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Bioactive flavones and biflavones from *Selaginella moellendorffii* Hieron

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ABSTRACT

Three new flavones named 5-carboxymethyl-4′,7-dihydroxyflavone (1), its ethyl ester (2) and butyl ester (3) were isolated from the herb *Selaginella moellendorffii* Hieron., together with ten known compounds. Their structures were elucidated on the basis of spectroscopic and chemical analysis. Selected compounds were evaluated for their anti-HBV and cytotoxic activity. Among them, compounds 2 and 3 displayed inhibitory activity *in vitro* on hepatitis B virus (HBV) surface antigen (HBsAg) secretion of the Hep G2.2.15 cell line with IC_{50} values of 0.17 mg/ml and 0.46 mg/ml, and on HBV e antigen (HBeAg) secretion with IC_{50} values of 0.42 mg/ml and 0.42 mg/ml, respectively. Compounds 7, 8, 10 and 12 exhibited selective cytotoxicity against the three human cancer cell lines tested.

1 . Introduction

Selaginella moellendorffii Hieron., a perennial herb of genus *Selaginella* (Selaginellacea), is mainly distributed in the southern area of Changjiang River in China, which is used extensively by the folks for the treatment of gonorrhea, jaundice, hepatitis, and bleeding [1]. Previous investigations of some other *Selaginella* species revealed the genus *Selaginella* to be a rich source of biflavonoids, which exhibited broad activities, including cytotoxic [2,3], antiviral [4], inhibition of nuclear factor-k B activation [5], antiplasmodial and leishmanicidal [6] activities; other types of compounds such as alkaloidal glycosides [7,8], phenylpropanones and lignans [9–11] were also reported from some *Selaginella* species. However, chemical analysis with *S. moellendorffii* has been limited yet [12–16]. Our search for bioactive metabolites of the *S. moellendorffii* herb led to the isolation of three new flavones and ten known compounds (Fig. 1). Selected compounds were evaluated for their anti-HBV activity *in vitro* using the HBV transfected Hep G2.2.15 cell line , as well as for cytotoxic activity against the non-small cell lung cancer (A549), stomach adenocarcinoma (BGC-823) and liver cancer (BEL-7402) human cell lines. This paper presents the details of isolation and structure elucidation of new compounds and results on the anti-HBV and cytotoxic activity.

2. Experimental

2.1 . General

Silica gel (200–300 mesh, Qingdao Marine Chemical Inc.; Qingdao, China), polyamide (100–200 mesh, Sinopharm Chemical Reagent Co., Ltds; Shanghai, China), HPD-100 resin (Changzhou Baoen Chemical Inc.; Hebei, China) and Sephadex LH-20 (Amersham Bioscience, Sweden) were used for column chromatography (CC). UV spectra were carried out on a Shimadzu UV 2401-PC spectrophotometer, λ_{max} in nm. IR spectra were measured on a Bruker Tensor 27 FT-IR spectrometer with KBr pellets, in cm^{-1}. Melting points (m.p.) were determined on a Yanaco MP-S_3 micro-melting point apparatus; uncorrected. MS data were obtained on a VG-Autospec-3000 mass spectrometer; in *m/z* (rel.%). NMR spectra were recorded on a Bruker AV-500

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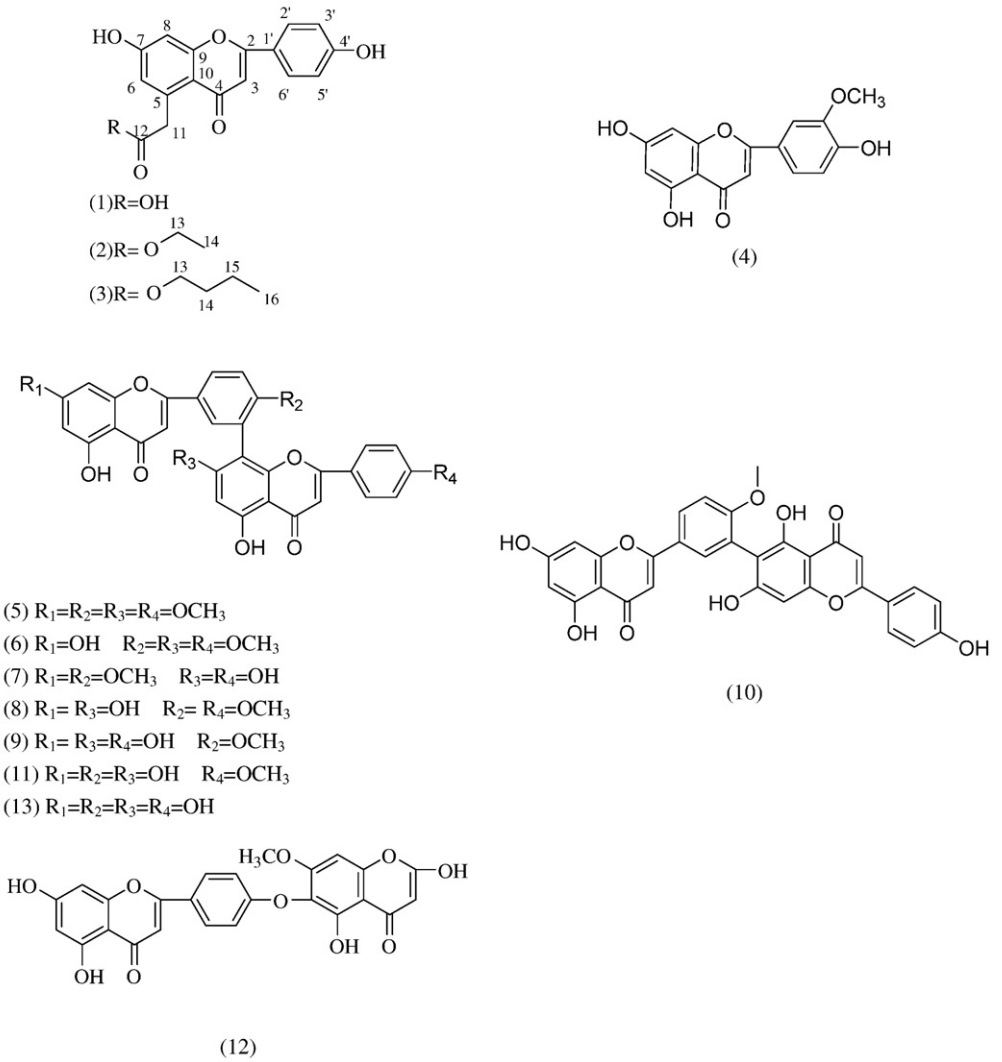


Fig. 1. Structures of compounds 1–13.

($^1H/^{13}C$, 500 MHz/125 MHz) spectrometers and chemical shifts were given in δ with TMS as internal reference.

2.2. Plant materials

The herb *S. moellendorffii* was collected from Zhejiang Province, PR China in October 2007 and identified by Dr. Pro. Qiang Wang, Department of Pharmacognosy, China Pharmaceutical University, where a voucher specimen (2007-10-003) was preserved.

2.3 . Extraction and isolation

The air-dried plant (5 kg) were cut into small pieces and extracted with 95% ethanol (50 L×3) under reflux. The ethanolic extracts were combined and concentrated *in vacuo* to yield a brown residue (250 g) which was defatted with petroleum ether (PE, 60–90 °C; 5 L×3) and then extracted with EtOAc (5 L×3) and n-BuOH (5 L×3), successively, to yield EtOAc fraction (108 g) and n-BuOH fraction (65 g), respectively.

A part of the EtOAc fraction (80 g) was subjected to CC (silica gel; 8×60 cm; 1.2 kg; $CHCl_3$/MeOH 1:0→1:1, and MeOH) to give six subfractions (Fr.). Fr. 2 (2.6 g) was further submitted to repeated CC (silica gel; $CHCl_3$/MeOH 1:0→100:2) to afford compounds 5 (15 mg) and 6 (6.2 mg). Fr. 3 (6.7 g) was separated by repeated CC (silica gel; $CHCl_3$/MeOH 100:1→100:5) to yield compounds 7 (28 mg) and 8 (25 mg). Compounds 9 (7.1 mg), 10 (18.2 mg) and 11 (7.8 mg) were obtained from Fr. 4 (10.5 g) by repeated chromatography over polyamide column (100–200 mesh). Fr. 5 (15.1 g) was subjected to CC (silica gel; 3.5×30 cm; $CHCl_3$/MeOH 96:4→50:50, and then MeOH), and further purified with Sephadex LH-20, produced compounds 12 (9.1 mg) and 13 (105 mg), respectively.

The n-BuOH extract (65 g) was separated by column chromatography over HPD-100 macroporous resin with a gradient from H_2O to 95% EtOH to give five fractions: H_2O

fraction, 20% EtOH fraction, 50% EtOH fraction, 70% EtOH fraction and 95% EtOH fraction. Evaporated 50% EtOH fraction (34.5 g) was subjected to repeated CC (silica gel; $CHCl_3$/MeOH 98:2 → 50:50) to afford compound 1 (49 mg). 70% EtOH fraction (3.1 g) was then subjected to CC (silica gel) and eluted with a $CHCl_3$–MeOH (99:1, 98:2, 96:4, 92:8, 80:20) gradient system. Fractions were combined based on TLC results to yield subfractions designated E1–10. After further repeated purification over Sephadex LH-20, eluting with MeOH, compound 2 (8.1 mg) came from E8, 3 (6.6 mg) from E5, and 4 (6.1 mg) from E3, respectively.

[7-Hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-5-yl]-acetic acid (1): pale yellow white feather crystal (MeOH). m.p. not detected. Decomposition point: 241 °C. UV (MeOH) λ_{max} nm 324 (3.16), 261 (3.05). IR (KBr): 3397, 2924, 2853, 1715, 1685, 1607, 1510, 1443, 837 cm^{-1}. For ^1H and ^{13}C NMR data see Table 1. FAB-MS *m/z* 311[M-H]$^-$, HR-FAB-MS *m/z* 311.0562[M-H]$^-$ (calcd. for $C_{17}H_{11}O_6^-$, 311.0555).

[7-Hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-5-yl]-acetic acid ethyl ester (2): pale yellow white solid. m.p. not detected. Decomposition point: 235 °C. UV (MeOH) λ_{max} nm 328 (3.40), 259 (3.12). IR (KBr): 3422, 3380, 2927, 2820, 1698, 1630, 1607, 1564, 1443, 1253, 836 cm^{-1}. For ^1H and ^{13}C NMR data see Table 1. FAB-MS *m/z* 341[M + H]$^+$, HR-FAB-MS *m/z* 341.1023[M + H]$^+$ (calcd. for $C_{19}H_{17}O_6^+$, 341.1025).

[7-Hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-5-yl]-acetic acid butyl ester (3): pale yellow white solid. m.p. not detected. Decomposition point: 186 °C. UV (MeOH) λ_{max} nm 222 (3.02), 261 (3.02). IR (KBr): 3415, 2924, 2853, 1628, 1606, 1508, 1251, 1176, 837 cm^{-1}. For ^1H and ^{13}C NMR data see Table 1. ESI-MS *m/z* [M-H]$^-$ 367.0 , ESI-MS^2 *m/z* [M-H]$^-$ 292.9.

2.4 . Anti-HBV assay

Three new flavones 1, 2, 3, and three biflavones 10, 12, 13 belonging to robusta-, hinoki- and amentoflavone series, respectively, isolated from *Selaginella moellendorffii* were tested for their potential anti-HBV activities, namely the abilities to inhibit the secretion of HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) in HBV-infected 2.2.15 cells using lamivudine (3TC, a clinically popular anti-HBV agent) as a positive control, according to previous reports [17,18]. The anti-HBV activity of each compound was expressed as the concentration of compound that achieved 50% inhibition (IC_{50}) to the secretion of HBsAg and HBeAg. The cytotoxicity of each compound, assayed by a modified MTT method [19], was expressed as the concentration of compound required to kill 50% (CC_{50}) of HepG 2.2.15 cells. The selectivity index (SI), a major pharmaceutical parameter that estimates possible future clinical development, was determined as the ratio of CC_{50} to IC_{50}. The bioactivity of each compound was evaluated by the combination of its IC_{50} and SI. The results are summarized in Table 2.

2.5. Cytotoxicity test (sulforhodamin B assay)

Due to insufficient material, seven compounds–1, 2, 3, 7, 8, 10, and 12–isolated from *Selaginella moellendorffii* were tested for their cytotoxic activity against A549, BGC-823 and BEL-7402 human cell lines by the sulfurhodamine B (SRB) assay with paclitaxel as positive control [20,21]. The cancer cells were cultured in RPMI-1640 containing 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Inhibition data were expressed as IC50 values and the results are summarized in Table 3.

3. Results and discussion

Compound 1 was obtained as a pale yellow white feather crystal (MeOH). The UV spectrum maxima at 324 nm (log ε 3.16) and 261 nm (log ε 3.05), and the positive result of the spray reagent of $AlCl_3$ suggested a flavone-like compound.

Table 1
^1H (500 MHz) and ^{13}C NMR(125 MHz) data for compounds 1,2 and 3 in DMSO-d_6.

Position	1		2		3	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
2	–	161.1	–	161.2	–	161.2
3	6.58 1H, s	105.5	6.59 (1H)	105.4	6.59 (1H)	105.4
4	–	178.0	–	178.0	–	178.1
5	–	137.9	–	137.5	–	137.2
6	6.68 (1H, d, 2.2 Hz)	118.0	6.70 (1H, d, 2.3 Hz)	118.1	6.70 (1H, d, 2.4 Hz)	118.1
7	–	161.0	–	161.1	–	161.1
8	6.89 (1H, d, 2.3 Hz)	101.7	6.90 (1H, d, 2.3 Hz)	101.8	6.90 (1H, d, 2.4 Hz)	101.9
9	–	158.5	–	158.6	–	158.6
10	–	114.6	–	114.5	–	114.5
11	4.06 (2H, s)	40.1	4.08 (2H, s)	40.1	4.08 (2H, s)	40.1
12	11.98 (OH)	172.1	–	170.5	–	170.7
13	–	–	4.05 (2H, q, 7.1 Hz)	59.8	3.99 (2H, t, 6.6 Hz)	63.4
14	–	–	1.18 (3H, t, 7.1 Hz)	13.6	1.53 (2H, qui. 7.0 Hz)	30.2
15	–	–	–	–	1.30 (2H, sex. 7.4 Hz)	18.5
16	–	–	–	–	0.86 (3H, t, 7.4 Hz)	13.5
1′	–	121.5	–	121.5	–	121.5
2′6′	7.88 (2H, d, 8.8 Hz)	128.0	7.88 (2H, d, 8.8 Hz)	128.0	7.88 (2H, d, 8.8 Hz)	128.0
3′5′	6.92 (2H, d, 8.8 Hz)	115.9	6.92 (2H, d, 8.9 Hz)	115.9	6.92 (2H, d, 8.9 Hz)	115.9
7-OH	10.67	–	10.70	–	10.70	–
4′-OH	10.20	–	10.20	–	10.20	–

Table 2
Anti-HBV activity, toxicity and selectivity index of selected compounds.

Compounds	CC_{50} [a]	HBsAg [b]		HBeAg [c]	
	(mg/ml)	IC_{50}(mg/ml) [d]	SI [e]	IC_{50}(mg/ml)	SI
1	>0.98	>0.98	–	>0.98	–
2	>1.02	0.17	>6.0	0.42	>2.4
3	>0.97	0.46	>2.1	0.42	>2.3
10	0.05	0.09	0.6	0.02	2.5
12	<0.02	<0.02	–	<0.02	–
13	<0.06	<0.06	–	<0.06	–
3TC [f]	6.00	4.04	1.5	9.80	0.6

[a] CC_{50}: 50% cytotoxic concentration.
[b] HBsAg: HBV surface antigen.
[c] HBeAg: HBV e antigen.
[d] IC_{50}: 50% effective concentration.
[e] SI (selective index) = CC_{50}/IC_{50}.
[f] 3TC: lamivudine, an antiviral agent used as positive control.

The IR spectrum showed the presence of hydroxyl group (3397 cm^{-1}), methylene group (2924, 2853 cm^{-1}), two carbonyl groups (1715, 1685 cm^{-1}), aromatic ring (1607, 1510, 1443 cm^{-1}) and *p*-disubstituted benzene (834 cm^{-1}). The negative ESI-MS and FAB-MS gave a molecular ion peak at *m/z* 311. The molecular formula was suggested as $C_{17}H_{12}O_6$ by the negative HR-FAB-MS at *m/z* 311.0562 [M-H]$^-$ (calcd. 311.0555). The ^{1}HNMR spectrum (DMSO-d_6) showed an AM coupling system signals at δ 6.68 (d, 1H, J = 2.2 Hz, H-6) and 6.89 (d, 1H, J = 2.3 Hz, H-8). No signal for chelated hydroxyl group was observed, but the singlet for a methylene appeared at δ 4.06. The ^{13}C NMR exhibited 17 carbons, among them are one methylene carbon (δ 40.1), one carbonyl carbon (δ 178.0) and one carboxylic carbon (δ 172.1). The HMBC correlations between methylene protons (δ 4.06) and C-5 (δ 137.9), C-6 (δ 118.0), C-10 (δ 114.6) and a carboxylic carbon (δ 172.1) indicated that a carboxymethyl group is attached to C-5. An AA′XX′coupling system signals at δ 6.92 (d, 2H, J = 8.8 Hz, H-3′, H-5′) and 7.88 (d, 2H, J = 8.8 Hz, H-2′, H-6′) were observed in the ^{1}HNMR spectrum, revealing that ring B was *p*-disubstituted. A singlet at 6.58 (1H) accounted for H-3, which in HMBC spectrum is correlated with C-2 (δ 161.1), C-4 (δ 178.0), C-10 (δ 114.6) and C-1′(δ 121.5) (Fig. 2). The ^{1}H and ^{13}C NMR signal assignments were achieved by combination of HSQC and HMBC spectral elucidation, and are in comparison with literature values of compound 5-carboxymethyl-4′-hydroxy-flavone-7-*O*-β-D-glucopyranoside [10]. Therefore, structure of compound 1 was elucidated as 7-Hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H- chromen-5-yl]-acetic acid.

Compound 2 was obtained as pale yellow white solid. The UV spectrum maxima at 328 nm (log ε 3.40) and 259 nm (log ε 3.12), and the positive result of the spray reagent of $AlCl_3$ implied that it was also a flavone-like compound. The IR spectrum showed characteristic absorption at 1253 cm^{-1} for $\nu_{c\text{-}o\text{-}c}$. The ^{1}H NMR and ^{13}C NMR of compound 2 was quite similar with that of compound 1 except for an additional ethyl group and the absence of OH group (δ 11.98), which suggested that the substituted group of CH_2COOH at C-5 in compound 1 be changed into $CH_2COOC_2H_5$. It was verified by the molecular formula of $C_{19}H_{16}O_6$ deduced from positive HR-FAB-MS *m/z* 341.1023[M + H]$^+$ (calcd. 341.1025).

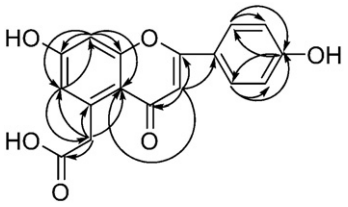


Fig. 2. Key HMBC correlations of compound 1.

Compound 3 was obtained as pale yellow white solid. The UV spectrum maxima at 322 nm (log ε 3.02) and 261 nm (log ε 3.02), and the positive result of the spray reagent of $AlCl_3$ indicated that it was also a flavone-like compound. The IR spectrum showed characteristic absorption at 1251 and 1176 cm^{-1} for $\nu_{c\text{-}o\text{-}c}$. It has been found that the 3 most shared structure, features with compounds 1 and 2 by careful comparison of their ^{1}H and ^{13}C NMR spectral data. The only difference between them could be rationalized that compound 1 was an acid while compound 2 was its ethyl ester, and compound 3 was its butyl ester. In addition, negative ESI-MS *m/z* 367.0 and ESI-MS^2 *m/z* 292.9, which also suggested the presence of a butyl group, consequently confirmed the structure of compound 3 as 7-Hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-5-yl]-acetic acid butyl ester.

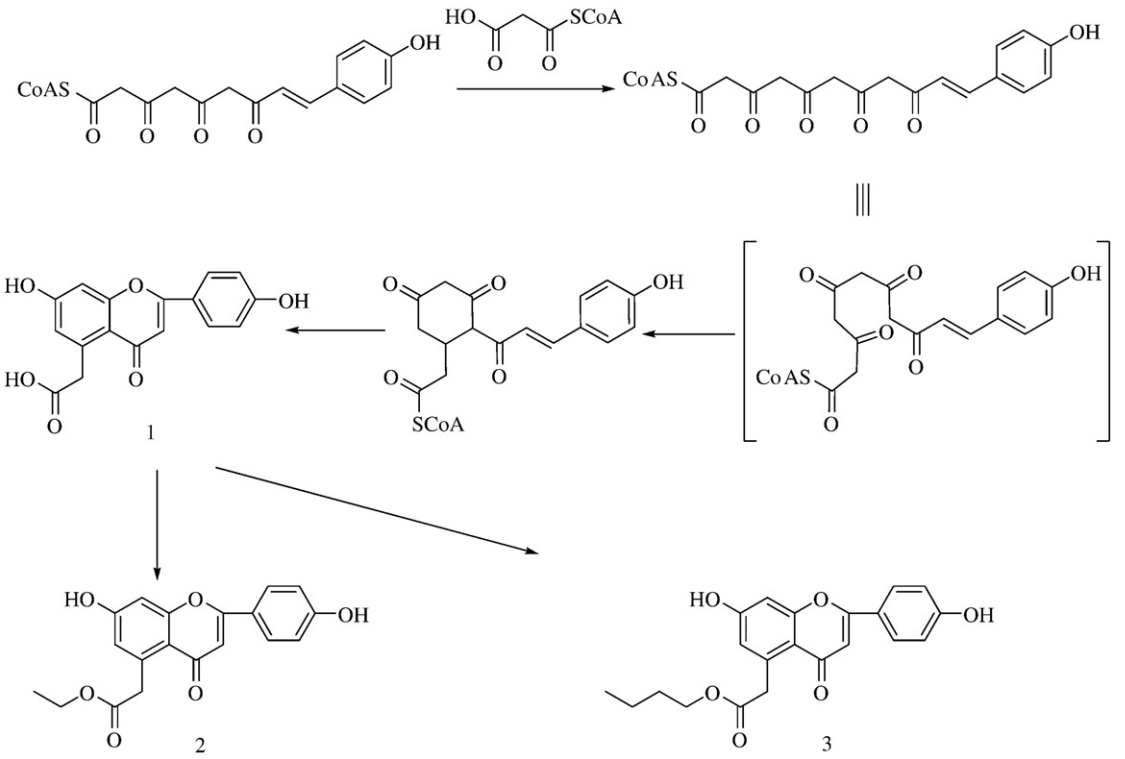
Comparison of the spectroscopic and physical data with those published allowed us to establish the structures of known compounds 4–13 as chrysoeriol [22,23], amentoflavone 7,4,7,4-tetramethyl ether, kayaflavone, ginkgetin [12,16], isoginkgetin, bilobetin [24], robustaflavone 4′-methyl ether [25], podocarpusflavone A [12,16], hinokiflavone [26] and amentoflavone [12,16], respectively. Among them, chrysoeriol and isoginkgetin were first reported from this genus, and bilobetin and robustaflavone 4′-methyl ether were first reported from this plant. These results show that *S. moellendorffii* biflavonoids possess mainly the C-3–C-8 interflavonoid linkage.

A plausible biosynthetic pathway was proposed for compounds 1–3, as shown in Scheme 1, in which 4-coumaroyl-tetraketide should be the precursor. It is known that most naturally occurring flavonoids possessed C-5-OH substitution, which came from phenylpropanoid pathway through chalcone synthase (CHS) type reaction. However, flavonoids with a carboxymethyl group attached to C-5 are very unusual, their biosynthetic process is still to be studied. To date, only one compound had been isolated with the similar structure features, also from *S. moellendorffii* [15]. This finding is important from a phylogenetic point of view, since the presence of such rare flavonoids in Selaginellacea supports the theory that the lycophyte in *S. moellendorffii* has evolved independently from other vascular plants [27,28].

Table 3
Cytotoxicity of selected compounds against cancer cell lines.

Cell lines	IC_{50}(μg/ml) [a]							
	1	2	3	7	8	10	12	Paclitaxel [b]
A549	NA [c]	NA	NA	1	NA	5.398	NA	0.075
BGC-823	NA	6.284	NA	NA	NA	NA	1.024	0.0014
BEL-7402	NA	NA	NA	5.001	6.077	6.772	1.406	0.58

[a] IC_{50}: The IC_{50} values are means of three experiments.
[b] Positive control.
[c] NA: not active.



Scheme 1. Biogenetic pathway for compounds 1–3.

Further chemical analysis of other *Selaginella* species would be of great interest as well as correlated DNA-based phylogenetic investigations.

The anti-HBV activities of the six compounds–1, 2, 3, 10, 12, 13–isolated from *S. moellendorffii* in this study were evaluated using the Hep G2.2.15 cell line stably transfected with the HBV genome. Anti-HBV activity, toxicity and selectivity index (SI) are summarized in Table 2.

It was concluded that compounds 2 and 3 showed anti-HBV activity at non-toxic concentrations with SI values of about 2 or more for HBsAg and about 2 for HBeAg. Interestingly, compound 1 was inactive, but its ethyl ester (compound 2) and butyl ester (compound 3), possessed inhibitory potency to the secretion of HBsAg and HBeAg. In addition, we chose three kinds of biflavones with different interflavonoid linkages, respectively, as candidates to test their toxicity and potential anti-HBV activity. All of them exhibited high cell toxicity, except that amentoflavone showed some degree of inhibitory potency to the secretion of HBeAg ($IC_{50} = 0.02$ mg/ml, $SI = 2.5$), which stimulated us to further test their cytotoxicity against human cancer cell lines. It is noticeable that all biflavonoids tested showed selective cytotoxic activity, with the results presented in Table 3.

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