a significant component of the oil. Additionally, six samples obtained at monthly intervals through the annual cycle of the shrub (November–April) were studied. The oil was characterized by large amounts of oxygenated sesquiterpenes (53.3–69.4%) and sesquiterpene hydrocarbons (14.3–27.9%), along with small amounts of monoterpenoids (1.4–2.9%). Lippifoli-1(6)-en-5-one (2), african-5-en-1α-ol (3), β-caryophyllene oxide, 4,5-*seco*-africanan-4,5-dione (10), *trans*-africanan-1α-ol (4), humulene epoxide II, integrifolian-1,5-dione (1) and lippifoli-1(6)-en-4β-ol-5-one (7) were the main oxygenated constituents. Although the qualitative composition appeared to be constant, there were large quantitative variations in the content of most components through the studied period. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: *Lippia integrifolia*; essential oil composition; seasonal variation; *trans*-africanan-1α-ol; new sesquiterpene alcohol

Introduction

The genus *Lippia* (family Verbenaceae) comprises some 200 species growing in the tropical and subtropical regions of South America, Central America and Africa.[1] In the Americas, members of this genus can be found from Mexico to the province of Buenos Aires in Argentina.[1,2] *Lippia integrifolia* (Griseb.) Hieronymus, commonly known as 'incayuyo' or 'té del inca', is a woody aromatic shrub native to central and northern Argentina, where infusions of the aerial parts are widely used in traditional medicine as a soft diuretic, emmenagogue, stomachic and nervine.[3,4] It is also used in Paraguay against asthma and as an abortive.[4] *L. integrifolia* has been shown to be a rich source of uncommon sesquiterpenoids. The unique ketones integrifolian-1,5-dione (**1**), lippifoli-1(6)-en-5-one (**2**) and closely related derivatives,[5–10] together with sesquitepenoids based on the rare africanane[11–13] and asteriscane[12,13] skeletons, constitute the bulk of its essential oil.[8–10]

The essential oil composition of *L. integrifolia* collected in different provinces of Argentina has been studied by several authors.[8–10,14] Forty-five components, accounting for 73.7% of the total oil, were identified in the essential oil from aerial parts of plants collected in the province of Cordoba,[9] which contained lippifoli-1(6)-en-5-one (**2**) (16.7%), camphor (16.2%) and an unidentified sesquiterpene (14.9%) as the main constituents, while in the flower oil (27 identified components, accounting for ~60%) (**2**) (18.9%), camphor (18.5%) and limonene (13.3%) predominated.[10] The major constituents from plants collected at Lujan, province of San Luis[14] (22 identified components, accounting for ~70%) were β-caryophyllene (18.4%), α-humulene (9.7%), limonene (8.2%), spathulenol (6.6%) and borneol (5.7%). Finally, air-dried aerial parts purchased from a local market in the

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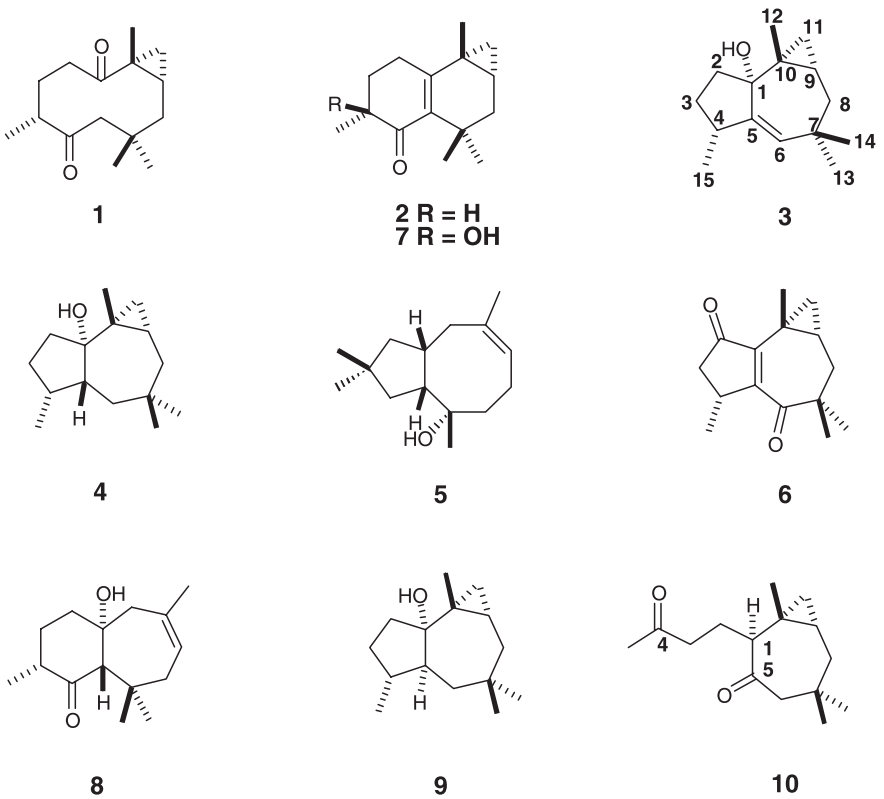

province of Tucumán yielded an essential oil (24 identified components, accounting for ~66%) dominated by **2** (21.8%), β-caryophyllene oxide (8.0%) and β-caryophyllene (5.8%).[8]

In the present paper we report the results of a detailed analysis carried out on the essential oil obtained from a selected population of *L. integrifolia* growing wild in the province of Catamarca, which permitted identification of 78 components which are listed in Table 1. One of these components resulted to be a new sesquiterpene alcohol, *trans*-africanan-1α-ol (**4**), whose structure was established by spectroscopic methods. The seasonal variation in the essential oil composition was also studied.

Materials and Methods

Plant Material

Aerial parts of *L. integrifolia* were collected from a large wild population growing at Dique Las Pirquitas, province of Catamarca, Argentina. The plant material was identified by Lic. Alberto Slanis of the Miguel Lillo Institute, Tucumán, Argentina, where a voucher specimen (LIL 605837) has been deposited. In order to study the seasonal variation, 26 plants were selected, labelled and harvested during November 2002–April 2003, as shown in Table 4.

Isolation of the Oil

The volatile oils were obtained by hydrodistillation in a Clevenger-type apparatus for 4 h. The aerial parts of *L. integrifolia* yielded orange-reddish oils which were dried over anhydrous sodium sulphate and stored at –5 °C.

Chemical Investigation

Chemical analysis of the essential oils, as well as of the various fractions obtained after column chromatography on silica gel and HPLC, was carried out by gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS) techniques. Pure compounds were also analysed by UV, IR, ^{1}H- and ^{13}C-NMR, using 1D and 2D techniques.

GC Analysis

They were carried out using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a capillary HP-5 column (30 m × 0.32 mm; 0.25 μm film thickness); injector and detector temperature were maintained at 250 °C and 270 °C, respectively; injection size, 0.5 μl, split mode; carrier gas,

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Table 1. Chemical composition of *L. integrifolia* essential oil

No.	Constituents	Amount (min–max) (%)	RI (HP-5)	Identification
1.	α-Thujene	tr	0928	MS, RI
2.	α-Pinene	0.2–0.5	0936	MS, RI
3.	Camphene	tr–0.1	0952	MS, RI
4.	5,5-Dimethyl-2-(5*H*)furanone	tr	0958	MS, RI
5.	Sabinene	tr	0972	MS, RI
6.	β-Pinene	tr	0977	MS, RI
7.	Myrcene	tr–0.1	0990	MS, RI
8.	α-Phellandrene	tr	1004	MS, RI
9.	δ-3-Carene	tr–0.1	1011	MS, RI
10.	α-Terpinene	tr	1015	MS, RI
11.	*p*-Cymene	0.1–0.2	1023	MS, RI
12.	Limonene	0.5–1.3	1028	MS, RI
13.	1,8-Cineole	tr	1031	MS, RI
14.	*Z*-β-Ocimene	tr–0.1	1036	MS, RI
15.	*E*-β-Ocimene	tr–0.2	1050	MS, RI
16.	γ-Terpinene	tr	1059	MS, RI
17.	*cis*-Sabinene hydrate*	—	1068	MS, RI
18.	*trans*-Linalool oxide*	—	1075	MS, RI
19.	*cis*-Linalool oxide*	—	1085	MS, RI
20.	Terpinolene	tr	1087	MS, RI
21.	*trans*-Sabinene hydrate*	—	1096	MS, RI
22.	Linalool	0.1–0.3	1099	MS, RI
23.	*cis*-*p*-Menth-2-en-1-ol*	—	1122	MS, RI
24.	*trans*-Pinocarveol*	—	1139	MS, RI
25.	1-Terpineol*	—	1140	MS, RI
26.	*trans*-Verbenol*	—	1144	MS, RI
27.	Camphor	tr	1145	MS, RI
28.	Borneol	0.1–0.2	1168	MS, RI
29.	Terpinen-4-ol*	tr	1177	MS, RI
30.	*p*-Cymen-8-ol*	—	1183	MS, RI
31.	α-Terpineol*	—	1190	MS, RI
32.	*cis*-Piperitol*	—	1196	MS, RI
33.	Verbenone*	—	1205	MS, RI
34.	*trans*-Piperitol*	—	1209	MS, RI
35.	*trans*-Carveol*	—	1218	MS, RI
36.	*cis*-*p*-Mentha-1(7),8-dien-2-ol*	—	1235	MS, RI
37.	Carvone*	tr	1242	MS, RI
38.	(*Z*)-3-Hexenyl-2-methylbutanoate	0.1–0.2	1247	MS, RI
39.	Bornyl acetate	tr	1288	MS, RI
40.	Tridecane	0.1–0.3	1300	MS, RI
41.	African-1-ene	0.7–1.1	1333	MS[a]
42.	African-5-ene	3.9–4.8	1347	MS[a]
43.	Africa-1,5-diene	3.1–6.3	1352	MS[a]
44.	α-Copaene	tr	1375	MS, RI
45.	Asterisca-3(15),6-diene	2.3–3.2	1376	MS[a]
46.	β-Elemene	tr–0.3	1390	MS, RI
47.	α-Cedrene*	—	1410	MS, RI
48.	β-Caryophyllene	2.9–8.6	1417	MS, RI

helium at a flow rate of 1 ml/min. The oven was programmed as follows: 60 °C for 5 min, rising at 0.8 °C/min to 100 °C, then rising at 0.4 °C/min to 140 °C, then rising at 10 °C/min to 275 °C and then kept for 5 min. This programme with three temperature ramps gave the best resolution for the very complex sesquiterpene region of the essential oil. For RI measurements, the oven temperature programme suggested by Adams[15] (60 °C to 246 °C at 3 °C/min) was used. Percentages (FID) in Table 1 and Table 5 are the means of three runs obtained from electronic integration measurements using an HP 3395 integrator.

GC–MS Analysis

Mass spectra were recorded on a 5973 Hewlett-Packard selective mass detector coupled to a Hewlett-Packard 6890 GC using HP-5MS (5% phenylmethylsiloxane) capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness). The injector, GC–MS interphase, ion source and selective mass detector temperatures were maintained at 250 °C, 275 °C, 280 °C and 150 °C, respectively; ionization energy, 70 eV; injection size: 1.0 μl (in split mode); carrier gas, helium at a flow rate of 1.0 ml/min. The oven was programmed as follows: 50 °C for 10 min,

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Table 1. (*Continued*)

No.	Constituents	Amount (min–max) (%)	RI (HP-5)	Identification
49.	Aromadendrene*	—	1440	MS, RI
50.	α-Humulene	1.3–4.5	1454	MS, RI
51.	*allo*-Aromadendrene*	—	1459	MS, RI
52.	α-Amorphene*	—	1485	MS, RI
53.	African-5-en-1α-ol (**3**)#	10.4–17.4	1485	MS[a], ^{1}H, ^{13}C
54.	α-Muurolene*	—	1498	MS, RI
55.	Pentadecane	tr	1500	MS, RI
56.	β-Bisabolene*	—	1505	MS, RI
57.	*trans*-Africanan-1α-ol (**4**)#	1.3–3.1	1525	MS, ^{1}H, ^{13}C
58.	*cis*-Calamenene*	—	1539	MS, RI
59.	Lippifoli-1(6)-en-5-one (**2**)#	14.2–27.9	1550	MS[b], ^{1}H, ^{13}C
60.	*trans*-Nerolidol*#	—	1564	MS, ^{1}H, ^{13}C
61.	Spathulenol*#	—	1578	MS, ^{1}H, ^{13}C
62.	β-Caryophyllene oxide#	5.4–13.1	1582	MS, ^{1}H, ^{13}C
63.	3α-Hydroxy-6-asteriscene (**5**)#	0.6	1584	MS[b], ^{1}H, ^{13}C
64.	Guaiol*	—	1601	MS, RI
65.	Humulene epoxide II	1.3–2.7	1607	MS, RI
66.	Lippifoli-1(6)-en-4β-ol-5-one (**7**)#	1.1–1.9	1610	MS[b], ^{1}H, ^{13}C
67.	Caryophylla-4(14),8(15)-dien-5β-ol#	0.2–0.8	1638	MS, ^{1}H
68.	African-1(5)-en-2,6-dione (**6**)#	tr	1644	MS[b], ^{1}H, ^{13}C
69.	β-Eudesmol#	—	1650	MS, RI, ^{1}H
70.	α-Eudesmol#	—	1653	MS, RI, ^{1}H
71.	1,6-*trans*-Lippifolian-1α-ol-5-one	0.6–1.7	1660	MS[b], ^{1}H, ^{13}C
72.	1,6-*cis*-Lippifolian-1α-ol-5-one	0.1–0.3	1665	MS[b], ^{1}H, ^{13}C
73.	Guai-1(10)-en-11-ol[c]*	—	1672	MS, RI
74.	Caryophylla-3,8(15)-dien-5β-ol[d]#	0.1–0.8	1675	MS, ^{1}H
75.	Compound **8**	0.2–0.3	1690	MS[b], ^{1}H, ^{13}C
76.	*trans*-Humul-9(*E*)-en-2,6-dione#	0.2–0.3	1739	MS[b]
77.	Integrifolian-1,5-dione (**1**)	1.4–2.3	1745	MS[b], ^{1}H, ^{13}C
78.	4,5-*seco*-Africanan-4,5-dione (**10**)	5.0–10.0	1756	MS[b], ^{1}H, ^{13}C

tr, trace (<0.05%).
* Identified in a fraction from column chromatography on silica gel.
# Isolated during this work.
[a] Identification by comparison with mass spectrum reported in the literature.[13]
[b] Identification by comparison with mass spectra reported in the literature[5,7,11,12,21] and in our files.
[c] Known also as bulnesol.
[d] Known also as caryophyllenol I.

Table 2. ^{1}H-NMR data for compounds 3, 4 and 9 (in $CDCl_3$)

H	3	4	4 (in C_6D_6)	9[a]
2α	1.96 dt (6, 12)	1.88 m	1.7–1.9 m	1.47
2β	1.80 m	1.92 m		1.97
3α	1.41 brdd (13, 6)	1.96 m	1.97 m	1.35 m
3β	1.54 brdd (13, 6)	1.17 m	1.08 m	1.68 m
4	2.35 m	1.74 m	1.7–1.9 m	1.31 m
5	—	1.05 ddd (11.7, 10.5, 2.7)	0.93[b]	1.09
6α	5.10 t (2.0)	1.28 dd (14.4, 11.7)	1.36 dd	0.99
6β		1.19 dd (14.4, 2.7, 2.1)	1.15 ddd	1.38
8α	1.87 brdd (14.4, 2.0)	1.89 dd (15.0, 11.8)	1.80	1.05
8β	1.74 ddd (14.4, 5.5, 2.0)	1.73 ddd (15.0, 5.5, 2.1)	1.63	1.47
9β	0.72 m	0.74 m[c]	0.63 m[c]	0.79
11α	1.02 dd (4.1, 5.0)			0.66
11β	0.39 dd (4.1, 8.5)	0.31 m[c]	0.23 m[c]	0.27
12 (Me)	1.00 s	1.12 s	1.11 s	1.03 s
13 (Me)	1.08 s	0.96 s	0.94 s	0.98 s
14 (Me)	0.95 s	0.94 s	0.91 s	0.84 s
15 (Me)	1.09 d (6.5)	0.93 d (6.5)	0.91 d	1.02 d

[a] Taken from ref. 18.
[b] Obscured by the intense methyl signals.
[c] Not first order.

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Table 3. ^{13}C-NMR data for compounds **3**, **4** and **9** (in $CDCl_3$)

C	3	4	9[a]
1	80.8 s	85.9 s	85.3 s
2	38.4 t	38.1 t	38.9 t
3	36.6 t	30.1 t	32.7 t
4	40.5 d	38.1 d	43.2 d
5	147.6 s	49.5 d	54.9 d
6	135.7 d	39.8[b] t	41.7 t
7	37.0 s	33.0 s	33.3 s
8	39.8 t	39.7[b] t	41.2 t
9	19.8 d	25.7 d	22.2 d
10	25.0 s	26.9 s	23.5 s
11	16.8 t	16.3 t	15.2 t
12	21.2 q	23.5 q	18.9 q
13	32.9 q	35.1 q	29.1 q
14	24.9 q	28.0 q	28.2 q
15	28.0 q	19.7 q	26.7 q

[a] Taken from ref. 18.
[b] Signals may be interchanged.

rising at 1.0 °C/min to 100 °C, rising at 0.5 °C/min to 150 °C, rising at 10 °C/min to 270 °C, then held for 5 min. The HR–MS was measured on a VG 7070 high-resolution mass spectrometer at the UCR Mass Spectrometry Facility, University of California, Riverside, CA, USA.

Identification

The component percentages were taken from capillary GC traces with FID. Identification of the individual components was based on (a) computer matching with commercial mass spectra libraries (NBS75K, NIST, WILEY),[15–17] comparison with spectra available in our files[5–8,11,12] and literature data;[13–18] (b) comparison of their GC retention indices (RI) on an HP-5 column.

^{1}H- and ^{13}C-NMR Analysis

NMR spectra were recorded on Varian Mercury 300 or Bruker DMX-500 spectrometers operating at 300 MHz and 500 MHz, respectively; ^{1}H using a 5 mm probe and $CDCl_3$ as solvent unless otherwise stated.

HPLC separations

For separation of mixtures, Gilson HPLC equipment with a differential refractometer was used. The following normal-phase and reversed-phase columns were employed: (a) Beckman Cyano (5 μm, 10 × 250 mm); (b) Beckman C18 (5 μm, 10 × 250 mm); and (c) Develosil 60-5 Silica (5 μm, 10 × 250 mm).

Results and Discussion

From 435 g air-dried aerial parts (leaves, flowers and tender branches) of *L. integrifolia* collected in March 2002, 5.2 ml oil (yield 1.2%) was obtained. A portion of this oil (4.7 ml) was chromatographed on silica gel 230–400 mesh using *n*-hexane with increasing amounts of Et_2O (0–40%); nine fractions (I–IX) were collected and analysed by GC–MS on a HP-5MS capillary column. Table 1 shows the identified constituents, their minimum and maximum percentages through the studied period, their retention indices and method of identification. Fractions I–IV were analysed separately by GC and GC–MS.

Fraction V contained significant amounts of a compound whose MS exhibited M^+ at *m/z* 220 and a base peak at *m/z* 179, which was identical to the MS corresponding to the main component of the essential oil obtained at the beginning of the season (21 November 2002). Rechromatography on silica gel followed by HPLC on column (C) using hexane:ethyl acetate 49:1 afforded 25.4 mg of the compound with M^+ 220 and 15.5 mg of a closely related product showing M^+ 222. These compounds were identified as african-5-en-1α-ol (**3**) and *trans*-africanan-1α-ol (**4**), respectively.

The new africanane derivative **4** was obtained as a colourless oil which showed no absorptions in the UV spectrum. The IR spectrum showed the presence of a hydroxyl group at 3450/cm, while the HREI–MS displayed M^+ at *m/z* 222.1987 (calcd. 222.1984), in agreement with a molecular formula $C_{15}H_{26}O$. The absence of signals for sp^2 carbons in the ^{13}C-NMR spectrum indicated that **4** was a tricyclic sesquiterpene alcohol. The ^{13}C-NMR spectrum of **4** was similar to that of african-5-en-1α-ol (**3**),[13] except for the signals corresponding to the

Table 4. Data of harvesting and essential oil yield of *L. integrifolia*

Sample	Growth phase and harvesting date	Essential oil yield (%, v/w of dry weight)
I	Young buds, 21 November 2002	0.9
II	Growing, 22 December 2002	1.2
III	Growing, first flowers appear 14 January 2003	1.4
IV	Growing, 19 February 2003	1.2
V	Growing, 2 March 2003	1.2
VI	Fully blooming, 5 April 2003	1.6

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Table 5. Seasonal variation in the essential oil composition (%) of *L. integrifolia*

Constituents	Composition (%)					
	I	II	III	IV	V	VI
α-Thujene	tr	tr	tr	tr	tr	tr
α-Pinene	0.5	0.4	0.4	0.3	0.4	0.2
Camphene	0.1	0.1	0.1	tr	0.1	0.1
5,5-Dimethyl-2-(5H)furanone	tr	tr	tr	tr	tr	tr
Sabinene	tr	tr	tr	tr	tr	tr
β-Pinene	tr	tr	tr	tr	tr	tr
Myrcene	0.1	0.1	0.1	tr	tr	tr
α-Phellandrene	tr	tr	tr	tr	tr	tr
δ-3-Carene	tr	0.1	tr	tr	tr	tr
α-Terpinene	tr	tr	tr	tr	tr	tr
p-Cymene	0.1	0.1	0.2	0.2	0.2	0.1
Limonene	0.8	1.3	0.8	0.5	1.3	0.8
1,8-Cineole	tr	tr	tr	tr	tr	tr
(*Z*)-β-Ocimene	tr	0.1	tr	tr	tr	tr
(*E*)-β-Ocimene	0.2	0.2	0.1	tr	tr	tr
γ-Terpinene	tr	tr	tr	tr	tr	tr
Terpinolene	tr	tr	tr	tr	tr	tr
Linalool	0.3	0.3	0.3	0.2	0.1	0.1
Camphor	tr	tr	tr	tr	tr	tr
Borneol	0.1	0.2	0.2	tr	0.2	0.2
Terpinen-4-ol	tr	tr	tr	tr	tr	tr
trans-Carveol	tr	tr	tr	tr	tr	tr
Carvone	tr	tr	tr	tr	tr	tr
(*Z*)-3-Hexenyl-2-methylbutanoate	0.2	0.1	0.1	0.1	0.1	0.1
Bornyl acetate	tr	tr	tr	tr	tr	tr
Tridecane	0.3	0.2	0.1	0.1	0.1	0.1
African-1-ene	1.1	1.0	1.1	0.9	0.7	0.8
African-5-ene	4.7	4.5	4.8	4.1	3.9	4.4
Africa-1,5-diene	5.7	6.3	5.1	4.0	3.1	4.1
α-Copaene	tr	tr	tr	tr	tr	tr
Asterisca-3(15),6-diene	3.1	2.9	3.1	2.6	2.3	3.2
β-Elemene	0.3	0.2	0.2	tr	tr	tr
β-Caryophyllene	8.6	6.1	5.4	3.8	2.9	4.4
α-Humulene	4.5	3.1	2.5	2.4	1.3	2.4
African-5-en-1a-ol (**3**)	17.4	14.1	17.2	10.4	10.8	12.5
Pentadecane	tr	tr	tr	tr	tr	tr
trans-Africanan-1α-ol (**4**)	3.1	2.3	2.7	1.3	1.5	2.1
Lippifoli-1(6)-en-5-one (**2**)	14.2	20.7	23.2	27.9	24.1	24.6
Spathulenol + β-caryophyllene oxide	6.4	5.4	6.8	10.6[a]	13.1[a]	9.0
3α-Hydroxy-6-asteriscene (**5**)	0.6	0.6	0.6	[b]	[b]	0.6
Humulene epoxide II	1.6	2.6[c]	1.3	2.7	2.3	2.2
Lippifoli-1(6)-en-4β-ol-5-one (**7**)	1.1	[d]	1.5	1.7	1.6	1.9
Caryophylla-4(14),8(15)-dien-5β-ol	0.5	0.5	0.6	1.0	1.3	0.7
African-1(5)-en-2,6-dione (**6**)	tr	tr	tr	tr	tr	tr
1,6-*trans*-Lippifolian-1α-ol-5-one	0.7	0.6	0.8	1.1	1.7	1.1
1,6-*cis*-Lippifolian-1α-ol-5-one	0.2	0.3	0.1	0.3	0.3	0.2
Caryophylla-3,8(15)-dien-5β-ol	0.1	0.2	0.5	0.8	0.7	0.5
Compound **8**	0.2	0.3	0.2	0.3	0.2	0.2
trans-Humul-9(*E*)-en-2,6-dione	0.2	0.2	0.2	0.3	0.2	0.2
Integrifolian-1,5-dione (**1**)	1.7	1.8	1.7	2.3	1.7	1.4
4,5-*seco*-Africanan-4,5-dione (**10**)	5.4	4.0	5.8	6.9	10.0	8.1

tr, trace (<0.05%).
[a] Includes 3α-hydroxy-6-asteriscene.
[b] Overlapped with spathulenol and β-caryophyllene oxide.
[c] Includes lippifoli-1(6)-en-4β-ol-5-one.
[d] Overlapped with humulene epoxide II.

sp^2 carbons, which were now replaced by two sp^3 carbons: a CH_2 and a CH (Table 3). These results indicated that we were dealing with a dihydroderivative of **3**. The ^{1}H-NMR spectrum of **4** (Table 2) showed signals for cyclopropane protons at δ 0.31 (1H, m, H-11β) and 0.74 (2H, m, H-11α, H-9β), three tertiary methyls at δ 0.94 (3H, s, Me-14), 0.96 (3H, s, Me-13) and 1.12 (3H, s, Me-12), a secondary methyl at δ 0.93 (3H, d, J = 6.5 Hz, Me-15) and a methyne proton at δ 1.05 (1H, ddd, 11.7, 10.5 and 2.7 Hz, H-5β). The stereochemistry of the molecule was established from ^{1}H-^{1}H COSY and NOESY correlations. The ^{1}H-^{1}H COSY spectrum showed long-range couplings of the axial Me-14 (0.94 ppm) with two antiperiplanar protons,

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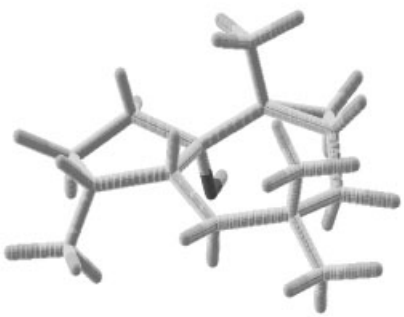
H-6α (1.28 ppm) and H-8α (1.89 ppm). Also, a long-range coupling (2.1 Hz) between H-6β and H-8β (W-type arrangement) was present, indicating a *trans*-fused africanane skeleton.[22] In the NOESY spectrum, H-11β (0.31 ppm) showed correlations with Me-12 (1.12 ppm) and H-9β/H-11α (0.74 ppm), while H-9β/H-11α showed correlations with Me-12, H-8β, Me-14 and H-11β, and H-5 (1.05) showed correlation with Me-14, indicating that it is β–orientated.

The *trans*-fusion of the 5,7-ring system was corroborated by the large ^{13}C chemical shift difference (Table 3) between the geminal methyl groups: δ (C-13) 35.1 ppm and δ (C-14) 28.0 ppm, $\Delta\delta_C$ = 7.1 ppm resulting from gauche interactions of C-14 with C-5 and C-9, while C-13 has none.[22,23] In *cis*-fused africananes the geminal methyls exhibit similar chemical shifts because each methyl group has one gauche interaction.

The α-orientation for the hydroxyl group of **4** was evident from the deshielding of the signal corresponding to H-11α (0.74 ppm), which appears at lower field than H-11β (0.31 ppm). In africanane derivatives unsubstituted at C-1, the signal for H-11α always appears at higher fields than the signal for H-11β, usually in the range 0.10–0.30 ppm.[13,22–24] Based on the same reasoning, the hydroxyl group of **3** should also be α-orientated. At this point, only the stereochemistry of the C-15 methyl group remains to be settled. The NOESY spectrum was not very helpful, owing to overlapping of the signals and crowding of cyclopentane protons. However, as $J_{4,5\beta}$ could be measured at the ddd corresponding to H-5β (δ 1.05): $J_{4,5\beta}$ = 10.5 Hz, $J_{5\beta,6\alpha}$ = 11.7 and $J_{5\beta,6\beta}$ = 2.7 (Table 2), the stereochemistry at C-4 could be determined by molecular modelling using the PCMODEL program (V 6.00, Serena Software, Bloomington, IN, USA). In this way, the geometry of the minimum energy conformer of both C-4 epimers was obtained and the calculated ^{1}H-NMR couplings for H-5β were compared with the observed values, as shown in Figures 1 and 2. As can be seen, the calculated coupling value for the epimer having the C-15 methyl group in the α position (Figure 1) matches very well with the observed coupling constant, while there is no agreement for the isomer having the secondary methyl group in the β position (Figure 2). Consequently, an α orientation was assigned to the secondary methyl group of alcohol **4**, in agreement with biogenetic considerations derived from the co-occurrence of **4** with its unsaturated analogue of known stereochemistry at C-4, african-5-en-1α-ol (**3**),[13] in *L. integrifolia*. The stereochemistry of **4**, depicted in Figure 1, which corresponds to 1*S*,4*R*,5*R*,9*S*,10*R*, is in further agreement with the absolute configuration recently determined[25] for lippifoliane and africanane derivatives isolated from *L. integrifolia*.

It is interesting to note that the *cis*-fused africanan-1α-ol (**9**) is a known compound isolated from the liverwort *Pellia epiphylla*.[18] The ^{1}H- and ^{13}C-NMR spectra reported for **9**[18] are included in Tables 2 and 3 for comparison purposes.

The EI–MS spectrum of **4** was partially similar to that reported[18] for the *cis*-fused isomer **9**. Both alcohols displayed a molecular ion at *m/z* 222 and a base peak at *m/z* 125. However, the spectrum of **4** showed significant and distinctive fragments at *m/z* 165 (15%), 151 (21%), 138 (33%), 109 (44%) and 96 (90%).



E_{MMX} = 35.00 kcal/mol

Protons	Dihedral angle (°)	J (Hz) calculated	J (Hz) observed
4β,5β	+0.3	10.4	10.5
5β,6α	–179.0	12.4	11.7
5β,6β	+65.9	2.4	2.7

Figure 1. Minimum energy conformation of (1*S*,4*R*,5*R*,9*S*,10*R*)-africanan-1-ol **(4)** and calculated H-C-C-H dihedral angles involving H-5

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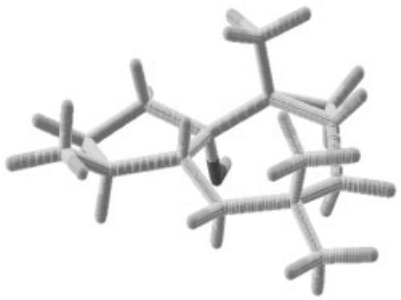

E_{MMX} = 33.52 kcal/mol

Protons	Dihedral angle (°)	J (Hz) calculated	J (Hz) observed
4α,5β	−139.0	7.3	10.5
5β,6α	−178.4	12.4	11.7
5β,6β	+64.5	2.6	2.7

Figure 2. Minimum energy conformation of (1*S*,4*S*,5*R*,9*S*,10*R*)-africanan-1-ol and calculated H-C-C-H dihedral angles involving H-5

African-5-en-1α-ol (**3**) was isolated as a solid, m.p. 40–41 °C, which was spectroscopically identical to the african-5-en-1-ol of unassigned stereochemistry at C-1, previously isolated from this plant by Fricke *et al.*[18] We have assigned α-stereochemistry to the hydroxyl group of **3,** based on the deshielding shown by the signal corresponding to H-11α, as discussed above. The GC–MS data of **3** recorded in our laboratory (see below) showed significant differences in the relative intensity of most fragments when compared with the reported values.[18] This could be due to the known tendency of tertiary alcohols to undergo dehydration and/or decomposition, depending on the experimental conditions and the presence of catalytic surfaces.

Fraction VI was processed by HPLC first on column (a) to give several subfractions, which in turn were rechromatographed on column (b), with 25% aqueous methanol to afford integrifolian-1,5-dione (**1**)[5], *trans*-humul-(9*E*)-ene-2,6-dione,[21] 1,6-*cis*- and 1,6-*trans*-lippifolian-1α-ol-5-one,[7] additional amounts of **4**, **6**, **7**, spathulenol, (*S*)-(+)-*trans*-nerolidol and 1α,7β,9α-1-hydroxy-3,6,6,9-tetramethylbicyclo[5.4.0]undec-3-en-8-one (**8**).[12]

Fraction VII, originally eluted with hexane containing 20% Et_2O, was processed by HPLC using column (a) with hexane:ethyl acetate 49:1 at 2 ml/min as eluting solvent, leading to three fractions (FVII.1–FVII.3).

Fraction VII.1 was rechromatographed by HPLC on column (b) with MeOH:H_2O 3:1 at 2 ml/min to give 5.5 mg spathulenol and 2.2 mg 3α-hydroxy-6-asteriscene (**5**),[12] which were identified by their MS, ^{1}H- and ^{13}C-NMR spectra.[12] Fraction VII.2 was processed by HPLC on column (b), as before, to give 6.2 mg african-1(5)-en-2,6-dione (**6**),[12] 3.4 mg lippifoli-1(6)-en-4β-ol-5-one (**7**),[7] 9.4 mg caryophylla-4(14),8(15)-dien-5b-ol, 6.2 mg β-eudesmol and 1.1 mg of α-eudesmol, which were identified from their MS and ^{1}H-NMR spectra. Fraction VII.3, processed by HPLC as before, yielded 3.2 mg caryophyllenol-I identified by ^{1}H- and ^{13}C-NMR.[19–20]

To study the seasonal variation, 26 specimens of a very large population of *L. integrifolia* located at Dique Las Pirquitas, province of Catamarca, were labelled; a small portion of the selected plants was harvested periodically, the plant material from each collection was combined and steam-distilled to obtain the essential oil, as summarized in Table 4. The samples were analysed separately and the results are summarized in Table 5.

The essential oil yield was in the range 0.9–1.6%, depending on the month of harvesting; the lowest content of oil (0.9%) was found at the beginning of the growing period and the highest yield (1.6%) was found at the end of the studied period (Table 4).

The oil was characterized by large amounts of oxygenated sesquiterpenes (53.3–69.4%) and hydrocarbon sesquiterpenes (14.3–27.9%) and small amounts of monoterpenes (1.4–2.9%); the variation in the amounts of the different types of constituents through the studied period is shown in Figure 3. The main constituents always were lippifoli-1(6)-en-5-one (**2**) (14.2–27.9%), african-5-en-1α-ol (**3**) (10.4–17.4%), β-caryophyllene oxide (5.4–13.1%) and 4,5-*seco*-african-4,5-dione (**10**) (5.0–10.0%). Other significant components were african-5-ene (3.9–4.8%), africa-1,5-diene (3.1–6.3%), asterisca-3(15),6-diene (2.3–3.2%), β-caryophyllene (2.9–8.6%), α-humulene (1.3–4.5%), *trans*-africanan-1α-ol (**4**) (1.3–3.1), humulene epoxide II (1.3–2.7%), lippifoli-1(6)-en-4β-ol-5-one (**7**) (1.0–1.9%) and integrifolian-1,5-dione (**1**) (1.4–2.3%), while the remaining components were

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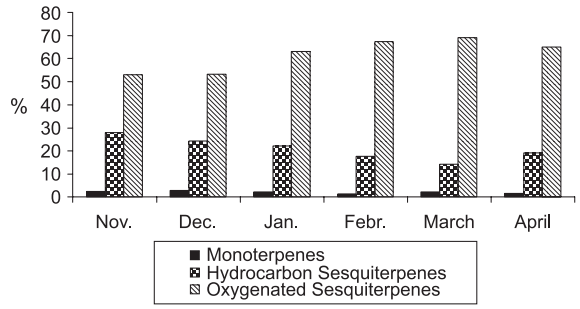

Figure 3. Fractional composition of the essential oil of *Lippia integrifolia* over a 6 months period

present as minor or trace constituents. Although significant quantitative variations were observed for most components, the qualitative composition of the oils appeared to be constant throughout the studied period.

The MS data for african-5-en-1α-ol (**3**), *trans*-africanan-1α-ol (**4**), 3α-hydroxy-6-asteriscene (**5**), african-1(5)-en-2,6-dione (**6**), lippifoli-1(6)-en-4β-ol-5-one (**7**) and 1α,7β,9α-1-hydroxy-3,6,6,9-tetramethylbicyclo[5.4.0]undec-3-en-8-one (**8**) are given below.

African-5-en-1α-ol (**3**). Crystalline solid, m.p. 40–41 °C; this alcohol decomposed completely after 2 months at −20 °C in a Pyrex glass container. ^{1}H- and ^{13}C-NMR data in Tables 2 and 3. EI–MS (70 eV); *m/z* (%):[M]$^+$ 220 (2); 205 (4); 202 (1); 187 (5); 179 (100); 164 (22); 159 (7); 149 (54); 145 (11); 135 (24); 131 (13); 121 (76); 107 (46); 105 (26); 99 (27); 91 (33); 79 (23); 77 (23); 55 (31); 43 (52); 41 (40).

trans-Africanan-1α-ol (**4**). Oil. $[\alpha]_{589}$ −41.2, $[\alpha]_{578}$ −41.3, $[\alpha]_{546}$ −49.2, $[\alpha]_{436}$ −82.5, $[\alpha]_{365}$ −136.5 (c 0.63, $CHCl_3$). ^{1}H- and ^{13}C-NMR data in Tables 2 and 3. EI–MS (70 eV); *m/z* (%): [M]$^+$ 222 (5), 207 (18), 204 (3), 189 (8), 179 (7), 165 (15), 151 (21), 138 (33), 125 (100), 109 (44), 96 (90), 81 (31), 69 (47), 55 (57), 41 (55).

3α-Hydroxy-6-asteriscene (**5**). Colourless oil. ^{1}H- and ^{13}C-NMR data in ref. 12. EI–MS (70 eV); *m/z* (%): [M]$^+$ 222 (8), 189 (7), 175 (5), 161 (8), 148 (16), 139 (100), 121 (16), 109 (12), 95 (28), 81 (22), 69 (15), 55 (17), 43 (50).

African-1(5)-en-2,6-dione (**6**). Colourless oil. ^{1}H- and ^{13}C-NMR data in ref. 12. EI–MS (70 eV); *m/z* (%): [M]$^+$ 232 (26), 217 (8), 204 (8), 191 (16), 175 (10), 161 (55), 148 (100), 133 (32), 127 (5), 119 (22), 106 (48), 91 (40), 77 (42), 65 (12), 53 (15), 41 (38).

Lippifoli-1(6)-en-4β-ol-5-one (**7**). ^{1}H- and ^{13}C-NMR data in ref. 7. EI–MS (70 eV); *m/z* (%): [M]$^+$ 234 (7), 219 (1), 206 (10), 191 (3), 176 (100), 161 (78), 148 (32), 133 (72), 119 (24), 105 (43), 91 (38), 77 (28), 65 (10), 55 (10), 43 (29).

1α,7β,9α-1-Hydroxy-3,6,6,9-tetramethylbicyclo-[5.4.0]undec-3-en-8-one (**8**). White solid; m.p. 56–59 °C. ^{1}H- and ^{13}C-NMR data in ref. 12. EI–MS (70 eV); *m/z* (%): [M-H_2O]$^+$ 218 (7), 174 (6), 167 (20), 147 (10), 139 (16), 127 (32), 110 (100), 95 (48), 83 (57), 77 (20), 69 (19), 55 (38), 41 (28).

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