Flavonoid Constituents from *Spiranthes australis* LINDL.

Mei-Ling DONG,[a,#] Guang CHEN,[b,#] and Zhi-Ming ZHOU*,[a]

[a] *School of Chemical Engineering and Environment, Beijing Institute of Technology; Beijing, 100081, P. R. China: and*
[b] *College of Life Science and Technology, Beijing University of Chemical Technology; Beijing, 100029, P. R. China.*
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Chemical investigation of the whole plant of *Spiranthes australis* (R. BROWN) LINDL. resulted in the isolation and characterization of three new flavonoid constituents, 5-hydroxy-4′-[(2-isopentenyl)oxy]-3,7,3′-trimethoxy-flavone (1), 3-*O*-{[*O*-*β*-D-xylopyranosyl]-(1→2)-*β*-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (2) and 3-*O*-{*O*-[2-*O*-(*E*)-*p*-coumaroyl-*β*-D-xylopyranosyl]-(1→2)-*β*-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (3), together with six known flavonoid compounds. The structures of new compounds were determined on the basis of spectroscopic analysis, including HR-ESI-MS, 1D- and 2D-NMR techniques and chemical methods.

Key words Orchidacae; *Spiranthes australis* LINDL.; flavonoid

The traditional Chinese medicine "Panlongcen" is derived from the dried root or whole plant of *Spiranthes australis* (R. BROWN) LINDL., which is an Orchidacea plant distributed in south China. The "Panlongcen" is used as a folk medicine for the treatment of bacterial and inflammatory diseases, such as cancer, blood and chest disorders.[1,2] Previous investigations of *Spiranthes australis* (R. BROWN) LINDL. have yielded flavanoids,[2,3] homocyclotriucallanes and dihydrophenanthrenes.[4,5]

In a previous paper, we had described the characterization of a new flavonoid compound from *Spiranthes australis* (R. BROWN) LINDL.[3] Our continuing chemical studies on this plant have resulted in the isolation of three new flavonoid compounds, 5-hydroxy-4′-[(2-isopentenyl)oxy]-3,7,3′-trimethoxyflavone (**1**), 3-*O*-{[*O*-*β*-D-xylopyranosyl]-(1→2)-*β*-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (**2**) and 3-*O*-{*O*-[2-*O*-(*E*)-*p*-coumaroyl-*β*-D-xylopyranosyl]-(1→2)-*β*-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (**3**), together with six known flavonoid compounds, 3-*O*-[*β*-D-xyloyranosyl-(1→2)-*β*-D-glucopyranosyl]-kaempferol (**4**),[6] 7-*O*-*β*-D-glucopyranosyl kaempferol (**5**),[7] paeonoside (**6**),[8] quercimeritrin (**7**),[9] retusine (**8**),[10] 3,5-dihydroxy-4′,7-dimethoxy flavone (**9**).[11] This paper reports the isolation and the structural elucidation of three new flavonoid compounds **1**—**3**.

Results and Discussion

The whole plant of *Spiranthes australis* (R. BROWN) LINDL. was extracted with 70% EtOH and the EtOH extract was then concentrated. The residue was dissolved in water and partitioned with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH, successively. The *n*-BuOH extract was then chromatographed by silica gel, Sephadex LH-20, HPLC to afford compounds **1**—**3**.

Compound **1** was obtained as yellow needles, whose molecular formula was deduced as $C_{23}H_{24}O_7$ by high resolution ESI-MS (HR-ESI-MS) (*m*/*z* 413.1588 $[M+H]^+$). In the ^{1}H-NMR spectrum of compound **1** (Table 1), a phenol proton appeared at δ_H 12.66 (1H, s), which was characteristic of 5-OH. The signals at δ_H 7.72 (1H, dd, J=8.2, 2.0 Hz), 7.70 (1H, d, J=2.0 Hz), 7.00 (1H, d, J=8.2 Hz), showed an ABX spin coupling system, indicating the 3′- and 4′-substitute structure of B-ring. The signals at δ_H 6.46 (1H, d, J=2.2 Hz) and 6.37 (1H, d, J=2.2 Hz) could be assigned to 8- and 6-protons of A-ring. In addition, an olefinic proton signal at δ_H 5.55 (1H, t, J=6.5 Hz), –CH_2O– signal at δ_H 4.69 (2H, d, J=6.5 Hz), two methyl signals at δ_H 1.81 (3H, s) and 1.78 (3H, s) were also noted in the ^{1}H-NMR spectrum, together with the signals of δ_C 138.3, 119.4, 65.8, 25.8 and 18.3 in the ^{13}C-NMR spectrum, an isopentenyl group can be deduced in the structure. Moreover, the signals at δ_H 3.96 (3H, s), 3.89 (3H, s), 3.87 (3H, s) in ^{1}H-NMR spectrum and δ_C 60.2, 56.1, 55.8 in ^{13}C-NMR spectrum suggested three methoxy groups

Table 1. The ^{1}H- and ^{13}C-NMR Data of Compound **1** in $CDCl_3$[a)]

Position	δ_H	δ_C	Position	δ_H	δ_C
2	—	155.9 (s)	4′	—	150.8 (s)
3	—	139.0 (s)	5′	7.00 (1H, d, J=8.2)	112.4 (d)
4	—	178.8 (s)	6′	7.72 (1H, dd, J=8.2, 2.0)	122.1 (d)
5	—	162.1 (s)	1″	4.69 (2H, d, J=6.5)	65.8 (t)
6	6.37 (1H, d, J=2.2)	97.8 (d)	2″	5.55 (1H, t, J=6.5)	119.4 (d)
7	—	165.5 (s)	3″	—	138.3 (s)
8	6.46 (1H, d, J=2.2)	92.2 (d)	4″	1.78 (3H, s)	18.3 (q)
9	—	156.8 (s)	5″	1.81 (3H, s)	25.8 (q)
10	—	106.1 (s)	5-OH	12.7 (1H, s)	—
1′	—	122.8 (s)	3-CH_3O	3.87 (3H, s)	60.2 (q)
2′	7.70 (1H, d, J=2.0)	111.6 (d)	7-CH_3O	3.89 (3H, s)	56.1 (q)
3′	—	149.2 (s)	4′-CH_3O	3.96 (3H, s)	55.8 (q)

a) δ in ppm and J in Hz. Run at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR.

* To whom correspondence should be addressed. e-mail: zzm@bit.edu.cn
# These authors contributed equally to this work.

in compound **1**. In the heteronuclear multiple bond correlation (HMBC) experiment, long-range correlation from the signal δ_H 4.69 (H-1″) to δ_C 150.8 (C-4′) placed the isopentenyl group to C-4′. Meanwhile, C-5 hydroxyl group was detected from the HMBC cross-peaks between 5-OH with δ_C 97.8 (C-6), 106.1 (C-10) and 162.1 (C-5). On the basis of correlations between δ_H 3.89 (MeO-7) with δ_C 55.8 (C-7) in the heteronuclear multiple quantum coherence (HMQC) analysis and cross peaks between δ_H 3.89 (MeO-7) with δ_C 165.5 (C-7), δ_H 6.46 (H-8), 6.37 (H-6) with δ_C 165.5 (C-7) in the HMBC spectrum, a methoxy group at C-7 position can be deduced. In addition, correlation signals based on the combined analysis of the HMQC and HMBC spectra provided good support for a C-3 methoxy group. A further nuclear Overhauser enhancement and exchange spectroscopy (NOESY) experiment was also carried out, since there was no correlation between H-5′ with another methoxy group, this methoxy group should locate at C-3′ position. Thus, the structure of compound **1** was established to be 5-hydroxy-4′-[(2-isopentenyl)oxy]-3,7,3′-trimethoxyflavone (Fig. 1).

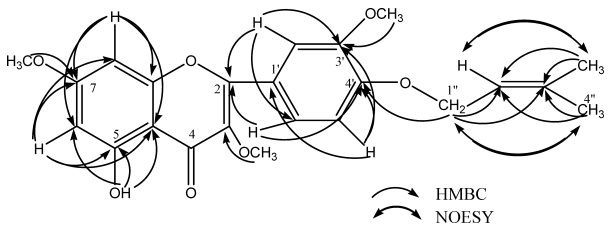

Fig. 1. Structure, Key HMBC and NOESY Correlations of Compound **1**

Compound **2**, isolated as yellow amorphous powder and gave positive reactions with $FeCl_3$ and HCl–Mg reagents. Its HR-ESI-MS showed a molecular-ion peak at *m/z* 709.1736 ($[M+Na]^+$) indicating the molecular formula $C_{33}H_{34}O_{16}$. Acid hydrolysis of **2** gave D-glucose and D-xylose identified by high-performance TLC (HP-TLC) and comparison with authentic samples. No methoxy signal was observed in the ^{1}H- and ^{13}C-NMR spectra (Table 2), indicating the hydroxyl

Table 2. The ^{1}H- and ^{13}C-NMR Data of Compounds **2** and **3** in DMSO-d_6 [a)]

Position	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C
2	—	155.1 (s)	—	154.9 (s)
3	—	132.8 (s)	—	133.0 (s)
4	—	177.7 (s)	—	177.8 (s)
5	—	159.2 (s)	—	159.2 (s)
6	6.34 (1H, s)	98.4 (d)	6.33 (1H, s)	98.3 (d)
7	—	161.9 (s)	—	161.7 (s)
8	—	106.1 (s)	—	106.0 (s)
9	—	153.7 (s)	—	153.5 (s)
10	—	104.0 (s)	—	104.0 (s)
1′	—	121.2 (s)	—	121.2 (s)
2′, 6′	8.00 (2H, d, *J*=8.8)	130.9 (d)	7.98 (2H, d, *J*=8.5)	130.9 (d)
3′, 5′	6.87 (2H, d, *J*=8.8)	115.3 (d)	6.86 (2H, d, *J*=8.5)	115.2 (d)
4′	—	160.0 (s)	—	160.0 (s)
1″	—	130.7 (s)	—	130.7 (s)
2″, 6″	7.00 (2H, d, *J*=8.3)	128.9 (d)	6.99 (2H, d, *J*=8.0)	128.9 (d)
3″, 5″	6.60 (2H, d, *J*=8.3)	115.1 (d)	6.60 (2H, d, *J*=8.0)	115.1 (d)
4″	—	155.5 (s)	—	155.5 (s)
7″	3.95 (2H, s)	27.0 (t)	3.94 (2H, s)	26.9 (t)
5-OH	12.6 (1H, s)	—	12.6 (1H, s)	—
Glc				
1	5.76 (1H, d, *J*=7.2)	98.0 (d)	5.74 (1H, d, *J*=7.5)	97.7 (d)
2	3.45 (1H, m)	81.9 (d)		79.3 (d)
3	3.10 (1H, m)	77.6 (d)		77.0 (d)
4	3.29 (1H, m)	69.5 (d)		69.8 (d)
5	3.48 (1H, m)	76.9 (d)		76.9 (d)
6	3.53 (1H, m) 3.29 (1H, m)	60.5 (t)		60.5 (t)
Xyl				
1	4.59 (1H, d, *J*=7.1)	104.7 (d)	4.99 (1H, d, *J*=7.6)	100.9 (d)
2	3.06 (1H, m)	74.0 (d)	4.66 (1H, dd, *J*=7.6, 8.0)	73.6 (d)
3	3.09 (1H, m)	76.2 (d)		74.3 (d)
4	3.09 (1H, m)	69.5 (d)		70.4 (d)
5	3.70 (1H, m) 3.06 (1H, m)	65.8 (t)		65.8 (t)
(*E*)-Coumaroyl				
1‴			—	125.4 (s)
2‴, 6‴			7.50 (2H, d, *J*=8.4)	130.2 (d)
3‴, 5‴			6.79 (2H, d, *J*=8.4)	115.8 (d)
4‴			—	159.7 (s)
7‴			7.55 (1H, d, *J*=16.4)	144.5 (d)
8‴			6.34 (1H, d, *J*=16.4)	115.2 (d)
9‴			—	165.9 (s)

a) δ in ppm and *J* in Hz. Run at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR.

substitute structure of compound **2**. The ^{1}H-NMR spectrum of **2** showed resonances for two AA′BB′ spin coupling systems at δ_H 8.00 (2H, d, J=8.8 Hz), 6.87 (2H, d, J=8.8 Hz) and 7.00 (2H, d, J=8.3 Hz), 6.60 (2H, d, J=8.3 Hz), which suggested two *p*-substitute benzenes in the structure. The phenol proton δ_H 12.63 (HO-5) showed correlations with δ_C 159.2 (C-5), 104.0 (C-10), 98.4 (C-6) in HMBC experiment, since the signal of δ_C 98.4 (C-6) correlated with the signal of δ_H 6.34 (H-6) in HMQC spectrum, which also correlated with δ_C 161.9 (C-7), 159.2 (C-5), 106.1 (C-8), 104.0 (C-10) in HMBC experiment, a 5,7-dihydroxy structure of A-ring could be deduced. The signal of δ_H 3.95 (H-7″) showed correlation with δ_C 27.0 (C-7″) in HMQC analysis, which also correlated with δ_C 130.7 (C-1″), 128.9 (C-2″, 6″), 106.1 (C-8) in HMBC experiment, the signal δ_H of 7.00 (H-2″, 6″) correlated with δ_C 128.9 (C-2″, 6″) in HMQC and showed correlations with δ_C 155.5 (C-4″), 130.7 (C-1″), 128.9 (C-2″, 6″), 27.0 (C-7″) in HMBC experiment. On the basis of above evidence, a *p*-hydroxy benzyl group was attributed to C-8 position of A-ring. Thus the other *p*-substitute benzene fragment could be assigned to the B-ring of compound **2**, and the 4′-hydroxy substitute structure of B-ring was then elucidated by the information of ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC spectra. Moreover, in the HMQC experiment, the anomeric protons at δ_H 5.76 (1H, d, J=7.1 Hz) and 4.59 (1H, d, J=7.1 Hz) were correlated with carbon signals at δ_C 98.0 and 104.7, respectively, which indicated two sugar units in the structure, combining with the acid hydrolysis result and their carbon shifts, the two sugar units were identified as β-D-glucopyranoside and β-D-xylopyranoside. In the HMBC spectrum, the glucose anomeric proton correlated with δ_C 132.8 (C-3), the xylose anomeric proton correlated with δ_C 81.9 (Glc C-2), which subsequently determined the sugar sequences of oligosaccharide chains. On the basis of aforementioned spectroscopic data, compound **2** was elucidated as 3-*O*-{[*O*-β-D-xylopyranosyl]-(1→2)-β-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (Fig. 2).

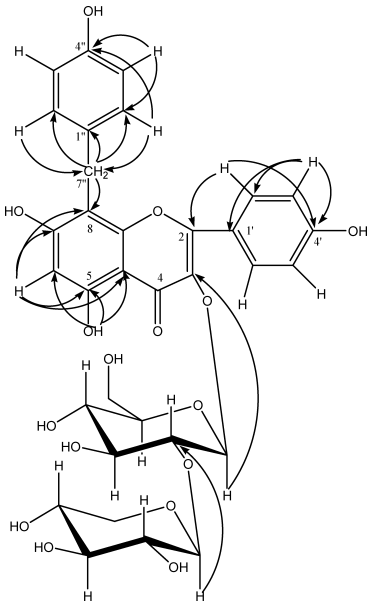

Fig. 2. Structure and Key HMBC Correlations of Compound **2**

Compound **3** was obtained as yellow amorphous powder, whose molecular formula was determined as $C_{42}H_{40}O_{18}$ by the quasimolecular ion peak at *m*/*z* 855.2110 ($[M+Na]^+$) in HR-ESI-MS spectrum. The acid hydrolysis of compound **3** also gave D-glucose and D-xylose identified by HP-TLC and comparison with authentic samples. Meanwhile, from the $CHCl_3$ extract of acid hydrolysis solution, the same aglycone with compound **2** was harvested. The alkaline hydrolysis test was further performed, which gave compound **2** and *p*-coumaric acid established by HP-TLC, these indicated a similar structure of **3** with **2** and the *p*-coumaroyl group should be located at oligosaccharide chains. This result could also be inferred by comparing the ^{1}H- and ^{13}C-NMR data (Table 2) of compound **2** with **3**. In the ^{1}H- and ^{13}C-NMR spectra of **3**, the signals at δ_H 7.50 (2H, d, J=8.4 Hz), 6.79 (2H, d, J=8.4 Hz), 7.55 (1H, d, J=16.4 Hz), 6.34 (1H, d, J=16.4 Hz) and δ_C 125.4, 130.2, 115.8, 159.7, 144.5, 115.2, 165.9 indicated a *p*-coumaroyl substituent in the structure. In the HMBC experiment of compound **3**, the correlation peak was observed between the signals of δ_H 4.66 (Xyl H-2) with δ_C 165.9 (C-9‴), from which the *p*-coumaroyl group could be placed to C-2 position of the xylose. Thus the structure of compound **3** was determined as 3-*O*-{*O*-[2-*O*-(*E*)-*p*-coumaroyl-β-D-xylopyranosyl]-(1→2)-β-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (Fig. 3).

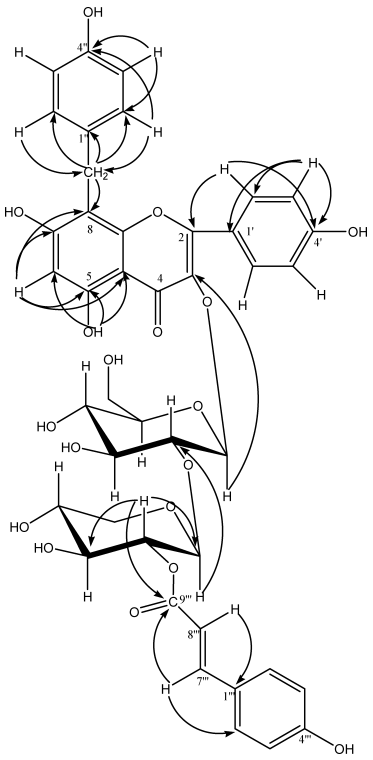

Fig. 3. Structure and Key HMBC Correlations of Compound **3**

The six known compounds 3-*O*-[β-D-xyloyranosyl-(1→2)-β-D-glucopyranosyl]kaempferol (**4**), 7-*O*-β-D-glucopyranosyl kaempferol (**5**), paeonoside (**6**), quercimeritrin (**7**), retusine (**8**), 3,5-dihydroxy-4′,7-dimethoxyflavone (**9**), were also isolated and identified by comparing their NMR and TLC data with authentic samples.

The cytotoxic activity of compounds **1**—**3** against human A549, Hep G2 and HT-29 cell lines were also examined, but there was no cytotoxic effect detected.

Experimental

General Experiment Procedures TLC was conducted on silica gel plates ($60F_{254}$, Merck). HPLC was performed on Waters-600 prep. HPLC instrument equipped with a Amersham Pharmacia Biotech-ODS (250×20 mm) column. Column chromatography (CC) was performed on silica gel (200—300 mesh; Qingdao Haiyang, Co., China), or Sephadex LH-20 (Pharmacia Biotech AB, S-Uppsala). Melting point was measured on a Yanaco MP-S3 micromelting point apparatus. Optical rotations were determined on a Perkin-Elmer 341 polarimeter. UV spectra were obtained on a UV-1201 Shimadzu spectrometer. IR spectra were recorded on a Perkin-Elmer Spectrum GX spectrometer. NMR spectra were obtained on a Bruker ARX-300 instrument. Chemical shifts are expressed as δ (ppm) with trimethylsilane (TMS) as the internal standard. ESI- and HR-ESI-MS were recorded on Bruker APEX II FT-ICR mass spectrometers.

Plant Material Dried whole plants of *Spiranthes australis* (R. Brown) Lindl. were purchased from the General Corporation of Medicinal Materials of Jiang-Su Province, P. R. China in March 2003. A voucher specimen (SAD20030310) was identified by Professor Y.-Z. Guo and has been deposited in the Department of Chinese Traditional Medicine Analysis, Shenyang Pharmaceutical University.

Extraction and Isolation The whole plants of *Spiranthes australis* (R. Brown) Lindl. (15 kg) was cut into small pieces and extracted successively with 70% EtOH under reflux to give an EtOH extract (800 g). The extract was concentrated *in vacuo* then suspended in 4 l of H_2O and partitioned successively with 4 l of petroleum ether, 4 l of EtOAc, and 4 l of *n*-BuOH. The *n*-BuOH extract (80 g) was adsorbed on silica gel (100 g) and subjected to CC (silica gel (2000 g) 9×100 cm column; $CHCl_3$/MeOH gradient) to yield 9 fractions (1—9). Fraction 1 was applied to CC (Sephadex LH-20 (55 g), 3×100 cm column using 55% MeOH). The collected fractions were purified by HPLC (MeOH/H_2O 75 : 25): **1** (32.5 mg). Fraction 2 was subjected to Sephadex LH-20 chromatography (60 g, 3×100 cm column using 55% MeOH): **8** (12.5 mg) and **9** (10.1 mg). Fraction 4 was also subjected to Sephadex LH-20 chromatography (50 g, 3×100 cm column using 55% MeOH): **5** (21.1 mg) and **7** (18.8 mg). Fraction 7 was applied to CC (Sephadex LH-20 (60 g), 3×100 cm column using 60% MeOH). The collected fractions were purified by HPLC (MeOH/H_2O 65 : 35): **2** (69.8 mg) and **3** (50.1 mg). Fraction 8 was applied to CC (Sephadex LH-20 (55 g), 3×100 cm column using 50% and 65% MeOH): **4** (18.1 mg) and **6** (21.4 mg).

5-Hydroxy-4′-[(2-isopentenyl)oxy]-3,7,3′-trimethoxyflavone (**1**): Yellow needles (MeOH), melting point (mp) 120—121 °C. UV λ_{max} (MeOH) nm (log ε): 254 (4.40), 354 (3.97); IR (KBr) cm^{-1}: 3430, 1668, 1601, 1495; ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) data see Table 1; ESI-MS (pos.) *m/z*: 413 (100, $[M+H]^+$); HR-ESI-MS: 413.1588 ($[M+H]^+$, $C_{23}H_{25}O_7^+$; Calcd 413.1594).

3-*O*-{[*O*-β-D-xylopyranosyl]-(1→2)-β-D-glucopyranosyl}-8-(*p*-hydroxybenzyl)-kaempferol (**2**): Yellow amorphous powder; $[\alpha]_D^{24}$ −40.5° (*c*=0.16, MeOH); UV λ_{max} (MeOH) nm (log ε): 225 (4.42), 271 (4.10); IR (KBr) cm^{-1}: 3388, 1653, 1611, 1511; ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) data see Table 2; ESI-MS (pos.; neg.) *m/z*: 709 (100, $[M+Na]^+$); 685 (65, $[M-H]^-$); HR-ESI-MS: 709.1736 ($[M+Na]^+$, $C_{33}H_{34}NaO_{16}^+$; Calcd 709.1739).

3-*O*-{*O*-[2-*O*-(*E*)-*p*-Coumaroyl-β-D-xylopyranosyl]-(1→2)-β-D-glucopyranosyl}-8-(*p*-hydroxy-benzyl)-kaempferol (**3**). Yellow amorphous powder; $[\alpha]_D^{24}$ −63.7° (*c*=0.12, MeOH); UV λ_{max} (MeOH) nm (log ε): 231 (4.36), 282 (4.02); IR (KBr) cm^{-1}: 3392, 1662, 1615, 1498; ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) data see Table 2; ESI-MS (pos.) *m/z*: 855 (100, $[M+Na]^+$); HR-ESI-MS: 855.2110 ($[M+Na]^+$, $C_{42}H_{40}NaO_{18}^+$; Calcd 855.2106).

Acid Hydrolysis of 2 and 3 Compounds **2** and **3** (8 mg each) were refluxed with 15% HCl/MeOH (10 ml) at 80 °C for 4 h. After cooling, the mixture was concentrated and the residue partitioned with $CHCl_3$/H_2O. The presence of glucose and xylose in this mixture was established by comparison with authentic samples. The HP-TLC in the solvent system MeCOEt/^{i}PrOH/Me_2CO/H_2O (20 : 10 : 7 : 6) resulted in the *Rf* 0.26 and 0.39 respectively. The aglycone of compound **3** was identified as same to compound **2** by polyamide TLC ($CHCl_3$/MeOH 15 : 1) with the *Rf* 0.43.

Alkaline Hydrolysis of 3 Compound **3** was dissolved in 20 ml solution (MeOH/H_2O, 1 : 1) containing appropriate NaOH and hydrolyzed under reflux (2 h) at 60 °C. Then the basic solution was evaporated to dryness. The HP-TLC of hydrolysis product in the solvent system $CHCl_3$/MeOH (2 : 1) resulted in the *Rf* 0.31 and 0.40 respectively. Compound **2** and *p*-coumaric acid were identified by comparison with compound **2** and authentic sample.

Cytotoxic Activity Compounds **1**—**3** were examined for their *in vitro* cytotoxic activity against human cell lines A549, Hep G2 and HT-29. The inhibition of growth was determined by *in vitro* treatment of the respective cell lines using MTT assay.[12)]

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