Antioxidative Flavanone Glycosides from the Branches and Leaves of *Viscum coloratum*

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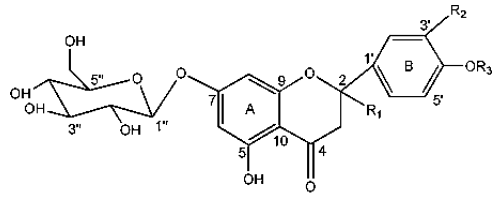
Two new flavanone glucosides, (2*S*)-homoeriodictyol 7,4′-di-*O*-*β*-D-glucopyranoside (4) and (2*R*)-eriodictyol 7,4′-di-*O*-*β*-D-glucopyranoside (5) were isolated from the branches and leaves of *Viscum coloratum* (KOMAR) NAKAI (Loranthaceae), along with three known flavanone glucosides: (2*S*)-homoeriodictyol 7-*O*-*β*-D-glucopyranoside (1), (2*S*)-eriodictyol 7-*O*-*β*-D-glucopyranoside (2), and (2*S*)-naringenin 7-*O*-*β*-D-glucopyranoside (3). The structures of these compounds were elucidated using spectroscopic methods. The antioxidant activities of these isolated compounds were evaluated by colorimetric methods based on their scavenging effects on hydroxyl radicals and superoxide anion radicals, respectively. All the compounds showed potent albeit varied degrees of antioxidative activities and the structure–activity relationship is discussed.

Key words *Viscum coloratum*; Loranthaceae; flavanone glycoside; antioxidative activity

Viscum coloratum (KOMAR) NAKAI (Loranthaceae) is a semi-parasitic plant distributed in southwest provinces of China. The branches and leaves of this plant are traditional Chinese medicines practiced mainly for the treatment of hypertension, atherosclerosis, rheumatism, and neuralgia.[1)] Previous phytochemical investigations revealed that flavonoids are the major secondary metabolites of this species.[2—6)] In our general screening for antioxidative natural substances from traditional Chinese medicine, the ethanol extract of *V. coloratum* was found to possess potent antioxidative activities. This finding prompted interest of a phytochemistry study aimed at identifying compounds responsible for the observed antioxidative activities. This paper reports the isolation and structural elucidation of two new flavanone glucosides, (2*S*)-homoeriodictyol 7,4′-di-*O*-*β*-D-glucopyranoside (**4**) and (2*R*)-eriodictyol 7,4′-di-*O*-*β*-D-glucopyranoside (**5**), along with three known compounds obtained from this plant. The antioxidative activities of these 5 compounds were evaluated based on their scavenging effects on hydroxyl radicals produced by H_2O_2/Fe^{2+}, and superoxide anion radicals produced by xathine/xanthine oxidase systems. An attempt to illustrate the structure–activity relationship was made based on these findings.



	R_1	R_2	R_3
1	β-H	OCH_3	H
2	β-H	OH	H
3	β-H	H	H
4	β-H	OCH_3	β-D-glucopyranosyl
5	α-H	OH	β-D-glucopyranosyl

Structures of compounds 1-5

Results and Discussion

The ethanol extract of the branches and leaves was re-suspended in water and partitioned with petroleum (60—90 °C). The water phase was filtered and the filtrate was loaded onto a D-101 resin column and then eluted sequentially with H_2O followed by 20%, 40%, 60%, 80% and 95% aqueous EtOH. The fraction eluted from 40% EtOH was chromatographed on a Sephadex LH-20 column which was eluted with aqueous MeOH (50—80%, gradient) to give four fractions. Fraction 2a was further separated on an ODS column eluted with 40% MeOH to yield compounds **4** and **5**. Fractions 2b, 2c, and 2d were each purified by Sephadex LH-20 to afford compounds **1**—**3**, respectively. Of the 5 compounds elucidated, the three known compounds were subsequently identified as (2*S*)-homoeriodictyol 7-*O*-*β*-D-glucopyranoside (**1**),[2)] (2*S*)-eriodictyol 7-*O*-*β*-D-glucopyranoside (**2**),[7)] and (2*S*)-naringenin 7-*O*-*β*-D-glucopyranoside (**3**),[8)] by comparisons of their spectral data (UV, IR, NMR, MS, and CD) with those reported in the literature. The purity of these compounds was confirmed by TLC and HPLC (purity >95% for all compounds).

Compound **4** was obtained as a pale yellow powder. Its molecular formula was established as $C_{28}H_{34}O_{16}$ by HR-ESI-MS at *m/z* 649.1739 $[M+Na]^+$ (Calcd 649.1735), and confirmed by ^{13}C-NMR spectrum. The characteristic UV absorption bands (282, 330sh nm) and the ABX system protons at δ_H 2.79 (1H, dd, J=17.0, 3.0 Hz), 3.15 (1H, m), and 5.55 (1H, dd, J=12.3, 3.0 Hz) in the ^{1}H-NMR spectrum showed **4** to be a flavanone.[9)] The ^{1}H-NMR spectrum showed two aromatic doublets at δ_H 6.15, 6.20 (each 1H, d, J=2.0 Hz) assignable to the A ring and three aromatic proton signals at δ_H 7.17 (1H, d, J=2.0 Hz), 7.13 (1H, d, J=8.5 Hz), and 7.01 (1H, dd, J=8.5, 2.0 Hz) attributable to the B ring, as well as one phenolic methoxyl group at δ_H 3.79 (3H, s) and one phenolic hydroxyl group at δ_H 12.05 (D_2O exchangeable). The ^{13}C-NMR spectrum resolved 28 carbon signals, corresponding to a flavanone skeleton bearing one methoxyl group, one hydroxyl group, and two sugar moieties. Two sugar moieties

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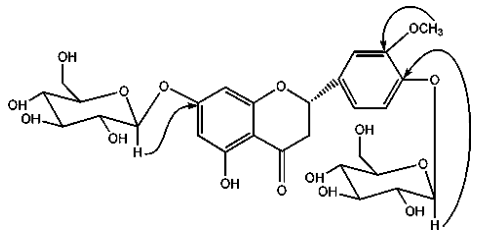

Fig. 1. Key HMBC Correlations of Compound **4**

were determined to be D-glucose by comparing the ^{13}C-NMR data (δ_C 100.1, 73.5, 76.8, 70.0, 77.5, 60.0; and 100.5, 73.6, 77.3, 70.1, 77.6, 60.1, respectively) with those reported in the literatures,[10] and their configurations were determined to be β-oriented as judged by the coupling constants of two anomeric protons at δ_H 4.96 (1H, d, J=7.0 Hz) and at δ_H 4.93 (1H, d, J=8.5 Hz) in the ^{1}H-NMR spectrum. The ^{1}H- and ^{13}C-NMR spectra were almost identical to those of viscumneoside I (homoeriodictyol-7-*O*-β-D-glucoside-4′-*O*-β-D-apioside) except the difference between one apioside and one glucoside moiety.[2] In the HMBC spectrum (Fig. 1), the proton at δ_H 4.96 (1H, d, J=7.0 Hz, H-1″) was correlated with C-7 (δ_C 165.8), and proton at δ_H 4.93 (1H, d, J=8.5 Hz, H-1‴) was correlated with C-4′ (δ_C 147.3), suggesting that two β-D-glucose moieties were located at C-7 and C-4′, respectively. In addition, the correlation of the proton at δ_H 3.79 (3H, s) with C-3′ (δ_C 149.4) indicated that the methoxyl group was located at C-3′. The stereochemistry at C-2 was determined to be *S* due to the presence of a positive Cotton effect at 333 nm and a negative Cotton effect at 287 nm in the CD spectrum.[11,12] Therefore, compound **4** was established as (2*S*)-homoeriodictyol 7,4′-di-*O*-β-D-glucopyranoside.

Compound **5** was also obtained as a pale yellow powder. Its molecular formula was established as $C_{27}H_{32}O_{16}$ by HR-ESI-MS at *m*/*z* 635.1583 $[M+Na]^+$ (Calcd 635.1578), and confirmed by ^{13}C-NMR spectrum. The characteristic UV absorption bands, ^{1}H-NMR, and ^{13}C-NMR spectra were resembled to those of **4**, except that a methoxyl group signal at C-3′ of **4** was replaced by a hydroxyl group signal of **5** (δ_H 8.74, br s, D_2O exchangeable) in ^{1}H-NMR. Two anomeric protons at δ_H 4.96 (1H, d, J=8.0 Hz) and at δ_H 4.72 (1H, d, J=7.1 Hz) in the ^{1}H-NMR spectrum and their corresponding carbons at δ_C 99.7 and 102.3 in the ^{13}C-NMR spectrum indicated that **5** also contained two β-oriented glucose moieties. Similarly, these two β-oriented glucose moieties were assigned to C-7 and C-4′, respectively, by HMBC analysis. The stereochemistry at C-2 of **5** was determined to be *R* due to the presence of a negative Cotton effect at 334 nm and a positive Cotton effect at 288 nm in the CD spectrum.[11,12] Therefore, compound **5** was established as (2*R*)-eriodictyol 7,4′-di-*O*-β-D-glucopyranoside.

In the assay of antioxidative activities, compounds **1**—**5** all exhibited significant scavenging effects on both hydroxyl radical and superoxide anion radical (Table 1). Comparatively, compounds **4** and **5** had no obvious differences in the antioxidative activities from their precursors, **1** and **2**. Compound **3** showed less antioxidative effects than did the other four flavanone glycosides. These results suggested that a hydroxyl or methoxyl substitution at C-3′ might have increased their antioxidative activities. This suggestion was consistent with reports that a catechol type B-ring was essential for a strong radical scavenging activity.[13,14] These flavonoids would be useful for the treatment of various diseases mediated by reactive oxygen species.

Table 1. Hydroxyl Radical and Superoxide Anion Radical Scavenging Activities of Compounds **1**—**5**

Compound	IC_{50} (mM)	
	Hydroxyl radical	Superoxide anion
1	0.25	0.23
2	0.28	0.30
3	0.33	0.49
4	0.21	0.39
5	0.18	0.25
EGCG	0.58	0.53

Experimental

General IR spectra were recorded on NEXUS47O FT-IR instrument with KBr pellets. UV spectra were recorded on a Lengguang Tech Spectrumlab 54 instrument. 1D NMR spectra were obtained on a Bruker DMX-500 with TMS as an internal standard and DMSO-d_6 as solvent. 2D NMR data were obtained using the standard XWIN-NMR 3.1 software package (Bruker). ESI-MS data were ontained on a Bruker APEXIII 7.0 TESLA FTMS mass spectrometer. CD data was recorded on a JASCO J-715 instrument. Column chromatography was performed on silica gel H (10—40 μm, Qingdao Marine Chemical Factory, Qingdao, Shandong, China), D-101 resin (Tianjin Farm Chemical Factory, Tianjing, China), and Sephadex LH-20 (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Materials The branches and leaves of *Viscum coloratum* (KOMAR) NAKAI were collected in July 2002, from Yunnan Province of China, and authenticated by Dr. Tong-Shui Zhou, School of Life Sciences, Fudan University, Shanghai, China. A voucher specimen (No. Z200298) was deposited in the Herbarium of Fudan University, Shanghai, China. Xanthine (X), xanthine oxidase (XO) and (−)-epigallocatechin gallate (EGCG) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). All other reagents were local products of analytical grade and were used without further purification.

Extraction and Isolation Dried branches and leaves (2 kg) were refluxed with 90% EtOH twice for 2 h. The combined liquid extracts were removed of solvent *in vacuo* to afford a residue (82 g), which was re-suspended in H_2O (10 l) and extracted with petroleum (2 l×5). The H_2O phase was filtered and the filtrate was loaded onto a column containing D_{101} resin (1 kg), and then eluted sequentially with H_2O followed by 20%, 40%, 60%, 80% and 95% aqueous EtOH (2.5 l each). The fraction eluted from 40% EtOH (24 g) was chromatographed on a Sephadex LH-20 column (5×45 cm) which was eluted with aqueous MeOH (50—80%, gradient) to give four fractions. Fraction 2a (60 mg) was further separated on an ODS column (2×25 cm) eluted with 40% MeOH to yield compound **4** (30 mg) and compound **5** (15 mg). Fractions 2b, 2c, and 2d were each purified by Sephadex LH-20 eluted with aqueous MeOH (60—80%, gradient), and compound **1** (400 mg), **2** (280 mg), and **3** (25 mg) were obtained.

(2*S*)-Homoeriodictyol 7,4′-di-*O*-β-D-Glucopyranoside (**4**): Pale yellow amorphous powder; UV λ_{max} (MeOH) nm (log ε): 282, 330 (sh); IR (KBr): ν_{max} cm^{-1}: 3385, 2918, 1639, 1578, 1516, 1452, 1425, 1371, 1298, 1269, 1196, 1173, 1082, 1043, 1028; CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 333 (+1.99), 287 (−13.84); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 12.05 (1H, br s, OH), 7.17 (1H, d, J=2.0 Hz, H-2′), 7.13 (1H, d, J=8.5 Hz, H-5′), 7.01 (1H, dd, J=8.5, 2.0 Hz, H-6′), 6.20 (1H, d, J=2.0 Hz, H-8), 6.15 (1H, d, J=2.0 Hz, H-6), 5.55 (1H, dd, J=12.3, 3.0 Hz, H-2), 4.96 (1H, d, J=7.0 Hz, H-1″), 4.93 (1H, d, J=8.5 Hz, H-1‴), 3.79 (3H, s, OMe-3′), 3.15 (1H, m, H_{trans}-3), 2.79 (1H, dd, J=17.0, 3.0 Hz, H_{cis}-3); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 42.6 (C-3), 56.3 (OMe-3′), 60.0 and 60.1 (C-6″, C-6‴), 70.0 and 70.1 (C-4″, C-4‴), 73.5 and 73.6 (C-2″, C-2‴), 76.8 and 77.3 (C-3″, C-3‴), 77.5 and 77.6 (C-5″, C-5‴), 79.1 (C-2), 96.0 (C-8), 97.1 (C-6), 100.1 (C-1″), 100.5 (C-1‴), 103.7 (C-10), 112.0 (C-2′), 115.7 (C-5′), 119.8 (C-6′), 132.4 (C-1′), 147.3 (C-4′), 149.4 (C-3′), 163.1 (C-9), 163.4 (C-5), 165.8 (C-7), 197.4 (C-4); ESI-MS: *m*/*z*: 649 $[M+Na]^+$, 627 $[M+H]^+$; HR-ESI-MS: *m*/*z*: 649.1739 $[M+Na]^+$ (Calcd for $C_{28}H_{34}O_{16}Na$: 649.1735).

(2*R*)-Eriodictyol 7,4′-di-*O*-β-D-Glucopyranoside (**5**): Pale yellow amorphous solid; UV λ_{max} (MeOH) nm (log ε): 282, 330 (sh); IR (KBr): ν_{max}

cm^{-1}: 3381, 2922, 1639, 1581, 1537, 1512, 1444, 1365, 1298, 1282, 1192, 1173, 1078, 1043, 1024 cm^{-1}; CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 334 (−1.99), 288 (+19.56); ^{1}H-NMR (500 MHz, DMSO-d_6,) δ: 12.03 (1H, br s, OH), 8.74 (1H, br s, OH), 7.14 (1H, d, J=8.5 Hz, H-5′), 6.98 (1H, d, J=2.0 Hz, H-2′), 6.89 (1H, dd, J=8.5, 2.0 Hz, H-6′), 6.17 (1H, d, J=2.0 Hz, H-8), 6.13 (1H, d, J=2.0 Hz, H-6), 5.51 (1H, dd, J=12.6, 3.0 Hz, H-2), 4.96 (1H, d, J=8.0 Hz, H-1″), 4.72 (1H, d, J=7.1 Hz, H-1‴), 3.15 (1H, m, H_{trans}-3), 2.80 (1H, dd, J=17.6, 3.0 Hz, H_{cis}-3); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 42.2 (C-3), 60.7 and 60.9 (C-6″, C-6‴), 69.7 and 70.0 (C-4″, C-4‴), 73.1 and 73.4 (C-2″, C-2‴), 76.0 and 76.4 (C-3″, C-3‴), 77.2 and 77.3 (C-5″, C-5‴), 78.4 (C-2), 95.6 (C-8), 96.7 (C-6), 99.7 (C-1″), 102.3 (C-1‴), 103.4 (C-10), 114.6 (C-2′), 116.8 (C-5′), 118.0 (C-6′), 133.2 (C-1′), 145.7 (C-4′), 147.0 (C-3′), 162.7 (C-9), 163.0 (C-5), 165.4 (C-7), 197.0 (C-4); ESI-MS: m/z: 635 $[M+Na]^+$, 613 $[M+H]^+$; HR-ESI-MS: m/z: 635.1583 $[M+Na]^+$ (Calcd for $C_{27}H_{32}O_{16}Na$: 635.1578).

Hydroxyl Radical Scavenging Assay The hydroxyl radical (·OH) scavenging assay was carried out by measuring the clearance of test compounds for hydroxyl radicals generated from the H_2O_2/Fe^{2+} system.[15] The reaction mixture contained 10 mmol salicylic acid, 4 mmol $FeSO_4$ dissolved in 2 mmol EDTA, 0.4 mol phosphate buffered saline (pH 7.4). After samples (100 μl) of various concentrations were added, 20 mmol H_2O_2 (100 μl) was added to activate the reaction. After co-incubation at 37 °C for 2 h, the absorption value at 510 nm was measured with a Shimadzu UV-260 spectrophotometer. EGCG was used as a positive control and the solvent dissolving samples was used as blank. Reactions were carried out in triplicates. The relative activity was calculated from the ratio of sample treated with all compounds *versus* blank at the same concentrations and time. And the effective activity of each sample was expressed in terms of IC_{50}.

Superoxide Anion Radical Scavenging Assay The scavenging assay of superoxide anion radicals ($O_2^{\cdot-}$) was evaluated as previously described by Beauchamp.[16] The reaction mixture contained 1 mmol xanthine (X), 10 μmol·min^{-1}·l^{-1} xanthine oxidase (XO), 25 μmol nitroblue tetrazolium (NBT) and 0.1 mmol EDTA, dissolved in 50 mmol sodium carbonate buffer (pH 10.2) to the final volume of 3.0 ml. Various concentrations of the test compounds were added into this reaction system. EGCG was used as positive control and the solvent used in dissolving samples was used as blank. The mixtures were incubated for 30 min at 25 °C. Absorbance of blue product was measured at 560 nm. The relative activity was calculated from the ratio of values of a sample treated with a compound *versus* blank at the same concentration and time. And the effective activity of each sample was expressed in terms of IC_{50}.

Acknowledgements This research was supported by Shanghai Commission of Science and Technology (grant No. 014319316).

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