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

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Two new flavanones, (2*S*)-pinocembrin 7-*O*-[β-D-apiosyl(1→2)]-β-D-glucoside (1), and (2*S*)-pinocembrin 7-*O*-[cinnamoyl(1→5)-β-D-apiosyl(1→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 articulactum*. 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 articulactum*; Loranthaceae; flavanone

The genus *Viscum*, plants of the Loranthaceae family, are photosynthetic shrubby, hemiparasites on the tree branches of the Moraceae, Theaceae, Ranunculaceae, Rutaceae, Rosaceae and Leguminosae families. In clinical use, *Lorthanthus* genus plants could be replaced by *Viscum* genus plants, but they belong to different genera of Loranthaceae. *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]

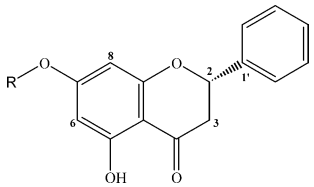
As a part of our ongoing phytochemical work on Chinese medicinal plants, we examined *Viscum articulactum* and isolated two new and eighteen known compounds. This paper deals with the structural determination of two new flavanones, (2*S*)-pinocembrin 7-*O*-[β-D-apiosyl(1→2)]-β-D-glucoside (**1**), and (2*S*)-pinocembrin 7-*O*-[cinnamoyl(1→5)-β-D-apiosyl(1→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→2)]-β-D-glucoside (**1**) was isolated as optically active colorless powder with a *pseudo*-molecular formula of $C_{26}H_{31}O_{13}$, 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 D_2O exchangeable sharp singlet integrated for one proton at δ 12.01 in the ^{1}H-NMR spectrum was characteristic of 5-OH. The ^{1}H-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 ^{13}C-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 proton 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 ^{13}C-NMR spectrum of **1**. The apiose was identified as β-form by ^{13}C-NMR data, which was in agreement with published data for the sugar moiety of (2*S*)-homoeriodictyol 7-*O*-[β-D-apiosyl(1→2)]-β-D-glucoside.[5] In the heteronuclear multiple bond connectivity (HMBC) spectrum, the signal at δ 5.08 (glucose anomeric proton) showed ^3J-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 ^3J-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→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 $C_{35}H_{37}O_{14}$, 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 ^{1}H- and ^{13}C-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→2)]-β-D-glucoside
2 R=−[cinnamoyl(1→5)-β-D-apiosyl(1→2)]-β-D-glucoside

Fig. 1

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

Compound	$O_2^{\cdot-}$ production (nmol/10^6 cell)			
	fMLP/CB			PMA
(μg/ml)	10	3	1	10
Control	30.41±0.16			32.12±0.89
1	29.91±1.03			29.74±2.92
2	24.35±1.26**			31.03±2.08
3	30.49±0.83			31.21±2.07
4	30.59±2.59			32.64±2.25
7	32.47±0.73			N
18	3.58±0.34***	7.55±0.73***	9.16±2.89***	29.82±1.12

Results are expressed as mean±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→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→5)-β-D-apiosyl(1→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*-hydroxy-benzoic acid (**11**),[13] protocatechuic acid (**12**),[15] 4-β-D-glucosyloxy-3-hydroxy-benzoic 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 ^{1}H- 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 authenticated 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×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 ($[\alpha]_D$ +6.5° (c=0.025, H_2O)) and D-glucose ($[\alpha]_D$ +24° (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 °C before use.

Measurement of Superoxide Anion ($O_2^{\cdot-}$) Generation The measurement of the generation of $O_2^{\cdot-}$ 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/ml) were equilibrated at 37 °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 μg/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* (ε=21.1/mM/10 mm).

(2*S*)-Pinocembrin 7-*O*-[β-D-Apiosyl(1→2)]-β-D-glucoside (**1**): Colorless powder (MeOH). mp: 204—205 °C. $[\alpha]_D$ −107.6° (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 $C_{26}H_{31}O_{13}$ m/z $[M+1]^+$ 551.1755, Found 551.1769. FAB-MS m/z (rel. int. %): 551 ($[M+1]^+$, 2), 419 (2), 307 (25), 257 (8), 154 (100), 136 (71). ^{1}H-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=6.0×10^{-5}, MeOH): $[\theta]_{(326)}$+169000, $[\theta]_{(283)}$−770700.

(2*S*)-Pinocembrin 7-*O*-[Cinnamoyl(1→5)-β-D-apiosyl(1→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 $C_{35}H_{37}O_{14}$ m/z $[M+1]^+$ 681.2172, Found 681.2180. FAB-MS m/z (rel. int. %): 681 ($[M+1]^+$, 2), 307 (16), 257 (27), 154 (100), 136 (71). ^{1}H-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=9.1×10^{-5}, MeOH): $[\theta]_{(331)}$+906200, $[\theta]_{(280)}$−4000000.

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References

1) Chiu S. T., "Flora of Taiwan," 2nd ed., Vol. II, Editorial Committee of the Flora of Taiwan, Taipei, 1996, pp. 282—285.
2) Chiu S. T., "Medicinal Resource of Formosan Mistletoes, Proceedings of International Symposium on Plant Biodiversity and Development of Bioactive Natural Products," Taichung, Nov. 18—20, Taiwan, 2001, pp. 103—116.
3) Mabry T. J., Markham K. R., Thomas M. B., "The Systematic Identification of Flavonoids," Springer-Verlag, New York, 1975, pp. 33—35.
4) Harborne J. B., Mabry T. J., "The Flavonoids," Chapman and Hall, London, 1975, pp. 45—77.
5) Fukunaga T., Kajikawa I., Nishiya K., Takeya K., Itokawa H., *Chem. Pharm. Bull.*, **37**, 1300—1303 (1989).
6) Lin J. H., Chiou Y. N., Lin Y. L., *J. Nat. Prod.*, **65**, 638—640 (2002).
7) Kyriakopoulou I., Magiatis P., Skaltsounis A.-L., Aligiannis N., Harvala C., *J. Nat. Prod.*, **64**, 1095—1097 (2001).
8) Mimaki Y., Kanmoto T., Sashida Y., Nishino A., Satomi Y., Nishino H., *Phytochemistry*, **41**, 1405—1410 (1996).
9) Prescott A. G., Stamford N. P. J., Wheeler G., Firmin J. L., *Phytochemistry*, **60**, 589—593 (2002).
10) Lin J. H., LinY. T., *J. Food Drug Anal.*, **7**, 185—190 (1999).
11) Kong D. Y., Luo S. Q., Li H. T., Lei X. H., *Acta Pharm. Sinica*, **23**, 593—600 (1988).
12) Liu K. C., Lee S. S., *Zhonghua Yaoxue Zazhi*, **45**, 231—235 (1993).
13) Wu T. S., Leu Y. L., Chan Y. Y., *Chem. Pharm. Bull.*, **47**, 571—573 (1999).
14) Wu T. S., Leu Y. L., Chan Y. Y., *Biol. Pharm. Bull.*, **23**, 1216—1219 (2000).
15) Zhang H. L., Nagatsu A., Okuyama H., Mizukami H., Sakakibara J., *Phytochemistry*, **48**, 665—668 (1998).
16) Cullmann F., Becker H., *Phytochemistry*, **52**, 1651—1656 (1999).
17) Chang M. H., Wang G. J., Kuo Y. H., Lee C. K., *J. Chin. Chem. Soc.*, **47**, 1131—1136 (2000).
18) Toyota M., Saito T., Matsunami J., Asakawa Y., *Phytochemistry*, **44**, 1265—1270 (1997).
19) Hanson K. R., *Biochemistry*, **4**, 2719—2731 (1965).
20) Leu Y. L., Shi L. S., Damu A. G., *Chem. Pharm. Bull.*, **51**, 599—601 (2003).
21) Fuchino H., Saito T., Tanaka N., *Chem. Pharm. Bull.*, **43**, 1937—1942 (1995).
22) Wood C. A., Lee K., Vaisberg A. J., Kingston D. G. I., Neto C. C., Hammond G. B., *Chem. Pharm. Bull.*, **49**, 1477—1478 (2001).
23) Dubreuil D., Cleophax J., Almeida M. V., Liaigre J., Gero G. S., *Tetrahedron*, **53**, 16747—16766 (1997).
24) Hwang T. L., Hung H. W., Kao S. H., Teng C. M., Wu C. C., Cheng S. J. S., *Mol. Pharmacol.*, **64**, 1419—1427 (2003).