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# Flavonol glycosides from *Costus spicatus*

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**Abstract**

Two flavonol diglycosides, tamarixetin 3-*O*-neohesperidoside, kaempferide 3-*O*-neohesperidoside and the known quercetin 3-*O*-neohesperidoside, together with six other known flavonoids were isolated from the leaves of *Costus spicatus* and their structures were elucidated by a combination of spectroscopic and chemical methods. The flavonol diglycosides were evaluated for inhibitory activity of nitric oxide production by activated macrophages (Fig. 1). © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* *Costus spicatus*; Costaceae; Leaves; Flavonol glycosides; Nitric oxide; Macrophage activation

## 1. Introduction

*Costus spicatus* Swartz (Costaceae), commonly called 'cana do brejo' in Brazil, is a medicinal plant found in wet coastal forests. The rhizome of this plant is used for the treatment of complaints of the bladder and urethra and to expel kidney stones (Manfred, 1947). An infusion of the aerial parts is taken to treat colds, sore throats, dysentery and diarrhea (Cruz, 1965). To our knowledge, no phytochemical studies on the aerial parts have been reported. Here, we describe the isolation and structure elucidation of two new flavonol diglycosides from the leaves of *C. spicatus*, named tamarixetin 3-*O*-neohesperidoside (**1**), kaempferide 3-*O*-neohesperidoside (**2**) and the known quercetin 3-*O*-neohesperidoside (**3**), together with tamarixetin 3-*O*-β-D-glucopyranoside, kaempferide 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-glucopyranoside, tamarixetin, kaempferide and quercetin. Compounds **1**–**3** here found to exhibit anti-inflammatory properties.

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## 2. Results and discussion

A methanolic extract of the leaves of *C. spicatus* was purified by Amberlite XAD-7 column chromatography. Compounds **1**–**3** were isolated from the purified leaf extract by column chromatography over silica gel and Sephadex LH-20 gel filtration followed by preparative HPLC. The pure flavonol diglycosides (**1**–**3**) were checked for homogeneity by analytical HPLC which afforded $R_t$ (min) for **1** (18.83), **2** (17.23) and **3** (15.30).

Compound **1** was assigned the molecular formula $C_{28}H_{32}O_{16}$ by analysis of LSIMS (neg. ion mode) $m/z$ 623 $(M\text{-}H)^-$ and $^{13}$C-NMR spectral data (Table 1). The IR spectrum showed bands of hydroxyl groups (3400 $cm^{-1}$), an α,β-unsaturated ketone (1651 $cm^{-1}$), and aromatic rings (1600, 1560, 1506 $cm^{-1}$). $^1$H-NMR spectral data taken in DMSO-$d_6$, revealed H-2-H-6 *meta*-coupling (1.8 Hz) at δ 7.54, H-6-H-5 *ortho*-coupling (8.4 Hz) at δ 7.54 and 6.56, respectively, and H-8-H-6 *meta*-coupling (1.8 Hz) at δ 6.48 and 6.22, respectively. As far as the disaccharide moiety is concerned, the doublet (7.2 Hz) at δ 5.80 has assigned to the

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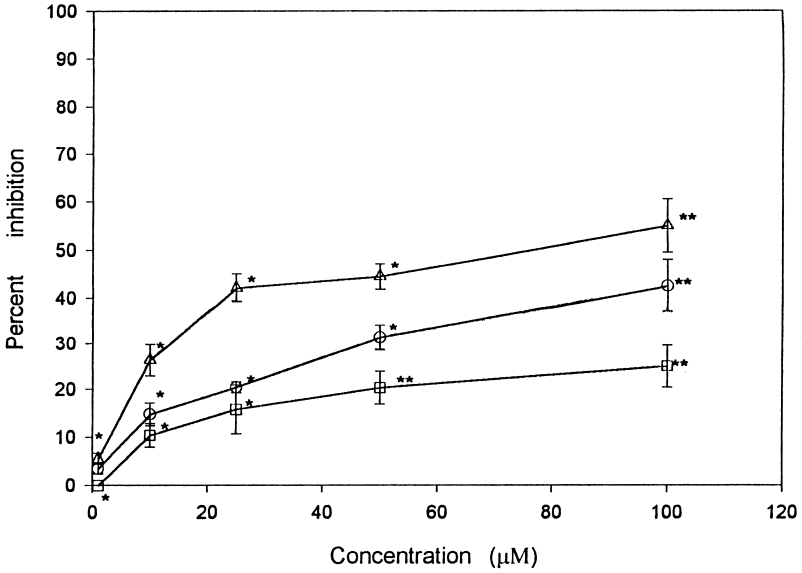



Fig. 1. Inhibitory activity on nitric oxide production of activated macrophages by compounds **1** (●), **2** (□) and **3** (△). Data points presented as mean ± SD, *p < 0.01, *p < 0.05. Significant difference from control group.

anomeric proton of 3-*O*-β-D-glucopyranoside and a broad singlet at 5.06, together with the doublet (6.2 Hz) at δ 0.68, to the anomeric and methyl group protons of α-L-rhamnopyranose, respectively (Barrero, Haidour, Muñoz-Dorado, Akssira, Sedqui & Mansour, 1998). In NOE difference experiments, separate saturation of the methoxyl signal at δ 3.83 resulted in the enhancement of H-5 (δ 6.86, *d*, $J = 8.4$ Hz), indicating that this methoxyl group was at C-4. The correlation peaks between the methoxyl proton signal, and its corresponding quaternary carbon resonance in the COLOC spectrum, allowed the carbon resonance at δ 149.38 to be assigned to C-4. Furthermore, the correlation peaks between H-2 and C-3 confirmed these assignments.

The $^{13}$C-NMR spectrum showed a singlet which resonated at δ 55.74, and was assigned to the carbon of the methoxyl-substituent at C-4. The signal at δ 177.30 was attributed to the carbonyl carbon. The signals of the aglycone were assigned by DEPT, $^{1}$H–$^{13}$C COSY and $^{1}$H–$^{13}$C COLOC and by comparison with data from the literature (Barrero et al., 1998; Kim, Higuchi, Kitamura & Komori, 1991). In the $^{13}$C-NMR spectrum, signals of a β-glucopyranosyl and an α-rhamnopyranosyl moiety were detected. The downfield shift of the C-2 and the upfield shift of C-1 suggested the position of attachment of the rhamnosyl moiety to be C-2 of glucose. This linkage was proven by methylation analysis of **1**, which showed a 2-linked glucopyranose and a terminal rhamnopyranose. The molar carbohydrate composition of **1** indicated the presence of two neutral monosaccharides, glucose : rhamnose (1.0 : 0.9). Their absolute configurations were determined by GC analysis of their TMSi (-)-2-butylglycosides. D-glucose and L-rhamnose were identified by GC–EIMS of the pertrimethylsilylated methylglycosides. The UV spectrum of **1** in MeOH was indicative of a 3-*O*-glycosidated flavonol (Markham, 1972) and UV spectrometry using shift reagents (NaOMe, NaOMe/$H_3BO_3$, $AlCl_3$ and $AlCl_3$/HCl) confirmed the structure of **1**.

On acid hydrolysis, compound **1** yielded tamarixetin, glucose and rhamnose. Mp, UV, IR, $^{1}$H- and $^{13}$C-NMR spectral data, and EIMS were consistent with the structure of tamarixetin (Barrero et al., 1998; Struck & Kirk, 1970). Hence, **1** was established as 3-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one, named tamarixetin 3-*O*-neohesperidoside.

Compound **2** was assigned the molecular formula $C_{28}H_{32}O_{15}$ by analysis of its LSIMS (neg. ion mode) *m/z* 607 $(M\text{-}H)^-$ and $^{13}$C-NMR data (Table 1). The IR spectrum was consistent with the proposed structure of **2**. The $^{1}$H spectrum displayed, in addition to a signal for a methoxyl group, two doublets at δ 6.92 and 8.06 for H-3, H-5 and H-2, H-6, respectively. Two doublets at δ 6.22 and 6.46 integrating for single protons were assigned to H-6 and H-8, respectively. A doublet at δ 5.68 ($J = 7.2$ Hz) and a broad singlet at δ 5.08 integrating for single protons were attributed to H-1 of a β-glucosyl unit and α-rhamnosyl unit, respectively (Table 1).

The $^{13}$C-NMR spectrum showed peaks for 28 carbon (Table 1) singlet which resonated at δ 55.95 was assigned to the carbon of a methoxyl-substituent at C-4. The signal at δ 177.29 was attributed to the carbonyl carbon. The resonance of the aromatic moiety was

Table 1
$^1$H- and $^{13}$C-NMR spectral data for compounds **1**–**3** in DMSO-$d_6$[a,b,c]

| Attribution | **1** | | **2** | | **3** | |
|---|---|---|---|---|---|---|
| | $\delta$ $^{13}$C | $\delta$ $^1$H | $\delta$ $^{13}$C | $\delta$ $^1$H | $\delta$ $^{13}$C | $\delta$ $^1$H |
| 2 | 156.34 | | 156.08 | | 156.41 | |
| 3 | 132.60 | | 132.74 | | 133.38 | |
| 4 | 177.30 | | 177.29 | | 177.31 | |
| 4a | 104.05 | | 104.03 | | 103.89 | |
| 5 | 161.24 | | 161.23 | | 161.21 | |
| 6 | 98.77 | 6.22 *d* (C-5, C-7) $J = 1.8$ Hz | 98.72 | 6.22 *d* (C-5, C-7) $J = 1.8$ Hz | 98.63 | 6.21 *d* (C-5, C-7) $J = 1.8$ Hz |
| 7 | 164.19 | | 164.20 | | 164.12 | |
| 8 | 93.71 | 6.48 *d* (C-7, C-8) $J = 1.8$ Hz | 93.70 | 6.46 *d* (C-7, C-8) $J = 1.8$ Hz | 93.55 | 6.42 *d* (C-7, C-8) $J = 1.8$ Hz |
| 8a | 156.32 | | 156.06 | | 156.45 | |
| 1′ | 121.08 | | 120.93 | | 121.61 | |
| 2′ | 113.56 | 7.98 *d* (C-3′) $J = 1.8$ Hz | 130.77 | 8.06 *d* $J = 8.4$ Hz | 115.33 | 7.70 *d* (C-3′) $J = 1.8$ Hz |
| 3′ | 146.88 | | 115.13 | 6.92 *d* (C-4′) $J = 8.4$ Hz | 144.68 | |
| 4′ | 149.38 | | 159.91 | | 148.38 | |
| 5′ | 115.59 | 6.86 *d* $J = 8.4$ Hz | 115.13 | 6.92 *d* (C-4′) $J = 8.4$ Hz | 116.21 | 6.80 *d* $J = 8.5$ Hz |
| 6′ | 121.89 | 7.54 *dd* $J = 1.8, 8.4$ Hz | 130.77 | 8.06 *d* $J = 8.4$ Hz | 121.21 | 7.72 *dd* $J = 1.8, 8.5$ Hz |
| 5-OH | | 12.52 *s* | | 12.53 *s* | | 12.52 *s* |
| 4′-$OCH_3$ | 55.74 | 3.83 *s* (C-4′) | 55.95 | 3.87 *s* (C-4′) | | |
| Glc-1″ | 98.77 | 5.80 *d* (C-3) $J = 7.2$ Hz | 98.37 | 5.68 *d* (C-3) $J = 7.2$ Hz | 98.48 | 5.75 *d* (C-3) $J = 7.2$ Hz |
| 2″ | 76.56 | | 76.54 | | 76.56 | |
| 3″ | 77.34 | | 77.32 | | 77.34 | |
| 4″ | 69.73 | | 69.71 | | 69.72 | |
| 5″ | 77.13 | | 77.13 | | 77.13 | |
| 6″ | 60.60 | | 60.58 | | 60.60 | |
| Rha-1‴ | 100.75 | 5.06 *bs* (C-2″) | 100.60 | 5.08 *bs* (C-2″) | 100.68 | 5.06 *bs* (C-2″) |
| 2‴ | 70.20 | | 70.18 | | 70.20 | |
| 3‴ | 70.62 | | 70.62 | | 70.62 | |
| 4‴ | 71.90 | | 71.88 | | 71.90 | |
| 5‴ | 68.29 | | 68.28 | | 68.28 | |
| 6‴ | 17.00 | 0.68 *d* $J = 6.2$ Hz | 17.25 | 0.76 *d* $J = 6.2$ Hz | 17.20 | 0.68 *d* $J = 6.2$ Hz |

[a] $^1$H–$^{13}$C COLOC correlations in parentheses.
[b] Proton and carbon signals were assigned by 2D-COSY, $^1$H $^{13}$C COSY and $^1$H and $^{13}$C COLOC experiments.
[c] Multiplicities were determined by DEPT experiments.

assigned by DEPT, $^1$H–$^{13}$C COSY and $^1$H–$^{13}$C COLOC and by comparison with data from the literature (Dauguet, Bert, Dolley, Bekaert & Lewin, 1993, Kofinas, Chinou, Loukis, Harvala, Maillard & Hostettmann, 1998; Wenkert & Gottlieb, 1997).

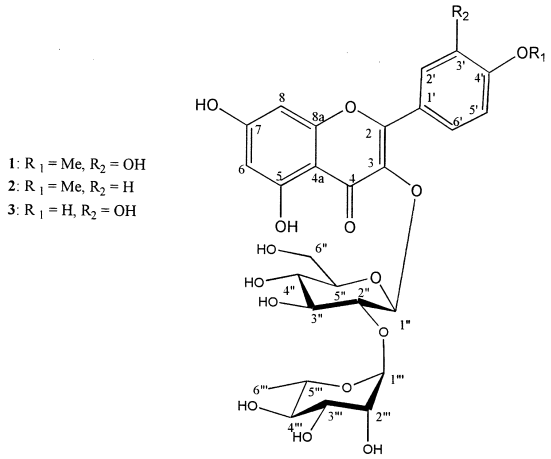
The UV spectral data in MeOH and with shift reagents were indicative of a 3-*O*-glycosidated flavonol (Markham, 1972). On acid hydrolysis, compound **2** yielded a flavonol aglycone, glucose and rhamnose. Mp, UV, IR, $^1$H- and $^{13}$C-NMR spectral data, and EIMS of the aglycone were in accordance with those reported in the literature for kaempferide (Majumder & Chattopadhyay, 1985; Tiwari & Srivastava, 1979). The molar carbohydrate composition, the absolute configurations of the sugars, the methylation analysis and $^1$H- and $^{13}$C-NMR spectral data indicated that compound **2** possessed the same sugar moiety as **1**. Hence **2** was established as 3-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5,7-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one, named kaempferide 3-*O*-neohesperidoside.

Flavonoid **3** was isolated and identified by spectral analyses and chemical reactions. UV, IR and $^1$H-NMR spectral data were similar to reported values for quercetin 3-*O*-neohesperidoside (Bacon, Mabry & Harborne, 1975; Williams, Harborne & Clifford, 1971). In the $^{13}$C-NMR spectrum (Table 1) the chemical shifts of the glycosidic moiety resonances were very

similar to those of **1** and **2**, indicating a neohesperidoside sequence. The LSIMS exhibited a $[M-H]^-$ at $m/z$ 607.

Six other known flavonoids were isolated from the leaves of *C. spicatus* and their structures were established by comparison with data from the literature as tamarixetin 3-*O*-β-D-glucopyranoside (Ishak, El Sissi, El Sherbieny & Nawwar, 1972), kaempferide 3-*O*-β-D-glucopyranoside (Tiwari & Srivastava, 1979), quercetin 3-*O*-β-D-glucopyranoside (Kim et al., 1991), tamarixetin (Struck & Kirk, 1970), kaempferide (Majumder & Chattopadhyay, 1985) and quercetin (Kim et al., 1991).

In order to investigate the anti-inflammatory properties of the flavonol diglycosides, compounds **1**–**3** were evaluated for their inhibitory activity on nitric oxide production by activated macrophages (Dirsch, Stuppner & Vollmar, 1998). Compound **3**, which was already enhance protein formation by endothelial cells and inhibition of endothelial tissue injury (Chen, Fang, Gu, Zhang & Zhao, 1990), exhibited an $IC_{50}$ value of 55 μM, showing moderate inhibitory activity on nitric oxide production. Nonetheless, compounds **1** and **2** showed $IC_{50}$ values higher than 100 μM, indicating slight inhibitory activity. The above results suggest that these compounds may be the potential therapeutic agents involved in inflammatory disorders, justifying the use of *C. spicatus* in Brazilian traditional medicine (Cruz, 1965).




## 3. Experimental

### 3.1. General

Melting points were determined by an Ellectrothermal 9200 micro melting point apparatus and are uncorrected. OR were measured on a Perkin Elmer 243B polarimeter. UV and IR spectra were measured on a Shimadzu UV-1601 and on a Perkin Elmer 599B, respectively. $^1$H- and $^{13}$C-NMR spectra were obtained on a Varian Gemini 200 NMR spectrometer operating at 200 MHz for $\delta_H$ and 50 MHz for $\delta_C$, in DMSO-$d_6$, TMS as international standard. GC was carried out with FID, using a glass capillary column (0.31 mm × 25 m) SE-30. EIMS and GC–MS: recorded at 70 eV. Negative LSIMS was carried out using HMPA-glycerol as matrix, 35 kV anodic voltage, 8 kV accelerating voltage using Cs ions.

Silica gel column (230–400 mesh ASTM, Merck), Amberlite XAD-7 nonionic polymeric adsorbent (20–60 mesh, Aldrich) and Sephadex LH-20 were used for CC. TLC was performed on silica gel coated plates (Merck) using the following solvent systems: (a) $CHCl_3$–MeOH–$H_2O$ (65 : 35 : 10, lower phase) for flavonol diglycosides and (b) $CHCl_3$–MeOH (19 : 1) for isoflavone aglycones, and (c) *n*-BuOH–pyridine–$H_2O$ (6 : 4 : 3) for sugars. Compounds **1**–**3** were detected under UV (254 and 366 nm) and by spraying with orcinol–$H_2SO_4$, sugars were detected by spraying with aniline–diphenylamine-85 orthophosphoric acid–MeOH (1 : 1 : 5 : 43).

### 3.2. Plant material

Leaves of *C. spicatus* Benth. were collected at Ilha do Fundão, Rio de Janeiro in September 1996, and identified by Luci S. Valle. A voucher specimen (no. R192950) is deposited at the herbarium of the National Museum, Rio de Janeiro, Brazil.

### 3.3. Extraction and isolation

Dried and powdered leaves of *C. spicatus* (250 g) were extracted with cold MeOH (2 l). Evaporation of the MeOH gave a residue (10 g), which was chromatographed on Amberlite XAD-7 (100 g). Fractions eluted with MeOH yielded a mixture of flavonol glycosides (1.75 g), which was submitted to CC (120 × 3 cm) on silica gel eluted with $CHCl_3$MeOH mixtures, of increasing polarity (up to 35% MeOH) to afford a mixture of three compounds (350 mg, $CHCl_3$–MeOH, 66 : 34) which were isolated by Sephadex LH-20 gel filtration followed by preparative HPLC to afford tamarixetin 3-*O*-neohesperidoside (**1**, 125 mg, $R_f$ 0.28), kaempferide 3-*O*-neohesperidoside (**2**, 70 mg, $R_f$ 0.41) and quercetin 3-*O*-neohesperidoside (**3**, 45 mg, $R_f$ 0.19).

### 3.4. High-performance liquid chromatography

The analytical HPLC system (Shimadzu, LC-10AD) was equipped with a diode-array detector, a 20 μl loop and a 200 × 4.6 mm ODS Hypersil column, 5 μm. For the preparative separation, a 250 × 10 mm Econosil C18 column, 10 μm, was used. Analytical and preparative HPLC systems were operated at room temperature

using the same solvents: (A) HCOOH–$H_2O$ (1 : 9) and (B) HCOOH–$H_2O$–MeOH (1 : 4 : 5). The elution profile for analytical HPLC consisted of isocratic elution (90% A, 10% B) for 10 min followed by a linear gradient from 10 to 100% B for 20 min and then isocratic elution (100% B) for 10 min followed by linear gradient from 100 to 10% B for 10 min. The flow rate was 1.0 ml $min^{-1}$ and aliquots of 15 μl were injected. The elution profile for preparative separations consisted of an isocratic elution (90% A, 10% B) for 10 min, a linear gradient from 10 to 100% B for 20 min, an isocratic elution for 15 min, followed by linear gradient from 100 to 10% B for 10 min. The flow rate was 4.0 ml $min^{-1}$.

### 3.5. Tamarixetin 3-O-neohesperidoside (*1*)

Yellow amorphous powder from MeOH, mp 180–190° dec, $[\alpha]_D^{20} - 78°$ (DMSO, *c* 0.001). UV $\lambda_{max}^{MeOH}$ nm (log ε): 254 (3.3), 286 (1.0), 373 (3.5); + $AlCl_3$: 268, 298, 386; + $AlCl_3$/HCl: 270, 300, 376; + NaOMe: 271, 328, 414; + NaOMe/$H_3BO_3$: 252, 282, 370. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400 (OH), 1651, 1600, 1560, 1506, 1289, 1205, 1166, 1129, 1050, 1030, 980. $^1$H- and $^{13}$C-NMR spectral data shown in Table 1. Negative LSIMS, *m/z* (rel. int.): 623 $[M-H]^-$ (30), 315 [M-309] (100). Compound **1** (40 mg) was heated in 2 M HCl (5 ml) until reflux began, this being continued for 2 h. Aglycone was extracted with EtOAc and evaporated to dryness in vacuo. The residue was dissolved in MeOH and the solution on concentration yielded a yellow compound which on further crystallization gave tamarixetin (18 mg). Melting point and UV, IR, $^1$H- and $^{13}$C-NMR spectral data and mass spectral analysis were in accordance with those reported in the literature (Struck & Kirk, 1970; Barrero et al., 1998). Aqueous layer was adjusted to pH 6 by addition of $NaHCO_3$. After lyophilization, sugars were dissolved in pyridine and analyzed by silica gel-TLC in the above-described system. After spraying, rhamnose gave a green spot at $R_f$ 0.75, and glucose gave a blue spot at $R_f$ 0.70.

### 3.6. Kaempferide 3-O-neohesperidoside (*2*)

Yellow amorphous powder from MeOH, mp 170–180° dec, $[\alpha]_D^{20} - 85$ (DMSO, *c* 0.001). UV $\lambda_{max}^{MeOH}$ nm (log ε): 252 sh, 266 (3.0), 353 (3.5); + $AlCl_3$: 254, 270, 418; + $AlCl_3$/HCl: 256, 270, 355; + NaOMe: 280, 323, 411; + NaOMe/$H_3BO_3$: 254, 268, 355. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3438 (OH), 1650, 1600, 1558, 1504, 1287, 1203, 1163, 1127, 1048, 1029, 975. $^1$H- and $^{13}$C-NMR spectral data shown in Table 1. Negative LSIMS, *m/z* (rel. int.): 607 $[M-H]^-$ (27), 299 [M-309] (100). Compound **1** (40 mg) was hydrolyzed by the procedure described above to afford kaempferide (15 mg), glucose and rhamnose. Melting point and UV, IR, $^1$H- and $^{13}$C-NMR spectral data and EIMS of kaempferide were in accordance with those reported in the literature (Tiwari & Srivastava, 1979; Majumder & Chattopadhyay, 1985).

### 3.7. Quercetin 3-O-neohesperidoside (*3*)

Physical constants, UV, IR and $^1$H-NMR spectral data of compound **3** were in accordance with those reported in the literature (Williams et al., 1971; Bacon et al., 1975). $^{13}$C-NMR spectral data are shown in Table 1. Negative LSIMS, *m/z* (rel. int.): 609 $[M-H]^-$ (72), 301 [M-309] (100). Compound **3** (40 mg) was hydrolyzed by the procedure described for **1** and **2** to afford quercetin (14 mg), glucose and rhamnose. Identity of quercetin was established by comparison with an authentic sample through analysis of mp, IR, $^1$H- and $^{13}$C-NMR, and EIMS.

### 3.8. Molar carbohydrate composition and D, L configurations

Monosaccharides were analyzed as their TMSi methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80°) and trimethylsilylation (Kamerling, Gerwig, Vliegenthart & Clamp, 1975). The configurations of the glycosides were established by capillary GC and GC–MS of their TMSi (−)-2-butylglycosides (Gerwig, Kamerling & Vliegenthart, 1978).

### 3.9. Methylation analysis

Compounds **1**–**3** were methylated with DMSO-lithium methylsulphinyl carbanion-$CH_3$**I** (Parente, Cardon, Leroy, Montreuil, Fournet & Ricart, 1985). The methyl ethers were obtained either (a) after hydrolysis (4 N TFA, 2 h, 100°) and analyzed as partially poliol-acetates by GC–MS (Fournet, Dhalluin, Leroy, Montreuil & Mayer, 1978) or (b) after methanolysis (0.5 M HCl in MeOH, 24 h, 80°) and analyzed as partially methylated methylglycosides by GC–MS (Fournet, Strecker, Leroy & Montreuil, 1981).

### 3.10. Measurement of nitric oxide production

Male BALB/c mice weighing from 20 to 25 g were used. Cells were collected from the peritoneal cavities by washing with culture medium, and were inoculated into flat-bottomed 96-well tissue culture plates ($1 \times 10^6$ cells/ml) in RPMI 1640 medium containing 10% fetal calf serum. After incubation for 24 h at 37°C in a $CO_2$ incubator, nonadherent cells were removed by washing with fresh culture medium. The cell suspensions were incubated with 1 μg/ml *Escherichia coli* lipopolysaccharide as stimulant and compounds **1**–**3** at different

concentrations for 48 h at 37°C. After this period, 100 μl of cell culture supernatant were removed and combined with 90 μl 1% sulfanilamide in 5% $H_3PO_4$ and 90 μl 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride followed by spectrophotometric measurement at 550 nm (Green, Wagner, Glogowski, Skipper, Wishnok & Tannenbaum, 1982).

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