The Inhibition of Superoxide Anion Generation in Human Neutrophils by *Viscum coloratum*

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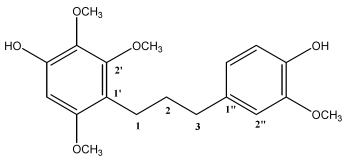
One new 1,3-diphenylpropane, viscolin (1), and one new flavanone, (2*S*)-7,4′-dihydroxy-5,3′-dimethoxyflavanone (2), together with thirty-nine known compounds, which included eleven known flavanones, two chromones, fourteen benzenoids, one inositol, two pyrimidines, four triterpenoids and five steroids, were isolated and characterized from *Viscum coloratum*. Structures of new compounds were determined by spectral analysis. Among them, viscolin (1) showed the most significant inhibition on superoxide anion generation by human neutrophils in response to fMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine).

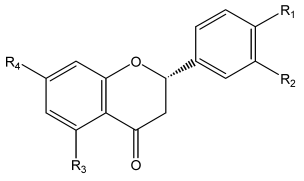
Key words *Viscum coloratum*; Loranthaceae; 1,3-diphenylpropane

In clinical use, *Taxillus* genus plants could be placed by *Viscum* genus plants. But, they belong to different genera of Loranthaceae. The genus of *Viscum*, the plants of Loranthaceae family, are photosynthetic shrubby, hemiparasites on the tree branches. *Viscum coloratum* has commonly been used for Chinese medicine as a curative for a number of ailments such as hemorrhage, pleurisy, gout, heart disease, epilepsy, arthritis and hypertension.[1,2] In our research, we found oleanolic acid showed significantly anti-inflammatory activitiy in *V. articulatum*.[3] Oleanolic acid concentration-dependently inhibited superoxide anion generation by human neutrophils in response to fMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine), but not to PMA (phorbol myristate acetate). It suggested that the inhibitory effect of oleanolic acid was through protein kinase C-independent pathway.

In a previous screening for the inhibitory activities on superoxide anion generation in fMLP-stimulated human neutrophils by *Viscum* and *Taxillus* medicinal plants, *V. coloratum* also exhibited the strong inhibition. From *V. coloratum*, two new and thirty-nine known compounds were isolated and characterized. This paper deals with the structural determination of two new compounds, viscolin (4′,4″-dihydroxy-2′,3′,6′,3″-tetramethoxy-1,3-diphenylpropane) (**1**) and (2*S*)-7,4′-dihydroxy-5,3′-dimethoxyflavanone (**2**) by spectral analysis. The inhibitory activities of crude extract and pure compounds on neutrophils stimulated by fMLP/CB were also tested.



1



2 R_1=OH, R_2=OMe, R_3=OMe, R_4=OH
3 R_1=OH, R_2=OH, R_3=OH, R_4=OH
4 R_1=OH, R_2=OH, R_3=OH, R_4=O.glc
5 R_1=OH, R_2=OMe, R_3=OH, R_4=OH
6 R_1=OH, R_2=OMe, R_3=OH, R_4=O.glc
7 R_1=OH, R_2=OMe, R_3=OH, R_4=O.glc(1->2)apiosyl
8 R_1=OH, R_2=H, R_3=OH, R_4=OH
9 R_1=OH, R_2=H, R_3=OH, R_4=O.glc
10 R_1=H, R_2=H, R_3=OH, R_4=OH
11 R_1=H, R_2=H, R_3=OH, R_4=O.glc
12 R_1=H, R_2=H, R_3=OH, R_4=O.glc(1->2)apiosyl
13 R_1=H, R_2=H, R_3=OH, R_4=O.glc(1->2)apiosyl(1->5)cinnamoyl

Results and Discussion

The crude extract and partitioned layers were examined for their anti-inflammatory effect using neutrophils. The chloroform soluble layer revealed significantly anti-inflammatory activities and the butanol soluble layer demonstrated slight functions in the results (Table 1). The resultant chloroform and butanol fractions were repeatedly subjected to silica gel and reverse phase column chromatographies and yielded two new compounds, viscolin (**1**) and (2*S*)-7,4′-dihydroxy-5,3′-dimethoxyflavanone (**2**).

Viscolin (**1**) was a pale yellow needles, and its chemical formula, $C_{19}H_{24}O_6$, was gained by HR-EI-MS (high resolution electron impact mass). UV spectrum of viscolin (**1**) revealed the maximum absorption at 282 and 229 (sh) nm and the absorption band at 3393 cm^{-1} showed hydroxyl group in its IR spectrum.

In the ^{1}H-NMR spectrum (Table 2) of viscolin (**1**), four methoxyl signals appeared at δ 3.88, 3.85, 3.82 and 3.74, respectively. In the aromatic region, an ABX splitting pattern and one singlet signals showed at δ 6.83, 6.72, 6.70 and 6.31 indicated a tri-substituted and a penta-substituted phenyl moieties in **1**. In addition, there were three mutual coupled methylene signals showed at δ 2.62, 2.60 and 1.79 confirmed

Table 1. Effects of Extracts from *V. coloratum* on the Generation of Superoxide Anion in fMLP/CB of PMA-Stimulated Human Neutrophils

Extracts (10 μg/ml)	$O_2^{\cdot -}$ production (nmol/10^6 cells)	
	fMLP/CB	PMA
Methanolic crude extract	19.48±1.14***	27.32±0.44
$CHCl_3$ layer	7.51±2.08***	29.21±0.90
n-BuOH layer	19.05±0.84***	28.28±0.83
H_2O layer	25.68±1.06	28.95±1.10
Control	31.43±0.31	32.46±0.22

Results are expressed as mean±S.E.M. of 3 separated experiments. *** $p<0.001$ compared to the control value.

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Table 2. ^{1}H- and ^{13}C-NMR Spectral Data, ^{1}H–^{13}C Long-Range Correlations and NOE Effects of **1** Recorded in $CDCl_3$

	^{1}H	^{13}C	^{2}J	^{3}J	NOE
1	2.60 (2H, t, J=7.6 Hz)	23.6	C-2, 1′	C-3, 2′, 6′	H-2
2	1.79 (2H, quint., J=7.6 Hz)	32.3	C-1, 3	C-1′, 1″	H-1, 3
3	2.62 (2H, t, J=7.6 Hz)	36.1	C-2, 1″	C-1, 2″, 6″	H-2, 2″
1′		116.5			
2′		151.7			
3′		133.9			
4′		147.8			
5′	6.31 (1H, s)	94.7	C-4′, 6′	C-1′, 3′	6′-OCH_3
6′		154.7			
1″		135.2			
2″	6.72 (1H, d, J=2.0 Hz)	111.4	C-3″	C-3, 4″, 6″	H-3, 3″-OCH_3
3″		146.6			
4″		143.9			
5″	6.83 (1H, d, J=8.4 Hz)	114.4	C-4″	C-1″, 3″	
6″	6.70 (1H, dd, J=8.4, 2.0 Hz)	121.3		C-2″, 4″	
OCH_3 (3″)	3.88 (3H, s)	56.3		C-3″	H-2″
OCH_3 (3′)	3.85 (3H, s)	61.3		C-3′	
OCH_3 (2′)	3.82 (3H, s)	61.0		C-2′	
OCH_3 (6′)	3.74 (3H, s)	56.1		C-6′	H-5′
OH	5.62 (2H, br s)				

by COSY spectrum.

The ^{13}C-NMR and HMQC spectra of viscolin (**1**) indicated four primary, three secondary, four tertiary and eight quaternary carbons. In the HMBC spectrum, the signal δ_H 2.62 (H-3) showed the ^{3}J correlations with δ_C 121.3 (C-6″), 111.4 (C-2″) and 23.6 (C-1), respectively, and the δ_H 2.60 (H-1) revealed the ^{3}J correlations with δ_C 154.7 (C-6′) and 151.7 (C-2′). These data showed that compound **1** had the 1,3-diphenylpropane skeleton. In addition, δ_C 154.7 (C-6′), 151.7 (C-2′), 133.9 (C-3′), 146.6 (C-3″) and methoxyl protons δ_H 3.74, 3.82, 3.85, 3.88 also showed ^{3}J correlations, respectively. Above these data, the nucleus of compound **1** could be determined.

The methoxyl substituted locations of compound **1** could be confirmed by NOESY experiment. From the foregoing spectral analyses, the structure **1** was assigned to viscolin (4′,4″-dihydroxy-2′,3′,6′,3″-tetramethoxy-1,3-diphenylpropane).

(2*S*)-7,4′-Dihydroxy-5,3′-dimethoxyflavanone (**2**) was isolated as optically active white powder with a molecular formula of $C_{17}H_{16}O_6$, deduced from its HR-EI-mass spectrum. Its UV absorption bands at 330 (sh), 282 and 228 (sh) nm indicated the presence of flavanone skeleton.[4] IR absorption bands at 3195 and 1652 cm^{-1} inferred the hydroxyl, and carbonyl groups, respectively. The ^{1}H-NMR spectrum of **2** showed the presence of three mutual coupled protons at δ 5.32 (1H, dd, J=12.4, 2.8 Hz), 3.02 (1H, dd, J=16.0, 12.4 Hz) and 2.53 (1H, dd, J=16.0, 2.8 Hz), corresponding to the moiety of flavanone H-2 and H_2-3. The presence of trisubstituted flavanone B-ring was confirmed by ABX splitting pattern coupled protons at δ 7.04 (1H, d, J=2.0 Hz), 6.87 (1H, dd, J=7.6, 2.0 Hz) and 6.77 (1H, d, J=7.6 Hz). The signals appeared at δ 6.05 (1H, d, J=2.0 Hz), 5.96 (1H, d, J=2.0 Hz) were the signals of H-6 and H-8. In the NOESY spectrum, the methoxyl signals at δ 3.77 and 3.73 showed NOE effects with H-2′ and H-6, separately. There was no hydrogen-bond signal appeared in the downfield region of ^{1}H-NMR spectrum. Thus, the two methoxyl groups were located at C-5 and C-3′ of compound **2**. The negative optical rotation of **2** inferred the *S* stereochemistry for C-2.[5] Consequently, the structure of (2*S*)-7,4′-dihydroxy-5,3′-dimethoxyflavanone was assigned as **2**.

The known compounds, (2*S*)-5,7,3′,4′-tetrahydroxyflavanone (**3**),[6] (2*S*)-5,3′,4′-trihydroxyflavanone-7-*O*-glucoside (**4**),[3] (2*S*)-homoeriodictyol (**5**),[3] (2*S*)-homoeriodictyol-7-*O*-glucoside (**6**),[3] (2*S*)-homoeriodictyol-7-*O*-[apiosyl(1→2)]-glucoside (**7**),[7] (2*S*)-naringenin (**8**),[8] (2*S*)-naringenin-7-*O*-glucoside (**9**),[3] (2*S*)-pinocembrin (**10**),[9] (2*S*)-pinocembrin-7-*O*-glucoside (**11**),[3] (2*S*)-pinocembrin-7-*O*-[apiosyl(1→2)]-glucoside (**12**),[3] (2*S*)-pinocembrin-7-*O*-[cinnamoyl(1→5)-apiosyl(1→2)]glucoside (**13**),[3] 5,7-dihydroxychromone (**14**),[10] 5-hydroxychromone-7-*O*-glucoside (**15**),[10] methyl-3-*O*-feruloylquinate (**16**),[11] 4-*O*-cinnamoylquinic acid (**17**),[3] oleanolic acid (**18**),[3] betulinic acid (**19**),[12] β-amyrin acetate (**20**),[3] lupeol (**21**),[13] β-sitostenone (**22**),[14] stigmasterol (**23**)+β-sitosterol (**24**) mixtures,[15] stigmasteryl-3-β-D-glucoside (**25**)+β-sitosteryl-3-β-D-glucoside (**26**) mixtures,[15] cinnamic acid (**27**),[16] coumaric acid (**28**),[15] ferulic acid (**29**),[15] caffeic acid (**30**),[15] 4-hydroxybenzaldehyde (**31**),[3] 4-hydroxybenzoic acid (**32**),[3] vanillin (**33**),[3] vanillic acid (**34**),[17] protocatechuic acid (**35**),[3] syringaldehyde (**36**),[17] acetovanillone (**37**),[18] 2,6-dimethoxy-*p*-benzoquinone (**38**),[19] 2-deoxy-*epi*-inositol (**39**),[3] thymine (**40**)[20] and uracil (**41**)[21] were also isolated and identified by comparison of their spectral data with corresponding literature values.

The inhibition of inflammatory responses in human neutrophils by two new components, eight flavanones and one chromone were further investigated. Our data (Table 3) suggest that the inhibition of inflammatory responses in human neutrophils by viscolin (**1**) is potent than oleanolic acid and cAMP/PKA-dependent, and that it occurs through inhibition of PDE activity. In addition, viscolin (**1**) also proved to be scavengers of the DPPH radical. The dual inhibition on PDE activity and radical generation might provide an attractive target in developing anti-inflammatory drugs. These findings thus reveal a new active ingredient and novel mechanism-mediated anti-inflammatory properties of *V. coloratum* in human neutrophils.

Table 3. Effects of Pure Compounds Isolated from *V. coloratum* on the Generation of Superoxide Anion in fMLP/CB or PMA-Stimulated Human Neutrophils

Compounds	$O_2^{\cdot -}$ production (nmol/10^6 cells)			
	fMLP/CB			PMA
Conc. (μg/ml)	10	3	1	10
1	1.75±0.47***	3.82±0.25***	6.42±0.77***	31.69±2.86
2	31.36±0.44	N	N	32.41±2.35
3[a)]	20.06±0.55	N	N	N
5	32.47±0.73	N	N	N
6	30.59±2.59	N	N	32.64±2.25
7	29.26±0.95	N	N	31.57±2.23
10	16.61±1.33**	21.73±0.80*	29.50±1.89	N
11	30.49±0.83	N	N	31.21±2.07
12	29.91±1.03	N	N	29.74±2.92
13	24.35±1.26**	N	N	31.03±2.08
15	29.61±2.17	N	N	32.64±2.43
18	3.58±0.34***	7.55±0.73***	9.16±2.89***	29.82±1.12
Control	30.41±0.16	N	N	32.12±0.89

Results are expressed as mean±S.E.M. of 3 separated experiments. $*p<0.1$ compared to the control value. $**p<0.01$ compared to the control value. $***p<0.001$ compared to the control value. N=no test. *a*) Alone induced superoxide anion generation by human neutrophils.

Experimental

General Experimental Procedures 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 Bruker Avance-400 spectrometer, with tetramethylsilane (TMS) as internal standard. Chemical shifts are shown in δ values. The mass spectra were performed in the EI or FAB (matrix: glycerol) mode on a VG 70-250S spectrometer. Specific rotations were determined on a Jasco P-1010 polarimeter.

Plant Material *V. coloratum* was supplied and authenticated by Chuang Song Zong pharmaceutical company at Pingtung, Taiwan. A voucher specimen (CGU-VC-1) was deposited in the herbarium of Chang Gung University, Taoyuan, Taiwan.

Extraction and Isolation Dry *V. coloratum* (3 kg) were extracted with MeOH (6 l×6) and concentrated to give brown syrup (1162.9 g). The syrup was suspended in H_2O and partitioned with $CHCl_3$ and *n*-BuOH, successively. The $CHCl_3$ extract (139.5 g) was subjected to column chromatography over silica gel and eluted with a $CHCl_3$ and MeOH stepwise gradients to afford eight fractions. Repeated column chromatography of the second fraction, over silica gel with *n*-hexane and acetone mixtures yielded β-amyrin acetate (**20**) (335 mg) and β-sitostenone (**22**) (2.3 mg). The third fraction was applied on silica gel column and eluted with a gradient of *n*-hexane and acetone to give viscolin (**1**) (142.3 mg), vanillin (**33**) (2.3 mg), acetovanillone (**37**) (0.8 mg), protocatechuic acid (**35**) (2.2 mg), syringaldehyde (**36**) (3.8 mg), lupeol (**21**) (4.5 mg) and stigmasterol (**23**)+β-sitosterol (**24**) mixtures (97.8 mg), separately. The fourth fraction was also subjected to column chromatography over silica gel and eluted with a $CHCl_3$ and MeOH step gradients to afford (2*S*)-pinocembrin (**10**) (30.4 mg) and betulinic acid (**19**) (3.1 mg). The fifth fraction was repeatedly chromatographed over silica gel with $CHCl_3$ and MeOH to get (2*S*)-homoeriodictyol (**5**) (61.8 mg), cinnamic acid (**27**) (3.2 mg), 2,6-dimethoxy-*p*-benzoquinone (**38**) (2.8 mg) and oleanolic acid (**18**) (18.9 mg). The sixth fraction was recrystalized to give stigmasteryl-3-β-D-glucoside (**25**)+β-sitosteryl-3-β-D-glucoside (**26**) mixture (35.4 mg). The seventh fraction was repeatedly chromatographed over silica gel with $CHCl_3$ and MeOH to afford 4-hydroxybenzoic acid (**32**) (1.0 mg). The *n*-BuOH layer (423.3 g) was applied on Diaion HP-20 gel and eluted with gradients of H_2O and MeOH to give eleven fractions. The second fraction recrystalized to afford 2-deoxy-*epi*-inositol (**39**) (285.9 mg). The forth fraction was chromatographed on silica gel column and eluted with gradients of EtOAc and MeOH to obtain 4-hydroxybenzaldehyde (**31**) (36.3 mg), thymine (**40**) (15.6 mg) and uracil (**41**) (0.7 mg). The sixth fraction was repeatedly column chromatographed over silica gel with $CHCl_3$: MeOH gradients to give 5-hydroxychromone-7-*O*-glucoside (**15**) (71.8 mg), methyl-3-*O*-feruloylquinate (**16**) (5.5 mg) and caffeic acid (**30**) (1.2 mg). The seventh fraction was also rechromatographed as above to obtain (2*S*)-5,3′,4′-trihydroxyflavanone-7-*O*-glucoside (**4**) (1.2 mg), (2*S*)-naringenin-7-*O*-glucoside (**9**) (2.2 mg), 4-*O*-cinnamoyl-quinic acid (**17**) (4.6 mg), vanillic acid (**34**) (0.8 mg), coumaric acid (**28**) (1.5 mg) and ferulic acid (**29**) (2.2 mg). The eighth fraction was treated as fraction seven to give (2*S*)-homoeriodictyol-7-*O*-glucoside (**6**) (8.5 mg), (2*S*)-homoeriodictyol-7-*O*-[apiosyl(1→2)]glucoside (**7**) (4.3 mg). (2*S*)-5,7,3′,4′-Tetrahydroxyflavanone (**3**) (11.3 mg), (2*S*)-pinocembrin-7-*O*-glucoside (**11**) (10.7 mg), (2*S*)-pinocembrin-7-*O*-[apiosyl(1→2)]glucoside (**12**) (8.3 mg), (2*S*)-7,4′-dihydroxy-5,3′-dimethoxyflavanone (**2**) (7.7 mg) and 5,7-dihydroxychromone (**14**) (1.2 mg) were purified by silica gel column chromatography from the ninth fraction. The tenth and eleventh fractions were recrystalized to gain (2*S*)-naringenin (**8**) (4.7 mg) and (2*S*)-pinocembrin-7-*O*-[cinnamoyl(1→5)-apiosyl(1→2)]glucoside (**13**) (2.5 mg), separately.

Preparation of Human Neutrophils Human neutrophils from venous blood of healthy, adult volunteers (18—32 years old) were isolated with 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*.[22] 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).

Viscolin (4′,4″-Dihydroxy-2′,3′,6′,3″-tetramethoxy-1,3-diphenylpropane) (**1**): Pale yellow needles powder ($CHCl_3$). mp: 118—121 °C. UV λ_{max} nm: 282 (3.95), 229 (sh) (4.05). IR ν_{max} cm^{-1}: 3393, 1615. EI-MS *m*/*z* (rel. int. %): 348 (M^+, 70), 197 (100), 184 (24), 137 (22). HR-EI-MS: Calcd for $C_{19}H_{24}O_6$ *m*/*z* $[M]^+$ 348.1567, Found 348.1573. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: see Table 2. ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: see Table 2.

(2*S*)-7,4′-Dihydroxy-5,3′-dimethoxyflavanone (**2**): White powder (MeOH). $[\alpha]_D$ −12.3° (*c*=0.01, MeOH). mp: 143—146 °C. UV λ_{max} nm: 330 (sh) (3.50), 282 (3.94), 228 (sh) (4.20). IR ν_{max} cm^{-1}: 3195, 1652. EI-MS *m*/*z* (rel. int. %): 316 (M^+, 57), 180 (28), 167 (100), 150 (68), 137 (35), 69 (16). HR-EI-MS: Calcd for $C_{17}H_{16}O_6$ *m*/*z* $[M]^+$ 316.0941, Found 316.0951. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 10.45 (1H, s, D_2O exchange disap., OH), 9.02 (1H, s, D_2O exchange disap., OH), 7.04 (1H, d, *J*=2.0 Hz, H-2′), 6.87 (1H, dd, *J*=7.6, 2.0 Hz, H-6′), 6.77 (1H, d, *J*=7.6 Hz, H-5′), 6.05 (1H, d, *J*=2.0 Hz, H-6), 5.96 (1H, d, *J*=2.0 Hz, H-8), 5.32 (1H, dd, *J*=12.4, 2.8 Hz, H-2), 3.77 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 3.02 (1H, dd, *J*=16.0, 12.4 Hz, H-3), 2.53 (1H, dd, *J*=16.0, 2.8 Hz, H-3).

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