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SHORT REPORT

Chemical constituents from *Selaginella sanguinolenta* with cytotoxic activity

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Abstract: A new piperazine alkaloid (**1**) and a new selaginellin derivative (**2**), along with six known compounds, selaginellin T (**3**), selaginellin D (**4**), selaginellin (**5**), selaginellin B (**6**), selariscinin C (**7**), and selaginpulvilin D (**8**), were isolated from *Selaginella sanguinolenta*. The structures of two new compounds were identified by UV, 1D, 2D NMR and HR-ESI-MS spectrum. Compound **1** represents the first natural product with a piperazine ring and a sulfonic acid group. Among these compounds, compound **2** exhibited moderate cytotoxicity against both HepG2 and H1975 cell lines, with IC_{50} values of 94.07 and 130.1 μM, respectively. Notably, compound **3** demonstrated more potent cytotoxicity against HepG2 cells, with an IC_{50} value of 41.97 μM, while the remaining compounds demonstrated no appreciable activity under the tested conditions.

Keywords: *Selaginella sanguinolenta*, natural products, alkaloids, selaginellin, cytotoxic activity

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1 Plant Source

The *Selaginella sanguinolenta (L.) Spring* was collected from Liangwang Mountain, Chenggong District, Kunming City, Yunnan Province, China, in 2023, and identified by Associate Professor Hui Zou (College of Pharmacy, Hunan Normal University). The identification was further confirmed by comparison with authenticated herbarium specimens of *S. sanguinolenta*, including the specimen deposited in the NYBG Steere Herbarium (Barcode No. 03865052). A voucher specimen (No. HZJB-2023) was deposited in the College of Pharmacy, Hunan Normal University.

2 Previous Studies

The genus *Selaginella* is an ancient and evolutionarily unique genus with a long history of use in TCM worldwide. Extensive phytochemical investigations have revealed that this genus produced a rich of structurally diverse secondary metabolites, including biflavonoids, lignans, polyacetylenes, alkaloids, phenols, and glycosides (Chen et al., 2025; Krizkovska et al., 2020; Reginaldo et al., 2020). These constituents have been confirmed to exhibit antitumor, anti-inflammatory, antioxidant, antimicrobial, antiviral, and neuroprotective effects, which making *Selaginella* as a valuable reservoir for drug discovery and development (Adnan et al., 2021; Kumar et al., 2021; Ren et al., 2023). However, despite these advances, studies on microcomponents and novel compounds in this genus remain insufficient (Cao et al., 2010; Rui et al., 2022). In particular, acetylenic phenols and alkaloids, which are regarded as characteristic chemotaxonomic markers of the genus, have been relatively rarely reported (Wang et al., 2009; Wen et al., 2021; Zhao et al., 2013).

Selaginella sanguinolenta (L.) Spring is a widely distributed representative species of the genus, often found in rocky and alpine habitats. To date, no phytochemical investigation has been reported on this species. Therefore, a detailed exploration of its chemical constituents is of considerable significance for enriching the chemical diversity of the genus, discovering structurally novel compounds, and providing new insights into the chemotaxonomic characteristics and potential pharmacological value of this species.

3 Present Study

5.0 Kg of air-dried whole plant of *Selaginella sanguinolenta* was refluxed twice with ethanol-H_2O (70:30). The combined

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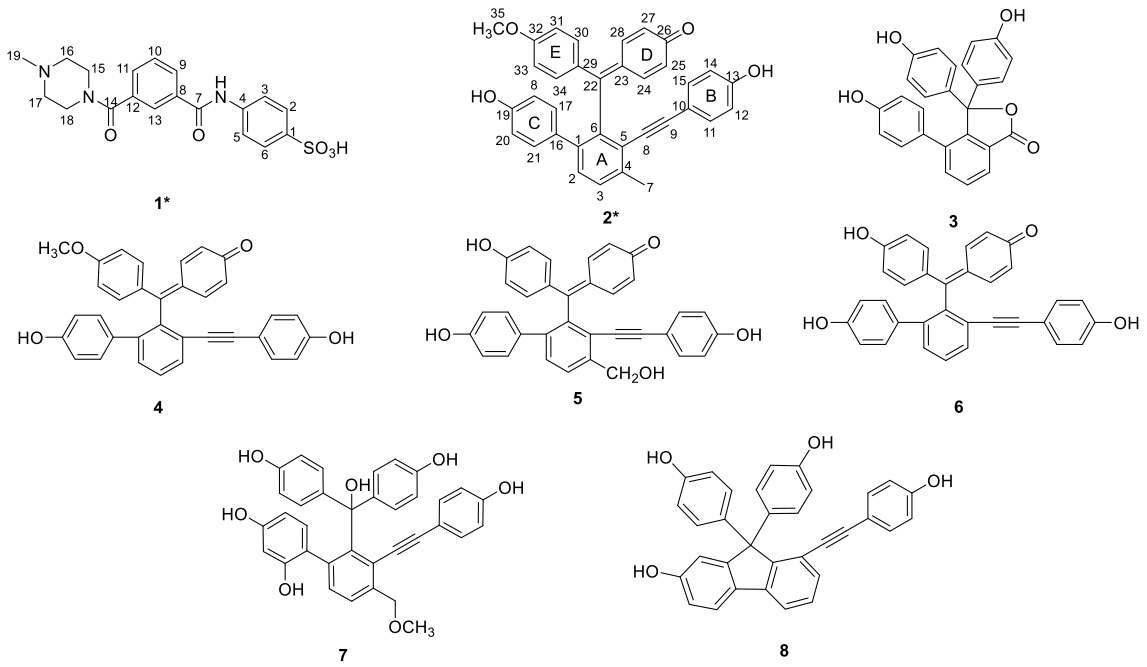

Figure 1. The chemical structures for compounds **1**–**8**

extracts were concentrated under reduced pressure to afford a crude extract (2.5 kg). The crude extract was load onto a silica gel column chromatography for separation. Gradient elution with a system of dichloromethane–methanol to yield five fractions (Fr.A-Fr.E). Fraction C was increasingly separated by ODS column chromatography using a H_2O-MeOH gradient system (100:0 → 0:100) to give several subfractions. Subfraction C3 was further purified by semi-preparative HPLC (ACN-H_2O, 70:30, 2.5 mL/min) to obtain compound **1** (2.7 mg). Subfraction C4 was further separated by silica gel column chromatography (CH_2Cl_2-MeOH, 20:1 → 10:1) and semi-preparative HPLC (ACN-H_2O, 75:25, 2.5 mL/min) to obtain compounds **3** (1.9 mg) and **4** (3.1 mg). Fraction Fr.B was subjected to a ODS column with a system of H_2O–MeOH (100:0 → 0:100), followed by semi-preparative HPLC (ACN-H_2O, 65:35, 2.5 mL/min) to afford compound **2** (2.6 mg). Fraction D was further purified by a silica gel column (CH_2Cl_2-MeOH, 15:1 → 8:1) and semi-preparative HPLC (CH_3CN-H_2O, 70:30, 2.5 mL/min) to obtain compounds **5** (1.8 mg) and **6** (3.3 mg). In addition, compounds **7** (2.9 mg) and **8** (1.7 mg) were obtained from fraction E by repeated silica gel column chromatography (CH_2Cl_2-MeOH, 12:1 → 6:1) and semi-preparative HPLC (ACN-H_2O, 68:32, 2.5 mL/min) (Figure 1).

*4-(3-(4-methylpiperazine-1-carbonyl)benzamido)benzenes ulfonic acid (**1**)*: White powder; UV (ACN) λmax: 229, 274, and 318 nM; HR-ESI-MS m/z calculated for $C_{18}H_{21}N_3O_5S$ $[M + H]^+$ 404.1266, found: 404.1280; ^{1}H NMR spectrum (400 MHz) and ^{13}C NMR (100 MHz) data see Table 1.

*4-((4′-hydroxy-4-(hydroxyphenyl)ethynyl)-4-methyl-[1,1′-biphenyl]-2-yl)(4-methoxyphenyl)methylene]-2,5-cyclohexadien-1-one (**2**)*: Yellow amorphous powder. UV (ACN) λmax: 223, 289, and 398 nM. HR-ESI-MS m/z calculated for $C_{35}H_{27}O_4$ 511.1903 $[M + H]^+$, Found: 511.1909; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data see Table 2.

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of Compound **1** (DMSO-d_6)

Position	δ_H (ppm) *J* (Hz)	δ_C (ppm)
1		129.6
2, 6	7.75 (2H, d, *J* = 8.9 Hz)	129.1
3, 5	8.08 (2H, d, *J* = 8.9 Hz)	120.6
4		143.8
7		165.8
8		135.1
9	8.21 (1H, dt, *J* = 7.7 Hz, 1.8 Hz)	132.5
10	7.69 (1H, t, *J* = 7.7 Hz)	129.4
11	8.17 (1H, dt, *J* = 7.7 Hz, 1.8 Hz)	133.0
12		131.9
13	8.55 (1H, t, *J* = 1.8 Hz)	129.1
14		167.4
15, 18	2.91 (4H, t, *J* = 5.3 Hz)	46.1
16, 17	2.42 (4H, t, *J* = 5.3 Hz)	53.9
19	2.17 (3H, s)	45.6
NH	10.87	

The structures of all isolates were identified by completed spectroscopic method, including UV, NMR, and HR-ESI-MS spectrum. The cytotoxic activities of all these compounds were evaluated by the MTT assay against HepG2, H1975, and 4T1 cell lines. Cells were treated with different concentrations of the tested compounds for 48 h, and cell viability was determined by measuring the absorbance at 490 nM. Tested compounds showing inhibition rates higher than 30% were further subjected to IC_{50} determination. The results

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Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of Compound **2** (DMSO-d_6)

Position	δ_H (ppm) *J* (Hz)	δ_C (ppm)
1		140.0
2	7.24 (1H, d, *J* = 8.7 Hz)	129.6
3	7.47 (1H, d, *J* = 8.7 Hz)	130.8
4		124.0
5		138.7
6		140.6
7	2.56 (3H, s)	21.1
8		85.6
9		98.6
10		112.3
11, 15	6.98 (2H, d, *J* = 8.7 Hz)	133.2
12, 14	6.66 (2H, d, *J* = 8.7 Hz)	116.1
13		158.6
16		130.8
17, 21	6.78 (2H, d, *J* = 8.7 Hz)	130.0
18, 20	6.55 (2H, d, *J* = 8.7 Hz)	115.2
19		160.0
22		159.1
23		130.9
24	7.40 (1H, dd, *J* = 2.0, 10.0 Hz)	138.6
25	6.37 (1H, dd, *J* = 2.0, 10.0 Hz)	128.6
26		186.1
27	6.32 (1H, dd, *J* = 2.0, 10.0 Hz)	128.3
28	7.22 (1H, dd, *J* = 2.0, 10.0 Hz)	140.6
29		131.0
30, 34	6.84 (2H, brs)	133.1
31, 33	6.84 (2H, brs)	114.0
32		160.8
35	3.72 (3H, s)	55.8

showed that compound **2** displayed moderate cytotoxicity against HepG2 and H1975 cells, whereas compound **3** displayed more potent cytotoxicity against HepG2 cells. These findings enrich the chemical diversity of *S. sanguinolenta* and provide preliminary insights into the cytotoxic potential of selaginellin-type compounds.

Compound **1** was obtained as a white powder. The UV spectrum showed absorption maxima at λmax 229, 274, and 318 nM. The HR-ESI-MS analysis exhibited a quasi-molecular ion peak at m/z 404.1266 $[M + H]^+$ (calcd for $C_{18}H_{21}N_3O_5S$, 404.1280), equivalent to the molecular formula $C_{18}H_{22}N_3O_5S$ and indicating 11 index of unsaturation (Ω = 11). In the ^{1}H NMR spectrum (400 MHz, DMSO-d_6), a singlet at δ_H 10.20 was observed and assigned to an amide NH proton. The HMBC correlation (Figure 2) of this proton with a carbonyl carbon indicated the presence of a secondary amide group. Two methylene signals at δ_H 2.91 (t, *J* = 5.3 Hz, 4H, 16, 17-CH_2) and 2.42 (t, *J* = 5.3 Hz, 4H, 15, 18-CH_2) indicated a piperazine ring. A singlet at δ_H 2.17 (3H, s, 19-CH_3) was assigned to an N-methyl group attached to the piperazine nitrogen. In the aromatic region, two doublets at δ_H 7.75 (2H, d, *J* = 8.9 Hz) and 8.08 (2H, d, *J* = 8.9 Hz) was belonged to the structrual characteristic of a para-disubstituted benzene ring.

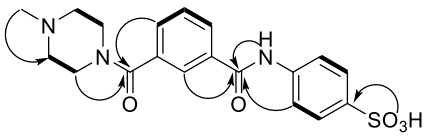

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of com-pound **1**

Additionally, four aromatic proton signals at δ_H 7.69, 8.17, 8.21 and 8.55 were consistent with a meta-disubstituted benzene ring, indicating the presence of the second aromatic ring. The ^{13}C NMR spectrum displayed two carbonyl carbons at δ_C 165.9 and 167.3, assignable to amide group. The DEPT analysis revealed eight aromatic CH carbons, four methylene carbons (δ_C 53.9 and 46.1), one methyl carbon (δ_C 45.6), and four quaternary carbons. The ^{1}H–^{1}H COSY spectrum established the coupling networks of two aromatic rings and a piperazine unit. Correlations between H-15/H-18 (δ_H 2.91) and H-16/H-17 (δ_H 2.42) confirmed the piperazine fragment. The ^{1}H–^{1}H COSY correlations of H-2/H-6 (δ_H 7.75) and H-3/H-5 (δ_H 8.08) were indicative of an AA′BB′ system corresponding to a para-disubstituted benzene ring, whereas the sequential correlations H-9 (δ_H 8.21) ↔ H-10 (δ_H 7.69) ↔ H-11 (δ_H 8.17) supported the presence of a meta-disubstituted benzene ring. In addition, the molecular formula was consistent with the presence of a sulfonic acid group. In the HMBC spectrum, the correlations from H-15, 18 (δ_H 2.91) to C-14 (δ_C 167.4) and H-11 (δ_H 8.17), H-13 (δ_H 8.55) to C-14 (δ_C 167.4) suggested the piperazine ring and the benzene ring were connected by the carbonyl carbon (C-14). The HMBC correlations from H-9 (δ_H 8.21) and H-13 (δ_H 8.55) to C-7 (δ_C 165.8) indicated the this benzene ring was connected with C-7. The HMBC correlations from NH (δ_H 10.87) to C-7 (δ_C 165.8) and C-3, 5 (δ_C 120.6) suggested that the second benzene ring was linked to the nitrogen atom. The chemical shift of H-2, 6 and H-3, 5 along with the HMBC correlations from H-3, 5 (δ_H 8.08) to C-1 (δ_C 129.6) indicated the sulfonic acid group should located at C-1.

Taken together, the above spectroscopic evidence established that compound **1** contains a para-substituted sulfonated benzene ring, a meta-substituted benzene ring, and a piperazine moiety connected through two amide linkages. Accordingly, compound **1** was determined to be 4-(3-(4-methylpiperazine-1-carbonyl)benzamido)benzene su lfonic acid. Compound **1** represents a novel and rare natural product containing a piperazine ring and a sulfonic acid group.

Compound **2**, a yellow amorphous powder, its UV spectrum showed characteristic absorption maxima at λmax 223, 289, and 398 nM. The HR-ESI-MS spectrum showed an ion paek at m/z 511.1903 $[M + H]^+$ (calcd for $C_{35}H_{27}O_4$, 511.1909), suggesting its molecular formula of $C_{35}H_{26}O_4$ with 23 index of unsaturation (Ω = 23). The ^{1}H NMR spectrum (400 MHz, DMSO-d_6) displayed two sets of AA′BB′ systems at δ_H 6.98 (2H, d, *J* = 8.7 Hz, H-11, 15)/6.66 (2H, d, *J* = 8.7 Hz, H-12, 14) and 6.78 (2H, d, *J* = 8.7

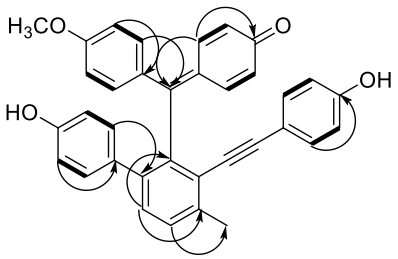

Figure 3. Key ^{1}H-^{1}H COSY and HMBC correlations for compound **2**

Hz, H-17, 21)/6.55 (2H, d, *J* = 8.7 Hz, H-18, 20) indicated the existence of two para-disubstituted benzene rings, and a 1,2,3,4-tetrasubstituted benzene ring at δ_H 7.24 (1H, d, *J* = 8.7 Hz, H-2) and 7.47 (1H, d, *J* = 8.7 Hz, H-3). The four aromatic proton signals of δ_H 7.40 (1H, dd, *J* = 2.0, 10.0 Hz, H-24), 6.37 (1H, dd, *J* = 2.0, 10.0 Hz, H-25), 6.32 (1H, dd, *J* = 2.0, 10.0 Hz, H-27), 7.22 (1H, dd, *J* = 2.0, 10.0 Hz, H-28) were attributed to ring D. Moreover, another four aromatic proton signals at δ_H 6.84 (4H. brs) were attributed to ring E. The signals at δ_H 3.72 (3H, s) and 2.56 (3H, s) were assigned to a methoxy group and a methyl group attached to a benzene ring, respectively. The ^{13}C NMR spectrum (100 MHz, DMSO-d_6) exhibited 35 carbon signals, including 30 aromatic carbons (corresponding to five benzene rings), one conjugated carbonyl carbon (δ_C 186.1), two acetylenic carbons (δ_C 85.6 and 98.6), one methoxy carbon (δ_C 55.8), and one methyl carbon (δ_C 21.1).

All these spectroscopic data suggested a selaginellin skeleton for compound **2**. The HSQC and ^{1}H–^{1}H COSY spectra further established this framework. The HMBC correlations (Figure 3) of the methyl protons H-7 (δ_H 2.56) to C-3 (δ_C 130.8) and C-4 (δ_C 124.0), together with the correlation from H-5 (δ_H 7.47) to C-7 (δ_C 21.1), established the attachment of the methyl group at C-4. The methoxy group was attached to C-32 based on the HMBC correlation from 35-OMe (δ_H 3.72) to C-32 (δ_C 159.1). In addition, the aromatic protons H-30/H-34 (δ_H 6.84) exhibited long-range correlations with C-32, further confirming the location of the methoxy substituent.

Thus, the above spectroscopic data established the structure of **2** as 4-((4′-hydroxy-4-(hydroxyphenyl) eth- ynyl) -4-methyl-[1,1′-biphenyl]-2-yl)(4-metoxyphenyl)meth ylen e] -2,5-cyclohexadien-1-one. We previously applied for a patent for compound **2** and this is the first report in paper.

The six known compounds (**3**–**8**) were identified as selaginellin T (**3**) (Liu et al., 2018), selaginellin D (**4**) (Li et al., 2021), selaginellin (**5**) (Yang et al., 2012), selaginellin B (**6**) (Cheng et al., 2008), selariscinin C (**7**) (Nguyen et al., 2015), and selaginpulvilin D (**8**) (Liu et al., 2014) by comparison of their spectroscopic data (^{1}H and ^{13}C NMR) with those reported in the literature and all these compounds were firstly isolated from *S. sanguinolenta*. All these compounds were evaluated for their cytotoxicity against HepG2, H1975, and 4T1 cells. The results showed that compound **2** exhibited moderate inhibition against HepG2 and H1975 cells, with IC_{50} values of 94.07 and 130.1 μM (Figures 4 and 5), respectively, indicating that this acetylenic phenolic framework possesses a certain degree of cytotoxic potential. Notably, compound **3** (selaginellin T) displayed more potent cytotoxicity against HepG2 cells, with an IC_{50} value of

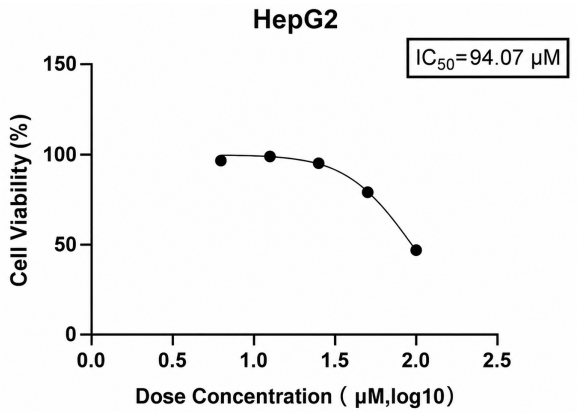

Figure 4. Dose–response curve of compound **2** against HepG2 cell line

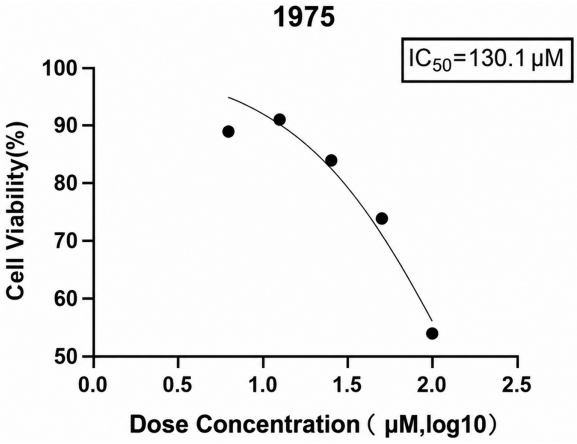

Figure 5. Dose–response curve of compound **2** against H1975 cell line

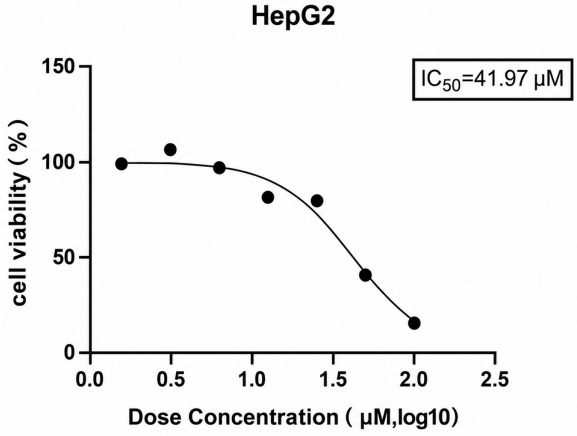

Figure 6. Dose–response curve of compound **3** against HepG2 cell line

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41.97 μM (Figure 6), whereas compound **1** and the remaining known compounds showed no significant activity under the tested conditions.

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Author Contributions

Yi-Yi Lu: Methodology, Data analysis, Writing original manuscript; Zi-Qi Zhou: Writing original manuscript; Format review & correction; Zi-Xuan Jiang: Methodology; Yong-Ming Xiao: Methodology; Jia-Yin Long: Methodology, Supervision; Hui Zou: Project design & promotion & supervision, Manuscript review & editing; Yin Xiao: Project design, supervision.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

Supporting Information

Supplementary data to this article can be found on line at https://www.acgpubs.org/journals/records-of-natural-products.

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