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Chemical constituents from *Cicuta virosa* Linnaeus and their reversal effects on doxorubicin-resistant human myelogenous leukemia (K562/A02) cells

Shu-Qi Wang [a], Qing-Wei Zhang [b], Xiao-Ling Wang [c], Xia-Xia Di [d], Xiao-Ning Wang [a,*], Hong-Xiang Lou [a,*]

[a] *School of Pharmaceutical Science, Shandong University, Jinan 250012, China*
[b] *Xintai Institute of Skin Diseases, Xintai 271200, China*
[c] *The Second Hospital of Shandong University, Jinan 250033, China*
[d] *Faculty of Pharmaceutical Sciences, School of Health Sciences, University of Iceland, Iceland*

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ABSTRACT

Two new compounds, 11,11′-dimer of scopoletin (**1**) and 11-*O*-*β*-glucopyranosylhamaudol (**2**), together with seven known compounds were isolated and identified from the whole grass of *Cicuta virosa*. The chemical structures of the isolated compounds were elucidated using different spectroscopic methods. In addition, the chemical constituents were evaluated for multidrug resistance reversing activity towards doxorubicin-resistant K562/A02 cells. Compounds **1**, **8**, and **9** were endowed with remarkable MDR reversing effects.

1. Introduction

Multidrug resistance (MDR) has long been recognized as a serious problem in cancer treatment. One of the major mechanisms of MDR in tumor cells is the over-expression of P-glycoprotein (P-gp) [1]. Therefore, there is great clinical interest in developing compounds that overcome resistances with lower host toxicity [2]. Several types of compounds have been identified as P-gp modulators, but most of them were reported to show toxicity at the pharmacological dose required to achieve significant MDR reversal [3,4]. Thus, P-gp inhibitors of plant origin have the potential to be developed as MDR reversing agents.

In a previous study, we reported that a series of coumarins from the methanol extract of *Cicuta virosa* L. possess potent multidrug resistance reversing activity [5]. In connection with our interest in this plant, two new compounds were isolated and identified, together with seven known compounds (Fig. 1). In addition, the chemical constituents were evaluated for their multidrug resistance reversing activity on human myelogenous leukemia cells.

2. Experimental

2.1. General

UV spectra were recorded in MeOH on a Agilent 8453E UV–vis spectroscopy system. The IR spectra were measured in KBr on a Thermo Nicolet NEXUS 470 FT-IR spectrometer. NMR spectra were recorded on a Bruker AV 600 MHz NMR spectrometer with TMS as an internal standard. HRESIMS data were obtained from Thermo-Fisher Scientific LTQ-Orbitrap XL. HPLC was performed on Agilent 1260 HPLC system. Optical rotation data were measured by a GYROMAT-HP polarimeter. The silica gel GF_{254} used for TLC was supplied by the Qingdao Marine Chemical Co., Ltd., Qingdao, China. Sephadex LH-20 and silica gel (200–300 mesh) used for column chromatography were supplied by Pharmacia Biotech AB, Uppsala, Sweden and Qingdao Marine Chemical Co., Ltd., Qingdao, China, respectively. Spots of TLC were visualized within I_2 vapor or by spraying with H_2SO_4/EtOH 1:9 followed by heating. All solvent ratios are measured v/v.

* Corresponding authors.
E-mail addresses: wangxn@sdu.edu.cn (X.-N. Wang), louhongxiang@sdu.edu.cn (H.-X. Lou).

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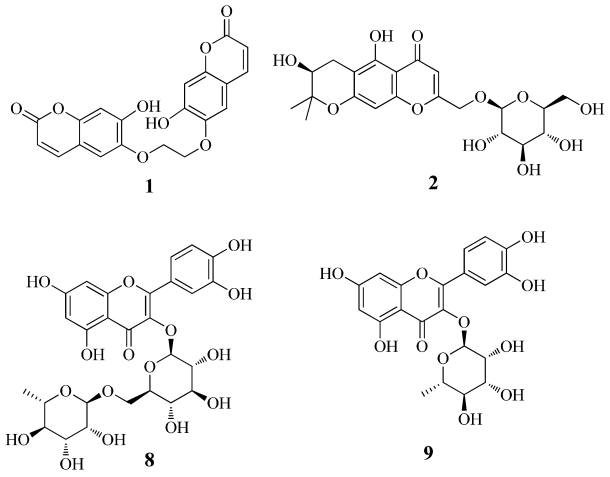

Fig. 1. Structures of compounds **1,2,8**, and **9** isolated from *C. virosa*.

2.2. Plant material

C. virosa Linnaeus was collected in July 2012 in Heilongjiang Province, China, and was identified by Prof. Xueshen Wen, School of Pharmaceutical Sciences, Shandong University. A voucher specimen (No. 201207CV) has been deposited at the Laboratory of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University, China.

2.3. Extraction and isolation

The air-dried powder of the plant material of *C. virosa* L. (3.0 kg) was extracted with EtOH/H_2O (3 × 3 L, 95:5, v/v) under condition of reflux. The combined EtOH extracts were concentrated in vacuum to yield the crude material (780 g), which was then successively partitioned with petroleum ether (PE; 210 g), AcOEt (140 g), and BuOH (240 g). The AcOEt fraction was chromatographed over silica gel, eluted with a solvent gradient system (PE/acetone 5:1–1:1) to produce fractions A–D. Fraction A (12 g) was subjected to column chromatography on silica gel eluted by PE/acetone (50:1–10:1), to give three sub-fractions (A1–A3). Sub-fraction A2 (220 mg) was separated by semi-preparative HPLC (MeOH/H_2O 80:20, 1.5 mL/min,) to give **4** (38.1 mg, t_R 21.5 min) and **5** (60.5 mg, t_R 24.2 min). Fraction C (345 mg) was further separated via column chromatography on silica gel with PE/acetone (30:1–10:1) to yield five sub-fractions (C1–C5). Sub-fraction C3 (35 mg) was purified by Sephadex LH-20, using CH_3Cl/CH_3OH (1:1) to yield compound **1** (25 mg). Fraction D (23 g) was further separated on column chromatography on silica gel with PE/acetone (10:1–5:1) to yield six sub-fractions (D1–D6). Sub-fraction D2 (80.4 mg) was purified by semi-preparative HPLC (MeOH/H_2O 70:30, 1.5 mL/min) to give **3** (46.3 mg, t_R 18.5 min). The BuOH extract was applied to column chromatography on silica gel eluted by CH_2Cl_2/CH_3OH (200:1–1:1) in gradient to give five fractions (E–I). Fraction E (18 g) was subjected to column chromatography on silica gel using CH_2Cl_2/CH_3OH (50:1–10:1), to yield mixtures (E1–E4). Sub-fractions E1 (150.0 mg) and E2 (95.7 mg) were purified by semi-preparative HPLC (CH_3OH/H_2O 65:35, 1.5 mL/min) to give **2** (35.2 mg, t_R 15.4 min) and **6** (43.1 mg, t_R 17.3 min), respectively. Fraction G (12.5 g) was applied to column chromatography on silica gel, with CH_2Cl_2/CH_3OH (20:1) as eluent to give two fractions (G1 and G2). Sub-fraction G2 (115 mg) was separated on a Sephadex LH-20 column with CH_2Cl_2/CH_3OH (1:1), to yield compound **8** (35.3 mg). Fraction H (520 mg) was further chromatographed on Sephadex LH-20 ($CHCl_3$/MeOH 1:1) and then purified by semi-preparative HPLC eluted with (MeOH/H_2O 50:50, 1.5 mL/min) to obtain **7** (55.8 mg, t_R 12.4 min) and **9** (78.3 mg, t_R 14.7 min).

11,11′-Dimer of scopoletin (**1**): Amorphous colorless powder. UV (MeOH): 230 (3.92), 254 (3.34). IR (KBr, cm^{-1}): 3650, 1710, 1620, 1511. ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): see Table 1. HRESIMS: 383.0765 ($[M + H]^+$; $C_{20}H_{15}O_8^+$, calcd. 383.0767).

11-*O*-*β*-Glucopyranosyl-hamaudol (**2**): Amorphous colorless powder. $[\alpha]_D^{20}$ −63 (*c* 0.11, MeOH). CD (MeOH): $\Delta\varepsilon_{254}$ + 5.2, $\Delta\varepsilon_{232}$ − 4.2. UV (MeOH): 230 (4.11), 252 (5.05). IR (KBr): 3499, 1655, 1580. ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): see Table 1. HRESIMS: 455.1547 ($[M + H]^+$; $C_{21}H_{27}O_{11}^+$, calcd. 455.1553).

2.4. Sugar identification.

D-Glucose (60 mg) and L-cysteine methyl ester hydrochloride (75 mg) were dissolved in pyridine (4 mL) and stirred at 60 °C for 1.5 h, and then *o*-tolyl isothiocyanate (330 μL) was added to the mixture and heated at 60 °C for 1.5 h. Separation by HPLC on an C-18 column eluted with H_2O (containing 0.2% TFA)–CH_3CN (70:30) gave the derivative S-1. The HRESIMS of S-1 gave a quasi-molecular ion $[M + H]^+$ peak at *m/z* 447.1246 (calcd. for $C_{18}H_{27}N_2O_7S_2$, 447.1254), which was well consistent with our previous experiment [6]. Compound **2** was hydrolyzed with HCl and extracted with CH_2Cl_2. The aqueous layer was passed through a Sephadex LH-20 column and the eluate was concentrated. The residue was dissolved in pyridine (0.4 mL) and stirred with L-cysteine methyl ester (10 mg) for 1.5 h at 60 °C, and then *o*-tolyl isothiocyanate (40 μL) was added to the mixture and heated at 60 °C for 1.5 h. The reaction mixture was analyzed by HPLC and detected at 250 nm. Analytical HPLC was performed on a Phenomenex C_{18} column

Table 1
^1H NMR and ^{13}C NMR data of **1** and **2** (600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR, in $CDCl_3$, δ in ppm, *J* in Hz).

1			**2**		
No.[a]	δ_H	δ_C	No.[a]	δ_H	δ_C
2/2′		160.6	2		166.7
3/3′	6.22 (d, 1H, *J* = 9.0 Hz)	113.4	3	6.39 (s, 1H)	106.5
4/4′	7.92 (d, 1H, *J* = 9.6 Hz)	143.3	4		181.8
5/5′	7.23 (s, 1H)	107.5	5		159
6/6′		144	6		158.6
7/7′		149.7	7		94.3
8/8′	6.78 (s, 1H)	103.2	8	6.53 (s, 1H)	155.1
9/9′		150	9		104
10/10′		111.5	10		103.7
11/11′	4.54 (s, 2H)	69.2	11	4.71 (d, 1H, *J* = 15.6 Hz) 4.60 (d, 1H, J = 15.6 Hz)	65.1
			2′		78.9
			3′	3.71 (m, 1H)	66.5
			4′	2.79 (dd, 1H, *J* = 4.8, 16.8 Hz) 2.48 (dd, 1H, *J* = 4.8, 16.8 Hz)	24.8
			5′	1.29 (s, 3H)	25
			6′	1.27 (s, 3H)	21.1
			1″	4.30 (d, 1H, *J* = 7.8 Hz)	102.3
			2	3.10 (m, 1H)	73.2
			3	3.18 (m, 1H)	76.4
			4	3.05 (m, 1H)	69.9
			5	3.12 (m, 1H)	76.9
			6	3.67 (dd,1H, *J* = 5.6, 12.5 Hz) 3.44 (dd, 1H, *J* = 2.5, 12.5 Hz)	60.9

[a] Atom numbering as indicated in Fig. 2.

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(4.6 × 250 mm) at 25 °C using a gradient of CH_3CN:0.2%TFA in H_2O: 0–25 min (32:68), 25–35 min (from 32:68 to 70:30), and 35–60 min (70:30) as the mobile phase. Peaks were detected with an Agilent DAD detector. D-Glucose was identified as the sugar moiety of **2** according to the same retention time (t_R 14.2 min).

2.5. Biological assays

The human myelogenous leukemia cell line K562, and its multidrug-resistant counterpart K562/A02 were obtained from the Department of Pharmacology, the Institute of Hematology of the Chinese Academy of Medical Sciences (Tianjin, China). K562/A02 cells were maintained in a complete RPMI-1640 medium containing 1 μg/mL doxorubicin (DOX) (Pfizer Italia S.r.l.) at 37° in a humidified atmosphere of 5% CO_2. The cells were cultured for two weeks in drug-free medium prior to their use in the experiments.

Cells were harvested and seeded into 96-well plates at 2×10^4 cells/well. For cytotoxicity experiments, different concentrations of compounds **1**–**9** were added into designated wells, and for MDR reversal experiments, different concentrations of DOX were added into designated wells with or without compounds **1**–**9** (10 μmol/L). After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, and the plate was further incubated for 4 h. The medium was discarded, and 100 μL of DMSO was added into each well to dissolve the formazan crystal. The absorbance in individual wells was determined at 570 nm. IC_{50} values for compounds **1**–**9** and doxorubicin (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. The reversal fold (RF) values, as potency of reversal, were obtained from fitting the data to RF = IC_{50} of cytotoxic DOX alone/IC_{50} of cytotoxic DOX in the presence of the tested compounds [7].

3. Results and discussion

Compound **1** was obtained as colorless, amorphous powder. The positive-ion-mode HRESIMS exhibited a quasi-molecular-ion peak at *m/z* 383.0765 ($[M + H]^+$; calcd. 383.0767) corresponding to the molecular formula $C_{20}H_{14}O_8$. The NMR spectra of **1** indicated a dimeric structure with a total of 15 C-atoms signals observed in the ^{13}C NMR spectra (Table 1). The spectral data of **1** was very similar to those of scopoletin with some notable differences [8,9]. The ^{13}C NMR spectra of **1** closely resembled that of scopoletin, except for the signal assignable to C-11. The C-11 signal at δ_C 69.2 showed a downfield shift by 12.8 ppm. The downfield shift of the signal assignable to H-11 was also observed in the ^1H NMR spectra. The H-11 signal at δ_H 4.54 (*s*, 2H, H-11/11′) showed a downfield shift by 0.72 ppm. All of the above data indicated that **1** might be the 11,11′-dimer of scopoletin. It was also supported by the NMR data of another coumarin dimer, diumancal, which was very similar to **1** [10]. Therefore, compound **1** was elucidated as 11,11′-dimer of scopoletin (Fig. 2).

Compound **2** was obtained as a colorless amorphous powder. The HRESIMS exhibited the $[M + H]^+$ peak at *m/z* 455.1547 (calcd. 455.1553), corresponding to the molecular formula $C_{21}H_{26}O_{11}$. The ^{13}C NMR spectra exhibited six signals due to a β-glucopyranosyl moiety, and 15 signals attributable to the aglycone moiety, which was similar to those of hamaudol (Table 1) [11]. The signals at δ_H 6.53 (s, 1H, H-8) and 6.39 (s, 1H, H-3) were characteristic for the chromone of **2**. The signals at δ_H 2.79 (dd, 1H, *J* = 4.8, 16.8 Hz, H-4′a) and δ_H 2.48 (dd, 1H, *J* = 4.8, 16.8 Hz, H-4′b), 3.71 (m, 1H, H-3′), and 1.29, 1.27 (s, each 3H, H-5′,6′) can be assigned to a trisubstituted pyran ring. In addition, the signal of anomeric H-atoms appeared at δ_H 4.30 (d, 1H, *J* = 7.8 Hz, H-1″) with coupling constants characteristic of a β-configuration. After sugar analysis, the presence of a D-glucose was confirmed [6]. The glucose residue in **2** was found to be linked to C-11 since the signal of anomeric proton at δ_H 4.30 showed a ^1H–^{13}C long range correlation with a signal of the C-11 at δ_C 65.1, in the HMBC spectrum (Fig. 2). The absolute configuration at C-3′ of **2** was confirmed as *S* by comparison of the circular dichrosim (CD) curve with those of hamaudol [11]. The basis of the evidence obtained, compound **2** was assigned to 11-*O*-β-glucopyranosyl-hamaudol.

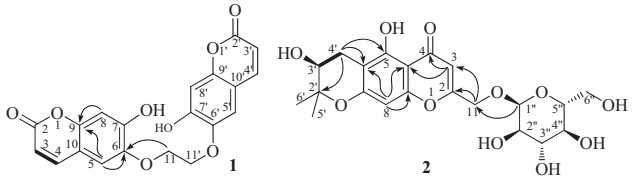

Fig. 2. The selected ^1H, ^1H-COSY (▬) and HMBCs (H → C) correlations of **1** and **2**.

Table 2
Effects of compound **1**–**9** on doxorubicin (DOX) cytotoxicity in K562 cells and K562/A02 cells.

Compounds	IC_{50} (μmol/L)		RF (K562/A02)
	K562	K562/A02	
DOX	0.37 ± 0.03	41.5 ± 0.12	
DOX + **1**	0.33 ± 0.12	13.3 ± 0.07*	3.12
DOX + **2**	0.39 ± 0.22	25.8 ± 0.11	1.61
DOX + **3**	0.33 ± 0.08	32.3 ± 0.24	1.28
DOX + **4**	0.41 ± 0.20	42.7 ± 0.14	0.97
DOX + **5**	0.43 ± 0.18	43.1 ± 0.09	0.96
DOX + **6**	0.29 ± 0.14*	21.7 ± 0.15*	1.91
DOX + **7**	0.35 ± 0.06	36.2 ± 0.07	1.15
DOX + **8**	0.31 ± 0.13	6.2 ± 0.06**	6.69
DOX + **9**	0.34 ± 0.18	8.3 ± 0.10**	5.00
DOX + Verapamil	0.29 ± 0.11*	4.7 ± 0.17**	8.83

Verapamil was used as a positive control agent.
Data were expressed as means ± SD of three independent experiments.
* $P < 0.05$ *vs.* doxorubicin treatment alone.
** $P < 0.01$ *vs.* doxorubicin treatment alone.

The seven known compounds were isobyakangelicin (**3**) [12], psoralene (**4**) [13], angelicin (**5**) [14], prim-*O*-glucosylangelicain (**6**) [15], apiosylskimmin (**7**) [16], rutin (**8**) [17], and quercetin-3-*O*-β-D-rhamnoside (**9**) [18], respectively.

The *in vitro* cytotoxic activities of the isolated compounds were evaluated against K562 and K562/A02. Compounds **1**–**9** possessed very weak cytotoxic activities (Table 2). The MDR reversal effects of compounds **1**–**9** were further investigated by using the revised 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method as described in the Experimental Part. Compounds **1**, **8**, and **9** were most active and exhibited significant MDR reversal effects on K562/A02 cell line, using verapamil as a reference compound.

4. Conclusion

In conclusion, nine compounds, including two new compounds (**1** and **2**), were isolated from the *C. virosa*. The present study about the isolation and identification of nine compounds shows the diversity of chemical constituents in *C. virosa*. In addition, some of these compounds showed remarkable MDR reversing effects.

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