Limonoids from the Endemic Brazilian Species *Raulinoa echinata*

Maique W. Biavatti[a,‡], Paulo C. Vieira[a,*], M. Fátima G. F. da Silva[a],
João B. Fernandes[a] and Sérgio Albuquerque[b]

[a] Departamento de Química, Universidade Federal de São Carlos, CP 676, 13565-905, São Carlos, SP, Brazil. *Fax: +55 16 260 8350. E-mail: paulo@dq.ufscar.br
[b] Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. do Café, 14040-903, Ribeirão Preto, São Paulo, Brazil
[‡] Permanent address: UNIVALI, Curso de Farmácia, CP 360, 88303-202, Itajaí, SC, Brazil

* Author for correspondence and reprint requests

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Phytochemical survey of stems and leaves of the South Brazilian endemic *Raulinoa echinata* Cowan, Rutaceae led to the isolation of five limonoid derivatives: the widespread limonin, limonexic acid, kihadalactone B, a methoxylated limonexic acid derivative and a degraded limonoid structurally related to fraxinellone. The two latter compounds have been isolated for the first time. These compounds displayed weak inhibitory activity when assayed *in vitro* against trypomastigote forms of *Trypanosoma cruzi*. In this paper, the isolation, structure elucidation and bioactivity of these compounds are reported.

Introduction

Raulinoa is a monospecific genus and the species *Raulinoa echinata* Cowan (Rutaceae) is endemic in the Itajaí Valley, SC, Brazil (Kaastra, 1982). This perennial woody shrub is characterized by the presence of spines and has been only found over a short distance (1000 m) on the frequently inundated banks of the Itajaí River in an approximate altitude of 100 m, showing a high degree of adaptation to the environment.

We have recently reported the isolation of some hexane extractable compounds of stems of *R. echinata* (Biavatti *et al.*, 2000). In continuation of our research on the chemistry of Rutaceae, methanol extracts of stems and leaves of *R. echinata* led to the isolation of typical rutaceous limonoids: the widespread limonin (**1**), limonexic acid (**2**), a limonexic acid derivative (**3**), a nomilin derivative (kihadalactone B) (**4**) and the degraded limonoid **5**.

A wide range of biological activities has been described for limonoids and its degraded derivatives: insecticidal, insect growth regulation (Jayaprakasha *et al.*, 1997), insect antifeedant (Okamura, *et al.*, 1997), antifungal (Zhao *et al.*, 1998), anticarcinogenic (Champagne *et al.*, 1992), antimutagenic (Miyazawa *et al.*, 1995), among others.

In this work, the isolated compounds were assayed *in vitro* against trypomastigote forms of *Trypanosoma cruzi* showing weak inhibitory activity.

Material and Methods

General experimental procedures

A Bruker DRX-400 spectrometer, operating at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C was used. All spectra were run in $CDCl_3$ or $CDCl_3$ with drops of DMSO-d_6 and with tetramethylsilane as internal standard. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Mass spectra were obtained from a Quattro LC Micromass spectrometer, APCI- (25 eV of colision energy). For HREIMS a Autospec-Micromass-EBE was used (Universidade Estadual de Campinas).

Plant material

Stems and leaves of *R. echinata* were collected in Ressacada, Ibirama, SC, Brazil and identified by A. Reis (Universidade Federal de Santa Catarina) and J. R. Pirani (Universidade de São Paulo). Voucher specimens [A. Reis & M. Biavatti 2.570 (26/07/98)] were deposited at the Herbário Barbosa Rodrigues (HBR), Itajaí, SC, Brazil.

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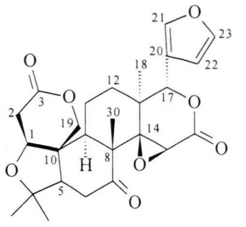

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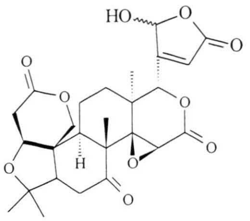

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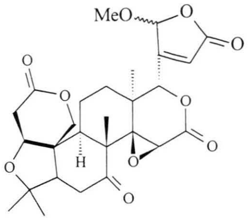

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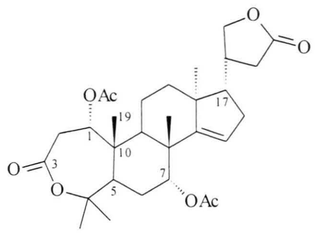

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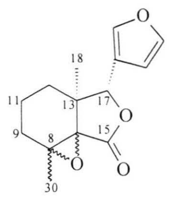

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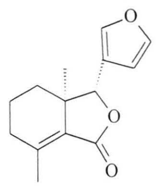

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Extraction and isolation of compounds

The dried and powdered stems (5 kg) and leaves (3 kg) were extracted with *n*-hexane and MeOH. Successively, half (100 g) of the MeOH crude extract of stems was fractionated over silica gel 60 (70–230 mesh) into four fractions: *n*-hexane, CH_2Cl_2, EtOAc and MeOH. The CH_2Cl_2 fraction (1.9 g) was successively chromatographed over silica gel (230–400 mesh) using as eluent appropriate ratios of hexane-acetone to furnish compound **5** (8 mg). The EtOAc (8.0 g) fraction was also chromatographed as described before, affording limonin (**1**) (1 g) and compound **4** (20 mg). From the MeOH fraction (90 g) compound **3** (50 mg) was also obtained after column chromatography.

Part of the MeOH crude extract of leaves (100 g) was submitted to liquid partition with solvents of increasing polarity, furnishing the following dry residues: hexane (35 g), CH_2Cl_2 (14 g), EtOAc (7 g) and MeOH (44 g). Successive column chromatography over silica gel (230–400 mesh) of the CH_2Cl_2 fraction yielded compound **2** (20 mg) and also limonin (**1**) (300 mg).

(−)-Limonin (**1**). White needles, mp 224–226°, $[\alpha]_D$ −101° (MeOH; *c* 0.07). Spectral data in agreement with the literature (Ng *et al.*, 1987).

(−)-Limonexic acid (**2**). White needles, mp 275–278°, $[\alpha]_D$ −43° (MeOH; *c* 0.03). APCI- MS/MS $[M]^-$ 502; with major fragments at *m/z* 458 (38), 414 (18), 399 (41), 381 (41), 345 (23), 263 (100), 150 (50), 134 (72), 110 (41). ^1H NMR: Table I. ^{13}C NMR: Table II. (Ng *et al.*, 1987).

(–)-21-O-Methyl-limonexic acid (**3**). White needles, mp 296–298°, $[\alpha]_D$ –54° (CH_2Cl_2; *c* 0.025). HREIMS: found: $[M]^{+\cdot}$ 516.23593; $C_{27}H_{32}O_{10}$ requires: 516.19956, with major fragments at *m/z* 505 (20), 411 (11), 320 (16), 264 (6), 97 (27), 55 (100). ^1H NMR: Table I. ^{13}C NMR: Table II.

(–)-Kihadalactone B (**4**). Amorphous solid, mp 178–180°, $[\alpha]_D$ –53° (CH_2Cl_2; *c* 0.01). HREIMS: found: $[M]^{+\cdot}$ 530.28797; $C_{30}H_{42}O_8$ requires: 530.28806; with major fragments at *m/z* 411 (15), 352 (90), 337 (100), 313 (35), 253 (27), 138 (30). ^1H NMR: Table I. ^{13}C NMR: Table II. (Kishi *et al.*, 1992).

(–)-8,14-Epoxyfraxinellone (**5**). Viscous oil, $[\alpha]_D$ –22.5° (CH_2Cl_2; *c* 0.02). HREIMS: found: $[M]^{+\cdot}$248.10486; $C_{14}H_{16}O_4$ requires: 248.10488, with major fragments at *m/z* 207 (13), 180 (4), 162 (23), 148 (19), 81 (43), 69 (100) 55 (92). ^1H NMR: Table I. ^{13}C NMR: Table II.

Bioactivity screening

In vitro trypomastigote forms of *T. cruzi* (Y strain) was performed according to established protocols (Alves *et al.*, 1999).

Results and Discussion

Compound **1** was identified as limonin by comparison with the spectral data described in the literature (Ng *et al.*, 1987). Compound **2** differed

Table I. ^1H NMR spectral data for compounds **2–5**.

H	2[a]	3	4	5
1	4.14 *br s*	4.07 *br s*	4.82 *t* (4.0)	
2	2.90 *dd* (16.7; 3.6)	3.02 *dd* (16.8; 3.6)	3.14 *m*	
2	2.79 *d* (16.7)	2.72 *dd* (16.8; 2.1)		
5	2.34 *dd* (15; 3)	2.22 *dd* (15.8; 3.1)	2.40 *m*	
6	3.01 *t* (15)	2.89 *br t* (15.8)		
			1.93 *m*	
6	2.41*dd* (15; 3)	2.48 *dd* (15; 3.1)		
7			5.15 *m*	
9				1.73 *m*
	2.54 *dd* (10; 3.7)	1.58 *m*	2.53 *m*	
9				2.13 *dt* (15; 2.8)
11				
	1.95 *m*	1.82 *m*		1.48 *m*
11				
12				1.31 *m*
	1.70 *m*	1.94 *m*	1.50 *m*	
12				1.60 *m*
15	3.87 *s*	3.84 *s*	5.28 *m*	
16			2.20 *m*	
17	5.32 *s*	5.24 *s*	1.70 *m*	5.19 *s*
18	1.17 *s*	1.16 *s*	0.96 *s*	0.89 *s*
19	4.49 *d (*13)	4.45 *d* (13)	1.15 *s*	
19	4.86 *d* (13)	4.79 *d* (13)		
20			2.67 *m*	
21			4.43 *t* (8.2)	
	6.01 *s*	5.65 *s*	3.90 *m*	7.48 *m*
21				
21-OMe		3.55 *s*		
22			2.50 *m*	6.35 *m*
	6.24 *s*	6.36 *s*		
22			2.23 *m*	
23				7.47 *br t* (1.7)
28	1.29 *s*	1.31 *s*	1.50 *s*	
29	1.16 *s*	1.19 *s*	1.39 *s*	
30	1.04 *s*	1.05 *s*	1.16 *s*	1.60 *s*
1-Ac			1.99 *s*	
7-Ac			2.11 *s*	

[a] In $CDCl_3$ with drops of $(CD_3)_2SO$.

Table II. ^{13}C NMR spectral data for compounds **2**–**5**.

C	**2**[a]	**3**	**4**	**5**
1	79.2	80.3	70.8	
2	35.5	35.7	34.8	
3	168.8	169.0	170.2	
4	77.1	78.9	85.4	
5	59.2	61.0	44.1	
6	34.9	36.4	26.3	
7	205.8	205.8	74.0	
8	44.9	46.1	41.8	66.1
9	46.8	48.2	35.5	31.5
10	50.6	51.8	44.2	
11	17.7	19.3	16.2	16.2
12	29.4	31.3	33.5	32.5
13	37.0	37.8	46.4	39.9
14	64.4	64.8	158.6	65.6
15	52.1	52.8	118.9	172.6
16	164.9	165.0	34.9	
17	77.9	77.9	57.9	82.7
18	20.4	21.5	18.8	16.2
19	64.4	65.5	15.2	
20	162.6	160.5	37.4	119.9
21	97.4	102.7	72.4	143.8
22	121.7	124.0	34.0	108.4
23	168.2	168.4	176.5	139.9
28	29.1	30.1	34.4	
29	19.9	21.5	23.6	
30	15.9	16.9	27.3	17.9
OMe		56.9		
1-Ac			21.1	
			169.9	
7-Ac			20.9	
			169.8	

[a] In $CDCl_3$ with drops of $(CD_3)_2SO$.

from limonin only in oxidation of the furan ring and was identified as limonexic acid on the basis of the following: (a) the ^1H NMR spectrum did not show the presence of a furan group; instead, resonances at δ 6.24 and 6.01 ppm (Table I) indicated a dihydrofuranone system, (b) DEPT 135 spectrum showed resonances at δ 97.4 and 121.7 ppm (Table II) consistent with the proposed system, (c) all the other signals related to limonoid skeleton were identical to compound **1**. (d) the mass spectrum showed a molecular ion at *m/z* 502, differing by 32 Da (2 × 16) from limonin ($[M]^{+\cdot}$470) molecular weight, attributed to two additional oxygen atoms in the furanoid ring.

The same pattern could be observed for compound **3**, with differences remaining on the furan ring skeleton. The ^1H NMR spectrum showed singlets at δ 6.36, 5.65 and 3.55 ppm (Table I), in accord with a methoxylated dihydrofuranone ring system and complemented by the ^{13}C NMR spectrum that presented resonances at δ 102.7 and 124.0 ppm (Table II). The methoxyl carbon (δ 56.9) showed correlation in the HMBC spectrum with the H-21 (δ 5.65), which also correlated with the C-22 (δ 124.0) (and vice-versa). H-17 (δ 5.24) correlated with the C-20 (δ 160.5), C-22 (δ 124.0), C-14 (δ 64.8), C-13 (δ 37.8) and C-18 (δ 21.5). H-1 and H-19 showed expected limonin-type correlations, as follows: H-1 (δ 4.07) correlated with C-3 (δ 169.0); H-19α/β (δ 4.45) correlated with C-9 (δ 48.2), C-5 (δ 61.0) and H-19α/β (δ 4.79) with C-4 (δ 78.9), C-8 (δ 46.1). The EIMS spectrum revealed a molecular weight of 516, signifying additional 46 Da on the limonin skeleton ($[M]^{+\cdot}$470) that was attributed to the presence of a methoxylated dihydrofuranone group. The occurrence of methoxylated dihydrofuranone ring on limonoids is not common; both compounds **2** and **3** were stereochemically pure and no mixture of α/β isomers were found, suggesting that they are true natural products and not artefacts caused by air or light oxidation.

Compound **4** was previously isolated from *Phellodendron amurense* (Kishi *et al.*, 1992) and named kihadalactone B. Its spectral data were completely in accordance with literature for the structure showing ^1H NMR resonances for five tertiary methyl groups (δ 0.96, 2 × 1.15, 1.39 and 1.50), two acetyl (δ 1.99 and 2.11), a vinyl hydrogen (δ 5.28 *br s*), two oxygen-bearing methines [δ 5.15 *br s* and 4.82 *t* (J = 4 Hz)] and a γ-lactone group [δ 4.43 *t* (J = 8.2 Hz), 3.90 *m*, 2.67 *m* and 2.50 *m*]. The ^{13}C NMR spectrum presented two lactone carbons (δ 170.2 and 176.5), two acetyl (δ 169.9 and 169.8) and two double bonded carbons (δ 158.6 and 118.9).

For compound **5**, the ^1H NMR spectrum showed the presence of methyl groups at δ 0.89 and 1.60 ppm and a furan ring [δ 7.48 *m*, 7.47 *br t* (J = 1.7 Hz) and 6.35 *m*]. The ^{13}C NMR spectrum of compound **5** showed carbon signals for five quaternary carbons similar to fraxinellone (**6**) (Blaise and Winternitz, 1985). Further comparison of the ^{13}C NMR data for **5** and **6** indicated the presence of epoxy quaternary carbons in **5** (δ 66.1 and 65.6) instead of double bonded carbons as in **6**. In the HMBC spectrum of **5**, several ^1H -^{13}C long-range correlation signals were observed, which also confirmed the attributed skeleton (Fig. 1).

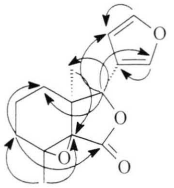

Fig. 1. Main ^1H-^{13}C long-range correlation signals in the HMBC spectrum of **5**.

An attempt to determine the relative configuration of compound **5** by NOE difference experiments failed because of the overlapping of signals and the difficulties to selectively irradiate each hydrogen.

Compounds **3** and **5** appear to be novel in the literature.

The isolated compounds **1–4** showed no activity against trypomastigote forms (Y strain) of *T. cruzi* with IC_{50} > 500.0 µg/ml. The compound **5** presented weak activity, with IC_{50} = 246.8 µg/ml.

Esenbeckia, Raulinoa, Metrodorea and *Pilocarpus* are genera belonging to the rutacean subtribe Pilocarpinae. So far, limonoids have been only isolated from the first genus of this group (Dreyer *et al.*, 1972). Based on these work findings, the genus *Raulinoa* shown closest affinity to *Esenbeckia*; due to the presence of several limonoids, which have not been found in *Metrodorea* and *Pilocarpus.*

Acknowledgements

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