Article

Chemical Constituents of *Aristolochia giberti*

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Foram isoladas quatorze substâncias de *Aristolochia giberti*, entre elas, um novo triterpeno, (−)-onocera-8,8'-diol. Além destas, 3-hidróxipropanoato, acetato e formato foram detectados por técnicas de RMN, o que sugere que estas substâncias sejam derivadas do 2-butinodioato, não detectável por RMN de [1]H. As análises dos óleos essenciais de caules e folhas, por CG-EM e quimiometria, mostraram grande similaridade entre as espécies cultivadas no Brasil e aquelas na Argentina, o que permitiu confirmar a identificação da espécie e diferenciar os óleos de acordo com as partes da planta.

Fourteen compounds were isolated from *Aristolochia giberti*. These included a new triterperne, (−)-onocera-8,8'-diol. In addition, 3-hydroxypropanoate, acetate, and formate were detected by NMR techniques, which suggests that they are derivatives from 2-butynedioate, which is not detected by ^{1}H NMR. GC-MS and chemometric analyses of essential oils from stems and leaves showed great similarity between this cultivated species in Brazil and that in Argentina, which allowed us to confirm the species identity and to differentiate the oils according to the plant parts.

Keywords: *Aristolochia giberti*, Aristolochiaceae, (−)-onocera-8,8'-diol, essential oil, 2-butynedioate

Introduction

Aristolochia species (Aristolochiaceae) are generally sold at Brazilian markets under their popular names, particularly as "One Thousand Man", and little distinction has been made among the species. They have been mainly used in Brazilian traditional medicine as abortifacients, stomachics, antiophidians, antiasthmatics, and expectorants, and recently in slimming therapies.[1,2] Aristolochic acids constitute a class of compounds that are characteristic of the *Aristolochia* genus. These acids have been associated with Chinese herb nephropathy, which is a kind of severe kidney disease caused by the intake of excessive aristolochic acids.[3] Therefore, it is essential, for health safety and quality control of related Brazilian herbal medicines, to know the chemical composition of these species, among them *Aristolochia giberti* Hook., and to develop efficient methods for species identification.

The chemical constituents of Brazilian Aristolochiaceae species, such as lignans, have shown antiplasmodial,[4] antimycobacterial,[5] insecticidal,[6,7] anti-inflammatory, and analgesic activities.[8]

Despite the significant number of Brazilian *Aristolochia* species (around 100), the volatile compounds they contain are known for only a few species.[9] In our previous studies on essential oils from roots of 10 *Aristolochia* species, we investigated the oil composition and correlated them to morphological groups by GC-MS and chemometric analyses, which could also help in the identification of these species.[9]

A total of 64 compounds were identified in the analysed essential oils from stems and leaves of *A. giberti* collected in Argentina and Paraguay, which corresponded to around 77% to 92% of the constituents in the oils.[10,11] Methanolic extracts of *A. giberti* protected against enzymatic and non-enzymatic lipid peroxidation in microsomal membranes of rat.[12]

The goals of the present study were to investigate the chemical composition of the leaf and stem extracts and the nature of the essential oils from stems and leaves of cultivated *A. giberti* in Brazil, to correlate its oil composition to those reported in the literature for plants collected in Argentina[10] and Paraguay,[11] and then to obtain information about interspecific variability as a function of provenance by using GC-MS and chemometric analyses as tools for plant identification.

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Experimental

Instrumentation

One-dimensional (^{1}H, ^{13}C, DEPT, and *g*NOESY) and two-dimensional (^{1}H-^{1}H *g*COSY, *g*HMQC, *g*HMBC, and *g*NOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 126 MHz (^{13}C), using the residual solvents as an internal standard. Mass spectra (electrospray ionization-mass spectroscopy (ESI-MS)) were performed on a Fisons Platform II, and flow injection into the electrospray source was used for ESI-MS. Infrared spectra (IR) were obtained on a Perkin Elmer 1600 FT-IR spectrometer using KBr disks. Ultraviolet (UV) absorptions were measured on a Perkin Elmer UV-Vis Lambda 14P diode array spectrophotometer. Optical rotations were measured on a Perkin Elmer 341-LC polarimeter. Melting points were recorded on a Microquímica MQAPF-301 melting point apparatus and were uncorrected. GC-MS analyses were performed on a Shimadzu GCMS-QP5050A system in EI mode (70 eV) equipped with a split/splitless injector (220 ºC), at a split ratio of 1/10, using a VF-1MS fused-silica capillary column (30 m by 0.25 mm i.d.; film thickness: 0.25 µm). The oven temperature was programmed from 60 ºC (5 min) to 280 ºC at a rate of 4 ºC min^{-1} and held at this temperature for 10 min. Helium was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The injection volume of each sample was 2 µL.

Solvents

Nanopure water (>18.2 MΩcm) was obtained using a Millipore purifier (Bedford, MA) and filtered through a Millipore membrane. All of the HPLC- and GC-grade solvents were purchased from Mallinckrodt Baker Inc. (Paris, KY); all organic solvents were filtered through Millipore PTFE membranes (0.5 µm, 47.0 mm) prior to use, and samples were filtered through Millipore polyvinylidene fluoride (PVDF) membranes (0.45 µm, 13.0 mm). CDCl$_3$, DMSO-d_6, and D$_2$O (≥ 99.98% D) for NMR analyses were purchased from Cambridge Isotope Laboratories, Inc. (CIL, Andover, MA).

Adsorbents

Silica gel 60 PF$_{254}$ for thin-layer chromatography (PTLC) was purchased from Aldrich (Milwaukee, WI), and silica gel 60H and silica gel 70-325 mesh for column chromatography (CC) were obtained from Merck (Darmstadt, Germany).

Plant material

The plant material was collected in Araraquara, SP, Brazil, in February, 2003, and identified as *Aristolochia giberti* Hook. (Aristolochiaceae) by Dr. Condorcet Aranha and Dr. Lindolpho Capellari Júnior (Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Piracicaba, SP, Brazil). A voucher specimen (ESA 88888) was deposited at the herbarium of the ESALQ, Piracicaba, SP, Brazil. The material was separated according to the plant parts, dried (*ca.* 45 °C), and ground.

Extraction and isolation of the chemical constituents

The leaves (306.2 g) and stems (1116.5 g) were extracted exhaustively at room temperature with hexane, acetone, and ethanol, successively. The residues were extracted with ethanol in a Soxhlet apparatus and the extracts were individually concentrated.

The stem crude hexane extract (10.0 g) was subjected to CC (5.0 by 35.0 cm; silica gel 70-325 mesh; 270.0 g; hexane/EtOAc gradient, 95:5 to 100% EtOAc) to give 16 fractions (*ca.* 200 mL each). Fractions 7, 8, and 11 gave **6** (858.5 mg), **10** (602.0 mg), and **8** (618.3 mg), respectively. Fractions 15 and 16 gave **2** (654.1 mg). Fractions 9 and 14 were individually subjected to PTLC (hexane/EtOAc, 4:1) to give **7** (48.4 mg) and **3** (10.7 mg), respectively (Figure 1).

The crude ethanol extract from leaves (9.09 g) was also subjected to CC (5.0 by 28.0 cm; silica gel 60 H; 220.0 g; CHCl$_3$/MeOH gradient, 95:5 to 100% MeOH) to give 25 fractions (*ca.* 150 mL each). Fractions 15, 20, and 25 gave **4** (87.0 mg), **5** (44.0 mg), and **2** (731.2 mg), respectively. Fraction 7 by PTLC (CHCl$_3$/EtOAc, 9:1) gave **8** (40.8 mg), and fraction 9 by PTLC (CHCl$_3$/EtOAc, 7:3) gave **1** (24.3 mg), **8** (31.5 mg), and **9** (60.9 mg) (Figure 1).

The crude ethanol Soxhlet extract from the leaves (14.3 g) was washed with CHCl$_3$ to give two fractions: a soluble (3.8 g) and an insoluble (10.5 g) in CHCl$_3$. A portion of the soluble fraction (1.1 g) was subjected to flash CC (4.0 by 5.0 cm; silica gel 60H; 24.0 g; hexane/EtOAc gradient 90:10 to 100% EtOAc, and then 100% MeOH) to give 18 fractions (*ca.* 100 mL each). Fraction 5 gave **4** (115.2 mg) and fraction 16 gave **2** (919.8 mg). Fraction 10, after PTLC (hexane/EtOAc, 7:3), gave **7** (1.0 mg). The insoluble CHCl$_3$ fraction (7.9 g) was partially dissolved in MeOH to give a MeOH subfraction (1.6 g) and a precipitate (4.4 g). The MeOH subfraction was subjected to CC [5.0 by 28.0 cm; silica gel 60H; 220.6 g; CHCl$_3$/(MeOH + 0.5% HOAc) gradient, 95:5 to100% (MeOH + 0.5% HOAc)] to give 15 fractions (*ca.* 100 mL each). Fractions 3, 4, 7, and 13 gave **2**
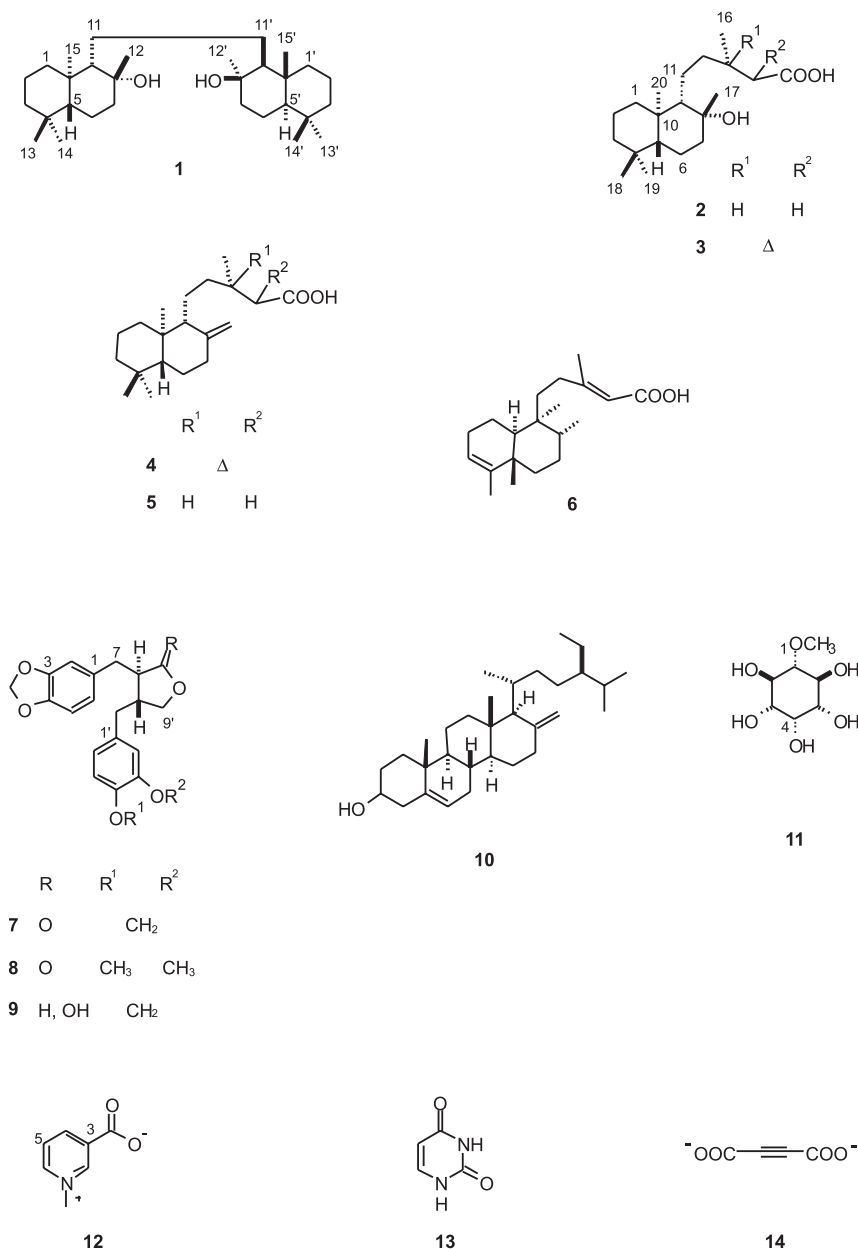
Figure 1. Chemical structures for compounds **1-14**.

(14.1 mg), **13** (4.0 mg), **11** (266.2 mg), and **12** (42.2 mg), respectively. Fraction 12 gave **14** as an amorphous solid (34.3 mg) which, after repetitive precipitation procedures from MeOH/H$_2$O, gave a mixture of **15**, **16**, and **17** in the soluble solutions that were reunited (Figure 1).

Mixture of 3-Hydroxypropanoate (**15**) + Acetate (**16**) + Formate (**17**) was analysed in D$_2$O and DMSO-d_6 solutions by ^{1}H and ^{13}C NMR.

3-Hydroxypropionate (15)

^{1}H NMR (D$_2$O) δ 3.77 (t, J 6.5 Hz, 2H-3), 2.40 (t, J 6.5 Hz, 2H-2); ^{13}C NMR (D$_2$O) δ 180.6 (C-1), 40.0 (C-2), 59.1 (C-3).

Acetate (16)

^{1}H NMR (D$_2$O) δ 1.89 (s, 3H-2); ^{13}C NMR (D$_2$O) δ 181.4 (C-1), 23.0 (C-2).

Formate (17)

^{1}H NMR (D$_2$O) δ 8.43 (s, H-1), ^{13}C NMR (D$_2$O) δ 167.0.

(–)-Onocera-8,8'-diol (1)

Colorless oil; $[\alpha]_D^{27}$ −4.0° (*c* 0.2, CHCl$_3$); for ^{1}H NMR (500 MHz, CDCl$_3$) and ^{13}C NMR (126 MHz, CDCl$_3$) spectra see Table 1; ESI-HR-TOF-MS (probe) ESI-MS, +50V, *m/z* (rel. int.): 469.4018 [M + Na]$^+$ (100) (calculated for C$_{30}$H$_{54}$O$_2$ + Na = 469.4016).

Table 1. NMR data for compound **1**[a]

Position	δ_C[b]	gHMBC	δ_H[c]
1, 1'	39.2 t	H-15,15'	1.59 m
			0.78 m
2, 2'	18.4 t	H-1,1'	1.52 m
			1.20 m
3, 3'	42.2 t	H-13,13', H-14,14'	1.33 m
			1.08 m
4, 4'	33.5 s	H-5,5', H-13,13', H-14,14'	-
5, 5'	56.0 d	H-13,13', H-14,14', H-15,15'	0.75 m
6, 6'	18.3 t	H-5,5'	1.52 m
			1.44 m
7, 7'	42.3 t	H-12,12'	1.68 m
			1.34 m
8, 8'	73.3 s	H-12,12'	-
9, 9'	59.5 d	H-1,1', H-11,11', H-12,12', H-15,15'	0.66 m
10, 10'	39.0 s	H-11,11', H-15,15'	-
11, 11'	20.1 t		0.89 m
12, 12'	30.7 q		1.05 s
13, 13'	33.3 q		0.80 s
14, 14'	21.7 q	H-13,13'	0.76 s
15, 15'	15.2 q		0.88 s

[a]The ^{1}H and ^{13}C NMR data were assigned with the assistance of DEPT 90° and 135°, gHMQC, gHMBC, and ^{1}H–^{1}H COSY experiments (11.7 T); [b]Recorded in CDCl$_3$, 126 MHz; [c]Recorded in CDCl$_3$, 500 MHz.

(−)-ent-8β-Hydroxy-labdan-15-oic acid (2)

Colorless oil; $[\alpha]_D^{25}$ −10.5° (*c* 0.2, CHCl$_3$); ^{1}H NMR (500 MHz, CDCl$_3$) δ 2.36 (1H, dd, *J* 15.0, 6.0 Hz, H-14), 2.16 (1H, ddq, *J* 15.0, 8.0, 1.0, Hz, H-14), 1.74 (1H, dd, *J* 13.0, 3.0 Hz, H-7), 1.67 (1H, m, H-6), 1.64 (1H, br, d, *J* 14.0, H-1), 1.57 (1H, m, H-11 or H-12), 1.50 (2H, m, H-2, H-6), 1.47 (1H, m, H-7), 1.37 (2H, m, H-3, H-12), 1.33 (1H, m, H-11),1.24 (1H, m, H-11 or H-12), 1.13 (2H, m, H-3, H-13), 1.11 (3H, s, H-17), 0.98 (1H, m, H-2), 0.98 (3H, dd, *J* 6.5, 1.0 Hz, H-16), 0.94 (3H, s, H-20), 0.86 (3H, s, H-18), 0.84 (1H, m, H-1), 0.82 (3H, s, H-19), 0.81 (1H, m, H-5), 0.74 (1H, br, s, H-9); ^{13}C NMR (126 MHz, CDCl$_3$) δ 178.4 (C-15), 73.5 (C-8), 59.3 (C-9), 55.9 (C-5), 42.1 (C-7), 42.0 (C-3), 41.3 (C-14), 40.6 (C-12), 39.2 (C-1), 38.9 (C-10), 33.4 (C-18), 33.2 (C-4), 31.1 (C-13), 30.4 (C-17), 22.5 (C-11), 21.6 (C-19), 19.7 (C-16), 18.3 (C-2), 18.1 (C-6), 15.1 (C-20); ESI-MS, *m/z* 325 [M + H]⁺.

(−)-ent-8β-Hydroxy-labd-13(E)-en-15-oic acid (3)

Colorless oil; $[\alpha]_D^{25}$ −10.0° (*c* 0.1, CHCl$_3$); ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.68 (1H, br, s, H-14), 2.28 (1H, m, H-12), 2.12 (3H, br, s, H-16), 2.10 (1H, m, H-12), 1.69 (1H, m, H-7), 1.60 (1H, m, H-1), 1.54 (2H, m, H-2, H-11), 1.50 (1H, m, H-6), 1.44 (2H, m, H-6, H-7), 1.37 (1H, m, H-11), 1.33 (1H, m, H-3), 1.08 (1H, m, H-3), 1.08 (3H, s, H-17), 0.90 (1H, m, H-2), 0.89 (3H, s, H-20), 0.81 (3H, s, H-18), 0.81 (1H, m, H-1), 0.78 (1H, m, H-5), 0.76 (3H, s, H-19), 0.74 (1H, m, H-9); ^{13}C NMR (126 MHz, CDCl$_3$) δ 170.0 (C-15), 163.0 (C-13), 114.8 (C-14), 72.9 (C-8), 58.4 (C-9), 55.8 (C-5), 44.5 (C-12), 42.2 (C-7), 41.8 (C-3), 39.0 (C-1), 33.0 (C-4, C-18), 30.3 (C-17), 21.5 (C-19), 19.6 (C-2, C-11), 18.8 (C-16), 17.8 (C-6), 14.4 (C-20); ESI-MS, *m/z* 323 [M + H]⁺.

(−)-Copalic acid (4)

Colorless oil; $[\alpha]_D^{25}$ −34.2° (*c* 0.5, CHCl$_3$) [lit. −33.7° (*c* 0.4, CHCl$_3$)][7]; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported;[7] ESI-MS, *m/z* 305 [M + H]⁺.

(−)-Eperuic acid (5)

Colorless oil; $[\alpha]_D^{25}$ −29.9° (*c* 1.0, CHCl$_3$); ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.73 (1H, d, *J* 1.5 Hz, H-17), 4.40 (1H, d, *J* 1.5 Hz, H-17), 2.30 (1H, dd, *J* 15.0, 5.5 Hz, H-14), 2.30 (1H, ddd, *J* 13.1, 4.0, 2.0 Hz, H-7), 2.04 (1H, dd, *J* 15.0, 8.5 Hz, H-14), 1.89 (1H, ddd, *J* 13.1, 12.5, 5.2 Hz, H-7), 1.70 (2H, m, H-1, H-11 or H-12), 1.65 (1H, dddd, *J* 12.5, 5.2, 2.5, 2.0 Hz, H-6), 1.46 (3H, m, H-2, H-9, H-11 or H-12), 1.41 (1H, m, H-2), 1.32 (1H, br, dt, *J* 12.5, 3.5 Hz, H-3), 1.24 (1H, qd, *J* 12.5, 4.0 Hz, H-6), 1.20 (2H, m, H-11, H-12), 1.11 (1H, td, *J* 12.5, 3.0 Hz, H-3), 1.02 (1H, dd, *J* 12.5, 2.5 Hz, H-5), 1.02 (1H, m, H-13), 0.95 (1H, td, *J* 13.0, 4.0 Hz, H-1), 0.90 (3H, d, *J* 6.5 Hz, H-16), 0.80 (3H, s, H-18), 0.73 (3H, s, H-19), 0.60 (3H, s, H-20); ^{13}C NMR (126 MHz, CDCl$_3$) δ 178.9 (C-15), 148.7 (C-8), 106.3 (C-17), 57.2 (C-9), 55.6 (C-5), 42.2 (C-3), 41.2 (C-14), 39.7 (C-10), 39.1 (C-1), 38.4 (C-7), 35.9 (C-12), 33.6 (C-4, C-18), 30.9 (C-13), 24.4 (C-6), 21.7 (C-19), 20.9 (C-11), 19.9 (C-16), 19.4 (C-2), 14.4 (C-20); ESI-MS, *m/z* 307 [M + H]⁺.

(−)-Kolavenic acid (6)

Colorless crystals: mp 97–98 °C; $[\alpha]_D^{25}$ −42.0° (*c* 1.0, CHCl$_3$) [lit. −41.4° (*c* 1.0, CHCl$_3$)][7]; IR, ^{1}H NMR, and ^{13}C NMR data were consistent with those previously reported;[7] ESI-MS, *m/z* 305 [M + H]⁺.

(−)-Hinokinin (7)

Yellow oil: $[\alpha]_D^{25}$ −31.5° (*c* 1.0, CHCl$_3$) [lit. −30.3° (*c* 0.6, CHCl$_3$)][7]; IR, UV, ^{1}H NMR, and ^{13}C NMR data were consistent with those previously reported;[7] ESI-MS, *m/z* 355 [M + H]⁺.

(−)-Kusunokinin (8)

Yellow oil: $[\alpha]_D^{25}$ −42.0° (*c* 1.1, CHCl$_3$) [lit. −40.0° (*c* 0.2, CHCl$_3$)][7]; IR, UV, ^{1}H NMR, and ^{13}C NMR data were consistent with those previously reported;[7] ESI-MS, *m/z* 371 [M + H]$^+$.

(−)-(8R,8′R,9R)-Cubebin + (−)-(8R,8′R,9S)-cubebin (9)

Colorless crystals: mp 126-128 °C [lit. 127-128 °C][7]; $[\alpha]_D^{25}$ −42.5° (*c* 1.0, CHCl$_3$) [lit. −41.5° (*c* 0.4, CHCl$_3$)][7]; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported;[8] ESI-MS, *m/z* 357 [M + H]$^+$.

β-*Sitosterol (10)*

Colorless crystals: mp 138-140 °C [lit. 137-139 °C][13]; ^{1}H and ^{13}C NMR data were consistent with those previously reported;[13] ESI-MS, *m/z* 415 [M + H]$^+$.

Sequoyitol (11)

Colorless crystals: mp 238-240 °C [lit. 232-234 °C][14]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.70 (1H, t, *J* 2.5 Hz, H-4), 3.43 (2H, t, *J* 9.5 Hz, H-2, H-6), 3.46 (3H, s, OCH$_3$), 3.14 (2H, dd, *J* 9.5, 2.5 Hz, H-3, H-5), 2.69 (1H, t, *J* 9.5 Hz, H-1). ^{13}C NMR data were consistent with those previously reported;[14] ESI-MS, *m/z* 195 [M + H]$^+$.

Trigonelline (12)

Yellow solid: mp 216-219 °C [lit. 218 °C dec.][15]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.20 (1H, s, H-2), 8.88 (1H, d, *J* 5.5 Hz, H-6), 8.76 (1H, d, *J* 8.0 Hz, H-4), 8.02 (1H, dd, *J* 8.0, 5.5 Hz, H-5), 4.36 (3H, s, N-CH$_3$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 162.3 (C-7), 145.9 (C-2), 144.7 (C-6), 144.0 (C-4), 139.8 (C-3), 126.7 (C-5), 47.6 (N-CH$_3$); ESI-MS, *m/z* 138 [M + H]$^+$.

Uracil (13)

Yellow solid, mp > 300 °C [lit. 335 °C][15]; ^{1}H and ^{13}C NMR data were consistent with those previously reported;[16] ESI-MS, *m/z* 113 [M + H]$^+$.

Essential oils

Stems (30 g) were cut into small pieces and stored at −8 °C until oil extraction. The essential oils were obtained by hydrodistillation in 250 mL H$_2$O for 4 h, with simultaneous extraction of the distillate with GC-grade *n*-hexane (1 mL), which enabled separation of the essential oil in an ice-cooled oil receiver, in a modified Clevenger apparatus to reduce hydrodistillation over-heating artifacts. The oils were collected with the addition of GC-grade *n*-hexane (1 mL) and dried over anhydrous Na$_2$SO$_4$. The solutions were then dried over a molecular sieve

and analysed by GC-MS. The composition of the volatile constituents was established by GC-MS analyses. Retention indices for all compounds were determined according to the equation proposed by Van den Dool and Kratz,[17] using *n*-alkanes as standards. Adjusted retention times for each peak were determined by subtracting the retention time of methane from the retention time of each peak. Components were identified based on comparison of their mass spectra with those at the Mass Spectrometry Data Centre,[18] Wiley and NBS Libraries[19] and those described by Adams,[20] as well as by comparison of their retention indices with data in the literature.[9] In several cases, the essential oils were co-injected with compounds that had been previously isolated from *Aristolochia* species or purchased standard compounds.

Standard compounds

Aldrich kits containing: 24 standard hydrocarbons/ C5–C30, straight-chain alkanes (Aldrich 29,850-6); 19 fatty acids/C6-C24, straight-chain (Aldrich 29,851-4); fatty acid methyl esters/C6–C24 straight-chain (Aldrich 29,851-4). Compounds isolated and identified by spectroscopic methods (mainly by MS, ^{1}H and ^{13}C NMR) from *Aristolochia* species: spathulenol, β-caryophyllene, caryophyllene oxide, *trans*-nerolidol, vanillin, kobusone, calarene, and 2-oxocalarene.

Statistical analysis

The agglomerative hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used as statistical methods to suggest the structure of the set and to analyse the variables in relation to the characteristics being studied. To reduce the scattering effects and to compare samples, the chromatograms from obtained oils were normalized by reducing the area under each chromatogram to a value of 1.[21] Overall, 68 characteristics (chemical compounds, of which 67 were identified and one was unknown) were analysed in 10 oils by HCA and PCA (Table 1 and Table S1-Supplementary Information). The chemical compositions were determined from the chromatographic profiles for 2 oils of the Brazilian cultivated species, and taken from data described in the literature for 2 oils from Paraguay, and 6 oils from Argentina. Plots defined by PC1 (score 1) and PC2 (score 2) for the 68 characteristics were obtained for chromatographic data using Pirouette® version 3.11.[22] The results were obtained using an original data matrix X (68 by 10) with 68 variables, 10 samples, 3 optimal factors, 1st derivative. Variances of PC1 (32.6168) and PC2 (3.9711) accounted for 79.90% and 9.73%, respectively, of the total PCA variance.

Results and Discussion

In the oils from cultivated species (fresh material), 17 compounds from the stem and 14 from the leaf were identified (Tables 2 and 3). The data from oils were compared with those described in the literature[10,11] for oils obtained from the stems and leaves of *A. giberti*, to obtain evidence that could contribute to the identification of species through significant interspecific variability. The variation in the chemical constitutions of the essential oils was examined by taking into account the part of the plant, the year that the plant was collected, and its provenance. Brazilian oils were characterized by the highest concentration of sesquiterpene hydrocarbons in oil from leaves (84.7%) and also the highest concentration of monoterpene hydrocarbons in oil from stems (78.3%). In this study, hierarchical cluster analysis (HCA) was used to search for sample provenance patterns and to create a classification scheme to differentiate plant parts or chemotypes. In this analysis, the data regarding the chemical composition of the oils described in the literature for *A. giberti* from Argentina (fresh material),[10] involving 36 compounds (one unknown), and Paraguay (dried material),[11] involving 46 compounds, were compared to those obtained in this study (25 compounds) (for further information see Supplementary Data). Based on similarities in the chemical composition, a dendogram was obtained (Figure 2). According to this dendogram, the oils could be separated into three distinct groups or chemotypes. The first and second groups (I and II) consisted of data of the stem and leaf oils, respectively, obtained from both Brazil and Argentina, and the third group (III) consisted of oils from Paraguay (I, II, and III; similarity index 0.652). The first group was greatly characterized by monoterpenes (α- and β-pinene, sabinene, camphene, tricyclene, and α-thujene). Thus, similar chemical profiles were detected

in stem samples collected in different years, which suggests a retention of unique chemical profiles in different populations from Argentina and Brazil.

Similarly, an analysis of the data regarding the chemical composition by PCA (Principal Component Analyses) of the oils was performed to obtain information about the characteristic compounds, which are the most discriminating for the samples observed in the plots (Figures 3 and 4). Except for stem oil from Paraguay (SP), they showed the highest positive scores of

Table 2. List of investigated oils with provenance, collection date, and abbreviation used

Code	Part of plant	Locality	Date
SA-1	stems	Argentina	July, 1994
SA-2	stems	Argentina	July, 1996
SA-3	stems	Argentina	June, 1997
SA-4	stems	Argentina	July, 1999
SB	stems	Brazil	February, 2004
SP	stems	Paraguay	December, 2000
LA-1	leaves	Argentina	May, 1994
LA-2	leaves	Argentina	June, 1994
LB	leaves	Brazil	February, 2004
LP	leaves	Paraguay	December, 2000

Table 3. Composition of essential oils of stems and leaves from Brazilian *A. giberti*

I^a / s	Compound	Species (code)	
		SB (%)[b]	LB (%)[c]
922	α-Thujene	0.6	-
934	α-Pinene	2.9	-
973	β-Pinene	0.7	-
984	β-Myrcene	6.5	-
1006	δ-3-Carene	4.5	-
1013	α-Phellandrene	25.6	-
1014	*o*-Cymene	20.4	-
1023	Limonene	15.1	-
1030	*cis*-Ocimene	2.0	-
1184	4(10)-Thujen-3-ol	1.2	-
1391	β-Elemene	-	1.3
1415	β-Caryophyllene	3.6	15.9
1446	α-Aromadendrene	-	1.8
1474	Acoradiene	-	1.2
1481	Germacrene D	2.5	14.3
1483	Isoledene	-	3.2
1485	β-Selinene	-	1.4
1492	Guaia-3,9-diene	-	1.4
1495	Germacrene A	3.5	35.7
1507	Valencene	-	4.2
1524	δ-Cadinene	0.7	4.3
1569	Spathulenol	5.3	11.7
1575	(−)-β-Caryophyllene oxide	3.1	1.9
1645	α-Cadinol	-	1.9
1674	Bulnesol	1.9	-
	Total	100.1	100.2
	Terpenoids		
	Monoterpene hydrocarbons	78.3	0.0
	Oxygenated monoterpenes	1.2	0.0
	Sesquiterpenes hydrocarbons	10.3	84.7
	Oxygenated sesquiterpenes	10.3	15.5

[a]I: linear retention index; [b]SB: stems, species collected in Brazil; [c]LB: leaves, species collected in Brazil.

Figure 2. Dendogram constructed following agglomerative hierarchical clustering analysis (HCA) of essential oils of *A. giberti* of different provenances and collection dates.

principal component 1 (PC1) and similar PC2 (principal component 2) values for stem oils, which allowed us to differentiate them from the leaf oils (Figure 3). Oils characterized by the highest positive score signals for PC1 corresponded to stems that showed a predominance of monoterpenes, such as those included in group I by HCA as well as β-myrcene, δ-3-carene, and α-phellandrene (Figure 4), whereas the highest negative score signals showed a predominance of oxygenated monoterpenes, such as bornyl acetate and isobornyl formate. Leaf oils from Argentina (LA) and Brazil (LB) were characterized by positive scores of PC2 and by oxygenated sesquiterpenes, such as aristolactone and α-cadinol. Oils from Paraguay (LP and SP) were characterized by negative loading of PC2 for sesquiterpenes, such as δ-cadinene and germacrene B (Figure 4). These plots also showed the similarity between stem oils from Argentina and Brazil, which provided evidence regarding the identity of the species and that these oils show insignificant annual chemical variability. However, as shown by HCA, the oils from Paraguay were not well-discriminated by PCA analysis, suggesting that the best results for discriminating the part of the plant and its provenance can be achieved by using fresh material.

Phytochemical studies on hexane and ethanol extracts from *A. giberti* led to the isolation of a new tetracyclic triterpene [(−)-onocera-8,8'-diol (**1**)], five diterpenes [(−)-*ent*-8β-hydroxy-labdan-15-oic acid (**2**), (−)-*ent*-8β-hydroxy-labd-13(*E*)-en-15-oic acid (**3**), (−)-copalic acid (**4**), (−)-eperuic acid (**5**), and (−)-kolavenic acid (**6**)], and three lignans [(−)-hinokinin (**7**), (−)-kusunokinin

(**8**), and (−)-cubebin (**9**)], as well as β-sitosterol (**10**), sequoyitol (**11**), trigonelline (**12**), and uracil (**13**). In addition, 3-hydroxypropanoate, acetate, and formate were detected by NMR techniques, which suggested that they are derived from a natural, yet undetectable compound of 2-butynedioate (**14**) (Figure 1).

Compounds **1**, **2**, **4-9**, and **11-14** were isolated by partition and chromatographic procedures from the ethanol extracts of the leaves, whereas compounds **2**, **3**, **6-8**, and **10** were obtained by chromatographic procedures from the hexane extract of stems. *A. giberti* was a rich source of the diterpene (−)-*ent*-8β-hydroxy-labdan-15-oic acid (**2**), which represents 8.0% of the ethanol and 21.9% of the ethanol Soxhlet extracts from the leaves.

The structures of compounds **4**, **6-11**, and **13** were identified by comparison of their spectroscopic data (ESI-MS, IR, 1D- and 2D-^{1}H and ^{13}C NMR) and optical activities (α_D) with those reported in the literature.[7, 13–16] Compounds **4** and **6-9** were also identified with reference to authentic samples obtained from *Aristolochia malmeana*.[7] Compound **12** was identified as trigonelline by 1D- and 2D-^{1}H and ^{13}C NMR and by comparison of its spectroscopic data with those reported for trigonellinium chloride and monohydrate.[23] Although labdane derivatives from **2**, **3**, and **5** have been known for some time, and have even been isolated from Aristolochiaceae species,[1,24,25] most of their spectroscopic data still remain to be described. A detailed analysis of ^{1}H-^{1}H *g*COSY, *g*NOESY, *g*HMQC, and *g*HMBC data of these diterpenes enabled us to assign the majority of their chemical shifts to carbons and hydrogens in the
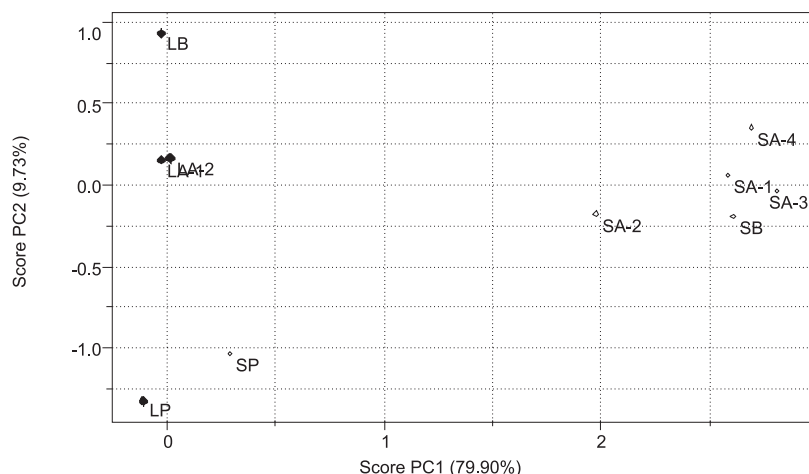
Figure 3. Principal component analysis (PCA) of chemical constituents of essential oils from stems and leaves of *A. giberti*. The principal components (PC1 and PC2) account for *ca.* 89.6% of the information (for abbreviations of oils, see Table 2).
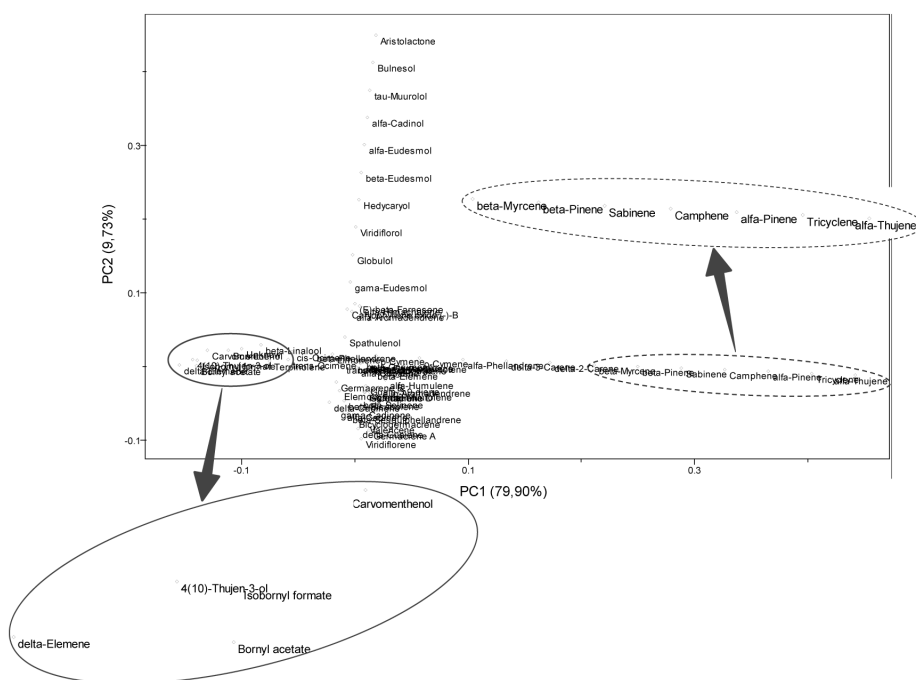

Figure 4. Loading plot of principal components (PC1 and PC2) obtained for essential oils from the stems and leaves of *A. giberti.*

structures (Figure 5) and also helped to determine their relative configurations. Moreover, the coupling constants (*J*) values determined for the hydrogens were confirmed by selective proton irradiation. The relative configuration established for **5** was corroborated by 1D *g*NOESY experiments that showed interactions between C$\underline{H}_3$-20 (δ_H 0.60) and H-6 (δ_H 1.24), H-17a (δ_H 4.40), and C$\underline{H}_3$-19 (δ_H 0.73), whereas H-17b (δ_H 4.73) showed interaction with H-17a (δ_H 4.40), H-7a (δ_H 2.30), and H-11a (δ_H 1.20). This latter also showed interaction with C$\underline{H}_3$-20 (δ_H 0.60). Compounds **2** and **3** showed characteristic chemical shifts for C$\underline{H}_3$-17 (δ_C *ca.* 30) in equatorial positions on the B rings. Consequently, OH-8 must be in axial positions. In

this case, hydrogens of C$\underline{H}_3$-20 were shifted downfield ($\Delta\delta_H$ *ca.* +0.16) in relation to those for labdanes in normal series, which present an opposite relative configuration at C-8 (e.g. they show δ_{H-20} *ca.* 0.78 and OH-8 in equatorial positions).[26] This deduction was further confirmed by 1D-gNOESY experiments for **2** and **3**, which showed spatial interactions between C$\underline{H}_3$-19 (δ_H *ca.* 0.82) and C$\underline{H}_3$-20 (δ_H *ca.* 0.94), C$\underline{H}_3$-18 (δ_H *ca.* 0.86), and H-3eq (δ_H *ca.* 1.50), as well as between C$\underline{H}_3$-17 (δ_H *ca.* 1.11) and H-9 (δ_H *ca.* 0.74), 2H-7 (δ_H *ca.* 1.47, 1.74), and H-11 (δ_H *ca.* 1.33). Based on these data and the optical activities, compounds **2** and **3** were identified as (−)-*ent*-8β-hydroxy-labdan-15-oic acid and (−)-*ent*-8β-hydroxy-labd-13(*E*)-en-15-oic acid,

respectively. Both were previously isolated from *Aristolochia galeata*,[25] and compound **5** was determined to be (−)-eperuic acid. Based on the identity of the observed optical activity of **5** with that reported for its methyl ester derivatives,[26] a 13(*S*) configuration could be established for **5**; while the optical activity of **2** differed from that report for its 13(*R*) diastereomer methyl ester.[27] Thus, diterpenes **2** and **5** should belong to *ent* labdane series and has a 13(*S*) configuration.
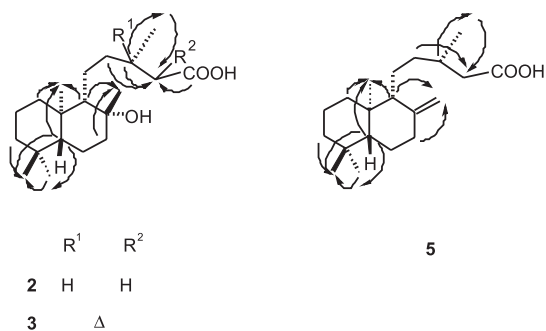
Figure 5. Selected *g*HMBC correlations (→) for diterpenes **2**, **3**, and **5**.

The ^{1}H NMR spectrum of compound **1** was similar to that of **2**. The main difference between them was the absence of a doublet corresponding to CH$_3$-16 in the spectrum of **1**. The ^{13}C NMR and DEPT (135° and 90°) spectra of **1** showed signals for 15 carbons, including four CH$_3$, six CH$_2$, two CH, and three quaternary carbons. These data (Table 1), together with the great similarity between ^{1}H and ^{13}C NMR spectra of **1** and those of **2** (for further information see Experimental and Supplementary Information, Figures S1 and S2), as well as the *J* values determined for the hydrogens by selective proton irradiation, suggested that, except for the substituents at C-9 (side chain), the A and B rings in the structures of both compounds were identical, including their relative configurations.

The HRMS spectra showed *quasi*-molecular ions [M+Na]$^+$ at *m/z* 469.4018 for **1**, which were consistent with the molecular formula $C_{30}H_{54}O_2$ + Na. Based on the HRMS and NMR experiments, particularly on the presence of a CH$_2$ (δ_C 20.1, δ_H 0.89), the structure of **1** was determined to be a triterpene, which consisted of two identical sesquiterpene units. Moreover, the correlations observed by *g*HMBC and 1D *g*NOESY experiments (irradiating at methyl hydrogen frequencies) allowed us to establish that the monomer units should be linked through C-11, C-11′ (Figure 6). The chemical shifts of C-12,12′ (δ_C 30.7) and methyl groups CH$_3$-15,15′ (δ_H 0.88) are in accordance with equatorial CH$_3$-12,12′ on the B and B′ rings. Thus, as observed for **2** and **3**, the hydroxyl groups at C-8,8′ (δ_C 73.3) should be in axial positions on the B and B′ rings, which was further supported by 1D-*g*NOESY experiments (Figure 6).
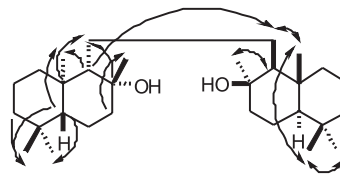

Figure 6. Selected *g*HMBC correlations (→) and nOe interactions (↔) for triterpene **1**.

Syntheses of onoceranols with diverse configurations have been described in the literature.[29-31] Vlad *et al.*[29] synthesized (5*S*,8*R*,9*R*,10*S*,5'*S*,8'*R*,9'*R*,10'*S*)-onoceranediol (**1a**) (Figure 7). However, the ^{13}C NMR data for C-8,8′ (δ_C 74.6) and its neighbouring carbons, such as C-12,12′ (δ_C 24.4), differ from those of **1**, which infers that these compounds have distinct relative configurations at C-8,8′ (Figure 7). Corey and Sauers[30] synthesized **1a** and its correspondent epimer at C-8,8′ (**1b**), which showed [α]$_D$ +13° and +42°, respectively. Since compound **1** showed [α]$_D$ − 4.0° and presents a symmetrical structure, as evidenced by its NMR spectra, its absolute configuration was determined to be (5*R*,8*R*,9*S*,10*R*,5'*R*,8'*R*,9'*S*,10'*R*). Hence, it should belong to the *ent*-series like the other diterpenes isolated from this species. Compound **1** was named (−)-onocera-8,8′-diol.
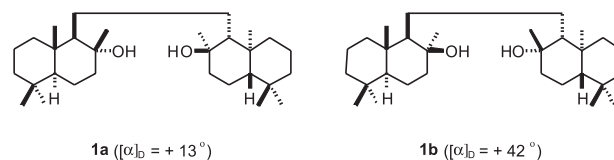

Figure 7. Structures for synthetic onocera-8,8′-diols **1a** and **1b**.

A colorless amorphous solid (**14**) was isolated from the ethanol Soxhlet extract of leaves, and its ^{1}H NMR spectrum did not show any signals for hydrogen in D$_2$O, suggesting that, in principle, this solid was an inorganic compound. However, its undetermined melting point (*ca.* 180 ºC) showed its decomposition as well as its organic character. The ^{1}H and ^{13}C NMR spectra in DMSO-*d$_6$*, obtained on consecutive days from the soluble solution resulting from precipitation procedures, showed signals for hydrogens and carbons with diverse intensities, indicating the chemical transformation of compound **14**. These spectra together with the results of DEPT, *g*HMBC, and *g*HMQC experiments clearly indicated the formation of 3-hydroxypropanoate (**15**, δ_C 177.3, 59.2, and 40.4, δ_H 3.45, and 2.08), acetate (**16**, δ_C 181.0, and 25.1, δ_H 1.63), and formate (**17**, δ_C 167.7, δ_H 8.50). Similarly, the formation of these metabolites by *in situ* ^{1}H NMR analysis of two biotransformations of undetected 2-butynedioate and propynoate ('invisible substrates') was observed Brecker *et al.*[32] using *Pseudomonas putida*.

They determined the metabolic pathways of these two α-alkynoates, in which the triple bonds in both 'invisible substrates' were initially hydrated, and 2-ketobutandioate as well as 3-ketopropanoate were then formed. These authors proposed that both β-keto acids were decarboxylated, resulting in pyruvate and acetaldehyde, respectively. Pyruvate was further hydrolyzed mainly to acetate and formate, whereas minor amounts were reduced to lactate. In the other biotransformation, acetaldehyde was oxidized to acetate accompanied by the reduction of 3-ketopropanoate to 3-hydroxypropanoate.[32] Based on this finding, we propose that compound **14** was 2-butynedioate (not detected by ^{1}H NMR), which, *via* decarboxylation, could give rise to propynoate, which in turn could give 3-hydroxypropanoate and acetate, and hydration of compound **14** followed by decarboxylation, could lead to acetate and formate (Figure 8). Thus, the difference between this proposal and those regarding biotransformations is that **14** could give propynoate as one of the intermediates.
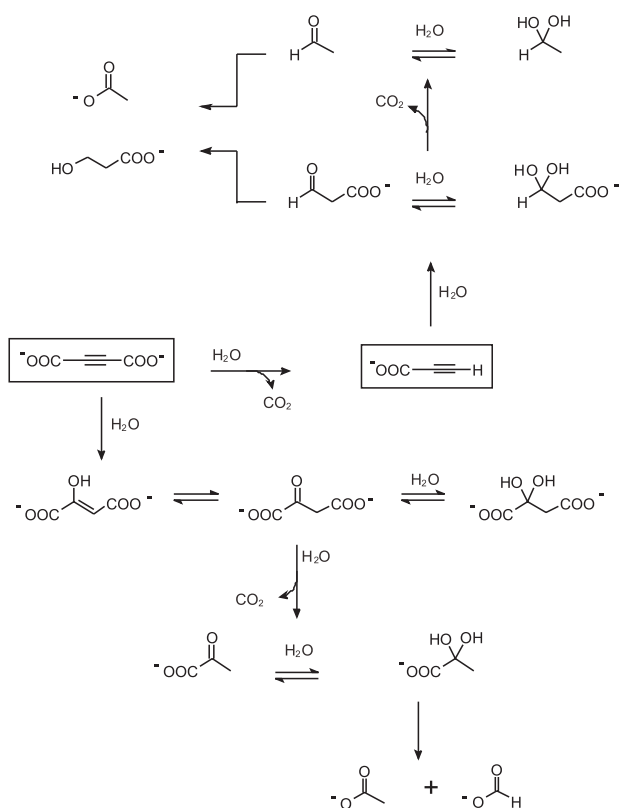
Figure 8. Proposed chemical pathways to 3-hydroxypropanoate, acetate, and formate from 2-butynedioate (**14**).

Sequoyitol (**11**), isolated from *Aristolochia cymbifera* and *Aristolochia gigantea*, among other species,[1] has been shown to exhibit antidiabetic properties.[33] Sequoyitol, (+)-pinitol, and aristolochic acids are oviposition stimulants for the pipevine swallowtail butterflies, *Battus philenor*

and *Atrophaneura alcinous* (Papilionidae), which use *Aristolochia* species as major hosts.[1] Trigonelline (**12**) is a natural zwitterion isolated from various plants, seeds and the western rock lobster.[23] It has been shown to have hypoglycemic, hypolipidemic, hypocholesterolemic, insulinotropic, and antioxidant activities.[34] These properties are related to the control of the Metabolic Syndrome, a disorder of carbohydrate and lipid metabolism which increases the risk of diabetes and cardiovascular disease.[34] Therefore, trigonelline is a potential natural antidiabetic agent, as well as an antimicrobial and anti-dementia agent.[35,36]

Conclusions

A. giberti is a rich source of the diterpene and lignans. From this species, 14 compounds were isolated. Among them, a new triterperne, (−)-onocera-8,8'-diol (**1**), was isolated together with known compounds that are potential agents against several diseases such as diabetes. In addition, 3-hydroxypropanoate, acetate, and formate were also detected and were suggested to be derivatives from 2-butynedioate, which could not be detected by ^{1}H NMR. Moreover, a total of 25 compounds were identified in the essential oils from stems and leaves. GC-MS and chemometric analyses showed the great similarity between this cultivated species in Brazil and that collected in Argentina, and allowed us to confirm the species identity and to differentiate the oils according to the different parts of the plant.

Acknowledgments

Supplementary Information

Supplementary data for compound **1** are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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