Determination of the Volatile and Semi-volatile Secondary Metabolites, and Aristolochic Acids in Aristolochia ringens Vahl

Elena E. Stashenko*, Sergio Andrés Ordóñez, Néstor Armando Marín, and **Jairo René Martínez**

Research Center of Excellence CENIVAM, Chromatography Laboratory, CIBIMOL, Universidad Industrial de Santander. Carrera 27 Calle 9. Bucaramanga, Colombia

Abstract

Volatile and semi-volatile secondary metabolites, as well as aristolochic acids (AA), present in leaves, stems, and flowers of *Aristolochia ringens* were determined by gas chromatography (GC)–mass spectrometry (MS) and high-performance liquid chromatography (HPLC) methods, respectively. Metabolite isolation was performed using different extraction techniques: microwave-assisted hydrodistillation (MWHD), supercritical fluid extraction, and headspace solid-phase microextraction (HS-SPME). The chemical composition of the extracts and oils was established by GC–MS. The determinations of AAI and AAII were conducted by methanolic extraction of different plant parts followed by HPLC analysis. Essential oil yields from leaves and stems were 0.008 ± 0.0022% and 0.047 ± 0.0026%, respectively. *Aristolochia ringens* flowers did not yield essential oil under MWHD. Sesquiterpene hydrocarbons (66%) were the main compounds in the essential oil isolated from leaves whereas monoterpene hydrocarbons (73%) predominated in the stems essential oil. Yields of extracts isolated by SFE from leaves, stems, and flowers were 4 ± 1.8%, 1.2 ± 0.25%, and 4 ± 1.8%, respectively. In vivo HS-SPME of flowers isolated compounds with known unpleasant smells such as volatile aldehydes and short-chain carboxylic acids. HPLC analysis detected the presence of AAII in the flowers of *Aristolochia ringens* at a concentration of 610 ± 47 mg/kg of dried flower.

Introduction

Phytochemical investigations of semiepiphytic vegetable species of the *Aristolochia* genus (Aristolochiaceae family) from different parts of the world have revealed antibacterial, antispasmodic, and antiviral activities of their extracts and essential oils (1,2). In the majority of its species, this genus is characterized by the presence of nitrophenantrene derivatives, known as aristolochic acids (AA), which have been found to be carcinogenic (3–7). In Colombia, *Aristolochia ringens* Vahl, popularly known as "guaco", is extensively spread in different zones of the country (Departments of Antioquia, Cundinamarca, Huila, Magdalena, Valle, and Santander). *A. ringens* Vahl is a climbing plant with long stems, leaves (17 cm), and flowers (18 cm). In this work, gas chromatography (GC)–mass spectrometry (MS) and high-performance liquid chromatography (HPLC) methods were applied to determine the chemical composition of *A. ringens*, the secondary metabolites present in leaves, stems, and flowers. Flower scent was sampled with in vivo and in vitro headspace solid-phase microextraction (HS-SPME) techniques. Microwave radiation-assisted hydrodistillation (MWHD) and supercritical CO_2 extraction (SFE) were used to obtain essential oils and extracts from stems and leaves.

*Author to whom correspondence should be addressed: elena@tucan.uis.edu.co.

Experimental

The taxonomic characterization of the *A. ringens* plant was carried out by Dr. Fabio González at the Institute of Natural Sciences (National University of Colombia, Bogotá). A herbarium specimen remained as permanent sample at the Colombian National Herbarium (COL, Bogota) labelled as: *Aristolochia ringens* (Vahl), number COL 519052.

Essential oil extraction

Fresh vegetable material (leaves or stems, 400 g) was submitted to MWHD, as described in literature (8). Briefly, a Clevenger-type distillation flask (containing plant material and 300 mL distilled water) with Dean-Stark reservoir was heated in a domestic microwave oven. The total time of extraction was 40 min in intervals of 10 min (4 × 10 min) at 60% of the maximum oven power (720 W). This procedure was repeated six times for leaves and stems in order to accumulate sufficient experimental material (oil). Subsequently, the oil was separated by decantation and weighed. The total set of extractions was carried out in triplicate. For their chromatographic analysis, individual essential oil samples (50 µL) were mixed with *n*-tetradecane (2 µL, internal standard) and diluted with dichloromethane to a final volume of 1.0 mL.

SFE

Leaves (4 g), stems (7 g), or flowers (2 g) were extracted for 2 h with CO_2 (300 g, 50ºC, 1100 psi) in a high-pressure Soxhlet system (J&W Scientific, Folsom, CA). Subsequently, the system was chilled until the pressure dropped to 500 psi in order to start depressurization by CO_2 release. The extract solutions in dichloromethane (1.0 mL, 1 µL *n*-tetradecane) were submitted to chromatographic analysis. SFE was performed in triplicate.

HS-SPME

For in vitro sampling, fresh *A. ringens* flowers (2.5 g) were manually chopped up with a sharp knife (~ 1 cm^2 pieces) and placed in a screw-cap vial (12 mL), equipped with a porous polytetrafluoroethylene (PTFE)-silicone rubber septum. After a pre-equilibration period (10 min) in a water bath (60ºC), the SPME fiber (PDMS, 100 µm, or PDMS/DVB, 65 µm) was exposed to the headspace inside the vial for 30 min. The SPME fiber was removed and transferred to the injection port of a GC (250°C, 5 min). The same procedure was used with a standard hydrocarbon mixture (C10–C25). Triplicate extractions were performed.

A transparent (polyacrylic) cylinder (15 cm i.d. × 40 cm, one end open) equipped with a side arm to support a SPME device was carefully brought next to an *A. ringens* vine and fixed in a position such that it surrounded a complete flower. After a temperature equilibration period (20 min), a SPME fiber (PDMS/DVB, 65 µm) was exposed for 30 min to the headspace inside the cylinder. The fiber was subsequently removed and transferred to the injection port of a GC. This in vivo HS-SPME sampling was performed in duplicate.

Chromatographic analysis

Compound identification was based on chromatographic (retention times, retention indices, and standards) and spectroscopic (spectral interpretation, comparison with databases, and standards) criteria (9,10). Two GC–MS systems were employed for essential oil and extract analysis: an Agilent Technologies 6890 Plus GC (Palo Alto, CA) equipped with an Agilent Technologies 5973N mass selective detector (EI, 70 eV, *m/z* 40–350) and an Agilent Technologies 6890 GC coupled to an Agilent Technologies 5975 mass selective detector (EI, 70 eV, *m/z* 40–350). Both systems were equipped with a split/splitless injector (split ratio 1:30), a 7863 automatic injector and an MS-ChemStation G1701-DA data system that included the spectral libraries WILEY 138K, NIST 2002, and QUADLIB 2004. A fused-silica 5% phenyl-poly(dimethylsiloxane) capillary column (DB-5MS, J&W Scientific, Folsom, CA) of 60 m × 0.25 mm i.d. × 0.25 µm, df, and a fused-silica cross-linked, bonded poly (ethyleneglycol) capillary column (DB-WAX, J & W Scientific, Folsom, CA) of 60 m × 0.25 mm, i.d. × 0.25 µm, df, were employed. The oven temperature program employed with the DB-5MS column was from 45°C (2 min) to 150°C (5 min) at 6°C/min, then to 275°C (10 min) at 8ºC/min. For the DB-WAX column, the oven temperature was programmed from 45°C (5 min) to 150°C (3 min) at 3°C/min, then to 220°C (5 min) at 4°C/min. The ionization chamber and the transfer line temperatures were kept at 230°C and 285°C, respectively. A 1:10 split ratio was employed when SPME samples were analyzed. A C10 – C25 mixture of *n*-alkanes was run under the same chromatographic conditions employed with the samples in order to provide retention time data required for the calculation of Kovàts indices.

The HPLC determination of aristolochic acids I (AAI) and II (AAII) followed the procedure of Jinbin et al. (11). *A. ringens* leaves, stems, and flowers were dried, powdered, and studied separately. Methanol (5 mL, HPLC-grade) was added to sample material (0.1 g) in a capped culture tube that was subsequently sonicated (15 min) and centrifuged (3000 rpm, 10 min). The supernatant was removed, and six additional cycles of methanol extraction were performed. The combined extracts were concentrated under gentle nitrogen flow to 10 mL. All extracts were filtered (0.45 µm, cellulose acetate) prior to HPLC analysis. The whole procedure was carried out in triplicate.

The AAI and AAII determinations were performed on an Agilent Technologies 1200 Series liquid chromatograph equipped with an UV–Vis diode array detector and an Agilent Technologies Zorbax Eclipse XDB-C_{18} column (4.6 mm × 150 mm). The mobile phase employed was a 3:2 mixture of methanol and aqueous acetic acid solution (0.1% v/v) at 1 mL/min under

Table I. Identification and Content of Secondary Metabolites Isolated with MWHD and SFE from *A. ringens* Leaves

Peak No*	Compound	IK†		Relative amount (%)‡		Conc. (g/L)‡
		DB-5	DB-WAX	MWHD	SFE	MWHD
1	(Z)-3-Hexenol§	–	1382	0.245 ± 0.001	–	3.0 ± 0.34
2	*n*-Hexanol	–	1352	0.092 ± 0.0071	–	1.1 ± 0.98
3	Limonene§	1032	1194	0.081 ± 0.005	–	1.0 ± 0.73
4	Linalool§	1100	1547	0.080 ± 0.009	–	1.0 ± 0.12
5	Bicycloelemene	1333	1485	1.39 ± 0.064	–	17.0 ± 0.96
6	δ-Elemene	1335	1473	0.54 ± 0.011	–	6.7 ± 0.21
7	α-Ylangene	1374	1490	0.58 ± 0.036	–	7.1 ± 0.52
8	α-Copaene	1380	1498	0.139 ± 0.0092	–	1.7 ± 0.13
9	β-Bourbonene§	1391	1598	4.3 ± 0.32	–	52 ± 4.5
10	α–Gurjunene	1414	1538	0.56 ± 0.033	–	6.9 ± 0.48
11	*trans*-β-Caryophyllene§	1433	1613	11.4 ± 0.17	1.2 ± 0.32	139.3 ± 0.60
12	β-Copaene	1439	1688	1.05 ± 0.048	–	12.9 ± 0.72
13	β-Gurjunene	1441	1599	0.230 ± 0.0064	–	2.81 ± 0.042
14	6,9-Guaiadiene	1448	1615	1.24 ± 0.047	–	15.2 ± 0.73
15	*cis*-3,5- Muurola-diene	1453	1606	0.45 ± 0.023	–	5.5 ± 0.34
16	allo-Aromadendrene	1457	1658	0.54 ± 0.024	–	6.6 ± 0.36
17	α-Humulene§	1467	1682	2.01 ± 0.073	–	25 ± 1.2
18	γ-Gurjunene	1471	1619	0.76 ± 0.033	–	9.4 ± 0.5
19	γ-Muurolene	1479	1700	0.74 ± 0.029	–	9.1 ± 0.45
20	Germacrene-D	1491	1717	–	2.2 ± 0.5	–
21	*trans*-4(14),5-Muuroladiene	1495	1728	13.0 ± 0.22	–	159 ± 1.1
22	Bicyclogermacrene	1510	1753	12.8 ± 0.28	1.5 ± 0.30	157 ± 1.7
23	γ-Cadinene	1522	1772	0.78 ± 0.081	–	9.6 ± 0.88
24	δ-Cadinene	1524	1767	1.09 ± 0.041	–	13.4 ± 0.65
25	(E)-Nerolidol§	1564	2039	3.05 ± 0.078	–	37 ± 1.4
26	Germacrene B	1572	1846	1.01 ± 0.036	–	12.4 ± 0.58
27	(Z)-3-Hexenyl benzoate	1578		0.17 ± 0.045	–	2.1 ± 0.52
28	Spathulenol	1593	2142	8.03 ± 0.081	0.42 ± 0.051	98 ± 2.0
29	Caryophyllene oxide	1598	2008	4.07 ± 0.037	–	49.82 ± 0.073
30	τ-Cadinol	1606	2032	0.74 ± 0.031	–	9.1 ± 0.47
31	α-Copaene-8-ol	1612		0.25 ± 0.016	–	3.1 ± 0.22
32	Humulene epoxide II	1624		0.20 ± 0.010	–	2.5 ± 0.15
33	Sclarene	1948		0.27 ± 0.034	–	3.3 ± 0.45
34	Manool	2075		0.53 ± 0.022	0.66 ± 0.033	6.5 ± 0.2
35	Sclareol	2223		0.82 ± 0.035	8.4 ± 0.32	10.1 ± 0.32
36	Methyl copalate	2331	2142	10.3 ± 0.81	67 ± 2.6	126 ± 8.6

* Peak number from Figure 1; † Experimental Kováts indices; ‡ Mean of three extractions; § Standard compound employed.

isocratic regime. The column temperature was maintained at 30ºC. Data acquisition used a 200–400 nm scan. The absorbance at 252 nm was employed for quantification. AA identification was based on comparison of retention times and UV spectra with those of certified standards (Sigma Aldrich, St. Louis, MO).

Results and Discussion

The present work contains examples of techniques employed to isolate secondary metabolites from different tissues found in the *A. ringens* vine, the delicate flower tissues, soft leaves, and the hard stems. Differences in solubility, vapor pressure, and diffusion rates among the metabolites within these diverse matrices and in the extracting fluid act as filters or selection criteria, which modulate the mass transfer processes. Thus, a single sampling technique does not provide a complete depiction of the secondary metabolite profile of a plant. Obtaining such a representative account requires a combination of sampling approaches. While HS-SPME is appropriate for sampling highly volatile substances, SFE is more suited to isolate much heavier, non-polar substances such as diterpenes.

Leaves

Typical chromatographic profiles of the secondary metabolites obtained from fresh *A. ringens* leaves with MWHD or SFE are presented in Figure 1. Peak assignments and relative amounts appear in Table I. While 36 identified substances represented 84% of the total chromatographic area of the essential oil, just seven components corresponded to 81% of the SFE extract chromatographic area. Much lower extraction yields were obtained with hydrodistillation (0.008 ± 0.0022%, n = 3) than with SFE (4 ± 1.8%, n = 3). The MWHD essential oil obtained from fresh *A. ringens* leaves presented a penetrating smell and a slightly yellow translucent color. Its main components were trans-4(14),5-muurola-diene (13%), bicyclogermacrene (12.8%), trans-β-caryophyllene (11.4%), and methyl copalate (10.3%). An interesting feature of this essential oil is its low monoterpene content. Sesquiterpenes (66%), oxygenated sesquiterpenes (19%), and diterpenes (14%) were the main compound families found in the essential oil obtained from fresh *A. ringens* leaves.

Stems

In comparison with leaves, fresh *A. ringens* stems afforded higher yields of essential oil (0.047 ± 0.0026%, n = 3) but lower yields of SFE extract (1.2 ± 0.25%, n = 3). Typical chromatographic profiles of the isolated secondary metabolite mixtures appear in Figure 2. A total of 46 essential oil components, representing 98% of the total chromatographic area, were positively identified (Table II). In contrast with the essential oil isolated from leaves, the oil from the stems was rich in monoterpenes (73%), whereas sesquiterpenes represented 13% of the oil components, followed by oxygenated monoterpenes (10%) and oxygenated sesquiterpenes (3%). Table II contains the identification of 20 components of the SFE fraction, which represent 86% of the total chromatographic area. This extract composition was qualitatively different from that obtained from leaves due to the presence of monoterpenes (limonene was its main component, 19,8%) and the lower relative amount of methyl copalate (11%). Larrahondo and Acevedo reported an ent-labdane diterpene as the main constituent of the *A. ringens* stems petroleum ether extract (12).

Flowers

No oil was obtained from *A. ringens* flowers by means of MWHD. SFE of dry flowers afforded an extract (4 ± 1.8%, n = 3) composed almost exclusively of methyl copalate (9.1 ± 0.66 mg/kg of dry flower, n = 2). *A. ringens* flowers have a fetid smell that attracts saprophagous insects, which help in pollination (13,14). Flower

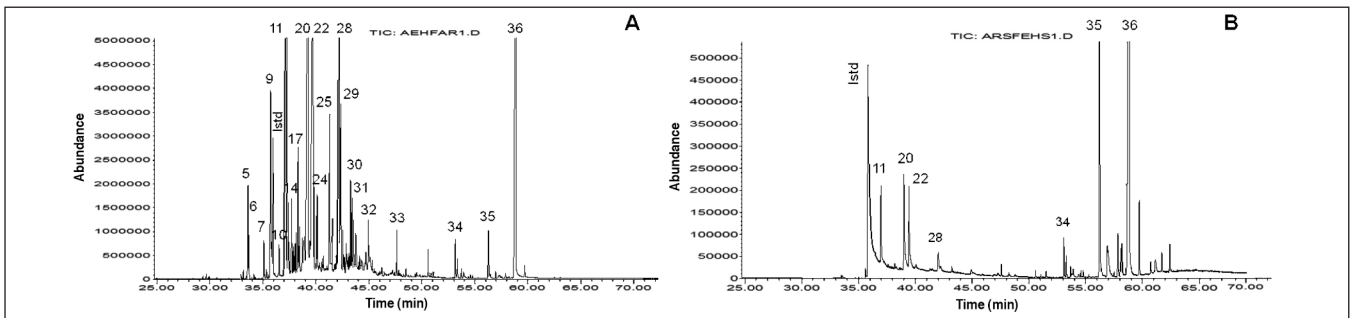

Figure 1. Typical chromatographic profiles of the essential oil and extract from *Aristolochia ringens* Vahl leaves with: MWHD, A; and SFE, B. Peak assignments appear in Table I.

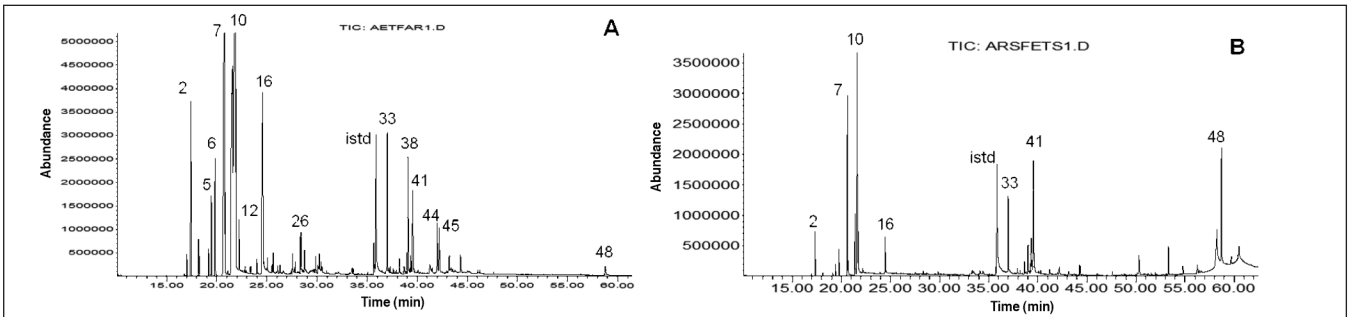

Figure 2. Typical chromatographic profiles of the essential oil and extract from *Aristolochia ringens* Vahl stems with: MWHD, A; and SFE, B. Peak assignments appear in Table II.

scent was sampled in vitro by HS-SPME with a PDMS (100 µm) or a PDMS/DVB (65 µm) coating. The chromatographic profiles obtained from both coating types differed both in number of volatile compounds extracted and in their relative amounts (Table III). While the 17 positively identified components of the volatile fraction isolated with the PDMS coating represented 82% of the total chromatographic area, 33 identified components represented 96% of the total area of the fraction isolated with the PDMS/DVB coating (Figure 3). As Table III shows, no aldehydes or low-molecular mass carboxylic acids were present in the volatile fractions isolated with HS-SPME. The identified constituents of these fractions were terpenes normally found in essential oils. On the other hand, the in vivo fraction that was sampled with HS-SPME using a PDMS-DVB coating, was composed mainly of substances of known unpleasant odor, such as undecanal, nonanal, decanal, and 2-methylbutanoic acid. The continuous emanation of these substances by the live flower permitted their accumulation on the SPME fiber in sufficient amounts to permit their chromatographic detection. On the other hand, in the in vitro sampling, the flowers are cut into small pieces, and a rapid change in coloration is apparent. Under these conditions, low-molecular mass, aldehydes are no longer generated in a continuous manner. The detection of the remaining traces would require the use of a more sensitive sampling method such as on-fiber derivatization (15). Further research into these in vivo and in vitro volatile profile differences is being conducted in our laboratory to clarify the contribution of tissue decay to the in vitro volatile profile.

Table II. Identification and Content of Secondary Metabolites Isolated with MWHD and SFE from *A. ringens* Stems

Peak N°*	Compound	Ik†		Relative amount (%)‡		Conc. (g/L)‡
		DB-5	DB-WAX	MWHD	SFE	MWHD
1	α-Thujene	928	1022	0.47 ± 0.032	–	2.6 ± 0.21
2	α-Pinene§	937	1019	4.9 ± 0.11	2.3 ± 0.28	27 ± 1.1
3	Camphene§	954	1060	0.99 ± 0.057	–	5.5 ± 0.21
4	Sabinene	976	1116	0.60 ± 0.035	–	3.3 ± 0.24
5	β-Pinene§	982	1102	1.89 ± 0.065	0.60 ± 0.07	10.4 ± 0.55
6	β-Myrcene§	991		3.62 ± 0.063	1.4 ± 0.16	20.0 ± 0.85
7	α-Phellandrene	1014	1164	16.1 ± 0.35	12.21 ± 0.08	89 ± 2
8	Δ^3-Carene	1015		1.09 ± 0.040	0.9 ± 0.20	6.0 ± 0.38
9	p-Cymene§	1034	1270	17.8 ± 0.66	4.0 ± 0.30	98 ± 6.4
10	Limonene§	1040	1202	20 ± 1.3	19.8 ± 0.87	113 ± 6.5
11	β-Phellandrene +Eucaliptol	1042	1208 + 1210	3.1 ± 0.23	3.0 ± 0.61	17 ± 1.3
12	trans-β-Ocimene§	1049	1250	1.04 ± 0.012	–	5.7 ± 0.18
13	γ-Terpinene	1063	1243	0.128 ± 0.0032	–	0.70 ± 0.028
14	cis-Sabinene hydrate + *cis*-linalool oxide	1076	1466	0.081 ± 0.0071	–	0.44 ± 0.044
15	Terpinolene + *trans*-linalool oxide	1090	1449	0.322 ± 0.0095	–	1.77 ± 0.052
16	Linalool§	1104	1547	6.5 ± 0.10	2.4 ± 0.29	36 ± 1.8
17	Isopentyl isovalerate	1106	1293	0.4 ± 0.12	–	2.2 ± 0.76
18	C11H18 (N.I.)	1113	1303	0.06 ± 0.044	–	0.3 ± 0.25
19	*trans*-p-Mentha-2,8-dien-1-ol	1124	1580	0.16 ± 0.019	–	0.9 ± 0.11
20	*cis*-p-Mentha-2-en-1-ol	1127		0.30 ± 0.018	–	1.63 ± 0.096
21	*trans*-Limonene oxide + p-mentha-2,8-dien-1-ol	1137	1447	0.22 ± 0.025	–	1.2 ± 0.15
22	**	1143	1415	0.22 ± 0.019	–	1.23 ± 0.093
23	Camphor§	1151	1521	0.11 ± 0.021	–	0.6 ± 0.13
24	Borneol*	1171	1708	0.49 ± 0.012	–	2.68 ± 0.057
25	Terpinen-4-ol§	1176		0.159 ± 0.008	–	0.88 ± 0.053
26	α-Terpineol§	1188		0.69 ± 0.065	–	3.8 ± 0.26
27	Metyl thymyl ether	1227		0.31 ± 0.013	–	1.68 ± 0.020
28	Bicycloelemene	1330	1485	0.22 ± 0.013	–	1.22 ± 0.070
29	δ-Elemene	1332	1473	0.250 ± 0.007	–	1.38 ± 0.06
30	β-Bourbonene	1387	1526	0.493 ± 0.009	–	2.72 ± 0.076
31	β-Elemene	1388	1591	0.26 ± 0.022	0.46 ± 0.08	1.41 ± 0.074
32	$C_{15}H_{24}$ (N.I.)	1423		0.090 ± 0.005	–	0.50 ± 0.015
33	trans-β-Caryophyllene§	1426	1609	3.8 ± 0.12	6.4 ± 0.29	20.7 ± 0.70
34	γ-Elemene	1430		0.128 ± 0.0061	–	0.70 ± 0.033
35	6,9-Guaiadiene	1444	1615	0.092 ± 0.0038	–	0.51 ± 0.038
36	α-Humulene	1463	1676	0.309 ± 0.0038	–	1.71 ± 0.057
37	β-Chamigrene	1484		tr	1.25 ± 0.086	–
38	Germacrene D	1488	1717	3.1 ± 0.10	2.2 ± 0.19	17 ± 1.1
39	Valencene	1493		0.140 ± 0.0030	0.68 ± 0.049	0.77 ± 0.015
40	β-Selinene	1496		0.42 ± 0.015	2.8 ± 0.26	2.31 ± 0.080
41	Bicyclogermacrene	1502	1746	3.41 ± 0.085	12 ± 1.7	18.8 ± 0.87
42	*trans*-Nerolidol	1562		0.37 ± 0.059		2.1 ± 0.40
43	Germacrene B	1569	1844	0.152 ± 0.0036		0.84 ± 0.053
44	Spathulenol	1587	2138	1.1 ± 0.22		6 ± 1.4
45	Caryophyllene oxide	1594	2008	1.2 ± 0.18	0.86 ± 0.076	7 ± 1.2
46	Selin-11-en-4-α-ol	1673	2271	0.270 ± 0.0075	0.84 ± 0.071	1.49 ± 0.077
47	Sclareol	2228		††	0.72 ± 0.084	–
48	Methyl copalate	2325	2142	0.25 ± 0.050	11 ± 1.2	1.4 ± 0.33

* Peak number from Figure 2; † Experimental Kováts indices; ‡ Mean of 3 extractions; § Standard compound employed.
** Not identified; †† Trace amounts (< 0.5%).

Aristolochic acids

Methanolic extracts of *A. ringens* leaves, stems, or flowers, were examined with the HPLC method described previously using a 200–400 nm scan. Comparison of retention times and UV spectra with those of AAI and AAII standards revealed the presence of AAII in the flower extract. The remaining extracts did not contain detectable amounts of aristolochic acids. Limits of detection of 0.18 and 0.16 mg/kg were calculated for AAI and AAII, respectively, based on the calibration curves obtained for the implemented analytical procedure. Replicate determinations of the AAI and AAII standards showed a reproducibility (CV) of 0.068 and 10% for retention times and chromatographic peak areas, respectively. The chromatographic peak area of AAII found in the flower extract permitted the calculation of the concentration of AAII in dry *A. ringens* flowers as 610 ± 47 mg/kg of flower. This is a high AAII content compared with those reported for other Aristolochia species, such as *A. debilis* and *A. fangchi* (3). An oral AAII dose of 25 mg/kg causes necrotic lesions in rats (6). Therefore, a cautionary recommendation is to exclude A. ringens flowers from phytomedicinal products. On the other hand, the potential application of *A. ringens* extracts in pest control has been investigated. The *A. ringens* petroleum ether extract performed very well as a biocide in the protection of maize grains from infestation and damage by *Sitophilus zeamais* (16).

Conclusion

The secondary metabolite profile of *Aristolochia ringens* Vahl, a climbing vine now recommended for gardening, is very diverse. While the essential oil obtained from the leaves is rich in sesquiterpenes, the oil obtained from the stems is dominated by monoterpenes. Among the 80 different secondary metabolites found in *A. ringens* leaves, stems, and flowers, only 25 were common in essential oils isolated from roots of other 10 different *Aristolochia* species in Brazil (17). The scents of collected or live *A. ringens* flowers differ dramatically. Volatiles emitted by flowers removed from the plant do not contain aldehydes at the levels detected by the GC–MS method employed, whereas live flowers have an unpleasant odor that results from the release of low molecular mass carboxylic acids and C9–C11 aliphatic aldehydes. AAII, a carcinogenic substance (7) was not found in leaves or stems, but *A. ringens* flowers contain it at the relatively high concentration of 610 ± 47 mg/kg.

Table III. Identification and Content of Secondary Metabolites Isolated with In Vitro HS-SPME from A. ringens Vahl Flowers

Peak N°*	Compound	IK†	Relative amount %‡	
		DB-5	PDMS/DVB	PDMS
1	β-Myrcene	990	0.40 ± 0.042	–
2	Limonene§	1040	0.3 ± 0.25	–
3	cis-β-Ocimene	1035	22 ± 2.2	1.4 ± 0.45
4	trans-β-Ocimene	1046	11.22 ± 0.039	0.8 ± 0.24
5	Linalool§	1096	3.1 ± 0.46	tr**
6	3-Methyl-3-butenyl 3-methylbutanoate	1116	0.23 ± 0.065	–
7	allo-Ocimene	1125	1.7 ± 0.24	0.4 ± 0.12
8	Bicycloelemene	1333	0.25 ± 0.011	6.2 ± 0.69
9	δ-Elemene	1335	0.51 ± 0.041	–
10	α-Cubenene	1347	0.230 ± 0.0014	–
11	α-Ylangene	1368	0.57 ± 0.093	0.43 ± 0.022
12	α-Copaene	1380	0.428 ± 0.0035	–
13	β-Bourbonene	1383	1.2 ± 0.77	4 ± 1.3
14	β-Elemene	1388	–	0.70 ± 0.092
15	α-Gurjunene	1406	0.40 ± 0.035	0.32 ± 0.010
16	trans-β-Caryophyllene§	1419	23 ± 2.0	33.3 ± 0.12
17	β-Copaene	1425	1.0 ± 0.16	1.81 ± 0.049
18	γ-Elemene	1427	0.36 ± 0.056	1.9 ± 0.21
19	α-Maailene	1437	0.40 ± 0.055	–
20	Aromadendrene	1441	5.5 ± 0.26	–
21	α-neo-Clovene	1443	–	0.38 ± 0.040
22	α-Humulene§	1450	1.5 ± 0.30	2.5 ± 0.17
23	Dihydro aromadendrene	1458	0.6 ± 0.16	–
24	allo-Aromadendrene	1459	–	0.27 ± 0.020
25	trans-1(6),4-Cadinadiene	1478	0.18 ± 0.040	–
26	γ-Muurolene	1479	2.3 ± 0.14	–
27	Germacrene D	1481	3.2 ± 0.27	18 ± 2.5
28	Viridiflorene	1497	5.3 ± 0.20	8.7 ± 0.72
29	α-Muurolene	1506	0.93 ± 0.033	–
30	δ-Amorphene	1512	0.67 ± 0.069	–
31	γ-Cadinene	1524	2.4 ± 0.24	–
32	δ-Cadinene	1525	4.4 ± 0.37	–
33	*trans*-1(2),4-Cadinadiene	1533	0.24 ± 0.079	–
34	α-Cadinene	1537	0.9 ± 0.13	–
35	Selina-3,7(11)-diene	1547	0.19 ± 0.046	–

* Peak number from Figure 3; † Experimental Kováts indices (DB-5 column, 60 m); ‡ Mean of three extractions; § Standard compound employed; ** Trace amounts (< 0.5%).

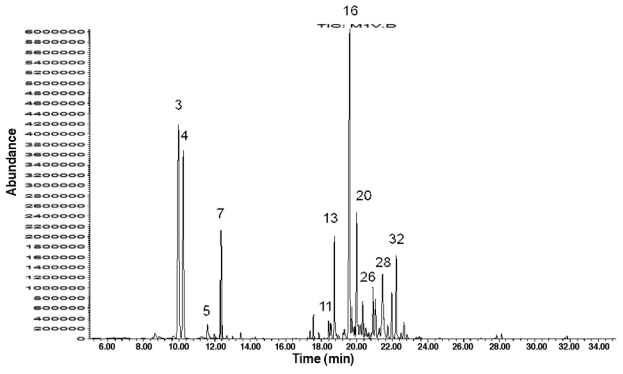

Figure 3. Typical chromatographic profiles of the volatile fraction collected from Aristolochia ringens Vahl flowers with HS-SPME using PDMS/DVB, 65 µm coating. Peak assignments appear in Table III.

Acknowledgements

Financial support from Colciencias (Grant RC-432-2004) is gratefully acknowledged.

References

1. T. Wu, A. Damu, C. Su, and P. Kuo. Terpenoids of Aristolochia and their biological activities. *Nat. Prod. Rep. Review* **21:** 594–624 (2004).
2. R. Otero, V. Núñez, J. Barona, R. Fonnegra, S. Jiménez, R. Osorio, M. Saldarriaga, and A. Díaz. Snakebites and ethnobotany in the northwest region of Colombia. Part III: Neutralization of the haemorrhagic effect of Bothrops atrox venom. *J. Ethnopharmacol.* **73:** 233–241 (2000).
3. K. Hashimoto, M. Higuchi, B. Makino, I. Sakakibara, M. Kubo, Y. Komatsu, M. Maruno, and M. Okada. Quantitative analysis of aristolochic acids, toxic compounds, container in some medicinal plants. *J. Ethnopharmacol.* **64:** 185–189 (1999).
4. J.R. Ioset, G. Raoelison, and K. Hostettmann. Detection of aristolochic acid in Chinese phytomedicines and dietary supplements used as slimming regimens. *Food Chem. Toxicol.* **41:** 29–36 (2003).
5. J.H. Jou, S. Chen, and T.S. Wu. Facile reversed-phase HPLC resolution and quantitative determination of aristolochic acid and aristolactam analogues in tradicional chinese medicine. *J. Liq. Chromatogr. Related Technol.* **26:** 3057–3068 (2003).
6. L. Chen, N. Mei, L. Yao, and T. Chen. Mutations induced by carcinogenic doses of aristolochic acid in kidney of Big Blue transgenic rats. *Toxicol. Lett.* **165:** 250–256 (2006).
7. N. Feldmeyer, H. Schmeiser, K.R. Muehlbauer, D. Belharazem, Y. Knyazev, T. Nedelko, and M. Hollstein. Further studies with a cell immortalization assay to investigate the mutation signature of aristolochic acid in human p53 sequences. *Mutation Research.* **608:** 163–168 (2006).
8. E. E. Stashenko, B. E. Jaramillo, and J. R. Martínez. Comparison of different extraction methods for the analysis of volatile secondary metabolites of Lippia alba (Mill) N. E. Brown, grown in Colombia, and evaluation of its in-vitro antioxidant activity. *J. Chromatogr. A* **1025:** 93–103 (2004).
9. P. Adams. Identification of essential oil components by gas chromatography/mass spectrometry. 4th Ed. Allured Publishing, Carol Stream, IL, USA, 2007, pp. 1–804.
10. D. Joulain and W. A. König. The atlas of spectral data of sesquiterpene hydrocarbons. E. B.Verlag, Hamburg, Germany, 1998, pp. 1–661.
11. Y. Jinbin, N. Lihua, Z. Deyu, L. Xubiao, T. Fei, D. Li, L. Qian, G. Manli, and Y. Shouzhuo. Simultaneous determination of nine aristolochic acid analogues in medicinal plants and preparations by high-performance liquid chromatography. *Talanta.* **73:** 644–650 (2007).
12. J. E. Larrahondo and C. Acevedo. Terpenoids of Aristolochia ringens. *Anales de la Asociacion Quimica Argentina* **78:** 355–358. (1990)
13. S. Sakai. Aristolochia spp. (Aristolochiaceae) pollinated by flies breeding on decomposing flowers in Panama. *Am. J. Bot.* **89:** 527–534 (2002).
14. M. Proctor, P. Yeo, A. Lack. The natural history of pollination. Timber Press, Portland, Oregon, U.S.A., 1996.
15. E. Stashenko, M. Puertas, W. Salgar, W. Delgado, and J. R. Martínez. Solid-Phase Microextraction with on-fibre derivatisation applied to the analysis of volatile carbonyl compounds. *J. Chromatogr. A* **886:** 175–181 (2000).
16. A.A. Elujoba. Female infertility in the hands of traditional birth attendants in South-Western Nigeria. *Fitoterapia* **66:** 239–248 (1995).
17. C. Francisco, G. Messiano, L. Lopes, A. Tininis, J. de Oliveira, and L. Capellari. Classification of Aristolochia species based on GC–MS and chemometric analyses of essential oils. *Phytochemistry* **69:** 168–175 (2008).

Manuscript received November 15, 2008;
Revision received April 5, 2009.