# Isolation and HPLC Quantitative Analysis of Flavonoid Glycosides from Brazilian Beverages (*Maytenus ilicifolia* and *M. aquifolium*)

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Aqueous infusions of Brazilian *Maytenus* leaves are used as beverages, foodstuffs, and phytomedicines. Previously, we isolated two new flavonoid tetrasaccharides from the infusion of *Maytenus aquifolium* leaves that showed antiulcer activity. In this investigation a new flavonoid tetrasaccharide, kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-*O*-[α-L-arabinopyranosyl (1→3)-*O*-α-L-rhamnopyranosyl (1→2)]-*O*-*β*-D-galactopyranoside (**3**), was isolated, together with kaempferol tri- and disaccharides and quercetin trisaccharides from the aqueous infusion of *Maytenus ilicifolia* leaves. All structures were elucidated by ES-MS and NMR spectroscopic methods. The quantitative analysis of the flavonoid glycosides from *Maytenus ilicifolia* and *M. aquifolium* has been performed by HPLC.

**Keywords:** *Maytenus ilicifolia; M. aquifolium Martius; Celastraceae; espinheira santa; infusion; beverage; flavonoids glycosides; 1D and 2D NMR; quantitative determination*

## INTRODUCTION

*Maytenus ilicifolia* Martius ex Reiss. and *M. aquifolium* Mart. (Celastraceae) are plant species widely used in Brazilian folk medicines in the form of aqueous infusions as antiulcers and against stomach diseases, and also as beverages in daily life instead of green tea. They are found in the local commerce as capsules, powders, dried leaves, fresh leaves, or as aqueous or aqueous−alcoholic preparations. *Maytenus ilicifolia* and *M. aquifolium* are popularly known as "espinheira-santa" (holly spines) because their leaves are spined. Many other species also have similar morphology (e.g., *Sorocea bomplandii* Baill., Moraceae and *Zolernia ilicifolia*, Leguminosae) and therefore many adulterations are commonly found in the Brazilian local commerce. *M. aquifolium* was also reported as being an adulterant of the "yerba-mate" (*Ilex paraguariensis* St. Hil., Aquifoliaceae) in Paraguay (*1*). This beverage is also widely used in South Brazil. In a previous work we isolated two new flavonoid tetra-glycosides from the infusion of the leaves of *M. aquifolium*: quercetin-3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[*β*-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside (**1**), and kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-*O*-[*β*-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside (**2**) (Figure 1); and we evaluated the antiulcer activity of the infusion (*2*, *3*). In this investigation of the aqueous infusion of the leaves of *M. ilicifolia* a new flavonoid tetra-glycoside, kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-*O*-[α-L-arabinopyranosyl (1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside (**3**) (Figure 1), was isolated, together with kaempferol tri- and disaccharides (**4** and **5**) and quercetin trisaccharides (**6**) (Figure 2). The quantitative analysis of flavonoids from *M. aquifolium* and *M. ilicifolia* extracts has been performed by HPLC, as a potential method for quality control purposes. The chemical information obtained is important not only for the correct understanding of this folk utilization but also for the future validation of these compounds as markers for the assessment of Brazilian *Maytenus* infusions (*4*).

## MATERIALS AND METHODS

**Biological Material.** The leaves of *Maytenus ilicifolia* and *M. aquifolium* were furnished by Ana Maria Soares Pereira, UNAERP, Ribeirao Preto, SP, Brazil. Voucher samples are deposited at the Herbario of the Universidade Estadual Paulista (UNESP). Commercial samples were also purchased in the local market and submitted to the same experimental procedures.

**Apparatus.** The ES-MS spectra were determined on a Fisons Platform spectrometer both in the positive (90 V) and negative (100 V) mode. The sample was dissolved in MeOH and injected directly.

UV spectra were measured in a HP 8472-A spectrometer (MeOH, $c = 1$). IR spectra: Nicolet Impact 400, KBr.

A Bruker DRX-600 spectrometer, operating at 599.19 MHz for $^1$H and 150.858 for $^{13}$C, with the UXNMR software package, was used for NMR experiments measured in $CD_3OD$. The DEPT experiments were performed using transfer pulse of 135° to obtain positive signals for CH and $CH_3$ and negative signals for $CH_2$. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. ROESY (*5*), $^1$H−$^1$H DQF−COSY (*6*, *7*), $^1$H−$^{13}$C HSQC, and HMBC (*8*) experiments were obtained using the conventional pulse sequences as described in the literature, and 1D TOCSY (*9*) was acquired using

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10.1021/jf010294n CCC: $20.00 
Published on Web 07/18/2001

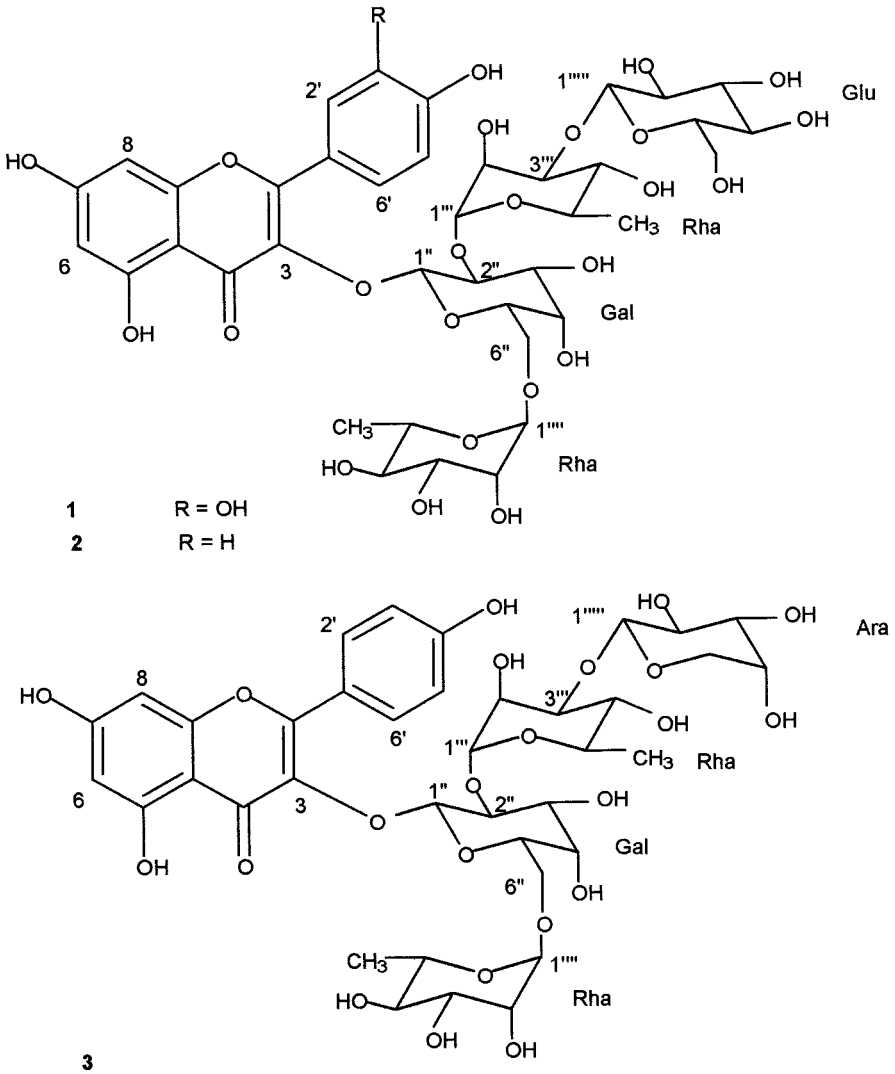



**Figure 1.** Flavonoid tetra-glycosides isolated from *Maytenus aquifolium* and *Maytenus ilicifolia* leaves: (**1**) quercetin-3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[*β*-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside, (**2**) kaempferol-3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[*β*-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside, and (**3**) kaempferol-3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[α-L-arabinopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-*β*-D-galactopyranoside. Gal = *β*-D-galactopyranosyl, Rha = α-L-rhamnopyranosyl, Glc = *β*-D-glucopyranosyl, and Ara = α-L-glucopyranosyl.

waveform, generator-based GAUSS shaped pulse, a mixing time ranging from 80 to 100 ms, and a MLEV-17 spin-lock field of 10 kHz preceded by a 2-ms trim pulse.

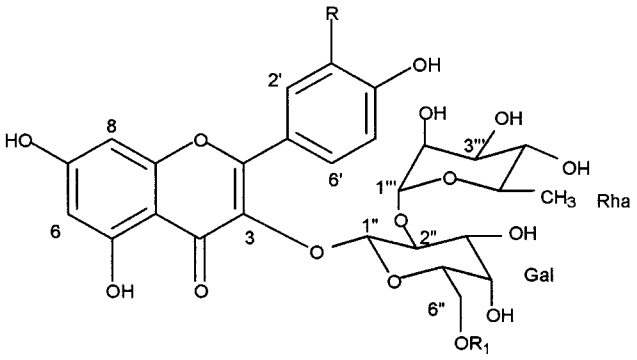
HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and with a Waters *μ*-Bondapak C-18 column and a U6K injector.

GC analyses were run using a Hewlett-Packard 5890 gas chromatograph equipped with a 5970 mass-selective detector and a HP-5 fused-silica column (25 m × 0.2 mm i.d.; 0.33 *μ*m film).

Quantitative HPLC analyses were performed on a Shimadzu liquid chromatograph, equipped with a LC-10AD pump, a Rheodyne injector valve (fitted with a 20-*μ*L loop), a SPD 10AV UV−vis spectrophotometric detector (set at *λ* 254 nm), and a Ultrasphere ODS 5 *μ*m (Altex) column (250 × 4.6 mm i.d.). Peak areas were calculated by a Shimadzu C-R6A integrator.

**Extraction and Isolation.** Leaves of *M. ilicifolia* were air-dried and milled. A 200-g portion of the powdered plant was boiled in water (1 L) for 8−9 min. The mixture was allowed to cool, filtered through filter paper, and evaporated to dryness affording 18 g of crude extract. An aliquot (2.0 g) was dissolved in 10 mL of MeOH and fractionated on a Sephadex LH-20 column (1 m × 3 cm i.d.) with a flow rate of 0.5 mL/min. A total of 100 fractions, of 8 mL each, was collected. After TLC analysis (Si-gel, n-BuOH−AcOH−$H_2O$ 65:15:25, $CHCl_3$−MeOH−$H_2O$ 70:30:3, v/v), fractions with similar $R_f$s were combined, giving 6 major fractions named A−F which were further purified by HPLC (C-18 *μ*-Bondpak column (30 cm × 7.8 mm, flow rate 2.5 mL/min). Fraction E (150 mg) was purified using MeOH−$H_2O$ (40:60, v/v) as the eluent to yield pure compound **3** (21.2 mg, $R_t$ = 16 min). Fraction D (200 mg) was purified using MeOH−$H_2O$ (50:50, v/v) as the eluent to yield pure compounds **4** (30.0 mg, $R_t$ = 14 min) and **5** (9.2 mg, $R_t$ = 12 min). Fraction C (150 mg) was purified using MeOH−$H_2O$ (60:40, v/v) as the eluent to yield pure compound **6** (18.1 mg, $R_t$ = 13 min).

**Quantitative Analysis.** Quantitative HPLC analyses were carried out on an Ultrasphere ODS 5-*μ*m (250 × 4.6 mm) column, using isocratic elution with MeOH−$H_2O$ (40:60, v/v) at a flow rate of 1.0 mL/min. Compounds **1** and **3** were used as external standards. For sample preparation, 500 mg of pulverized plant material was extracted twice with 80 mL of methanol−water (80:20, v/v) at 60 °C for 15 min. After filtration, the filtrate was adjusted to a final volume of 200 mL in a volumetric flask. A standard solution containing 50 *μ*g/mL of flavonoid tetragycosides **1** and **3** in methanol−water (80:20, v/v) was also prepared, and 1 mL of this solution was diluted to 10 mL with the same solvent. A linear relationship between peak area and concentration (1−10 *μ*g/mL) was observed with a correlation coefficient $r$ = 0.9997 for each glycoside. The relationship between peak areas ($y$) and con-




| Compound | R | $R_1$ |
|---|---|---|
| **4** | H | Rha |
| **5** | H | H |
| **6** | OH | Rha |

**Figure 2.** Known flavonoid glycosides isolated from *Maytenus ilicifolia* leaves: (**4**) kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-[L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside, (**5**) kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside, and (**6**) quercetin-3-O-α-L-rhamnopyranosyl hf(1→6)-*O*-[α-L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside.

centrations in μg/mL ($x$) was $y = 10240x - 984$ (**1**) and $y = 9593x - 842$ (**3**). The minimum detection limit was 0.2 ng, which resulted in a signal-to-noise ratio of 3:1. Reproducibility was verified with 5 extracts of an identical sample. Relative standard deviations (%) were 4.79 and 4.95, respectively; for retention times the standard deviation was less than 1%.

**Acid Hydrolysis of Compounds 3−6.** A solution of each compound (3.0 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 their $R_f$ values on TLC and also by their $^1$H NMR spectra.

**Methanolysis of Compounds 3−6.** Each compound (1.0 mg) was heated in a vial for 24 h at 80 °C in MeOH−2% HCl (2 mL). After MeOH and HCl evaporation in a $N_2$ stream, $Ag_2CO_3$ and MeOH were added until $CO_2$ production stopped. The centrifugate was dried over $P_2O_5$. The resulting monosaccharides were treated with Trisil-Z (Pierce) and analyzed by GC−MS.

**Compound 3.** Yellowish amorphous solid. mp 269−271 °C. [α]D$^{25}$ −63° (*c* 0.1, $CH_3OH$); UV λ max (nm, MeOH): 265, 352; + KOH: 271, 327, 396; +$AlCl_3$: 270, 301 sh, 350; +$AlCl_3$ + HCl: 272, 301 sh, 348, 393; + NaOAc: 271, 363; + NaOAc + $H_3BO_3$: 265, 352. IR (KBr): 3376 (OH), 1650 (C=O) cm$^{-1}$. ES-MS, *m/z* (rel int.) (100 V, negative mode): 871 [M − H]$^-$ (100), 725 [M − H − rha]$^-$ (6), 749 [M − H − ara]$^-$ (2), 285 [M − H − ara − 2 rha − gal]$^-$ = [A − H]$^-$ (79); (90 V, positive mode): 911 [M + K]$^+$ (40), 895 [M + Na]$^+$ (47), 873 [M + H]$^+$ (18), 595 [M + H − ara − rha]$^+$ (20), 449 [M + H − ara − 2 rha]$^+$ (16), 287 [A + H]$^+$ = [M + H − glu − 2 rha − gal]$^+$ (100). Anal. Calcd for $C_{38}H_{48}O_{23}$: C 52.29%, H 5.54%, O 42.16%. Found: C 52.11%, H 5.48%, O 41.03%. $^1$H and $^{13}$C NMR data are provided in Tables 1 and 2.

**Compound 4.** Colorless amorphous solid. UV λ max (nm, MeOH): 265, 352; +KOH: 270, 326, 395; +$AlCl_3$: 269, 300 sh, 350; +$AlCl_3$ + HCl: 270, 300 sh, 347, 392; + NaOAc: 270, 362; +NaOAc + $H_3BO_3$: 267, 352. IR (KBr): 3374 (OH), 1653 (C=O) cm$^{-1}$. ES-MS, *m/z* (rel int.) (100 V, negative mode): 739 [M − H]$^-$ (100), 593 [M − H − rha]$^-$ (7), 447 [M − H − 2 rha]$^-$ (7), 285 [M − H − 2 rha − gal]$^-$ = [A − H]$^-$ (69); (90 V, positive mode): 779 [M + K]$^+$ (39, 763 [M + Na]$^+$ (53), 741 [M + H]$^+$ (14), 595 [M + H-rha]$^+$ (18), 449 [M + H − 2 rha]$^+$ (15), 287 [A + H]$^+$ = [M + H − 2 rha − gal]$^+$ (100); Anal. calcd for $C_{33}H_{40}O_{19}$: C 53.51%, H 5.44%, O 41.04%. Found: C 52.38%, H 5.41%, O 40.89%. $^1$H and $^{13}$C NMR data are presented in Tables 1 and 2.

**Compound 5.** Colorless needles. UV λ max (nm, MeOH): 267, 351; +KOH: 270, 325, 395; +$AlCl_3$: 271, 302 sh, 352; +$AlCl_3$ + HCl: 270, 302 sh, 346, 393; + NaOAc: 270, 362; +NaOAc + $H_3BO_3$: 264, 353. IR (KBr): 3374 (OH), 1653 (C=O) cm$^{-1}$. ES-MS, *m/z* (rel int.) (100 V, negative mode): 593 [M − H]$^-$ (100), 447 [M − H − rha]$^-$ (5), 285 [M − H − rha − gal]$^-$ = [A − H]$^-$ (83); (90 V, positive mode): 633 [M + K]$^+$ (30), 627 [M + Na]$^+$ (60), 595 [M + H]$^+$ (14), 449 [M + H − rha]$^+$ (15), 287 [A + H]$^+$ = [M + H − rha − gal]$^+$ (100). Anal. calcd for $C_{27}H_{30}O_{15}$: C 54.51%, H 5.09%, O 40.37%. Found: C 54.38%, H 4.81%, O 40.19%. $^1$H and $^{13}$C NMR data are presented in Tables 1 and 2.

**Compound 6.** Colorless needles. UV λ max (nm, MeOH): 267, 352; +KOH: 272, 330, 392; +$AlCl_3$: 271, 305 sh, 349; +$AlCl_3$ + HCl: 273, 305 sh, 346, 393; + NaOAc: 271, 363; +NaOAc + $H_3BO_3$: 269, 353. IR (KBr): 3380 (OH), 1655 (C=O) cm$^{-1}$. ES-MS, *m/z* (rel int.) (100 V, negative mode): 755 [M − H]$^-$ (100), 609 [M − H −rha]$^-$ (10), 301 [M − H − 2 rha − gal]$^-$ = [A − H]$^-$ (80); (90 V, positive mode): 795 [M + K]$^+$ (40), 779 [M + Na]$^+$ (45), 757 [M + H]$^+$ (11), 611 [M + H − rha]$^+$ (18), 465 [M + H − 2 rha]$^+$ (13), 303 [A + H]$^+$ = [M + H − 2 rha − gal]$^+$ (100); Anal. calcd for $C_{33}H_{40}O_{20}$,: C 52.38%, H 5.33%, O 42.29%. Found: C 52.19%, H 5.21%, O 42.03%. $^1$H and $^{13}$C NMR data are provided in Tables 1 and 2.

## RESULTS AND DISCUSSION

The infusion from the leaves of *M. ilicifolia* was prepared as described in the previous section. Carlini (*10*) has previously established the antiulcer activity of the aqueous infusion and did not detect any toxic effect.

The infusion was fractionated by GPC to investigate its chemical constituents as described above. Fractions were further purified on reversed-phase HPLC to yield pure flavonoid glycosides **3**−**6** as the major constituents of this infusion. Acid hydrolysis of **3**−**6** released kaempferol for **3**−**5** and quercetin for **6**. The aglycons were identified by $^1$H and $^{13}$C NMR spectra. The gas-chromatographic analysis of the methanolysis products showed the presence of galactose, rhamnose, and arabinose, in the ratio 1 gal:2 rha:1 ara for **3**, 1 gal:2 rha for **4** and **6**, and 1 gal:1 rha for **5**. Retention times were identical to those of the authentic Trisil sugars.

The ES-MS (100 V, negative ion) mass spectrum of **3** gave as base peak the [M − H]$^-$ ion at *m/z* 871. The fragment at *m/z* 285 corresponds to the deprotonated aglycon [A − H]$^-$. Fragment ions occurred at *m/z* 725 [(M − H) − 146]$^-$ and at *m/z* 739 [(M − H) − 132]$^-$, which were interpreted as independent losses of terminal deoxyhexose and pentose units. In the ES-MS spectrum in the positive-ion mode (90 V) we observed the pseudomolecular ion [M + H]$^+$ at *m/z* 873. The adducts [M + Na]$^+$ at *m/z* 895 and [M + K]$^+$ at *m/z* 911 were also observed. The fragment at *m/z* 595 [M + H − 146 − 132]$^+$ corresponds to the loss of the two terminal sugars. The fragments at *m/z* 449 [M + H − 146 − 132 − 146]$^+$ and the base peak at at *m/z* 287 [M + H − 146 − 132 − 146 − 162]$^+$ = [A + H]$^+$ correspond to the subsequent losses of deoxyhexose and hexose moieties.

The complete structure of **3** was elucidated by 1D and 2D NMR experiments at 600 MHz. The $^1$H NMR spectrum of **1** (Table 1) displayed signals for two meta-coupled protons at δ 6.18 (d, $J$ = 1.5 Hz, H-6) and δ 6.35 (d, $J$ = 1.5 Hz, H-8) and also for an ortho-coupled system at δ 8.08 (d, $J$ = 8.5 Hz, H-2′ and H-6′) and δ 6.92 (d, $J$ = 8.5 Hz, H-3′ and H-5′) indicating a kaempferol derivative (*11*).

The $^{13}$C NMR shifts of the aglycon part of **3** (Table 2) corresponded well with the shifts for kaempferol, with

**Table 1. $^1$H NMR Assignments ($\delta_H$ in $CD_3OD$) of Compounds 3−6[a]**

| proton | 3 | 4 | 5 | 6 |
|---|---|---|---|---|
| 6 | 6.18 (d, 1.5) | 6.20 (d, 1.5) | 6.17 (d, 1.5) | 6.17 (d, 1.5) |
| 8 | 6.35 (d, 1.5) | 6.40 (d, 1.5) | 6.35 (d, 1.5) | 6.35 (d, 1.5) |
| 2′ | 8.08 (d, 8.5) | 8.09 (d, 8.5) | 8.10 (d, 8.5) | 7.72 (d, 1.5) |
| 3′ | 6.92 (d, 8.5) | 6.92 (d, 8,5) | 6.91 (d, 8.5) | - - - - - - |
| 5′ | 6.92 (d, 8.5) | 6.92 (d, 8.5) | 6.91 (d, 8.5) | 6.90 (d, 8.5) |
| 6′ | 8.08 (d, 8.5) | 8.09 (d, 8.5) | 8.10 (d, 8.5) | 7.61 (dd, 1.5, 8.5) |
| 3-Gal | | | | |
| 1″ | 5.57 (d, 7.5) | 5.63 (d, 7.5) | 5.55 (d, 7.5) | 5.63 (d, 7.5) |
| 2″ | 3.96 (dd, 7.5, 9.7) | 3.95 (dd, 7.5, 9.6) | 3.57 (dd, 7.5, 9.5) | 3.94 (dd, 7.5, 9.5) |
| 3″ | 3.73 (dd, 7.5, 3.6) | 3.73 (dd, 7.5, 3.5) | 3.74 (dd, 7.5, 3.6) | 3.75 (dd, 7.5, 3.5) |
| 4″ | 3.51 (dd, 3.5, 1.5) | 3.51 (dd, 3.5, 1.5) | 3.49 (dd, 3.5, 1.5) | 3.50 (dd, 3.5, 1.5) |
| 5″ | 3.67 (ddd, 1.5, 5.0, 7.0) | 3.66 (ddd, 1.5, 5.0, 7.0) | 3.44(ddd, 1.5, 5.0, 7.0) | 3.66 (dd,5.0,7.0) |
| 6a″ | 3.45 (dd, 12.0, 7.0) | 3.45 (dd, 12.0, 7.0) | 3.58 (dd, 12.0, 7.0) | 3.45 (dd, 12.0, 7.0) |
| 6b″ | 3.76 (dd, 12.0, 5.0) | 3.77 (dd, 12.0, 5.0) | 3.73 (dd, 12.0, 5.0) | 3.76 (dd, 12.0, 5.0) |
| (6−1) Rha | | | | |
| 1‴ | 4.55 (d, 1.5) | 4.55 (d, 1.5) | | 4.53 (d, 1.5) |
| 2‴ | 3.60 (dd, 3.5, 1.5) | 3.60 (dd, 3.5, 1.5) | | 3.61 (dd, 3.5, 1.5) |
| 3‴ | 3.55 (dd, 9.5, 3.5) | 3.53 (dd, 9.5, 3.5) | | 3.54 (dd, 9.5, 3,5) |
| 4‴ | 3.31 (t, 9.5, 9.5) | 3.29 (t, 9.5, 9.5) | | 3.34 (t, 9.5, 9.5) |
| 5‴ | 3.57 (dq, 9.5, 6.0) | 3.56 (dq, 9.5, 6.0) | | 3.58 (dq, 9.5, 6.0) |
| 6‴ | 1.20 (d, 6.6) | 1.20 (d, 6.6) | | 1.22 (d, 6.0) |
| (3−1) Ara | | | | |
| 1⁗ | 4.52 (d, 5.2) | | | |
| 2⁗ | 3.68 (dd, 5.2, 8.5) | | | |
| 3⁗ | 3.59 (dd, 8.5, 3.0) | | | |
| 4⁗ | 3.86 (m) | | | |
| 5⁗ | 3.93 (dd 12.0, 3.0) | | | |
| | 3.67 (dd 12.0, 2.0) | | | |
| (2−1) Rha | | | | |
| 1‴ | 5.27 (d, 1.5) | 5.24 (d, 1.5) | 5.24 (d, 1.5) | 5.27 (d, 1.5) |
| 2‴ | 4.26 (dd, 3.0, 1.5) | 4.02 (dd, 3.0, 1.5) | 4.01 (dd, 3.0, 1.5) | 4.26 (dd, 3.0, 1.5) |
| 3‴ | 3.95 (dd, 9.5, 3.0) | 3.82 (dd, 9.5, 1.5) | 3.80 (dd, 9.5, 1.5) | 3.81 (dd, 9.5, 1,5) |
| 4‴ | 3.58 (t, 9.5, 9.5) | 3.36 (t, 9.5, 9.5) | 3.33 (dd, 9.5, 9.5) | 3.34 (dd, 9.5, 1.5) |
| 5‴ | 4.17 (dq, 9.5, 6.0) | 4.09 (dq, 9.5, 6.0) | 4.05 (dq, 9.5, 6.0) | 4.15 (dq, 9.5, 6.0) |
| 6‴ | 1.02 (d, 6.0) | 1.01 (d, 6.0) | 0.97 (d, 6.0) | 1.00 (d, 6.0) |

[a] Chemical shift values are in ppm and *J* values in Hz are presented in parentheses. All signals were assigned by 2D-HOHAHA, DQF−COSY, HSQC, and HMBC studies. Gal = $\beta$-D-galactopyranosyl, Rha = α-L-rhamnopyranosyl, and Ara = α-L-arabinopyranosyl.

the only significant difference being those corresponding to C-2 and C-3. These shifts are analogous to those reported when the 3-hydroxy group is glycosylated in a flavonol glycoside (*12*). Four anomeric protons were easily identified in the spectra of **3**. They resonated at $\delta$ 5.57 (d, $J$ = 7.5 Hz), $\delta$ 5.27 (d, $J$ = 1.5 Hz), $\delta$ 4.55 (d, $J$ = 1.5 Hz), and $\delta$ 4.52 (d, $J$ = 5.2 Hz), and they correlated to carbons at $\delta$ 101.1, $\delta$ 102.1, $\delta$ 101.8, and $\delta$ 106.6, respectively. From the assigned aglycon and sugar values (Tables 1 and 2), it was apparent that a tetrasaccharide unit was attached to C-3 of the aglycone. The structure of tetrasaccharide chain has been determined by a combination of 1D TOCSY, 2D DFQ-COSY, HSQC, and HMBC experiments. The isolated anomeric signals resonating at uncrowded regions of the spectrum, between 5.57 and 4.64 ppm were the starting points for the 1D-TOCSY experiments. Because of the selectivity of multistep coherence transfer, the 1D-TOCSY subspectra of the single monosaccharide unit could be extracted from the overlapping region of the spectrum (between 3.0 and 4.0 ppm). Each subspectra could be attributed to one set of coupled protons such as H−C (1) to H−C (4) for arabinose or H−C (1) to H−C (4) for galactose of a carbohydrate moiety.

The irradiation of the signal at $\delta$ 5.57 showed a set of coupled resonances in a sugar ascribable from H-1 to H-4 of a galactose unit linked at C-3 of the aglycone. 1D TOCSY subspectra obtained by irradiating at $\delta$ 4.55 and 1.20 ppm allowed the identification of one rhamnose moiety, whereas 1D TOCSY subspectra obtained by irradiating at $\delta$ 5.27 and 1.02 ppm led to the identification of the second rhamnose unit. The irradiation at $\delta$ 4.52 showed connectivities to three methine protons ($\delta$ 3.68, 3.59, and 3.86 ppm). The coherence transfer to H-5 was not obtained because of the small $J_{H4-H5}$ value of this arabinose unit. The sequential assignments of these sugar protons as shown in Table 1 derived from their distinctive DQF−COSY patterns. The assignments of all proton resonances for the sugar moieties immediately allowed assignment of the resonances of the linked carbon atoms by HSQC (Table 2).

Information about the sequence of the tetrasaccharide chain was deduced from an HMBC experiment. Key correlation peaks were observed between the anomeric proton of the galactose ($\delta$ 5.57) and the C-3 of the kaempferol ($\delta$ 134.4), the anomeric proton signal of the inner rhamnose ($\delta$ 5.27) and the C-2 of galactose ($\delta$ 77.2), the anomeric proton of the arabinose ($\delta$ 4.52) and the C-3 of the inner rhamnose ($\delta$ 82.7), and the anomeric proton of the outer rhamnose ($\delta$ 4.55) and the C-2 of the galactose ($\delta$ 77.2).

The $\beta$-configuration at the anomeric position for the galactopyranosyl unity ($J_{H1-H2}$ = 7.5 Hz) was easily seen from their relatively large $^3J_{H1-H2}$ coupling constants (7−8 Hz). The α-configuration in the rhamnose residues was clear from their H-1 unsplitting pattern and their distinct C-3 and C-5 chemical shift differences from that of methyl $\beta$-L-rhamnopyranoside (*12*), whereas the α-configuration for the arabinopyranosyl unit ($J_{H1-H2}$ = 5.2 Hz) was established by the results of ROESY experiments as previously reported (*13*). These data suggested that the structure of **3** is kaempferol-3-

**Table 2. $^{13}C$ NMR Assignments ($\delta_C$ in $CD_3OD$) of Compounds 3−6[a]**

| carbon | DEPT | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 2 | C | 158.5 | 158.4 | 158.5 | 158.2 |
| 3 | C | 134.4 | 134.6 | 134.4 | 134.3 |
| 4 | C | 179.7 | 179.5 | 179.7 | 179.0 |
| 5 | C | 163.1 | 163.1 | 163.2 | 163.4 |
| 6 | CH | 101.0 | 101.7 | 101.8 | 99.7 |
| 7 | C | 167.5 | 167.5 | 167.5 | 165.8 |
| 8 | CH | 95.5 | 95.6 | 95.7 | 94.4 |
| 9 | C | 158.5 | 158.7 | 158.6 | 158.2 |
| 10 | C | 106.6 | 106.6 | 106.6 | 105.6 |
| 1′ | C | 122.9 | 123.1 | 122.9 | 123.1 |
| 2′ | CH | 132.2 | 132.3 | 132.2 | 115.9 |
| 3′ | CH | 116.2 | 116.3 | 116.4 | 145.6 |
| 4′ | C | 161.0 | 161.0 | 161.1 | 149.4 |
| 5′ | CH | 116.2 | 116.3 | 116.4 | 117.1 |
| 6′ | CH | 132.2 | 132.3 | 132.2 | 122.8 |
| 3-Gal | | | | | |
| 1″ | CH | 101.1 | 102.5 | 104.3 | 102.4 |
| 2″ | CH | 77.2 | 77.8 | 72.8 | 77.3 |
| 3″ | CH | 75.7 | 75.4 | 75.0 | 75.1 |
| 4″ | CH | 70.7 | 70.5 | 70.1 | 70.7 |
| 5″ | CH | 75.3 | 75.6 | 75.3 | 75.5 |
| 6″ | $CH_2$ | 67.1 | 67.2 | 67.3 | 66.8 |
| (6−1) Rha | | | | | |
| 1‴ | CH | 101.8 | 101.8 | 101.8 | 101.6 |
| 2‴ | CH | 72.0 | 72.1 | 72.0 | 71.9 |
| 3‴ | CH | 72.2 | 72.2 | 72.3 | 72.1 |
| 4‴ | CH | 73.9 | 73.7 | 73.8 | 73.7 |
| 5‴ | CH | 69.7 | 69.6 | 69.7 | 69.6 |
| 6‴ | $CH_3$ | 17.9 | 17.8 | 17.8 | 17.1 |
| (3−1) Ara | | | | | |
| 1⁗ | CH | 106.6 | | | |
| 2⁗ | CH | 73.1 | | | |
| 3⁗ | CH | 74.0 | | | |
| 4⁗ | CH | 69.7 | | | |
| 5⁗ | $CH_2$ | 67.1 | | | |
| (2−1) Rha | | | | | |
| 1‴ | CH | 102.1 | 100.3 | | 100.8 |
| 2‴ | CH | 72.0 | 72.3 | | 72.1 |
| 3‴ | CH | 82.7 | 72.4 | | 72.2 |
| 4‴ | CH | 72.9 | 73.9 | | 73.9 |
| 5‴ | CH | 69.5 | 69.7 | | 69.6 |
| 6‴ | $CH_3$ | 17.6 | 17.9 | | 17.8 |

[a] All signals were assigned by 2D-HOHAHA, DQF−COSY, HSQC, and HMBC studies. Gal = β-D-galactopyranosyl, Rha = α-L-rhamnopyranosyl, and Ara = α-L-arabinopyranosyl.

*O*-α-L-rhamnopyranosyl (1→6)-*O*-[α-L-arabinopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside.

Compounds **4** and **5** presented $^1H$ and $^{13}C$ NMR spectra almost superimposable to those of compound **3**. The main differences were the absence of the arabinose signal at $\delta$ 4.52 for compound **4** and the absence of signals of arabinose at $\delta$ 4.52 and of rhamnose at $\delta$ 4.55 for compound **5** in the $^1H$ NMR spectra, together with the absence of their respective signals in the $^{13}C$ NMR spectra (see Tables 1 and 2). From these considerations the structure of kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-[α-L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside was assigned to **4**, and the structure of kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside was assigned to **5** (*14*). Minor amounts of the quercetin-3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[α-L-rhamnopyranosyl(1→2)]-*O*-β-D-galactopyranoside **6** were also detected by its NMR data (Tables 1 and 2) and ES-MS spectra (*14*).

The quantitative analysis of the flavonoids glycosides from *M. ilicifolia* (1 and 2) and *M. aquifolium* (3−6) extracts was performed by HPLC. The concentrations of each compound in the extracts, calculated from the experimental peak areas by interpolation to standard calibration curves, were 1.75% for compound **1**, 1.50% for **2**, 1.18% for compound **3**, 1.62% for **4**, 0.98% for **5**, and 0.52% for **6**, corresponding to 157.5 mg/100 g dried weight for compound **1**, 135.0 mg for **2**, 106.2 mg for **3**, 145.8 mg for **4**, 88.2 mg for **5**, and 46.8 mg for **6**. Relative standard deviations were in the range of 3.76−4.12% calculated as mean of five replications, whereas for retention times the relative standard deviation was less than 1%.

Similar quercetin and kaempferol tetra-glycosides were isolated from the aqueous infusion of *M. aquifolium* leaves (*2, 3*), but with a terminal glucose unit instead of arabinose. There are few other reports of the isolation of flavonoid tetra-saccharides from plant infusions. Kijima et al (*15*) reported the presence of kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1→6)]-*O*-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside from the water soluble fraction of the extract of *Alangium premnifolium*, whereas Hu et al (*16*) described the occurrence of anhydroicaritin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside from the aerial parts of *Epimedium acuminatum*.

Polyphenolic compounds, including flavonoids, have been the subject of increasing interest since in vitro and in vivo biological assays indicated that flavonoids can mediate a range of mechanisms related to anticancer, antitumor, and anti-oxidant activities, among others (*17*). The contribution of the flavonoids to the dietary intake of polyphenolic compounds is considerable. In fact, cereals, legume seeds, fruits, wine, and tea contain significant amounts of flavonoids and their derivatives (*18*). The 3-*O*-glycosides of quercetin and kaempferol are the most common group of flavonoids. The sugar moiety is an important factor for the bioavailability of the flavonoid derivatives (*19*).

The occurrence of tetra-glycosylated flavonoids in these two species may confirm their strong botanical correlation and afford a valuable chemical marker for the quality control of the Brazilian *Maytenus* marketed as phytomedicines, foodstuffs, or beverages in daily life.

## LITERATURE CITED


(1) Gonzalez, G. A. Componentes Mayoritarios del *Maytenus aquifolium* y sintesis parcial del acido 3-oxo-20-hidroxilup-28-oico. *Rev. Latinoam. Quim.* **1986**, *17*, 51−53.

(2) Sanommiya, M.; Vilegas, W.; Rastrelli, L.; Pizza, C. Flavonoid glycoside from *Maytenus aquifolium*. *Phytochemistry* **1998**, *54*, 237−239.

(3) Vilegas, W.; Sannomiya, M.; Rastrelli, L.; Pizza, C. Isolation and structure elucidation of two new flavonoid glycosides from the infusion of *maytenus aquifolium* leaves. Evaluation of the antiulcer activity of the infusion. *J. Agric. Food Chem.* **1999**, *47*, 403−406.

(4) Vilegas, J. H. Y.; Lancas, F. M.; Cervi, A. C. HRGC Analysis of adulteration of "espinheira-santa", a Brazilian medicinal plant. *Phytother. Res.* **1994**, *8*, 241−244.

(5) Bax, A.; Davis, D. G. Practical aspects of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson.* **1985**, *63*, 207−213.

(6) Bodenhausen, E.; Ruben, D. J. Unexpected cross-peaks in two-dimensional homonuclear-relayed coherence-transfer spectroscopy of a peptide. *J. Magn. Reson.* **1986**, *69*, 185−186.

(7) Homans, S. W. *Programs in NMR Spectroscopy*; Pergamon Press: Oxford, 1990; pp 55−81.

(8) Martin, G. E.; Crouch, R. C. Inverse detected two-dimensional NMR methods: application in natural products chemistry. *J. Nat. Prod.* **1991**, *54*, 1−70.
(9) Davis, D. G.; Bax, A. Simplification of $^1$H NMR spectra by selective excitation of experimental subspectra. *J. Am. Chem. Soc.* **1985**, *107*, 7198−7199.
(10) Carlini, E. A. *Estudos de acao antiulcera gastrica de plantas brasileiras: Maytenus ilicifolia (Espinheira Santa) e outras*; CEME/AFIF: Brasilia, 1988.
(11) Harborne, J. B., Ed. *The Flavonoids − Advances in Research since 1980*; Chapman and Hall: London, 1988.
(12) Agrawal, P. K., Ed. *Carbon 13 NMR of Flavonoids*; Elsevier: Amsterdam, The Netherlands, 1989.
(13) Rastrelli, L.; Aquino, R.; Abdo, S.; Proto, M.; De Simone, F.; De Tommasi, N. Studies on the constituents of *Amaranthus caudatus* leaves. Isolation and characterization of new triterpene saponins and ionol glycosides. *J. Agric. Food Chem.* **1998**, *46*, 1797−1804.
(14) Rastrelli, L.; Saturnino, P.; Schettino, O.; Dini, A. Studies on the constituents of *Chenopodium pallidicaule* (cañihua) seeds. Isolation and characterization of two new flavonol glycosides. *J. Agric. Food Chem.* **1995**, *43*, 2020−2024.
(15) Kijima, H.; Ide, T.; Otsuka, H.; Takeda, Y. Alangiflavoside, a new flavonol glycoside from the leaves of *Alangium premnifolium*. *J. Nat. Prod.* **1995**, *58*, 1753−1755.
(16) Hu, B.; Zhou, L.; Liu, Y. New tetrasaccharide flavonol glycoside from *Epimedium acuminatum*. *J. Nat. Prod.* **1992**, *55*, 672−675.
(17) Duthie, G. G.; Duthie, S. J.; Kyle, J. A. M. Plant polyphenols in cancer and heart disease: Implications as nutritional antioxidants. *Nutr. Res. Rev.* **2000**, *13*, 79−106.
(18) Middleton, E., Jr. Effect of plant flavonoids in immune and inflammatory cell function. **1998**, *439*, 175−182.
(19) Hollman, P. C. H.; Arts, I. C. W. Flavonols, flavones and flavanols − nature, occurrence and dietary burden. *J. Sci. Food. Agric.* **2000**, *80*, 1081, 1093.

Received for review March 6, 2001. Revised manuscript received June 1, 2001. Accepted June 4, 2001. The authors are grateful to FAPESP (Fundaçao de Amparo à Pesquisa do Estado de Sao Paulo) that has sponsored part of this work and to CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnologico) for fellowships to W.V. and J.H.Y.V.

JF010294N