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THREE NEW ISOFLAVONES FROM THE ROOT OF *PUERARIA LOBATA* AND THEIR BIOACTIVITIES

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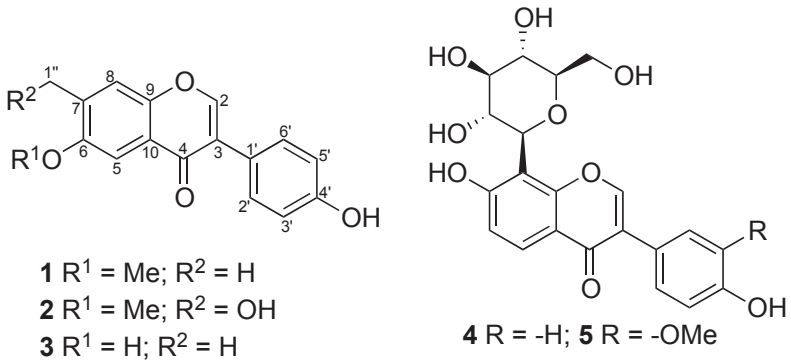
Abstract – Three new isoflavones, 4′-hydroxy-6-methoxy-7methylisoflavone (**1**), 4′,6-dihydroxy-7-methylisoflavone (**2**), and 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), together with two known isoflavones (**4** and **5**), were isolated from the root of *Pueraria lobata*. Their structures were elucidated by spectroscopic methods, including extensive ^{1}D- and ^{2}D NMR techniques. Compounds **1-5** were evaluated for their anti-tobacco mosaic virus (anti-TMV) activities. The results showed that compounds **1** and **2** exhibited comparable anti-TMV activities with inhibition rates of 34.2 and 33.5%. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 21.8-25.6%, respectively. The cytotoxicities of compounds **1-5** against five human tumor cell lines (NB4, A549, SHSY5Y, PC3, and MCF7) were also tested. The results revealed that compounds **1-5** showed weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 3.9-9.2 μM.

The dried root of *Pueraria lobata* (Wild.) Ohwi (Yege) belonging to the family of Fabaceae or Leguminosae, which is a twining perennial herb with woody base native to South East Asia regions, such as China, Korea and Japan.[1-3] Since 2005, this herbal medicine is also called Gegen in the Chinese Pharmacopeia.[4] Gegen has traditionally been used in TCM for improving the body function, such as promoting circulation and increasing the blood flow.[1,4] Puerarin, the first isoflavone isolated from the root of *Pueraria lotaba* in the late 1950s bearing a specific carbon-glycoside bond,[5] has proven to be responsible for the pharmacological actions on the cardiovascular systems of this herbal medicine.[1,4]

Actually its high medicinal value and nutritional value have rightfully earned Gegen herb a reputation as the "Asian ginseng" and "longevity powder" in Japan, where it is honored as the "Royal Special food".[6] Previous phytochemical studies of root of *Pueraria lobata* have shown the presence of isoflavones,[7-9] triterpenes,[2,10,11] coumarins,[12,13] steroids,[14] and the homologous. Today's research also discovered that the concentration and activity of isoflavones contained in the root of *Pueraria lobata* are far more than that found in soybeans, and it is enjoying great popularity in the west and Japan for a variety of purposes, e.g. weight loss, breast enlargement, hair loss treatment, alcoholism prevention, liver tonic, and so on.[15,16] In continuing efforts to the phytochemistry research on the root of *Pueraria lobata* led to the isolation of three new (**1-3**) and two known (**4** and **5**) isoflavones. This paper deals with the isolation, structural elucidation, and bioactivities of these compounds, as well as their anti-tobacco mosaic virus (anti-TMV) activities and cytotoxicities.



1 R^1 = Me; R^2 = H
2 R^1 = Me; R^2 = OH
3 R^1 = H; R^2 = H

4 R = -H; **5** R = -OMe

Figure 1. Isoflavones from the root of *Pueraria lobata*

A 90% aq. MeOH extract prepared from the root of *Pueraria lobata* was subjected repeatedly to column chromatography and preparative HPLC to afford three new isoflavones, 4′-hydroxy-6-methoxy-7-methylisoflavone (**1**), 4′,6-dihydroxy-7-methylisoflavone (**2**), and 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), and two known isoflavones (**4** and **5**). The structures of the compounds **1-5** were as shown in **Figure 1**, and the ^{1}H and ^{13}C NMR data of **1-3** were listed in **Table 1**. The known compounds, compared with literature, were identified as puerarin (**4**),[17] and 3′-methoxypuerarin (**5**).[18]

Compound **1** was obtained as an orange-yellow gum. The molecular formula of $C_{17}H_{14}O_4$ was determined from the HRESIMS spectra showing the sodiated molecular ion at *m/z* 305.0798 $[M+Na]^+$ (calcd 305.0790). The ^{1}H and ^{13}C NMR spectrum of **1** (**Table 1**) along with analysis of the DEPT spectra displayed 17 carbon signals and 14 proton signals, respectively, corresponding to 4′,6,7-trisubstituted isoflavones nucleus (C-2 ~ C-10 and C-1′ ~ C-6′; H-5, H-8, H-2′,6′, and H-2′,6′),[19] one methyl group (δ_C 16.6 q; δ_H 2.73 s), one methoxy groups (δ_C 55.9 q; δ_H 3.86 s), and one phenolic

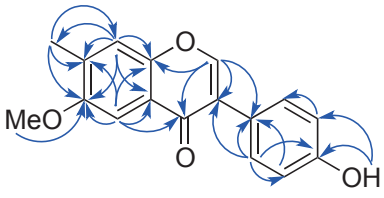


Figure 2. key HMBC (↷) correlations of **1**

hydroxy group (δ_H 11.20 s). The 4′,6,7-trisubstituted isoflavones nucleus was also supported by the HMBC correlations (**Figure 2**) of H-5 with C-4, C-6, C-7, C-9, C-10, of H-8 with C-6, C-7, C-9, C-10, of H-2 with C-1′, C-3, C-4, C-9, and of H-2′,6′ with C-3. The location of methyl group was assigned to C-7 position on the basis of HMBC correlations of H_3-1″ (δ_H 2.37) with C-6 (δ_C 154.1), C-7 (δ_C 132.0), C-8 (δ_C 117.8), and of H-8 (δ_H 6.69) with C-1″ (δ_C 16.6). The HMBC correlations from the methoxy proton (δ_H 3.86) to C-6 (δ_C 154.1) concluded the linkage of the methoxy group at C-6. Finally, The phenolic hydroxy group located at C-4′ was supported by the HMBC correlations of phenolic hydroxy proton (δ_C 157.5) with C-4′ and C-3′,5′ (δ_C 116.0). In addition, the typical proton signals of H-5 (δ_H 7.13 s), H-8 (δ_H 6.69 s), H-2′,6′ (δ_H 7.73, d, J = 8.8), and H-3′,5′ (δ_H 6.78, d, J = 8.8) also supported the above substituents pattern. Thus, the structure of **1** was established as 4′-hydroxy-6-methoxy-7-methylisoflavone.

Table 1. ^{1}H and ^{13}C NMR data for compounds **1-3** ($CDCl_3$, 125 and 500 MHz)

No.	Compound (**1**)		Compound (**2**)		Compound (**3**)	
	δ_C (mult.)	δ_H (mult, *J*, Hz)	δ_C (mult.)	δ_H (mult, *J*, Hz)	δ_C (mult.)	δ_H (mult, *J*, Hz)
2	152.6 d	7.85 s	152.3 d	7.85 s	152.8 d	7.87 s
3	123.6 s		123.9 s		123.1 s	
4	176.2 s		176.5 s		176.1 s	
5	114.9 d	7.13 s	116.3 d	7.07 s	115.5 d	7.18 s
6	154.1 s		151.2 s		153.3 s	
7	132.0 s		133.3 s		134.9 s	
8	117.8 d	6.69 s	118.2 d	6.65 s	116.9 d	6.71 s
9	149.0 s		149.2 s		149.6 s	
10	121.4 s		121.8 s		122.7 s	
1′	124.9 s		125.0 s		124.4 s	
2′,6′	130.5 d	7.73 (d) 8.8	130.2 d	7.74 (d) 8.8	130.2 d	7.79 (d) 8.8
3′,5′	116.0 d	6.78 (d) 8.8	115.9 d	6.80 (d) 8.8	115.9 d	6.81 (d) 8.8
4′	157.5 s		157.4 s		157.1 s	
1″	16.6 q	2.37 s	16.2 q	2.34 s	63.2 t	4.61 s
OMe-6	55.9 q	3.86 s			56.3 q	3.85 s
Ar-OH-6				11.21 s		
Ar-OH-4′		11.20 s		10.91 s		10.84 s

4′,6-Dihydroxy-7-methylisoflavone (**2**) was isolated as orange gum and it gave an $[M+Na]^+$ peak at *m/z* 291.0628, consistent with a molecular formula of $C_{16}H_{12}O_4$. The data of ^{1}H NMR were assigned to ^{13}C NMR with the help of HSQC spetrum (**Table 1**). Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of **1**, which suggested that compound **2** was structurally related to **1**. The marked differences between them were due to the inexistence of a methoxy group, and appearance of a phenolic hydroxy group (δ_H 10.91 s) in compound **2**. These changes indicated that a methoxy group in **1** was replaced by a phenolic hydroxy group in compound **2**. In addition, the obvious chemical shift differences of the upfield shift of C-6 from δ 154.1 ppm to δ 151.2 ppm suggested the substituent groups should be varied at C-6.

This was also supported by the HMBC correlations of the phenolic hydroxy proton signal (δ_H 10.91) with C-5 (δ_C 116.3), C-6 (δ_C 151.2), and C-7 (δ_C 133.3). According to above informations, the structure of compound **2** was assigned.

Compound **3** was assigned a molecular formula of $C_{17}H_{14}O_5$ as supported by the HRESIMS (*m/z* 321.0744 $[M+Na]^+$), corresponding to 11 degrees of unsaturation. Its ^1H and ^{13}C NMR spectroscopic data were also similar to those of compound **1**, except for the presence of a hydroxymethyl group signal (δ_H 4.61 s), and the absence of a methyl proton signal. These changes indicated that the methyl group in **1** was substituted by a hydroxymethyl group in compound **3**. The HMBC correlations from the methoxy protons (δ_H 3.85) to C-6 (δ_C 153.3) suggested that the methoxy groups located at C-6. The phenolic hydroxy group located at C-4′ was supported by the HMBC correlations of the phenolic proton signal (δ_H 10.84) with C-3′,5′ (δ_C 115.9) and C-4′ (δ_C 157.1). Finally, the location of hydroxymethyl group located at C-7 was supported by the HMBC correlations of H_2-1″ (δ_H 4.61) with C-6 (δ_C 153.3), C-7 (δ_C 134.9), and C-8 (δ_C 116.9), and of H-8 (δ_H 6.71) with C-1″ (δ_C 63.2). Accordingly, the structure of 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**) was determined as shown.

Since certain of the isoflavones exhibit potential anti-TMV activity,[20-22] compounds **1-5** were tested for their anti-TMV activity. The inhibitory activity of compounds **1-5** against TMV replication were tested using the half-leaf method.[23] Ningnanmycin (with inhibition rate of 31.5%), a commercial product for plant disease in China, was used as a positive control. The antiviral inhibition rates of compounds **1-5** at the concentration of 20 μM were listed in **Table 2**. The results showed that compounds **2** and **3** exhibited comparable anti-TMV activities with inhibition rates of 34.2% and 33.5%. The inhibition rates are higher than that of positive control. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 21.8-25.6%, respectively.

Table 2. The TMV Inhibition rates compounds **1-5**

Compounds	Inhibition rates (%)	Compounds	Inhibition rates (%)
1	25.6 ± 2.8	**4**	23.4 ± 2.9
2	34.2 ± 3.2	**5**	21.8 ± 2.6
3	33.5 ± 3.3	ningnanmycin	31.5 ± 3.2

All results are expressed as mean ± SD; n = 3 for all groups.

Since certain of the isoflavones exhibit potential cytotoxic activity,[24-26] the cytotoxicities of compounds **1-5** were also tested using a previously reported

Table 3. Cytotoxic activity of compounds **1-5**

Compounds	Cell lines and IC_{50} (μM)				
	NB4	A549	SHSY5Y	PC3	MCF7
1	>10	>10	9.2 ± 0.8	>10	>10
2	>10	7.8 ± 0.5	8.5 ± 0.5	>10	>10
3	6.4 ± 0.6	4.8 ± 0.4	3.9 ± 0.4	5.5 ± 0.4	7.3 ± 0.7
4	8.8 ± 0.5	>10	>10	7.6 ± 0.6	9.2 ± 0.8
5	>10	7.9 ± 0.6	6.8 ± 0.7	8.2 ± 0.5	7.1 ± 0.5
Taxol	0.03	0.02	0.05	0.05	0.05

NB4, human leukemia cell; A549, carcinomic human alveolar basal epithelial cell; SHSY5Y, human neuroblastoma cell; PC3, human prostate cancer cell; MCF7, human breast adenocarcinoma cell.
All results are expressed as mean ± SD; n = 3 for all groups.

procedure.[27] the cytotoxic abilities against five human tumor cell lines (NB4, A549, SHSY5Y, PC3, AND MCF7) by MTT-assay were summarized in **Table 3**. the results revealed that compounds **1-5** showed weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 3.9-9.2 μM.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on DRX-500 spectrometers with TMS as internal standard, and the chemical shifts (δ) were expressed in ppm. HRESIMS was performed on an API QSTAR time-of-flight spectrometer and a VG Autospec-3000 spectrometer, respectively. Preparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with a ZORBAX PrepHT GF (21.2 mm × 25 cm, 7 μm) column or a Venusil MP C_{18} (20 mm × 25 cm, 5 μm) column. Column chromatography was performed with silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China). The fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant Material. The root of *Pueraria lobata* was collected fromYuxi Prefecture, Yunnan province, China, in September 2016 and identified by Prof. Ning Yuan. A voucher specimen (YNNI-16-09-28) has been deposited in the Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan University.

Extraction and Isolation. The air-dried and powdered root of *Pueraria lobata* (6.8 kg) were extracted four times with 90% aqueous MeOH (3 × 5 L) at room temperature and filtered. The solvent was evaporated in vacuo, and the crude extract was dissolved in H_2O and partitioned with EtOAc. The EtOAc partition (182 g) was applied to silica gel (200-300 mesh) column chromatography, eluting with a $CHCl_3$-MeOH gradient system (9:1, 8:2, 7:3, 6:4, 5:5, 4:6), to give six fractions A-F. Further separation of fraction B (8:2, 12.5 g) by silica gel column chromatography, eluted with $CHCl_3$-Me_2CO (9:1-1:2), yielded mixtures B1–B7. Fraction B3 (7:3, 1.15 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (50% MeOH-H_2O, flow rate 20 mL/min) to give **1** (12.2 mg) and **3** (10.8 mg). Fraction B4 (6:4, 1.86 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (44% MeOH-H_2O, flow rate 20 mL/min) to give **2** (12.6 mg). Fraction B5 (5:5, 1.86 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (38% MeOH-H_2O, flow rate 20 mL/min) to give **4** (33.6 mg) and **5** (42.1 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method,[23] and Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control.

Cytotoxicity Assay. The cytotoxicity tests for the isolates were performed by against NB4, A549,

SHSY5Y, PC3, and MCF7 tumor cell lines by MTT-assay (with taxol as the positive control).[27]

4′-Hydroxy-6-methoxy-7-methylisoflavone (**1**), $C_{17}H_{14}O_4$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 328 (3.46), 262 (3.85), 210 (4.32) nm; IR (KBr) ν_{max} 3310, 3068, 2935, 1642, 1610, 1558, 1506, 1440, 1365, 1257, 1148, 1059, 962, 847 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 305; HRESIMS (positive ion mode) *m/z* 305.0798 $[M+Na]^+$ (calcd 305.0790 for $C_{17}H_{14}NaO_4$).

4′,6-Dihydroxy-7-methylisoflavone (**2**), $C_{16}H_{12}O_4$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 325 (3.52), 262 (3.76), 210 (4.41) nm; IR (KBr) ν_{max} 3318, 3065, 2938, 1640, 1610, 1554, 1502, 1436, 1362, 1254, 1143, 1050, 938, 862 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 291; HRESIMS (positive ion mode) *m/z* 291.0628 $[M+Na]^+$ (calcd 291.0633 for $C_{16}H_{12}NaO_4$).

4′-Hodraxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), $C_{17}H_{14}O_5$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 332 (3.48), 265 (3.80), 210 (4.36) nm; IR (KBr) ν_{max} 3356, 3060, 2934, 1715, 1645, 1612, 1561, 1507, 1432, 1369, 1248, 1146, 1059, 972, 844 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 321; HRESIMS (positive ion mode) *m/z* 321.0744 $[M+Na]^+$ (calcd 321.0739 for $C_{17}H_{14}NaO_5$).

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