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ORIGINAL ARTICLE

Polysubstituted Isoflavonoids from *Spatholobus suberectus*, *Flemingia macrophylla*, and *Cudrania cochinchinensis*
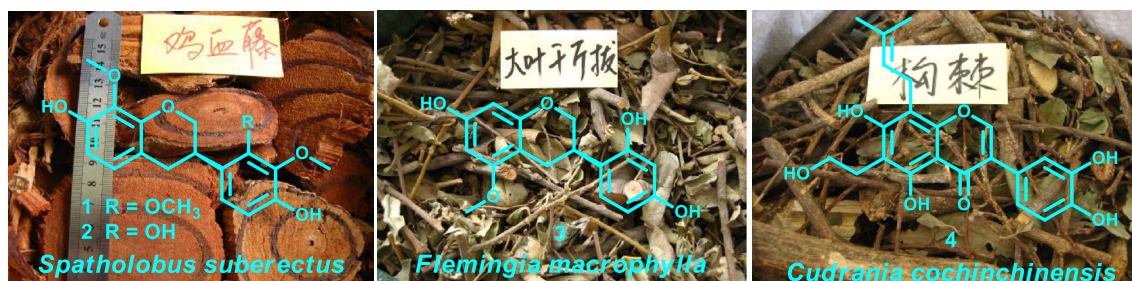
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Abstract Four hitherto unknown polysubstituted isoflavonoids, including three isoflavans: 7,4′-dihydroxy-8,2′,3′-trimethoxyisoflavan (1), 7,2′,4′-trihydroxy-8,3′-dimethoxyisoflavan (2), and 7,2′,4′-trihydroxy-5-methoxyisoflavan (3), and one prenylated isoflavone cudraisoflavone M (4) were isolated from the ethanol extracts of *Spatholobus suberectus* (for 1 and 2), *Flemingia macrophylla* (for 3), and *Cudrania cochinchinensis* (for 4), respectively. Their structures were established on the basis of extensive spectroscopic analysis. Compounds 1 and 4 exhibited weak cytotoxic activity against five human cancer cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW-480).

Graphical Abstract

1 Introduction

Isoflavonoids are a large group of secondary metabolites with diverse biological activities that occur widely in plants, which can be subdivided into isoflavones, isoflavans, isoflavanones, rotenoids, pterocarpans, etc. An overwhelming number of isoflavonoids reported come from the Leguminosae family, and some non-leguminous families such as Moraceae are also relatively abundant in isoflavonoids [1, 2]. Some isoflavans and prenylated isoflavones possess potent biological activities, especially in cytotoxicity [3–6], anti-inflammatory [7], and neuroprotective activity [8]. As part of a BioBioPha (http://www.chemlib.cn) objective to assemble a large-scale natural product library valuable in the discovery of new drug leads from nature [9–12], the phytochemical investigations on *Spatholobus suberectus* (Leguminosae), *Flemingia*

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macrophylla (Leguminosae), and *Cudrania cochinchinensis* (Moraceae) led to the isolation of three new isoflavans 7,4′-dihydroxy-8,2′,3′-trimethoxyisoflavan (**1**), 7,2′,4′-trihydroxy-8,3′-dimethoxyisoflavan (**2**), and 7,2′,4′-trihydroxy-5-methoxyisoflavan (**3**), and one new prenylated isoflavone namely cudraisoflavone M (**4**), respectively (Fig. 1). Herein we report the structure elucidation of new isoflavonoid and their cytotoxicity evaluation against five human cancer cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW-480).

2 Results and Discussion

Compound **1** was obtained as white amorphous powder, and its molecular formula was determined to be $C_{18}H_{20}O_6$ from the positive HRESIMS at m/z 355.1163 [M+Na]$^+$ (calcd. for $C_{18}H_{20}O_6Na$, 355.1158) with nine degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) displayed five aliphatic proton signals due to two methylene groups [δ_H 2.86 (1H, ddd, J = 15.8, 5.3, 1.9 Hz) and 2.94 (1H, dd, J = 15.8, 11.3 Hz), 4.00 (1H, t, J = 10.5 Hz) and 4.37 (1H, ddd, J = 10.5, 3.7, 1.9 Hz)], and one methine [δ_H 3.53 (1H, dddd, J = 11.3, 10.5, 5.3, 3.7 Hz)], which were characteristic of an isoflavan [13]. The ^{1}H NMR spectrum also exhibited two pairs of *ortho*-coupled aromatic doublets [δ_H 6.52 (1H, d, J = 8.3 Hz), 6.72 (1H, d, J = 8.3 Hz), 6.71 (1H, d, J = 8.5 Hz), and 6.75 (1H, d, J = 8.5 Hz)], three methoxy groups [δ_H 3.87, 3.92 and 3.93 (each 3H, s)], and two phenolic hydroxy protons [δ_H 5.66 and 5.71 (each 1H, s)]. The ^{13}C NMR spectrum (Table 2) showed a total of 18 carbon signals, including 12 aromatic carbons for two phenyl units (rings A and B), the ring C carbons at δ_C 31.4 (d), 31.5 (t), 70.5 (t), and three methoxy carbons at δ_C 60.6, 60.8, 61.0 (each q). The above NMR spectroscopic features were very similar to those of isoduartin (=7,2′-dihydroxy-8,3′,4′-trimethoxyisoflavan) [14], except for an obvious downfield shift for H-5′ (Δ = +0.27 ppm), which hinted a different substituted

pattern in ring B. The pattern can be determined as 4′-hydroxy-2′,3′-dimethoxy by the following HMBC correlations (Fig. 2): from 4′-OH (δ_H 5.71) to C-3′ (δ_C 139.8), C-4′ (δ_C 148.5) and C-5′ (δ_C 110.5); from H-3 (δ_H 3.53) and 2′-OCH$_3$ (δ_H 3.87) to C-2′ (δ_C 150.8); and from 3′-OCH$_3$ (δ_H 3.93) to C-3′. The particular downfield shifts ($\Delta \approx$ +5 ppm) of aromatic methoxy carbons also implied the location of these groups. According to the empirical rule, the carbon signals of the methoxy groups with substituents in both *ortho* positions will appear at 60–62 ppm, while those sterically non-hindered at 55–57 ppm [15]. On the basis of the above analysis, the structure of **1** was established as 7,4′-dihydroxy-8,2′,3′-trimethoxyisoflavan.

Compound **2**, isolated as white amorphous powder, had a molecular formula of $C_{17}H_{18}O_6$ by the positive HRESIMS at m/z 341.1002 [M+Na]$^+$ (calcd. for $C_{17}H_{18}O_6Na$, 341.1001). Comparison of the ^{1}H and ^{13}C NMR spectral data (Tables 1, 2) with those of **1** revealed that a methoxy group in the ring B was replaced by a hydroxy. From the HMBC correlations of the OH signal at δ_H 5.72 (1H, s) to C-1′ (δ_C 119.9), C-2′ (δ_C 147.1), and C-3′ (δ_C 134.5), we could conclude that the emerging hydroxy was positioned at C-2′ (Fig. 2). Hence, the structure of **2** was established as 7,2′,4′-trihydroxy-8,3′-dimethoxyisoflavan.

Compound **3** was isolated as white amorphous powder, and its molecular formula was determined to be $C_{16}H_{16}O_5$ according to the positive HRESIMS at m/z 289.1075 [M+H]$^+$ (calcd. for $C_{16}H_{17}O_5$, 289.1076). Five diagnostic ring C proton signals [δ_H 2.59 (1H, dd, J = 16.3, 10.8 Hz), 2.76 (1H, ddd, J = 16.3, 5.5, 1.9 Hz), 3.34 (1H, dddd, J = 10.8, 10.2, 5.5, 3.1 Hz), 3.86 (1H, dd, J = 10.2, 10.2 Hz), and 4.16 (1H, ddd, J = 10.2, 3.1, 1.9 Hz)], an ABX-type aromatic proton system [δ_H 6.30 (1H, d, J = 2.4 Hz), 6.24 (1H, dd, J = 8.3, 2.4 Hz), and 6.85 (1H, d, J = 8.3 Hz)], two *meta*-coupled aromatic doublets [δ_H 5.88 (1H, d, J = 2.1 Hz) and 5.99 (1H, d, J = 2.1 Hz)], and one methoxy signal [δ_H 3.74 (3H, s)] were observed in the ^{1}H NMR spectrum (Table 1), which revealed that **3** was
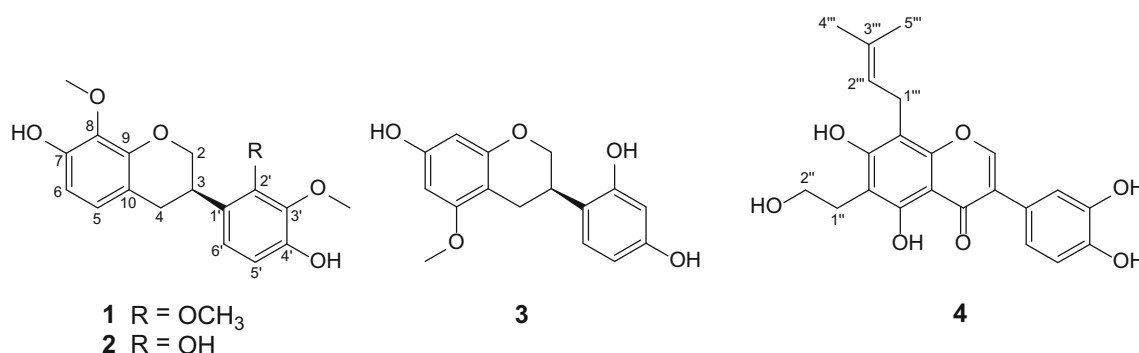

1 R = OCH$_3$
2 R = OH

3

4

Fig. 1 Structures of new compounds **1–4**

Table 1 ^{1}H NMR spectroscopic data of compounds **1–3**

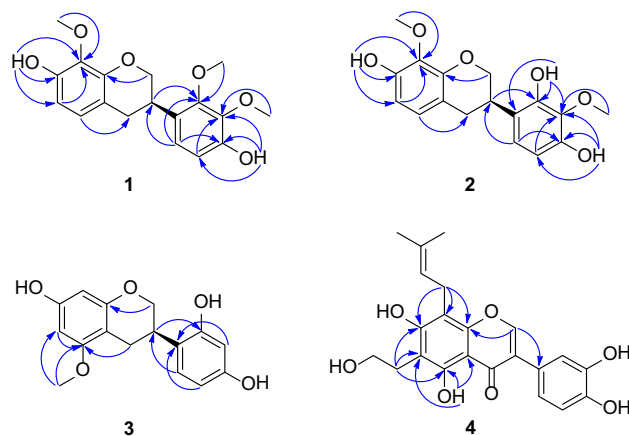| No.	**1**[a]	**2**[a]	**3**[b]
2-H$_{eq}$	4.37 (ddd, 10.5, 3.7, 1.9)	4.43 (ddd, 10.2, 3.4, 2.0)	4.16 (ddd, 10.2, 3.1, 1.9)
2-H$_{ax}$	4.00 (dd, 10.5, 10.5)	4.07 (dd, 10.2, 10.2)	3.86 (dd, 10.2, 10.2)
3	3.53 (dddd, 11.3, 10.5, 5.3, 3.7)	3.52 (dddd, 10.9, 10.2, 5.3, 3.4)	3.34 (dddd, 10.8, 10.2, 5.5, 3.1)
4-H$_{ax}$	2.94 (dd, 15.8, 11.3)	3.01 (dd, 15.8, 10.9)	2.59 (dd, 16.3, 10.8)
4-H$_{eq}$	2.86 (ddd, 15.8, 5.3, 1.9)	2.90 (ddd, 15.8, 5.3, 2.0)	2.76 (ddd, 16.3, 5.5, 1.9)
5	6.72 (d, 8.3)	6.72 (d, 8.3)	
6	6.52 (d, 8.3)	6.52 (d, 8.3)	5.99 (d, 2.1)
8			5.88 (d, 2.1)
3′			6.30 (d, 2.4)
5′	6.71 (d, 8.5)	6.47 (d, 8.5)	6.24 (dd, 8.3, 2.4)
6′	6.75 (d, 8.5)	6.74 (d, 8.5)	6.85 (d, 8.3)
7-OH	5.66 (s)	5.69 (s)	
2′-OH		5.72 (s)	
4′-OH	5.71 (s)	5.30 (br s)	
5-OCH$_3$			3.74 (s)
8-OCH$_3$	3.92 (s)	3.91 (s)	
2′-OCH$_3$	3.87 (s)		
3′-OCH$_3$	3.93 (s)	3.88 (s)	

[a] Measured in CDCl$_3$ (δ_H 7.26 ppm)

[b] Measured in CD$_3$OD (δ_H 3.30 ppm)

Table 2 ^{13}C NMR spectroscopic data of compounds **1–3**

No.	**1**[a]	**2**[a]	**3**[b]
2	70.5 (t)	69.8 (t)	71.0 (t)
3	31.4 (d)	31.8 (d)	32.6 (d)
4	31.5 (t)	30.3 (t)	26.1 (t)
5	124.3 (d)	124.3 (d)	160.1 (s)
6	107.0 (d)	106.9 (d)	92.3 (d)
7	147.5 (s)	147.4 (s)	157.7 (s)
8	134.8 (s)	134.7 (s)	96.5 (d)
9	147.1 (s)	147.1 (s)	156.9 (s)
10	115.3 (s)	115.3 (s)	104.1 (s)
1′	126.3 (s)	119.9 (s)	120.4 (s)
2′	150.8 (s)	147.1 (s)	157.2 (s)
3′	139.8 (s)	134.5 (s)	103.5 (d)
4′	148.5 (s)	147.4 (s)	157.9 (s)
5′	110.5 (d)	107.8 (d)	107.6 (d)
6′	122.0 (d)	122.7 (d)	128.8 (d)
5-OCH$_3$			55.8 (q)
8-OCH$_3$	61.0 (q)	60.9 (q)	
2′-OCH$_3$	60.8 (q)		
3′-OCH$_3$	60.6 (q)	61.2 (q)	

[a] Measured in CDCl$_3$ (δ_C 77.0 ppm)

[b] Measured in CD$_3$OD (δ_C 49.0 ppm)

Fig. 2 Key HMBC (⁀) correlations of **1–4**

methoxy group at C-5 (Fig. 2). Therefore, the structure of **3** was established as 7,2′,4′-trihydroxy-5-methoxyisoflavan.

Absolute configurations of the isoflavans (**1–3**) were established by comparison of the CD curves with those reported earlier for closely related analogues. All the CD spectra showed a negative Cotton effect in the transition region (215–240 nm), similar with those of 7-*O*-methylisomucronulatol, (3*R*)-isomucronulatol, and abruquinone L [16–19], which indicated the absolute configurations at C-3 of **1–3** being *R*-form. And optical rotation measurements were also taken to assign the absolute configuration. We carefully examined the specific rotation

a monomethyl ether derivative of 5,7,2′,4′-tetrahydroxy-isoflavan. The HMBC correlations from the methoxy and H-4 proton signals to C-5 (δ_C 160.1) confirmed the

values of some 2′,3′,4′-trisubstituted isoflavans (Table 3), and the results suggested that the methoxy group substituted at C-2′ had a huge impact on the specific rotation, probably on account of the spatial proximity. For 2′-methoxyisoflavans, R-/S-form result in positive/negative specific rotation values, respectively, but for 2′-hydroxyisoflavans, R-form gives negative values [14, 17, 20–25]. Our current research results were consistent with the above empirical rule.

Compound **4**, yellowish amorphous powder, had a molecular formula of $C_{22}H_{22}O_7$ according to the positive HRESIMS at m/z 399.1440 $[M+H]^+$ (calcd. for $C_{22}H_{23}O_7$, 399.1444). The NMR spectra (Table 4) showed an olefinic signal (δ_H 8.23, 1H, s; δ_C 154.3, d) and a chelated hydroxy group at δ_H 13.48 (1H, s), characteristic of a 5-hydroxy-isoflavone skeleton [8, 13]. In addition, an ABX-type aromatic proton system [δ_H 7.15 (1H, d, $J = 2.0$ Hz), 6.86 (1H, d, $J = 8.2$ Hz), and 6.94 (1H, dd, $J = 8.2$, 2.0 Hz)], a prenyl group [δ_H 1.63, 1.79 (each 3H, s), 3.45 (2H, d, $J = 7.1$ Hz), and 5.22 (1H, br t, $J = 7.1$ Hz)], and a

hydroxyethyl moiety [δ_H 2.97, 3.90 (each 2H, t, $J = 5.2$ Hz)] were also detected in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum displayed a total of 22 carbon resonances, including two methyls, three sp^3 methylenes, five sp^2 methines, and 12 sp^2 quaternary carbons. These spectroscopic features were very similar to those of cud-raisoflavone L, which was recently isolated from the same genus and shared the same molecular formula with **4** [26]. Their structural difference was only due to the location of the prenyl group and the hydroxyethyl moiety. The hydroxyethyl unit was located at C-6 from the HMBC correlations of H-1″ (δ_H 2.97) and 5-OH (δ_H 13.48) to C-5 (δ_C 158.5) and C-6 (δ_C 111.2), while the prenyl group at C-8 by the correlations of H-1‴ (δ_H 3.45) and H-2 (δ_H 8.23) to C-9 (δ_C 154.6). Thus the structure of **4** was established and named as cudraisoflavone M. It is worth mentioning that cudraisoflavone M (**4**) was also obtained in our phytochemical investigation on the plant *Derris robusta* (Leguminosae).

Table 3 Specific rotation values of some 2′,3′,4′-trisubstituted isoflavans

Compound	Substitution pattern							$[\alpha]_D$	References
	3	6	7	8	2′	3′	4′		
(−)-Duartin	S	–	OH	OMe	OMe	OH	OMe	−18.5 (CHCl₃)	[20]
Mucronulatol	S	–	OH	–	OMe	OH	OMe	−18.5 (Me₂CO)	[21]
Bryaflavan	S	OH	OH	–	OMe	OH	OMe	−17.3 (MeOH)	[22]
2′-O-Methylisomucronulatol	R	–	OH	–	OMe	OMe	OMe	+6.5 (CHCl₃)	[23]
(+)-Duartin	R	–	OH	OMe	OMe	OH	OMe	+3.4 (CHCl₃)	[14]
8,3′-Dihydroxyvestitol	R	–	OH	OH	OH	OH	OMe	−5.2 (MeOH)	[24]
Isomucronulatol	R	–	OH	–	OH	OMe	OMe	−19.4 (EtOH)	[25]
7-O-Methylisomucronulatol	R	–	OMe	–	OH	OMe	OMe	−11.0 (CHCl₃)	[17]

Table 4 NMR spectroscopic data of cudraisoflavone M (**4**) in acetone-d_6 (δ_H 2.04 ppm, δ_C 29.8 ppm)

No.	δ_H	δ_C	No.	δ_H	δ_C
2	8.23 (s)	154.3 (d)	4′		146.1 (s)
3		123.5 (s)	5′	6.86 (d, 8.2)	115.8 (d)
4		182.0 (s)	6′	6.94 (dd, 8.2, 2.0)	121.4 (d)
5		158.5 (s)	1″	2.97 (t, 5.2)	26.1 (t)
6		111.2 (s)	2″	3.90 (t, 5.2)	63.7 (t)
7		161.8 (s)	1‴	3.45 (d, 7.1)	22.4 (t)
8		107.7 (s)	2‴	5.22 (br t, 7.1)	123.3 (d)
9		154.6 (s)	3‴		131.8 (s)
10		105.8 (s)	4‴	1.63 (s)	25.8 (q)
1′		123.8 (s)	5‴	1.79 (s)	17.9 (q)
2′	7.15 (d, 2.0)	117.2 (d)	5-OH	13.48 (s)	
3′		145.5 (s)			

Considering the potent cytotoxic activity of some isoflavonoids, the cytotoxicity of these new isoflavonoids (**1**–**4**) was evaluated against five human cancer cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW-480) using the MTS method. DDP (cisplatin) and paclitaxel were used as positive controls. The results showed that **1** and **4** exhibited weak cytotoxic activity (Table 5), while **2** and **3** were inactive (IC_{50} values >40 μM) for all cell lines.

3 Experimental Section

3.1 General Experimental Procedures

Optical rotations were measured on Jasco P-1020 automatic digital polarimeter. CD spectra were recorded on a Chirascan spectropolarimeter (Applied Photophysics, Leatherhead, Surrey, UK). UV data were obtained from HPLC online analysis. IR spectra were obtained on a Bruker Tensor-27 infrared spectrophotometer with KBr pellets. NMR spectra were carried out on a Bruker Avance III 600 or DRX-500 spectrometer with deuterated solvent signals used as internal standards. ESIMS and HRESIMS were measured using Agilent G6230 time-of-flight mass spectrometer. Preparative MPLC was performed on a Büchi apparatus equipped with Büchi fraction collector C-660, Büchi pump module C-605 and manager C-615. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China), MCI gel CHP-20P (75–150 μm, Mitsubishi Chemical Corporation, Japan), Chromatorex C-18 (40–75 μm, Fuji Silysia Chemical Ltd., Japan) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used for column chromatography. Fractions were monitored and analyzed using TLC, in combination with an Agilent 1200 series HPLC system equipped by an Extend-C18 column (5 μm, 4.6 × 150 mm).

3.2 Plant Material and Isolation (Table 6)

The retention times (t_R) of **1**–**4** on an analytical HPLC Extend-C18 column (20% → 100% MeOH in H_2O over 8.0 min followed by 100% MeOH to 13.0 min, 1.0 mL/min, 25 °C) were 7.51, 6.85, 6.61 and 9.12 min, respectively.

3.2.1 7,4′-Dihydroxy-8,2′,3′-trimethoxyisoflavan (**1**)

White amorphous powder; UV (MeOH) λ_{max}: 228 (sh), 278 nm; $[\alpha]_D^{25}$ +11.0 (c 0.20, MeOH); IR (KBr) ν_{max}: 3396, 2944, 1602, 1497, 1464, 1292, 1198, 1170, 1097, 1068, 1040, 1010, 963 cm^{-1}; ^{1}H NMR data: see Table 1; ^{13}C NMR data: see Table 2; ESIMS (pos.): m/z 355 [M+Na]$^+$; HRESIMS (pos.): m/z 355.1163 [M+Na]$^+$ (calcd. for $C_{18}H_{20}O_6Na$, 355.1158).

3.2.2 7,2′,4′-Trihydroxy-8,3′-dimethoxyisoflavan (**2**)

White amorphous powder; UV (MeOH) λ_{max}: 229 (sh), 277 nm; $[\alpha]_D^{25}$ −13.2 (c 0.20, MeOH); IR (KBr) ν_{max}: 3404, 2929, 1619, 1509, 1470, 1328, 1184, 1091, 1058, 1037, 1001, 804 cm^{-1}; ^{1}H NMR data: see Table 1; ^{13}C NMR data: see Table 2; ESIMS (pos.): m/z 341 [M+Na]$^+$; HRESIMS (pos.): m/z 341.1002 [M+Na]$^+$ (calcd. for $C_{17}H_{18}O_6Na$, 341.1001).

3.2.3 7,2′,4′-Trihydroxy-5-methoxyisoflavan (**3**)

White amorphous powder; UV (MeOH) λ_{max}: 230 (sh), 279 nm; $[\alpha]_D^{25}$ −24.0 (c 0.20, MeOH); IR (KBr) ν_{max}: 3406, 2937, 1620, 1606, 1520, 1501, 1472, 1459, 1200, 1142, 1118, 1037, 974, 819 cm^{-1}; ^{1}H NMR data: see Table 1; ^{13}C NMR data: see Table 2; ESIMS (pos.): m/z 289 [M+H]$^+$; HRESIMS (pos.): m/z 289.1075 [M+H]$^+$ (calcd. for $C_{16}H_{17}O_5$, 289.1076).

Table 5 Cytotoxic activities of compounds **1**–**4** against five human cancer cell lines

Compd.	HL-60	A-549	SMMC-7721	MCF-7	SW-480
1	19.27	>40	19.11	39.72	>40
4	20.60	18.77	25.25	24.30	24.53
DDP	1.49	22.30	18.60	30.10	20.50
Paclitaxel	<0.008	<0.008	<0.008	<0.008	<0.008

Table 6 Plant material and isolation

Compd. no.	Family	Species	Plant parts	Place of origin	Plant wt. (kg)	Extract wt. (g)	Compd. wt. (mg)
1	Leguminosae	*S. suberectus*	stems	Kunming, Yunnan, CN	10.0	800	63
2							10
3	Leguminosae	*F. macrophylla*	twigs and leaves	Yuanyang, Yunnan, CN	10.0	930	56
4	Moraceae	*C. cochinchinensis*	twigs and leaves	Puer, Yunnan, CN	13.0	1000	58

3.2.4 Cudraisoflavone M (4)

Yellowish amorphous powder; UV (MeOH) λ_{max}: 212, 270, 346 (sh) nm; IR (KBr) ν_{max}: 3425, 2926, 1644, 1582, 1522, 1435, 1380, 1306, 1209, 1116, 1083, 1030 cm^{-1}; ^{1}H and ^{13}C NMR data: see Table 4; ESIMS (pos.): m/z 399 $[M+H]^+$; HRESIMS (pos.): m/z 399.1440 $[M+H]^+$ (calcd. for $C_{22}H_{23}O_7$, 399.1444).

3.3 Cytotoxicity Assays

Five human tumor cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW-480) obtained from ATCC (Manassas, VA, USA) were used in the cytotoxicity assay. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of MTS (Sigma, St. Louis, MO, USA). Briefly, 100 μL of adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with an initial density of 1×10^5 cells/mL in 100 μL medium. Each cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with cisplatin and paclitaxel as positive controls. After the incubation, 20 μL MTS and 100 μL medium was added to each well after removal of 100 μL medium, and the incubation continued for 2–4 h at 37 °C. The optical density was measured at 492 nm using a Multiskan FC plate reader (Thermo Scientific, USA). The IC_{50} value of each compound was calculated according to the Reed and Muench method.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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