Antioxidant Flavonoid Glycosides from *Daphniphyllum calycinum*[1]

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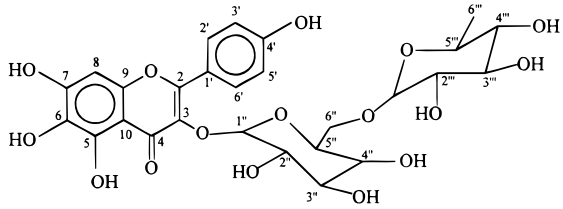
A novel flavonoid diglycoside, 5,6,7,4′-tetrahydroxyflavonol 3-*O*-rutinoside (**1**), and a previously known compound, kaempferol 3-*O*-neohesperidoside (**2**), were isolated from an ethyl acetate extract of *Daphniphyllum calycinum* leaves that showed significant activity in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical assay. The structure of **1** was elucidated by a combination of spectroscopic methods, and compounds **1** and **2** were found to be moderately active as antioxidants in the DPPH assay.

The shrub *Daphniphyllum calycinum* Benth. is native to the People's Republic of China, where it has a number of folkloric uses, such as for the healing of wounds and as an antiinflammatory remedy.[2] A previous phytochemical investigation performed on this species resulted in the isolation of alkaloids of the daphniphylline group, comprising calycine, daphnicadine, daphnicaline, and daphnicamine.[3] As part of our continuing search for plant-derived cancer chemopreventive agents, an ethyl acetate-soluble extract of the leaves of this plant was found to exhibit significant antioxidant effects, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.[4] Bioassay-guided fractionation of this extract utilizing this antioxidant assay resulted in the isolation of a novel active flavonoid glycoside, 5,6,7,4′-tetrahydroxyflavonol 3-*O*-rutinoside (**1**), which was purified along with the known kaempferol 3-neohesperidoside (**2**), using solvent extraction and repeated column chromatography over silica gel. The present paper deals with the structure elucidation of **1** and the antioxidant evaluation of flavonoid glycosides **1** and **2**.



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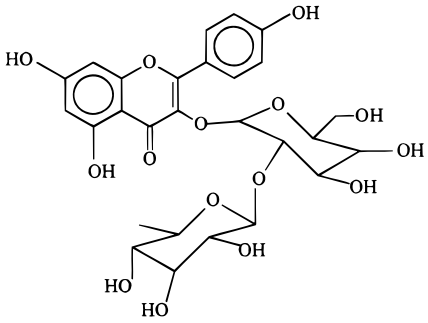

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A molecular formula of $C_{27}H_{30}O_{16}$ was assigned to compound **1** from its HRFABMS data (*m*/*z* 611.4531 [M + 1]$^+$). The UV spectrum (λ_{max} 350 nm), which exhibited a bathochromic effect in alkali, was indicative of the presence of a phenolic functionality, and together with absorption bands at 3435, 1657, 1593, and 1465 cm^{-1} in the IR spectrum suggested that **1** was a flavonoid glycoside.[5] The presence of two anomeric protons (δ 5.12, d, J = 7.3 Hz; δ 4.51 brs) in the ^{1}H NMR spectrum and observations from the DQ-COSY spectral data implied that **1** contained β-glucose and α-rhamnose moieties.[6] Indeed, the presence of a rhamnosyl unit was evidenced in the ^{1}H and ^{13}C NMR spectra by a 3H-doublet (J = 6.2 Hz) at δ 1.12 and a peak at δ 17.9, respectively.[7] This was confirmed unambiguously by a ^{1}H−^{1}H DQ-COSY correlation between this methyl group signal and H-5‴. The small coupling constant (brs) between the anomeric proton of the rhamnosyl moiety and H-2‴ supported an α configuration for the anomeric proton. The presence of six signals in the ^{13}C NMR spectrum of **1** at δ_C 68.6 (t, C-6″), 71.4 (d, C-4″), 75.7 (d, C-2″), 77.2 (d, C-5″), 78.1 (d, C-3″), and 104.6 (d, C-1″), respectively, was indicative of a glucosyl moiety substituted at C-6″.[7] The aromatic part of the ^{1}H NMR spectrum of **1** along with the analogous ^{13}C NMR and APT spectral data suggested that the aglycon was a flavonol.[7] In the ^{1}H NMR spectrum of **1**, the occurrence of an AA′BB′ spin system supported the presence of a para-substituted B ring, and it was inferred that ring A was trisubstituted from the appearance of a single aromatic proton singlet at δ 6.39 (H-8). This signal exhibited three-bond correlations with C-6 (δ_C 158.6) and C-10 (δ_C 105.5) in the HMBC spectrum. On the basis of the evidence obtained, the aglycon was assigned as 5,6,7,4′-tetrahydroxyflavonol. The disaccharide unit of **1** was shown to be attached at C-3 of the aglycon as demonstrated by an HMBC correlation between H-1 (δ

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5.12) of the glucosyl portion and C-3 of the aglycon, which appeared at an unusually low field (δ_C 135.5). Furthermore, a three-bond HMBC correlation between the anomeric proton of the rhamnosyl moiety and C-6 of the glucosyl unit, along with the downfield shift of C-6″ in the ^{13}C NMR spectrum (δ_C 68.6), suggested a 1→6 linkage between the rhamnosyl group and the glucosyl unit, consistent with a rutinoside structure.[7] Thus, the structure of **1** was established as 5,6,7,4′-tetrahydroxyflavonol 3-*O*-rutinoside [(5,6,7,4′-tetrahydroxyflavonol 3-*O*-(6-α-L-rhamnopyranosyl-β-D-glucopyranoside)].

A known compound was also isolated in this investigation as an additional antioxidant flavonoid glycoside and identified by comparison with published physical and spectral data as kaempferol 3-*O*-neohesperidoside (**2**).[8,9]

Compounds **1** and **2**, with IC_{50} values of 43.2 and 79.6 μg/mL, respectively, exhibited moderate activities as free-radical scavengers in the DPPH assay, in comparison with reference antioxidants such as ascorbic acid (IC_{50}: 22 μg/mL), 2(3)-*tert*-butyl-4-hydroxyanisole (IC_{50}: 21 μg/mL), caffeic acid (IC_{50}: 12 μg/mL), gallic acid (IC_{50}: 5 μg/mL), and nordihydroguaiaretic acid (12 μg/mL). The less potent antioxidant activity of **2** in comparison with that of **1** must be associated with the one less phenolic group present in the molecule. This observation is in accordance with conclusions drawn from a larger study of the antioxidant activity of about 40 flavones and flavonols.[10] In addition, the lack of catechol functionalities in ring B of compounds **1** and **2** presumably results in a significant decrease of free-radical scavenging potency, respectively, in comparison with a literature value for quercetin.[11]

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 241 polarimeter at 25 °C. UV and IR spectra were recorded on a Beckman DU-7 and a Midac Collegian FT-IR spectrometer, respectively. ^1H and ^{13}C NMR data were recorded with TMS as internal standard, using a Varian XL-300 instrument operating at 300 and 75.4 MHz, respectively. APT, $^1H-^1H$ DQ-COSY, and $^1H-^{13}C$ HETCOR NMR experiments were performed on the same instrument, using standard pulse sequences, while $^1H-^{13}C$ HMQC and $^1H-^{13}C$ HMBC spectra were measured on a GE Omega-500 (500 MHz) instrument. FAB-MS spectra were recorded by direct inlet using a Finnigan MAT 90 spectrometer.

Plant Material. Leaves of *D. calycinum* Benth. were collected at the Guangzhou Botanical Garden, Wushan, Guangzhou, People's Republic of China, in June, 1994. The plant material was collected and identified by one of us (L.F.Z.), and a voucher specimen has been deposited in South China Institute of Botany, Academia Sinica, Guangzhou, People's Republic of China.

Extraction and Isolation. The air-dried powdered leaves of *D. calycinum* (5 kg) were extracted exhaustively with MeOH (2 × 25 L) for 48 h by maceration, and the resultant extracts were combined *in vacuo* at 45 °C to afford 300 g of a residue. After defatting this residue with petroleum ether (2 × 1 L) and partitioning between H_2O and EtOAc (3 × 2 L), 190 g of a dried ethyl acetate-soluble extract was obtained. When tested for antioxidant potency, this extract exhibited activity with an IC_{50} value of 85 μg/mL and was, therefore, fractionated over a glass column containing silica gel (1 kg), eluting with $CHCl_3$ and gradient mixtures of $CHCl_3$−MeOH to give 10 fractions. Fractions 4−7, eluted with $CHCl_3$−MeOH (70:30), demonstrated antioxidant activity and were pooled to afford 47.3 g of residue on drying. Further purification was carried out by placing this residue on an open column packed with 1.5 kg of silica gel suspended in $CHCl_3$ and elution with mixtures of $CHCl_3$ and MeOH (5 → 30%), 100% MeOH, and H_2O. This column afforded two major active subfractions that were pooled for additional chromatographic purification with $CHCl_3$−MeOH−H_2O (65:35:10, lower phase) as eluant to produce 152 mg of impure **1**. Elution of this same column with $CHCl_3$−MeOH−H_2O (80:20:3) afforded 25 mg of impure **2**. Column chromatography applied to this first crude mixture using $CHCl_3$−MeOH−H_2O (65:35:10, lower phase), followed by preparative TLC on silica gel plates (silica gel 60 F_{254} Merck; plates 20 × 20 cm, 0.25 mm layer thickness), using this same solvent mixture for elution, gave 47 mg of **1** (0.00094% w/w) (R_f 0.35). The impure mixture containing **2** was purified by column chromatography as described for compound **1**. Final preparative TLC by elution with $CHCl_3$−MeOH−H_2O (80:20:3) afforded 5 mg of **2** (0.0001% w/w) (R_f 0.50).

5,6,7,4′-Tetrahydroxyflavonol 3-*O*-rutinoside (1): amorphous compound: $[\alpha]^{20}_D$ −27.4° (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ϵ) 350 (4.14) nm; (MeOH + NaOH) 405 nm λ_{max} (MeOH + NaOAc) 410 nm; λ_{max} (MeOH + $AlCl_3$) 423 nm; IR ($AgNO_3$) ν_{max} 3435, 2930, 1657, 1593, 1465, 1369, 1208, 1080, 840, 808 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 8.06 (2H, d, *J* = 8.7 Hz, H-2′, H-6′), 6.88 (2H, d, *J* = 8.7 Hz, H-3′, H-5′), 6.39 (1H, s, H-8), 5.12 (1H, d, *J* = 7.3 Hz, H-1″), 4.51 (1H, brs, H-1‴), 1.12 (3H, d, *J* = 6.2 Hz, CH_3-6‴); ^{13}C NMR (75.4 MHz, CD_3OD) δ 17.9 (q, C-6‴), 68.6 (t, C-6″), 69.7 (d, C-5‴), 71.4 (d, C-4″), 72.1 (d, C-2‴), 72.3 (d, C-3‴), 73.9 (d, C-4‴), 75.7 (d, C-2″), 77.2 (d, C-5″), 78.1 (d, C-3″), 95.0 (d, C-8), 102.4 (d, C-1‴), 104.6 (s, C-1″), 105.5 (s, C-10), 116.1 (2d, C-3′, C-5′), 122.8 (s, C-1′), 132.4 (2d, C-2′, C-6′), 135.5 (s, C-3), 158.6 (s, C-2), 158.6 (s, C-6), 159.3 (s, C-9), 161.5 (s, C-5), 163.0 (s, C-4′), 166.6 (s, C-7), 179.3 (s, C-4); FABMS (negative-ion mode) *m/z* 609 [M − H]$^-$; HRFABMS (positive-ion mode) *m/z* calcd for $C_{27}H_{31}O_{16}$ 611.4527, found 611.4531.

Kaempferol 3-*O*-neohesperidoside (2): amorphous powder; $[\alpha]^{20}_D$ −94.4° (*c* 0.1, MeOH). The compound exhibited spectral (UV, IR, ^1H NMR, ^{13}C NMR, FABMS) data comparable to published values.[8,9]

Evaluation of Antioxidant Activity. The potential antioxidant activity of plant extracts and pure compounds was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.[4] Reaction mixtures containing test samples (dissolved in DMSO) and 300 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37 °C for 30 min, and absorbances were measured at 515 nm. Percent inhibition by sample treatment was determined by comparison with a DMSO-

treated control group. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

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