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Three new flavonoids from *Millettia pachyloba*

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ABSTRACT

Two new prenylated isoflavones, pachyloisoflavone A (**1**), pachyloisoflavone B (**2**), and a new pterocarpan, pachylobin A (**3**), together with ten known flavonoids (**4**–**13**), were isolated from the vine stems and leaves of *Millettia pachyloba*. The structures of new compounds were elucidated on the basis of extensive spectroscopic data interpretation, including 1D, 2D NMR and HREIMS. The absolute configuration of **2** was established by the comparison of experimental and calculated electronic circular dichroism (ECD) spectra.l

1. Introduction

The vine stems of several *Millettia* species (Leguminosae), locally known in Chinese herbal medicine as "*ji-xue-teng*," are useful in promoting blood circulation and relieving stasis and have been used to treat pain or numbness in wrists, knees, or other joints and irregular menstruation (Jiang Su New Medical College, 1977). Plants of the genus *Millettia* are well known for elaborating prenylated flavones and isoflavones with annellated furan and pyran rings (Kamperdick et al., 1998). *Millettia pachyloba* Drake is a climb vine distributed in Guangdong, Guangxi, Hainan and Yunnan Province, China (Editorial Committee of Flora of China, 1994). The roots and seeds of this plant have been used by the local people for the treatment of rheumatism, numbness and apocatastasis (Wu et al., 1998). The previous study on chemical constituents of the grains of *M. pachyloba* afforded six isoflavones, 5-methoxybarbigerone, 6-methoxybarbigerone, calopogoniumisoflavone B, durmillone, jamaicin, ichthynone; two pterocarpans, pachylobin and (−)-pisatin; and one rotenone, (−)-rotenone. 6-methoxybarbigerone and pachylobin exhibited cytotoxicity against KB cells with IC_{50} values of 2.0 and 17.6 μM, respectively (Mai et al., 2010). To the best of our knowledge, no phytochemical work on the vine stems and leaves of this species has been reported. As part of our continuing chemical investigation on *Millettia* species (Na et al., 2014, 2013), thirteen flavonoids (**1**–**13**) including two new isoflavones (**1–2**) and a new pterocarpan (**3**), were isolated from the vine stems and leaves of *Millettia pachyloba*. In the present paper, the isolation and structure elucidation of the new compounds are reported.

2. Results and discussion

Repeated column chromatography (including normal-phase silica gel, reverse-phase RP-18 and Sephadex LH-20) of the EtOH extract of the vine stems and leaves of *M. pachyloba* has led to the isolation of two new prenylated isoflavones, named pachyloisoflavone A (**1**), pachyloisoflavone B (**2**), and a new pterocarpan, named pachylobin A (**3**) (Fig. 1), together with ten known flavonoids, 6-methoxycalpogonium isoflavone A (**4**) (Yenesew et al., 1997), ichthynone (**5**) (Dagne et al., 1989), millesianin C (**6**) (Gong et al., 2009), durallone (**7**) (Yenesew et al., 1996), millesianin D (**8**) (Gong et al., 2009), genistein (**9**) (Feng et al., 2007), afromosin (**10**) (Kou et al., 2012), hernancorizin (**11**) (Zidorn, 2015), 5-hydroxy-2′, 4′, 5′, 7-tetramethoxyflavone (**12**) (Encarnacion et al., 1994) and hydnocarpin (**13**) (Afifi et al., 1993) (Supplementary data Fig. S1).

Compound **1** was obtained as white amorphous powder. The HREIMS exhibited a molecular ion peak at *m*/*z* 426.1674 ([M]$^+$, calcd. for 426.1679), suggesting the molecular formula of $C_{24}H_{26}O_7$. The IR spectrum exhibited strong absorption band of hydroxyl group at 3417 and conjugated carbonyl at 1635 cm^{-1}. The UV spectrum showed absorption maxima at 300, 254 and 218 nm. The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) showed low-field resonances due to an sp^2 methine at δ_H 8.02 (1H, *s*) and δ_C 154.1, which were characteristic of H-2 and C-2, respectively, on an isoflavone skeleton (Ito et al., 2004). The ^{1}H NMR spectra further revealed the signals of four methoxy groups (δ_H 3.79, 3.85, 3.93 and 3.99, each 3H, *s*), one dimethylallyl unit (or prenyl) (δ_H 1.70 and 1.83 each 3H,

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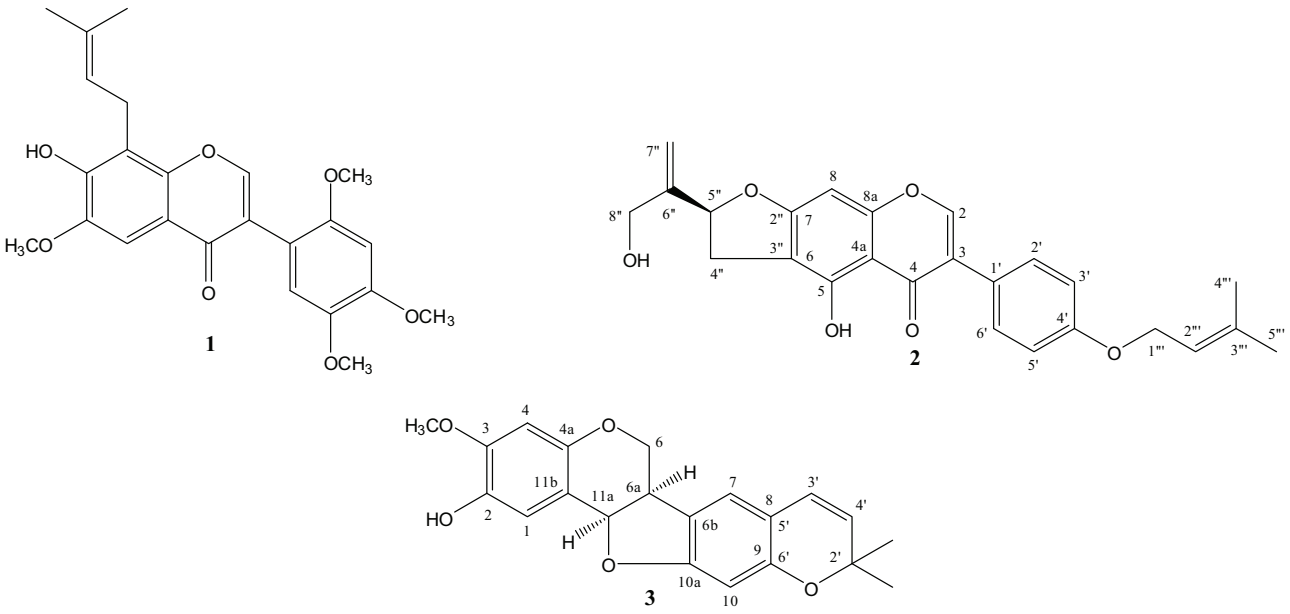

Fig. 1. Structures of compounds **1**–**3**.

s; δ_H 3.59, 2H, *d*, *J* = 7.2 Hz; δ_H 5.28, 1H, *t*, *J* = 7.2 Hz) and four singlet hydrogens (δ_H 6.35, 6.63, 6.96 and 7.54, each 1H, *s*). Two singlet protons at δ_H 6.63 and 6.96, were assigned to the two *para*-positioned aromatic protons H-3′ and H-6′ of ring B. Its similarity to the ^{1}H NMR data of millesianin C (Gong et al., 2009) indicated a ring B of **1** with 2′,4′,5′-trimethoxy substituent. This was also confirmed by the three-bond correlation in the HMBC spectrum (Fig. 2). The fourth methoxyl should be situated at C-6 as its proton (δ_H 3.99) showed ^3J correlation with C-6 (δ_C 145.0) in the HMBC spectrum. The HMBC correlations of H_2-1″ (δ_H 3.59) with C-7 (δ_C 148.5), C-8 (δ_C 115.6), and C-8a (δ_C 150.9) implied that the dimethylallyl group was attached to the C-8 position. The aromatic proton at δ_H 7.54 should be at C-5 due to HMBC correlations H-5 (δ_H 7.54) with C-4 (δ_C 176.0), C-7 (δ_C 148.5) and C-8a (δ_C 150.9). Beside the partial structures mentioned above, the molecular formula requires a hydroxyl group which should be connected with C-7 according to an HMBC experiment from the correlations between the hydoxyl proton at δ_H 6.35 to C-6 (δ_C 145.0) and C-8 (δ_C 115.6). Based on the above spectral evidence, compound **1** was assigned as 7-hydroxy-8-(3-methylbut-2-enyl)-2′, 4′, 5′, 7-tetramethoxy isoflavone and has been given the trivial name pachyloisoflavone A.

Table 1
^{1}H NMR and ^{13}C NMR data of **1** and **2** (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, in $CDCl_3$, δ in ppm, *J* in Hz).

No.	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
2	154. 1	8.02 (*s*)	152.5	7.85 (*s*)
3	120.9		123.6	
4	176.0		181.0	
4a	117.6		106.7	
5	102.3	7.54 (*s*)	157.3	
6	145.0		108.5	
7	148.5		165.8	
8	115.6		89.1	6.39 (*s*)
8a	150.9		158.3	
1′	112.6		122.8	
2′	151.9		130.1	7.44 (‘d’, 8.8)
3′	98.3	6.63 (*s*)	114.8	6.99 (‘d’, 8.8)
4′	149.7		159.1	
5′	143.0		114.8	6.99 (‘d’, 8.8)
6′	115.3	6.96 (*s*)	130.1	7.44 (‘d’, 8.8)
1″	22.4	3.59 (2H, *d*, 7.2)		
2″	121.0	5.28 (*t*, 7.2)		
3″	132.9			
4″	25.8	1.70 (3H, *s*)	31.1	3.14 (*dd*, 7.8, 15.6) 3.44 (*dd*, 9.6, 15.6)
5″	17.9	1.83 (3H, *s*)	85.8	5.48 (*brdd*, 7.8, 9.6)
6″			146.7	
7″			112.8	5.28 (*brs*) 5.30 (*brs*)
8″			62.9	4.28 (2H, *s*)
1‴			64.9	4.54 (2H, *d*, 6.8)
2‴			119.5	5.52 (*t*, 6.8)
3‴			138.5	
4‴			25.9	1.80 (3H, *s*)
5‴			18.2	1.75 (3H, *s*)
6-OMe	56.4	3.99 (*s*)		
2′-OMe	56.9	3.79 (*s*)		
4′-OMe	56.2	3.93 (*s*)		
5′-OMe	56.5	3.85 (*s*)		
5-OH				13.13 (*s*)
7-OH		6.35 (*s*)		

Compound **2** was isolated as white amorphous powder. The molecular formula of **2** was determined to be $C_{25}H_{24}O_6$ by HREIMS (*m*/*z* 420.1573 [M]$^+$, calcd. for 420.1566). Its IR spectrum showed the presence of a chelated hydroxyl (3381 cm^{-1}) and a conjugated carbonyl (1672 cm^{-1}). The UV spectrum exhibited absorption maxima at 276 and 218 nm. The ^{1}H and ^{13}C NMR spectra of **2** (Table 1) exhibited low-field resonances at δ_H 7.85 (1H, *s*) and δ_C 152.5, which were characteristic of H-2 and C-2, respectively, of an isoflavone nucleus (Ito et al., 2004). The ^{1}H NMR spectrum displayed *ortho*-coupled AA′BB′-type protons at δ_H 7.44 and 6.99 (each 2H, *d*, *J* = 8.8 Hz) assignable to H-2′, 6′ and H-3′, 5′ of ring B, respectively, which indicated that the aromatic ring B was 1, 4-substituted. The signals at δ_H 5.52 (1H, *t*, *J* = 6.8 Hz), 4.54 (2H, *d*, *J* = 6.8 Hz), 1.80 (3H, *s*) and 1.75 (3H, *s*), assigned to methine, methylene and *gem*-dimethyl protons, respectively, revealed the presence of an oxyprenyl residue in **2** (Sritularak and Likhitwitayawuid, 2006). The oxyprenyl moiety was connected to C-4′ as the oxygenated methylene protons at δ_H 4.54 (CH_2-1‴) correlated to C-4′ (δ_C 159.1) of the isoflavone nucleus in the HMBC spectrum (Fig. 2). A downfield signal at δ_H 13.13 was ascribed to 5-OH due to the formation of intramolecular hydrogen bond with carbonyl group at C-4.

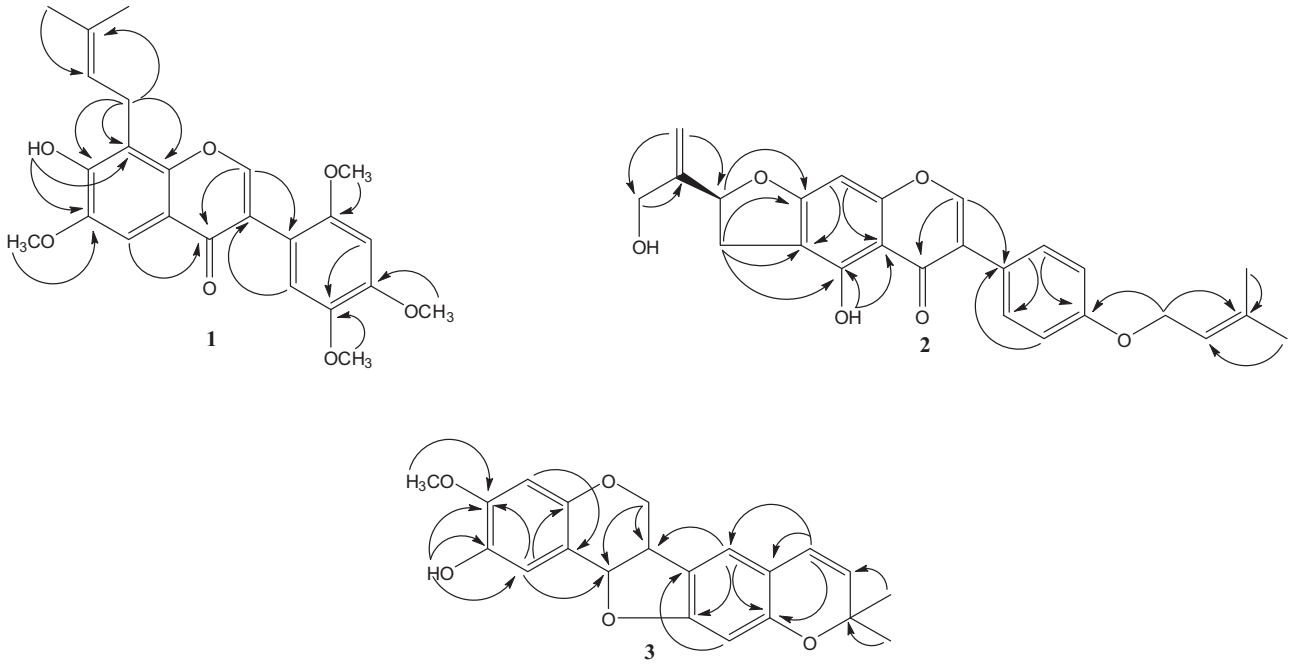

Fig. 2. Key HMBC (H → C) correlations of compounds **1**–**3**.

Proton signals at δ_H 3.14 (1H, *dd*, *J* = 7.8, 15.6 Hz, Ha-4″), 3.44 (1H, *dd*, *J* = 9.6, 15.6 Hz, Hb-4″), (δ_H 4.28, 2H, *s*), 5.28 (1H, *brs*, Ha-7″), 5.30 (1H, *brs*, Hb-7″), and 5.50 (1H, *brdd*, *J* = 7.8, 9.6 Hz, H-5″) were almost identical to those of an isopropenyl-dihydrofuran group (Morel et al., 2001; Li et al., 2001; Awouafack et al., 2011), except for the absence of methyl and the presence of an sp^3 oxymethylene group (δ_H 4.28, 2H, *s*; δ_C 62.9). The methyl group of isopropenyl-dihydrofuran was replaced by a hydroxymethyl group and the partial structure was 3-hydroxyprop-1-en-2-yl-dihydrofuran. The HMBC spectrum showed correlations between the methylene protons [δ_H 3.14 (Ha-4″); 3.44 (Hb-4″)] of the dihydrofuran moiety and some carbons [δ_C 157.3 (C-5); 108.5 (C-6); 165.8 (C-7)] of the A-ring. The dihydrofuran side chain was therefore assigned as located at C-6 and C-7 on the basis of the HMBC experiment. The absolute configuration of **2** was elucidated by electronic circular dichroism (ECD) calculations (Xu et al., 2016). The two enantionmers of **2** (*S* and *R*) were calculated for ECD spectra. As a result, the pattern of the calculated ECD spectra of *S* was in good correspondence with the experimental data of **2** (Fig. 3). Thus, the absolute configurations of the compound **2** had an *S* configuration at C-5″ and the structure of **2** was determined as 5″ –(*S*)-5-hydroxy-4′-*O*-(3-methylbut-2-enyl)-5″-(3-hydroxyprop-1-en-2-yl)-4″, 5″- dihydrofurano [2″, 3″:6, 7] isoflavone, and named pachyloisoflavone B.

Compound **3** was obtained as colorless amorphous powder, and its molecular formula was determined to be $C_{21}H_{20}O_5$ on the basis of the HREIMS data (*m*/*z* 352.1316 $[M]^+$, calcd. for 352.1311). The IR spectrum showed the presence of hydroxyl group at 3380 cm^{-1}. The ^1H and ^{13}C NMR spectra of **3** (Table 2) showed signals for one oxymethylene (δ_H 3.60, 1H, *t*, *J* = 11.0 Hz, δ_H 4.20, 1H, *dd*, *J* = 5.0, 11.0 Hz) and two methines [δ_H 3.49 (1H, *m*) and 5.43 (1H, *d*, *J* = 7.0 Hz)], and these signals were characteristic of a pterocarpan skeleton (Sakurai et al., 2006). The ^1H and ^{13}C NMR of **3** also revealed signals for a 2, 2-dimethylpyran moiety at δ_H 6.25 (1H, *d*, *J* = 9.8 Hz), 5.45 (1H, *d*, *J* = 9.8 Hz), and 1.40, 1.41 (each 3H, *s*); one methoxy group [δ_H 3.86 (3H, *s*)] and four aromatic protons [δ_H 6.33, 6.45, 6.83 and 7.04 (each 1H, *s*)]. The HMBC correlations (Fig. 2) of the olefinic proton (H-4′, δ_H 6.25) of the 2, 2-dimethylpyran moiety with C-7 (δ_C 121.9), C-8 (δ_C 114.8) and C-9 (δ_C 154.5) indicated that the pyran ring was fused at C-8 and C-9. The HMBC three-bond correlations also indicated the positions of four protons on C-1 (H-1 with C-3, C-4a, and C-11a), C-4 (H-4 with C-2 and C-11b), C-7 (H-7 with C-9 and C-10a) and C-10 (H-10 with C-8 and C-6b), respectively (Fig. 2). In the A ring, the presence of two singlet aromatic protons at δ_H 7.04 (H-1) and 6.45 (H-4) required that C-2 and C-3 were substituted. The methoxyl should be situated at C-3 as its proton (δ_H 3.86) showed ^3J correlation with C-3 (δ_C 147.9) in the HMBC spectrum. Considering the molecular formula of $C_{21}H_{20}O_5$, it suggested that one hydroxyl group was attached to

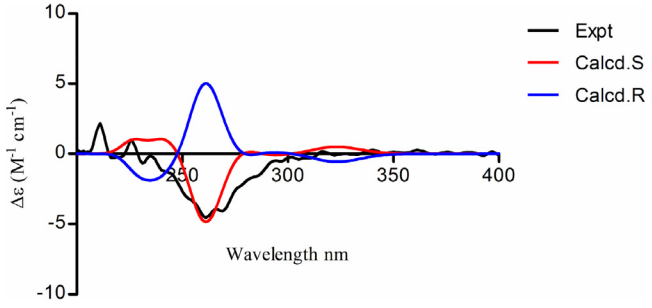

Fig. 3. Experimental and calculated ECD spectra for compound **2.**

Table 2
^1H NMR and ^{13}C NMR data of **3** (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR, in $CDCl_3$, δ in ppm, *J* in Hz).

No.	δ_C	δ_H	No.	δ_C	δ_H
1	115.0	7.04 (*s*)	9	154.5	
2	140.6		10	99.4	6.33 (*s*)
3	147.9		10a	160.3	
4	100.0	6.45 (*s*)	11a	78.4	5.43 (*d*, 7.0)
4a	149.2		11b	111.8	
6	66.8	4.20 (*dd*, 5.0, 11.0) 3.60 (*t*, 11.0)	2′	76.5	
6a	39.7	3.49 (*m*)	3′	127.6	5.45 (*d*, 9.8)
6b	119.0		4′	122.2	6.25 (*d*, 9.8)
7	121.9	6.83 (*s*)	2′-Me_2	27.8	1.41 (3H, *s*)
				28.0	1.40 (3H, *s*)
8	114.8		3-OMe	56.0	3.86 (3H, *s*)

the pterocarpan nucleus and the hydroxyl had to be located at the C-2 position. Placement of hydroxyl to C-2 was further confirmed by an HMBC experiment, revealing correlation between the hydrogen signal from δ_H 5.29 (1H, *s*) to the carbon at δ_C 115.0 (C-1), 140.6 (C-2) and 147.9 (C-3).

Pterocarpans contain two chiral centers (C-6a and C-11a), but only the (6a *R*, 11a *R*) and (6a *S*, 11a *S*) configurations are found in nature. It is accepted that all laevorotatory pterocarpans have the (6a *R*, 11a *R*) absolute configuration, and dextrorotatory ones the (6a *S*, 11a *S*) configuration (Slade et al., 2005). In the case of compound **3**, because the high negative optical rotation value (−216.0, c 0.13, MeOH + $CHCl_3$), the absolute configuration of **3** was determined to be 6a *R*, 11a *R*. The absolute configuration of **3** can also be deduced as (6a *R*, 11a *R*) from the negative Cotton effect of the CD curve (MeOH, [Δε] −30.71) at 223 nm by comparison with the CD data of related known compounds (Tanaka et al., 1997; Kikuchi et al., 2007). Based on the above mentioned evidence, the structure of compound **3** was established as 2-hydroxy-3-methoxy-2′, 2′-dimethylpyrano-[5′, 6′:8, 9]-(6a *R*, 11a *R*) pterocapan, and named pachylobin A.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Horiba SEPA-300 polarimeter; UV spectra were obtained using a Shimadzu UV-2401 PC spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Bruker Tensor-27 infrared spectrophotometer with KBr pellets. HREIMS was performed on a Waters AutoSpec Premier P776 instrument. All NMR experiments were run on a Bruker DRX-500 instrument with tetramethylsilane (TMS) as the internal standard. Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical Co. Ltd., China), Lichroprep RP-18 gel (40–63 μm, Merck, Germany), MCI gel CHP-20P (75–150 μm, Mitsubishi Chemical Co., Japan) and Sephadex LH-20 (GE healthcare, Sweden). Fractions were monitored by TLC (GF_{254}, Qingdao Marine Chemical Co. Ltd., China), and spots were visualized under UV light or by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

3.2. Plant material

The vine stems and leaves of *Millettia pachyloba* Drake were collected from Xishuangbanna, Yunnan Province, China in October 2015, and authenticated by Prof. Hong Wang, herbarium of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (No. 20151001) was deposited in the ethnomedicine research group of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

3.3. Extraction and isolation

The sun dried and powdered vine stem and leaves of *M. pachyloba* (7.5 kg) were extracted three times (3, 5 and 7 days, respectively) by maceration with 95% EtOH at room temperature, to afford crude extract after evaporation under vacuum. The crude extract was suspended in water and successively extracted with petroleum ether, $CHCl_3$ and EtOAc. The combined petroleum ether extract was evaporated under reduced pressure to afford a deep-brown gum (115 g) and the $CHCl_3$ extract was evaporated to give a brown gum (118 g). The petroleum ether fraction showed similar TLC to that of the $CHCl_3$ fraction; hence they were combined and subjected to silica gel column chromatography (CC) with petroleum ether–EtOAc step-gradient elution (9:1–3:7) to yield 5 fractions (A–E). Fraction B (6 g) was separated by reversed-phase C_{18} (RP-18) CC eluted with MeOH–H_2O (70–90%) to give compound **2** (15 mg) and two sub-fractions (B1 and B2). Sub-fractions B1 (1 g) was further purified by CC on Sephadex LH-20 eluted with MeOH to afford compound **3** (18 mg). Fraction E (18 g) was separated by CC on MCI gel CHP-20P eluted with MeOH-H_2O (9:1), followed by Sephadex LH-20 CC eluted with MeOH to provide compound **1** (32 mg).

3.3.1. Pachyloisoflavone A (*1*)

White amorphous powder; UV (MeOH) λ_{max} nm (log ε): 300 (4.15), 254 (4.31), 218 (4.57); IR (KBr): ν_{max} 3417, 2934, 1635, 1605, 1515, 1467, 1433, 1217, 1150, 1032 cm^{-1}; HREIMS: *m/z* 426.1674 (calc. for $C_{24}H_{26}O_7$, 426.1679, deviation −1.2 ppm); ^1H and ^{13}C NMR see Table 1.

3.3.2. Pachyloisoflavone B (*2*)

White amorphous powder, $[\alpha]_D^{26}$ −86.8 (*c* 0.089, MeOH + $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 276 (4.65), 218 (4.55); CD (MeOH, $c = 1.34 \times 10^{-4}$ mol L^{-1}); λ_{max} nm (Δε): 226 (+2.81), 261 (−10.46) nm; IR (KBr): υ_{max} 3381, 2915, 1672, 1621, 1566, 1513, 1469, 1299, 1250, 1063 cm^{-1}; HREIMS: *m/z* 420.1573 (calc. for $C_{25}H_{24}O_6$, 420.1566, deviation −1.7 ppm); ^1H and ^{13}C NMR see Table 1.

3.3.3. Pachylobin A (*3*)

Colorless amorphous powder; $[\alpha]_D^{25}$ −216.0 (*c* 0.13, MeOH + $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 308 (4.03), 220 (4.57); CD (MeOH, $c = 2.07 \times 10^{-4}$ mol L^{-1}); λ_{max} nm (Δε): 223 (−30.71), 313 (+7.64) nm; IR (KBr): υ_{max} 3380, 2967, 1624, 1510, 1479, 1268, 1133, 1078 cm^{-1}; HREIMS: *m/z* 352.1316 (calc. for $C_{21}H_{20}O_5$, 352.1311, deviation 1.4 ppm); ^1H and ^{13}C NMR data see Table 2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2017.02.002.

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