Chemical Constituents from the Infusion of *Zollernia ilicifolia* Vog. and Comparison with *Maytenus* Species

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The new flavonoid glycoside kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-O-[α-L-rhamnopyranosyl(1→6)]-O-β-D-galactopyranoside-7-O-α-L-rhamnopyranoside was isolated together with (*S*)-zierin from the leaves of *Zollernia ilicifolia* (Fabaceae), a medicinal plant used as analgesic and antiulcerogenic effects in Brazilian Tropical Atlantic Rain Forest. The structures were established on the basis of ^{1}H, ^{13}C NMR and 2D NMR (COSY, HMBC, HMQC), UV, MS and IV spectra. The infusion of *Zollernia ilicifolia* was qualitatively compared to the infusion of the espinheiras-santas (*Maytenus aquifolium* and *Maytenus ilicifolia*) by HPLC-DAD.

Key words: Zollernia ilicifolia, Flavonol Glycoside, *Maytenus* Species

Introduction

In Brazil many plants are used in folk medicine as aqueous infusions because of their biological activities. This is the case of *Maytenus ilicifolia* and *M. aquifolium* (Celastraceae), whose leaves are widely employed and have shown anti-ulcer properties (Souza-Formigoni *et al.*, 1991). Because of the similar phenotypic characteristics, some plant species were incorporated as medicines by traditional communities of Brazilian Tropical Atlantic Forest (Di Stasi *et al.*, 2002). Some of these species have been commercialized with the same traditional uses of the true *Maytenus* species (Vilegas *et al.*, 1995; Sanommiya *et al.*, 1998). One of these species is *Zollernia ilicifolia* (Fabaceae), which is also popularly known as "espinheira-santa".

In a recent comparative study, Gonzalez *et al.* (2001) investigated the analgesic and antiulcerogenic effects of 70% methanolic extract of the leaves of *Z. ilicifolia*, *Sorocea bomplandii* and *Maytenus aquifolium.* A methanolic extract of *Zollernia ilicifolia* showed analgesic and antiulcerogenic effects on gastric lesions induced by indomethacin/bethanechol in mices. In the same study, Gonzalez *et al.* (2001) show that leaves of *Zollernia ilicifolia* reacted positively for catechins, fixed acids, flavones, flavonols, saponins, steroids, tannins, triterpenoids and xanthones, but no chemical compounds was isolated. Another reports found in the literature for plants from this genus deals with the presence of flavonoids, isoflavonoids and chalcones from the wood of *Zollernia paraensis* (Ferrari *et al.*, 1983, 1984).

Therefore, we decided to chemically investigate the leaves of *Z. ilicifolia* because chemical information is important not only for understanding the folk utilization but also for the validation of compounds as markers for the assessment of the infusion of different Brazilian 'espinheira-santas' In this paper we report the isolation and structure elucidation of a new flavonol tetrasaccharide from the leaves of *Zollernia ilicifolia* by HPLC-DAD (high performance liquid chromatography coupled to diode array detector) analyses.

Materials and Methods

Biological material

The plant material was collected in December in the Intervales State Park, Saibadela, Sete Barras, Vale do Ribeira, State of São Paulo, Brazil (collectors: Di Stasi, L. C., Reis, M. S. and Mariot, A.). Plant material used was *Zollernia ilicifolia* Vog. (Fabaceae) as authenticated by Dr. Ademir Reis, Herbario Barbosa Rodrigues, Itajaí, State of

Santa Catarina, Brazil, where voucher specimens were deposited. *Maytenus ilicifolia* and *M. aquifolium* were obtained as previously described (Leite *et al.*, 2001; Vilegas *et al.*, 1999).

Apparatus

ES-MS was performed spectrometer on a Fisons VG Platform in positive (70 V) mode. The sample was dissolved in MeOH and injected directly.

IR spectrum was performed in a FT-IR-Nicolet Impact IMACT-400, KBr. The RMN spectra in DMSO d_6 were obtained using a Varian INOVA 500 spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C and 2D-NMR ($^1H-^1H$ COSY, HMQC, TOCSY, HMBC).

Preparative HPLC separations and analytical HPLC analyses were performed on a Varian ProStar 330 chromatograph (Sugar Land, Texas, USA) managed by a Varian workstation equipped with a Varian ProStar 220 diode array detector (DAD) operating from 200 a 600 nm and a Rheodyne injector with a 1 mL loop for preparative analyses and 20μl loop for analytical analyses. For the preparative analyses a Phenomenex column C_{18} 250 mm × 10 mm was used at flow rate of 2 ml/min using MeOH/H_2O 60:40 v/v as eluent. For the analytical analyses the column used was a Phenomenex C_{18} 250 × 4.6 mm i.d., at 30 °C. Gradient elution was performed starting from MeOH 20% until MeOH 100%, during 30 min, as mobile phase at a flow rate of 1.0 ml/min. Monitoring wavelength were 205 and 254 nm. The infusion was filtered on a 0.45 μm Millex filter and directly injected. Standard solutions of compounds **1** and **2** and flavonoids isolated from *M. ilicifolia* (Leite *et al.*, 2001) and *M. aquifolium* (Vilegas *et al.*, 1999) were dissolved in the mobile phase and injected into the HPLC for qualitative purposes.

TLC analyses were carried out on silica gel 60G (Merck) (20 cm × 20 cm × 0.2 mm) plates eluted either with n-BuOH-AcOH-H_2O 65:15:25 v/v/v or with $CHCl_3$-MeOH-H_2O-n-PrOH (5:6:4:1 v/v/v), lower layer). The spots were visualized separatedly by spraying with NP/PEG reagent or 10% H_2SO_4 followed by heating at 110 °C for 5 min (Wagner *et al.*, 1984). Sephadex LH-20 (Pharmacia) was used for GPC.

Preparation of infusions for qualitative HPLC analysis

One gram of air-dried leaves of *Zollernia ilicifolia*, *Maytenus aquifolium* and *Maytenus ilicifolia* were separately milled and put into 50 ml Erlenmeyer flasks. Boiled water (10 ml) was added to each sample. The infusion was centrifuged, and the supernatant was filtered through a 0.45 μm Millex filter. The filtered solution (10 μl) was directly injected into the analytical HPLC system.

Extraction and isolation of **1** *and* **2**

The fresh leaves of *Zollernia ilicifolia* were detached from the stems, dried in an oven at 37 °C and powdered in a mill. The dried powder (110 g) was boiled for 8–9 min with water (1.1 l). The mixture was allowed to cool, filtered through filter paper, and evaporated to dryness, affording 5 g of crude extract of plant. This extract was dissolved in 1.0 l water and fractionated by XAD-2 resin CC (30 cm × 3 cm) eluted with 1.5 l H_2O fraction, followed by 500 ml pure MeOH and finally with 300 ml pure acetone. An aliquot (2.0 g) of the methanolic fraction was dissolved in 10 ml MeOH and fractionated by Sephadex LH-20 CC (100 cm × 3 cm) eluted with MeOH at 0.5 ml/min flow rate. 108 fractions of 5 ml were collected. The fractions were combined according to their behavior by TLC [silica gel plates, n-BuOH/HOAc/H_2O 60:15:30 v/v/v], was purified on reversed phase HPLC on a C-18 Phenomenex column (flow rate = 2 ml/min). Fraction containing cyanogenic glycoside was purified using MeOH/H_2O 50:50 v/v as the eluent to yield pure compound **1** (20 mg, t_R = 9 min), identified by its spectral data compared to those already reported (Seigler, 1975), and the flavonoid was purified using MeOH/H_2O 60:40 as eluent to afford the compound **2** (15 mg, t_R = 14 min),

Compound **1**

$C_{14}H_{17}NO_7$, UV λ max (MeOH): 217 nm. IR (KBr): 3264 cm^{-1} (OH), 2476 cm^{-1} (C ≡ N). ES-MS *m/z* (rel. int.) (70 V, positive ion): 316 $[M+H]^+$ (100), 154 $[M-162+H]^+$ (30). ^1H NMR (DMSO d_6) δ 5.90 (s, H-2), 6.92 (dd, *J* = 1.5 and 1.5 Hz, H-4), 6.83 (ddd, *J* = 8.0, 1.5 and 1.5 Hz, H-6), 7.24 (dd, *J* = 8.0 and 8.0 Hz, H-7), 6.96 (ddd,

J = 8.0, 1.5 and 1.5 Hz, H-8), 4.18 (d, J = 8.5 Hz, H-1 glucose). $\alpha_{[D]} = -28.5$.

Compound **2**

$C_{39}H_{50}O_{23}$, UV λ max (MeOH): 264, 346. IR (KBr): 3378 (OH), 1650 cm^{-1} (C = O). ES-MS *m/z* (rel. int.) (70 V, positive ion): 887 $[M+H]^+$ (49), 287 $[A+H]^+$ (100), 741 $[M\text{-}146+H]^+$ (29), 595 $[M\text{-}146-146+H]^+$ (34), 431 $[M\text{-}146-146-146\text{-}H_2O+H]^+$ (84). For ^1H and ^{13}C NMR data see Table I.

Acid Hydrolysis of Compound **2**

A solution of compound (3 mg) in 6% HCl (3.5 ml) was refluxed for 2 h. The reaction mixture was diluted with H_2O and then extracted with EtOAc. The resulting products were identified by TLC comparison according to their R_f and also to their ^1H NMR spectra.

Results and Discussion

The infusion from the leaves of *Zollernia ilicifolia* was prepared as described in the Materials and Methods and was first submitted to CC on Amberlite XAD-2 resin and eluted with H_2O, MeOH and acetone, respectively. Chromatographic work up by GPC (gel permeation chromatography) followed by reversed-phase HPLC yield the known cyanogenic glycoside **1** and the new flavonoid **2**.

Compound **2** was obtained as a yellow solid and showed a bright yellow spot on TLC observed in UV light after being sprayed with NP/PEG (natural products-polyethylene glycol) reagent, thus indicating a kaempferol derivative (Wagner *et al.*, 1984). Acid hydrolysis of **2** released kaempferol, L-rhamnose and D-galactose, identified by TLC compared to authentic samples. The IR spectrum showed a strong absorption at 1650 cm^{-1} (C = O) and a broad band at 3378 (ν_{OH}). The UV spectrum was obtained through the HPLC-DAD analysis and presented bands at 264 nm and 346 nm, showing good correlation to those of a kaempferol derivative (Mabry *et al.*, 1970).

The ES-MS (70 V, positive ion) mass spectrum gave as base peak the $[M+H]^+$ ion at *m/z* 887 (49%), corresponding to the molecular formula $C_{39}H_{50}O_{23}$. The fragment at *m/z* 287 (100%)corresponds to the protonated aglycone $[A+H]^+$, thus reinforcing that compound **2** is a kaempferol derivative. Fragment ions at *m/z* 741 $[M\text{-}146+H]^+$ (29%), at *m/z* 595 $[M\text{-}146-146+H]^+$ (34%) and at *m/z* 431 $[M\text{-}146-146-146\text{-}H_2O+H]^+$ (15%), correspond to independent losses of terminal deoxyhexose units.

The complete structure of **2** was elucidated by 1D and 2D-NMR experiments at 500 MHz. The ^1H NMR spectra (Table I) displayed signals for two *meta*-coupled protons at δ 6.44 (*d*, J = 2.0 Hz, 1H) and δ 6.79 (*d*, J = 2.0 Hz, 1H) corresponding to H6 and H8, respectively (Harborne *et al.*, 1996). An ortho-coupled system at δ 8.02 (*d*, J = 8.5 Hz, 2H) and δ 6.87 (*d*, J = 8.5 Hz, 2H) corresponds to H2′/H6′ and H3′/H5′, respectively, and confirmed the structure of a kaempferol derivative (Harborne *et al.*, 1996). The ^{13}C NMR spectrum showed 39 signals, 15 from which could be assigned to the kaempferol aglycone (Harborne *et al.*, 1996; Agrawal, 1989) (Table I).

The presence of four anomeric signals at δ 5.58 (*d*, J = 8.0 Hz), δ 5.55 (*d*, J = 1.5 Hz), δ 5.05 (*d*, J = 1.5 Hz) and δ 4.37 (*d*, J = 1.5 Hz) in the ^1H NMR spectra and four anomeric carbons at δ 100.5, 99.9, 98.9 and 98.4 in the ^{13}C NMR spectra suggested **2** to be a tetrasaccharide (Table I). Furthermore, the presence of doublets with J = 7 Hz at δ 0.79, δ 1.05, δ 1.13 in the ^1H-NMR spectrum and at δ 17.2, δ17.6 and δ 17.8 in the ^{13}C NMR spectrum indicated the presence of three rhamnose moieties.

To determine the nature of the monosaccharides, the sequences of the oligosaccharide chain and the site of attachment to the kaempferol skeleton we performed DFQ-COSY, 1D-TOCSY, HMBC and HMQC experiments. Analysis of the DFQ-COSY spectrum combined with the TOCSY data secured the assignments of the spin systems for each sugar moiety of the tetraglycoside moiety of **2** as presented in Table I. Irradiation of the anomeric signals of the rhamnose unities led to the observation of the H1-H2 spin system, whereas irradiation over the anomeric signal of the galactose moiety led to the observation of signals corresponding to H1 to H4.

The HMQC experiment allowed to establish the direct ^1H-^{13}C correlations (Table I), while the connecting position of the sugars were established using HMBC experiments. Correlations were ob-

Table I. ^{1}H NMR and ^{13}C NMR spectral data for **2** (in DMSO $d_6$6, 500 MHz).

Position	δ ^{13}C	δ ^{1}H
2	155.9	–
3	132.9	–
4	177.6	–
5	161.6	–
6	99.3	6.44 (d, 2.0)
7	160.8	–
8	94.5	6.79 (d, 2.0)
9	156.8	–
10	105.5	–
1′	120.6	–
2′, 6′	130.9	8.09 (d, 8.5)
3′, 5′	115.0	6.87 (d, 9.0)
4′	160.0	8.09 (d, 8.5)
3-Gal		–
1	98.9	5.58 (d, 8.0)
2	74.9	–
3	73.7	–
4	68.1	–
5	73.3	–
6	65.0	–
		–
(2–1)Rha		–
1	100.5	5.05 (d, 1.5)
2	70.6	–
3	70.5	–
4	71.5	–
5	71.5	–
6	17.2	0.79 (d, 6.5)
(6–1)Rha		
1	99.9	4.37 (d, 1.5)
2	70.5	–
3	70.2	–
4	71.8	–
5	71.8	–
6	17.8	1.05 (d, 6,0)
7-Rha		
1	98.4	5.55 (d, 1.5)
2	70.0	–
3	70.3	–
4	71.8	–
5	69.8	–
6	17.8	1.13 (d, 6.0)

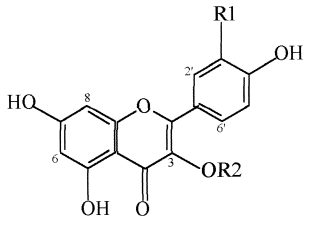
served between the anomeric signal of galactose (δ 5.58, *d*, *J* = 8.0 Hz) and the C-3 of the kaempferol (δ 132.9), the anomeric hydrogen signal of the inner rhamnose (δ 5.05, *d*, *J* = 1.5 Hz) and the C-2 of galactose (δ 74.9), the anomeric hydrogen of the outer rhamnose (δ 4.37, *d*, *J* = 1.5 Hz) and the C-6 of the galactose (δ 65.0). Additional correlation was observed between the hydrogen anomeric of the third rhamnose moiety (δ 5.55, *d*, *J* = 1.5 Hz) and the C-7 of kaempferol (δ 160.8).

The β-configuration at the anomeric position for the galactopyranosyl unity ($J_{H1\text{-}H2}$ = 8.0 Hz) was easily seen from their relatively large $^{3}J_{H1\text{-}H2}$ coupling constants (7–8 Hz). The α-configuration in the rhamnose residues was clear from their H-1 non-splitting pattern and their distinct C-3 and C-5 chemical shift differences from that of methyl β-L-rhamnopyranoside (Agrawal, 1989).

These findings indicated that the structure of compound **2** is the new kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-O-β-D-galactopyranosyl-7-O-α-L-rhamnopyranoside (Fig. 1).

A quercetin tetraglycoside with a similar sugar chain was previously isolated from the leaves of *Rhazya stricta* (Apocynaceaae) (Andersen *et al.*, 1987). Few other studies report the isolation of flavonoid tetraglycosides in plants. Vilegas *et al.* (1999) identified the presence of kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnospyranosyl(1→2)-*O*-β-D-galactopyranoside and quercetin 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnospyranosyl(1→2)-*O*-β-D-galactopyranoside in the infusion of the leaves from *Maytenus aquifolium* that presented significant anti-ulcer activity (Vilegas *et al.*, 1999). Therefore, the observed anti-ulcer activity of the MeOH 70% extract of *Z. ilicifolia* could be related to the presence of the flavonoid tetraglucoside **2**, since the structures are similar to those found in species of *Maytenus*, plants with analgesic and antiulcerogenic effects. Further studies are in progress to check this hypothesis and to identify the analgesic and antiulcerogenic mechanisms of actions.

The main objective of this study was to investigate the chemical compounds from *Zollernia ilicifolia* because this species is used as a herbal remedy in traditional medicine of the Brazilian tropical Atlantic Forest Region, State of São Paulo, Brazil (Di Stasi *et al.*, 2001). However, previous pharmacological tests have also shown that animals treated with *Z. ilicifolia* extract exhibited increase of irritability and respiratory rate, loss of corneal reflex and decrease of motor activity (Gonzalez *et al.*, 2001). Although the cyanogenic glycoside was not pharmacologically evaluated, the presence of this compound in the infusion is an alert against a possible misuse of this species as medicinal plant or instead of the true 'espinheiras-santas'.



		R1	Sugar sequence (R2)	R_t[min]
Zollernia ilicifolia	**2**	H	3-[-Rha(1→6)Rha(1→2)Gal], 7-Rha	17.6
Maytenus aquifolium	**3**	H	3-Rha(1→6)Glu(1→3)Rha(1→2)Gal	18.9
	4	OH	3-Rha(1→6)Glu(1→3)Rha(1→2)Gal	21.8
Maytenus ilicifolia	**5**	H	3-Rha(1→6)Ara(1→3)Rha(1→2)Gal	19.2

Fig. 1. Flavonoid tetraglycosides isolated from *Zollernia ilicifolia*, *Maytenus aquifolium* and *Maytenus ilicifolia* leaves: kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-O-[α-L-rhamnopyranosyl(1→6)]-O-β-D-galactopyranoside-7-O-α-L-rhamnopyranoside **2**, kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnospyranosyl(1→2)-*O*-β-D-galactopyranoside **3**, quercetin 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnospyranosyl(1→2)-*O*-β-D-galactopyranoside **4**, and kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[α-L-arabinopyranosyl(1→3)-*O*-α-L-rhamnospyranosyl(1→2)-*O*-β-D-galactopyranoside **5**.

Therefore, to check the chemical differences between the infusions of *Zollernia ilicifolia* and of the true 'espinheiras-santas' (*Maytenus aquifolium* and *Maytenus ilicifolia*) we developed a method based on the comparative HPLC-DAD analysis.

The chromatography profiles of the infusions could be established by comparing the retention times and UV spectra of the peaks with those of isolated compounds from *Zollernia ilicifolia* and *Maytenus* species (Fig. 1).

Concerning flavonoidic compounds, checked by their UV spectra, the three infusions show peaks in a narrow range of retention time, between 17.6 min and 21.8 min. These small differences probably arises from the different sugar moieties attached to the aglycones, but these flavonoids are useful markers to differenciate each infusion.

The chromatogram of the infusion of *Zollernia ilicifolia* at 205 nm shows an additional intense peak due to the cyanogenic glycoside (*S*) – zierin **1** (t_R = 9 min), absent in the infusion of the *Maytenus* species.

Therefore, not only the presence of the flavonoids but also of the cyanogenic glycoside **1** can be used to establish chemical differentiation between the infusions of *Maytenus* species and the false espinheira-santa *Zollernia ilicifolia* affording valuable chemical markers for the assessment of the quality control of these plants as phytomedicines.

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