The Inhibition of Superoxide Anion Generation by Neutrophils from *Viscum articulatum*

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Two new flavanones, (2*S*)-pinocembrin 7-*O*-[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (1**), and (2*S*)-pinocembrin 7-*O*-[cinnamoyl(1 $\rightarrow$ 5)]- β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (**2**) together with eighteen known compounds, which include five known flavanones, nine benzenoids, one inositol and three triterpenoids, were isolated and characterized from fresh *Viscum articulatum*. Structures of new compounds were determined by spectral analysis. Among them, oleanolic acid (**18**) showed a significant inhibition effect on superoxide anion generation by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP).**

Key words *Viscum articulatum*; Lorantheaceae; flavanone

The genus *Viscum*, plants of the Lorantheaceae family, are photosynthetic shrubby, hemiparasites on the tree branches of the Moraceae, Theaceae, Ranunculaceae, Rutaceae, Rosaceae and Leguminosae families. In clinical use, *Loranthus* genus plants could be replaced by *Viscum* genus plants, but they belong to different genera of Lorantheaceae. *Viscum articulatum* BURM. has commonly been used in Chinese medicine as a curative for a number of ailments such as hemorrhage, pleurisy, gout, heart disease, epilepsy, arthritis and hypertension.^{1,2} Previous investigations of *Viscum* have shown that the major components are flavonoids, triterpenoids and organic acids.²

As a part of our ongoing phytochemical work on Chinese medicinal plants, we examined *Viscum articulatum* and isolated two new and eighteen known compounds. This paper deals with the structural determination of two new flavanones, (2*S*)-pinocembrin 7-*O*-[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (**1**), and (2*S*)-pinocembrin 7-*O*-[cinnamoyl(1 $\rightarrow$ 5)]- β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (**2**) by means of spectral analysis. The inhibition activity on neutrophils stimulated by fMLP (formyl-L-methionyl-L-leucyl-L-phenyl-alanine)/CB (cytochalasin B) was tested.

Results and Discussion

(2*S*)-Pinocembrin 7-*O*-[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (**1**) was isolated as optically active colorless powder with a *pseudo*-molecular formula of C₂₆H₃₁O₁₃, deduced from its high resolution (HR)-FAB mass spectrum. Its UV absorption bands at 326 (sh) and 283 nm indicated the presence of a flavanone skeleton.^{3,4} IR absorption bands at 3500, 3422 and 1637 cm⁻¹ inferred the hydroxyl and carbonyl groups, respectively. Accordingly, a D₂O exchangeable sharp singlet integrated for one proton at δ 12.01 in the ¹H-NMR spectrum was characteristic of 5-OH. The ¹H-NMR spectrum of **1** showed the presence of three mutually coupled protons at δ 5.65 (1H, dd, *J*=12.4, 3.2 Hz), 3.30 (1H, dd, *J*=17.2, 12.4 Hz) and 2.83 (1H, dd, *J*=17.2, 3.2 Hz), corresponding to the moiety of flavanones H-2 and H-3. The presence of mono-substituted flavanone B-ring was confirmed by five mutually coupled protons at δ 7.56 (2H, d, *J*=7.6 Hz), and 7.41 (3H, m). In the ¹³C-NMR spectrum of **1**, the chemical shifts of the aglycone and glucose conformed to those of (2*S*)-pinocembrin 7-*O*- β -D-glucoside (**3**). An anomeric pro-

ton resonating as a doublet at δ 5.08 (1H, d, *J*=7.2 Hz) indicated the presence of β -glucose. There were five signals of apiose at δ 109.2, 79.7, 76.6, 74.4 and 64.6 in the ¹³C-NMR spectrum of **1**. The apiose was identified as β -form by ¹³C-NMR data, which was in agreement with published data for the sugar moiety of (2*S*)-homoeriodictyol 7-*O*-[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside.⁵ In the heteronuclear multiple bond connectivity (HMBC) spectrum, the signal at δ 5.08 (glucose anomeric proton) showed ³*J*-correlation with C-7 (δ _C 165.5) and the signals at δ 5.32 (apiose anomeric proton) and C-2 of glucose (δ _C 76.3) also showed ³*J*-correlation, suggesting that apiose moiety was attached to C-2 of glucose. This was confirmed by the fragments of FAB-MS at *m/z* 419 [M-133+H]⁺ and 257 [M-132-162+H]⁺. Acidic hydrolysis of **1** yielded pinocembrin, glucose and apiose detected by TLC.⁶ The D-apiose and D-glucose could be confirmed by their specific optical rotation showing +6.5° and +24°, respectively.^{7–8} The circular dichroism (CD) spectrum of **1** exhibited a positive Cotton effect at 326 nm and a negative Cotton effect at 283 nm. Therefore, C-2 was assigned as the *S*-configuration.⁹ Consequently, the structure of (2*S*)-pinocembrin 7-*O*-[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside was assigned as **1**.

Compound **2** was obtained as a white optically active colorless powder with a *pseudo*-molecular formula of C₃₅H₃₇O₁₄, determined by its HR-FAB mass spectrum. The UV absorption bands at 331 (sh) and 280 nm also indicated the presence of a flavanone skeleton.^{3,4} The hydroxyl and carbonyl groups were confirmed at 3500, 3422 and 1637 cm⁻¹ in its IR absorption bands, respectively.

Comparison of the ¹H- and ¹³C-NMR spectral data of compound **1** and **2** showed that **2** had a more cinnamic acid moiety. In the HMBC spectrum of **2**, the C-7 (δ _C 165.4) and

1 R = -[β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside

2 R = -[cinnamoyl(1 $\rightarrow$ 5)]- β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside

Fig. 1

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Table 1. Effects of Pure Compounds Isolated from *Viscum articulatum* on the Generation of Superoxide Anion in fMLP/CB or PMA-Stimulated Human Neutrophils

Compound ($\mu\text{g/ml}$)	$\text{O}_2^{\cdot -}$ production (nmol/ 10^6 cell)			
	fMLP/CB			PMA
	10	3	1	10
Control	30.41 $\pm$ 0.16			32.12 $\pm$ 0.89
1	29.91 $\pm$ 1.03			29.74 $\pm$ 2.92
2	24.35 $\pm$ 1.26**			31.03 $\pm$ 2.08
3	30.49 $\pm$ 0.83			31.21 $\pm$ 2.07
4	30.59 $\pm$ 2.59			32.64 $\pm$ 2.25
7	32.47 $\pm$ 0.73			N
18	3.58 $\pm$ 0.34***	7.55 $\pm$ 0.73***	9.16 $\pm$ 2.89***	29.82 $\pm$ 1.12

Results are expressed as mean $\pm$ S.E.M. of 3 separate experiments. **: $p < 0.01$, ***: $p < 0.001$ compared to the control value. N=no test.

anomeric proton of glucose (δ_{H} 5.10), C-2 of glucose (δ_{C} 76.0) and anomeric proton of apiose (δ_{H} 5.37), carbonyl group of cinnamic acid moiety (δ_{C} 166.3) and H-5 of apiose (δ_{H} 4.08) all showed 3J -correlation. Therefore, the sugar moiety of **2** was proved to be apiosyl(1 $\rightarrow$ 2)glucose and the cinnamic acid moiety was attached to C-5 of apiose. Acidic hydrolysis of compound **2** gave pinocembrin, glucose, apiose and cinnamic acid, respectively. The CD spectrum of **2** exhibited a positive Cotton effect at 331 nm and a negative Cotton effect at 280 nm. Therefore, C-2 of **2** was also assigned the *S*-configuration.⁹⁾ On the basis of the above results, the structure of compound **2** was established to be (2*S*)-pinocembrin 7-*O*-[cinnamoyl(1 $\rightarrow$ 5)- β -D-apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside.

The known compounds, (2*S*)-pinocembrin 7-*O*- β -D-glucoside (**3**),¹⁰⁾ (2*S*)-homoeriodictyol 7-*O*- β -D-glucoside (**4**),¹¹⁾ (2*S*)-5,3',4'-trihydroxyflavanone 7-*O*- β -D-glucoside (**5**),¹²⁾ (2*S*)-naringenin 7-*O*- β -D-glucoside (**6**),¹²⁾ (2*S*)-homoeriodictyol (**7**),¹¹⁾ *p*-hydroxybenzaldehyde (**8**),¹³⁾ vanillin (**9**),¹⁴⁾ methylparaben (**10**),¹³⁾ *p*-hydroxybenzoic acid (**11**),¹³⁾ protocatechuic acid (**12**),¹⁵⁾ 4- β -D-glucosyloxy-3-hydroxybenzoic acid (**13**),¹⁶⁾ 2-phenylethanol (**14**),¹⁷⁾ cinnamic acid methyl ester (**15**),¹⁸⁾ 4-*O*-cinnamoyl quinic acid (**16**),¹⁹⁾ β -amyrin acetate (**17**),²⁰⁾ oleanolic acid (**18**),²¹⁾ lupenyl acetate (**19**),²²⁾ and 2-deoxy-*epi*-inositol (**20**)²³⁾ were also isolated and identified by comparison of their spectral data with corresponding literature values.

Based on the screening results (Table 1), oleanolic acid (**18**) showed significantly anti-inflammatory activities and compound **2** demonstrated slight functions. Oleanolic acid (**18**) concentration-dependently inhibited superoxide anion generation by human neutrophils in response to fMLP, but not to PMA (phorbol myristate acetate). This data suggests that the inhibitory effect of oleanolic acid (**18**) is through the protein kinase c-independent pathway.

Experimental

Melting points were measured on a Yanagimoto MP-S3 micromelting point apparatus and were uncorrected. The UV spectra were recorded on a Hitachi U-3010 spectrophotometer in MeOH solution. The IR spectra were recorded on a Jasco IR Report-100 spectrophotometer as KBr discs. The ^1H - and ^{13}C -NMR spectra were recorded on a Bruker Avance-400 spectrometer. Chemical shifts are shown in δ values with tetramethylsilane as internal reference. The mass spectra were performed in the EI or FAB (matrix: glycerol) mode on a VG 70—250 S spectrometer. Specific rotations were determined on a Jasco P-1010 polarimeter.

Plant Material *Viscum articulatum* BURM. was collected and authenti-

cated by Prof. C. T. Chiu at Nantou, Taiwan. A voucher specimen (CGU-VA-1) was deposited in the herbarium of Chang Gung University, Taoyuan, Taiwan.

Extraction and Isolation Fresh *Viscum articulatum* BURM. (50 g) was extracted with MeOH (100 ml $\times$ 6) and concentrated to give brown syrup (8.496 g). The syrup was suspended in H_2O and partitioned with CHCl_3 . The CHCl_3 extract (2.104 g) was subjected to column chromatography over silica gel and eluted with CHCl_3 and MeOH step gradients to afford nine fractions. Repeated column chromatography of the second fraction over silica gel with *n*-hexane and chloroform mixtures yielded **17** (5 mg) and **19** (12 mg). The third fraction was applied on silica gel column and eluted with a gradient of *n*-hexane and acetone to give **9** (1 mg), **10** (1 mg) and **18** (4.5 mg). The fourth fraction was purified by recrystallisation to afford **7** (10 mg). The fifth fraction was repeatedly chromatographed over silica gel with CHCl_3 to get **8** (0.5 mg). The H_2O layer (6.392 g) was applied on Diaion HP-20 gel and eluted with gradients of H_2O and MeOH to give six fractions. The second fraction was chromatographed on Sephadex LH-20 column and eluted with gradients of H_2O and MeOH to afford **11** (2 mg), **12** (3 mg), and **13** (1.5 mg), successively. The fifth fraction was repeatedly column chromatographed over silica gel with CHCl_3 : MeOH gradients to give **4** (25 mg), **5** (1 mg), and **6** (3.5 mg), successively. The sixth fraction was also rechromatographed as above to obtain **1** (5.0 mg), **2** (4.5 mg), and **3** (6.0 mg).

Acidic Hydrolysis of 1 and 2 Compound **1** and **2** (each 3 mg) was dissolved in 5% HCl/ H_2O (2 ml) and refluxed for 1 h, separately. The sugar components were identified by TLC (on Si gel, developed with *n*-butyl acetate/2-butanone/acetic acid/ H_2O =6.0:2.5:1.2:0.3) as apiose and glucose in comparison with authentic samples. The reaction mixtures were also applied on Sephadex G-10 column to gain D-apiose ($[\alpha]_{\text{D}} + 6.5^\circ$ ($c=0.025$, H_2O)) and D-glucose ($[\alpha]_{\text{D}} + 24^\circ$ ($c=0.03$, H_2O)), respectively.

Preparation of Human Neutrophils Human neutrophils from venous blood of healthy, adult volunteers (18—32 years old) were isolated by a standard method of dextran sedimentation prior to centrifugation in Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by trypan blue exclusion, were resuspended in HBSS buffer at pH 7.4 and kept at 4 $^\circ\text{C}$ before use.

Measurement of Superoxide Anion ($\text{O}_2^{\cdot -}$) Generation The measurement of the generation of $\text{O}_2^{\cdot -}$ was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*.²⁴⁾ In brief, after supplement with ferricytochrome *c* (0.5 mg/ml), neutrophils ($10^6/\text{ml}$) were equilibrated at 37 $^\circ\text{C}$ for 2 min and incubated with either control or different concentrations of tested compounds for 5 min. Cells were activated by fMLP or PMA for 10 min. When fMLP was used as stimulant, CB (1 $\mu\text{g/ml}$) was incubated for 3 min before peptide activation. The changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring. Calculation is based on the difference of the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\epsilon=21.1/\text{mm}/10\text{ mm}$).

(2*S*)-Pinocembrin 7-*O*-[β -D-Apiosyl(1 $\rightarrow$ 2)]- β -D-glucoside (**1**): Colorless powder (MeOH). mp: 204—205 $^\circ\text{C}$. $[\alpha]_{\text{D}} -107.6^\circ$ ($c=0.01$, MeOH). IR ν_{max} cm^{-1} : 500, 3422, 1637, 1611, 1218, 1194, 1082. UV λ_{max} nm: 326 (sh) (3.94), 283 (4.22). HR-FAB-MS: Calcd for $\text{C}_{26}\text{H}_{31}\text{O}_{13}$ m/z $[\text{M}+1]^+$ 551.1755, Found 551.1769. FAB-MS m/z (rel. int. %): 551 ($[\text{M}+1]^+$, 2), 419 (2), 307 (25), 257 (8), 154 (100), 136 (71). ^1H -NMR (DMSO- d_6 , 400 MHz)

δ : 12.02 (1H, s, 5-OH), 7.53 (2H, d, $J=7.6$ Hz, H-2', H-6'), 7.42 (3H, m, H-3', H-4', H-5'), 6.18 (1H, d, $J=2.0$ Hz, H-8), 6.12 (1H, d, $J=2.0$ Hz, H-6), 5.65 (1H, dd, $J=12.8, 2.4$ Hz, H-2), 5.32 (1H, s, api. H-1), 5.24 (1H, d, $J=5.2$ Hz, glc. 3-OH), 5.10 (1H, d, $J=5.2$ Hz, glc. 4-OH), 5.08 (1H, d, $J=7.2$ Hz, glc. H-1), 5.03 (1H, d, $J=5.6$ Hz, api. 2-OH), 4.62 (1H, t, $J=6.0$ Hz, api. 5-OH), 4.56 (1H, dd, $J=5.6, 4.8$ Hz, glc. 6-OH), 4.48 (1H, s, api. 3-OH), 3.85 (1H, d, $J=9.2$ Hz, api. H-4), 3.73 (1H, d, $J=5.6$ Hz, api. H-2), 3.66 (1H, m, glc. H-6), 3.62 (1H, d, $J=9.2$ Hz, api. H-4), 3.47 (1H, m, glc. H-2), 3.45 (1H, m, glc. H-3), 3.40 (1H, m, glc. H-6), 3.37 (1H, m, glc. H-5), 3.32 (1H, dd, $J=17.2, 12.8$ Hz, H-3), 3.27 (2H, d, $J=6.0$ Hz, api. H-5), 3.14 (1H, m, glc. H-4), 2.84 (1H, dd, $J=17.2, 2.8$ Hz, H-3). ^{13}C -NMR (DMSO- d_6 , 100 MHz) δ : 197.2 (C-4), 165.5 (C-7), 163.4 (C-5), 163.0 (C-9), 138.9 (C-1'), 129.1 (C-4'), 129.0 (C-3', C-5'), 127.1 (C-2', C-6'), 109.2 (api. C-1), 103.8 (C-10), 98.3 (glc. C-1), 97.1 (C-6), 95.8 (C-8), 79.7 (api. C-3), 79.1 (C-2), 77.4 (glc. C-5), 77.1 (glc. C-3), 76.6 (api. C-2), 76.3 (glc. C-2), 74.4 (api. C-4), 70.2 (glc. C-4), 64.6 (api. C-5), 61.0 (glc. C-6), 42.7 (C-3). CD ($c=6.0 \times 10^{-5}$, MeOH): $[\theta]_{(326)} +169000$, $[\theta]_{(283)} -770700$.

(2S)-Pinocembrin 7-O-[Cinnamoyl(1 $\rightarrow$ 5)- β -D-xylopyranosyl(1 $\rightarrow$ 2)]- β -D-glucoside (2): White powder (MeOH). mp: 170–172 °C. $[\alpha]_D -129.7$ ($c=0.035$, MeOH). IR ν_{max} cm^{-1} : 3447, 3355, 1698, 1650, 1181, 1090. UV λ_{max} nm: 331 (sh) (3.50), 280 (4.25). HR-FAB-MS: Calcd for $\text{C}_{35}\text{H}_{37}\text{O}_{14}$ m/z $[\text{M}+1]^+$ 681.2172, Found 681.2180. FAB-MS m/z (rel. int. %): 681 ($[\text{M}+1]^+$, 2), 307 (16), 257 (27), 154 (100), 136 (71). ^1H -NMR (DMSO- d_6 , 400 MHz) δ : 11.94 (1H, s, 5-OH), 7.59 (2H, m, H-2', H-6'), 7.52 (1H, d, $J=16.0$ Hz, H-3'), 7.40 (8H, m, H-3'–5', H-5'–9'), 6.44 (1H, d, $J=16.0$ Hz, H-2''), 6.12 (1H, d, $J=2.0$ Hz, H-8), 6.09 (1H, d, $J=2.0$ Hz, H-6), 5.43 (1H, dd, $J=12.8, 2.8$ Hz, H-2), 5.42 (1H, d, $J=5.2$ Hz, api. 2-OH), 5.37 (1H, s, api. H-1), 5.30 (1H, d, $J=5.2$ Hz, glc. 3-OH), 5.11 (1H, d, $J=5.2$ Hz, glc. 4-OH), 5.10 (1H, d, $J=7.2$ Hz, glc. H-1), 4.93 (1H, s, api. 3-OH), 4.56 (1H, dd, $J=5.6, 5.2$ Hz, glc. 6-OH), 4.08 (2H, s, api. H-5), 4.01 (1H, d, $J=9.6$ Hz, api. H-4), 3.76 (1H, d, $J=5.2$ Hz, api. H-2), 3.65 (1H, m, glc. H-6), 3.62 (1H, d, $J=9.6$ Hz, api. H-4), 3.50 (1H, m, glc. H-2), 3.48 (1H, m, glc. H-3), 3.43 (1H, m, glc. H-5), 3.39 (1H, m, glc. H-6), 3.17 (1H, dd, $J=17.2, 12.8$ Hz, H-3), 3.15 (1H, m, glc. H-4), 2.68 (1H, dd, $J=17.2, 2.8$ Hz, H-3). ^{13}C -NMR (DMSO- d_6 , 100 MHz) δ : 196.9 (C-4), 166.2 (C-1'), 165.4 (C-7), 163.5 (C-5), 162.9 (C-9), 144.9 (C-3''), 138.9 (C-1'), 134.4 (C-4''), 130.8, 129.4, 129.1, 129.0, 128.7, 127.1 (C-2'–6', C-5'–9''), 118.1 (C-2''), 108.7 (api. C-1), 103.7 (C-10), 98.0 (glc. C-1), 96.8 (C-6), 95.7 (C-8), 79.0 (C-2), 77.9 (api. C-2), 77.4 (C-5), 77.1 (glc. C-3, api. C-2), 76.0 (glc. C-2), 74.2 (api. C-4), 70.2 (glc. C-4), 67.2 (api. C-5), 60.9 (glc. C-6), 42.6 (C-3). CD ($c=9.1 \times 10^{-5}$, MeOH): $[\theta]_{(331)} +906200$, $[\theta]_{(280)} -4000000$.

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