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Cytotoxicity of cardenolides and cardenolide glycosides from *Asclepias curassavica*

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ABSTRACT

A new cardenolide, 12β,14β-dihydroxy-3β,19-epoxy-3α-methoxy-5α-card-20(22)-enolide (**6**), and a new doubly linked cardenolide glycoside, 12β-hydroxycalotropin (**13**), together with eleven known compounds, coroglaucigenin (**1**), 12β-hydroxycoroglaucigenin (**2**), calotropagenin (**3**), desglucouzarin (**4**), 6′-O-feruloyl-desglucouzarin (**5**), calotropin (**7**), uscharidin (**8**), asclepin (**9**), 16α-hydroxyasclepin (**10**), 16α-acetoxycalotropin (**11**), and 16α-acetoxyasclepin (**12**), were isolated from the aerial part of ornamental milkweed, *Asclepias curassavica* and chemically elucidated through spectral analyses. All the isolates were evaluated for their cytotoxic activity against HepG2 and Raji cell lines. The results showed that asclepin (**9**) had the strongest cytotoxic activity with an IC$_{50}$ value of 0.02 μM against the two cancer cell lines and the new compound **13** had significant cytotoxic activity with IC$_{50}$ values of 0.69 and 1.46 μM, respectively.

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Asclepias curassavica L. (Asclepiadaceae) is a common garden plant and has been used as a treatment for tumor, hemostasia, anti-inflammation, acesodyne in traditional medical practice.[1] Previous phytochemical investigations have led to the characterization of cytotoxic cardenolides and cardenolide glycosides, 3,4-*seco*-triterpenoids, pregnanes, and pregnane glycosides from this plant.[2–6] In our ongoing research for new cytotoxic compounds from medicinal plants from Yunnan flora, we had the opportunity to investigate the chemical constituents of *A. curassavica*. However, oxypregnane glycosides which we isolated from the plant were almost no cytotoxicity.[7] Further investigation on extracts of *A. curassavica* resulted in the isolation of one new cardenolide (**6**) and one new doubly linked cardenolide glycoside (**13**), as well as eleven known compounds (**1–5**, **7–12**).[8] These known compounds were identified as coroglaucigenin (**1**),[2] 12β-hydroxycoroglaucigenin (**2**),[2] calotropagenin (**3**),[9] desglucouzarin (**4**),[10] 6′-O-feruloyl-desglucouzarin (**5**),[11] calotropin (**7**),[12] uscharidin (**8**),[13] asclepin (**9**),[4] 16α-hydroxyasclepin (**10**),[4] 16α-acetoxycalotropin (**11**),[14] and 16α-acetoxyasclepin (**12**),[11] respectively, by comparison of their MS, ^{1}H, and ^{13}C NMR spectroscopic data with those reported. The structural elucidation of the two new compounds and cytotoxicity of the isolated compounds are reported in this Letter (Fig. 1).

Compound **6**,[15] obtained as a white amorphous powder, displayed the molecular formula C$_{24}$H$_{34}$O$_6$ from the HR-ESI-MS (*m/z* 419.2436 [M+H]$^+$). Its IR spectrum exhibited absorption bands for

hydroxyl (3426 cm^{-1}), carbonyl (1727 cm^{-1}), and olefinic (1629 cm^{-1}) groups. The UV spectrum indicated the presence of an α,β-unsaturated carbonyl group (λ_{max} 220 nm). A 5α-cardenolide skeleton was evident from the characteristic ^{1}H and ^{13}C NMR spectra of **6** (Table 1) displaying the presence of an α,β-unsaturated-γ-lactone [δ_H 6.24 (s), 5.24 (dd, *J* = 1.5, 18.0 Hz), 5.11 (dd, *J* = 1.5, 18.0 Hz); δ_C 176.4 (s), 174.6 (s), 117.4 (d), 74.0 (t)], one tertiary methyl (δ_C 10.1), one methoxyl (δ_C 49.1), nine methylenes (including an oxygenated methylene, δ_C 67.0), five methines (including an oxygenated methines, δ_C 74.3), three quaternary carbons (including an oxygenated quaternary carbon, δ_C 84.9), and a ketal carbon (δ_C 98.2). Inspection of NMR spectral data of **6** revealed their similarity to those of **2**. The major differences in the ^{13}C NMR spectral data between **6** and **2** were the presence of a ketal group in **6** instead of one oxygenated methane in **2**. The HMBC cross-peaks between the ketal carbon (δ_C 98.2) and H$_2$-1 (δ_H 1.25, 2.16), H$_2$-2 (δ_H 1.70, 2.09), H$_2$-4 (δ_H 1.61, 1.95), H-5 (δ_H 1.51), H$_2$-19 (δ_H 3.96, 4.27), and OMe (δ_H 3.33), and between the oxygenated methylene (δ_C 67.0) and H$_2$-1 (δ_H 1.25, 2.16), H-5 (δ_H 1.51) suggested that the ketal linkage was located between C-19 and C-3. The relative configuration of **6** was assigned by the ROSEY correlations (Fig. 2) observed among the protons OMe/H-5, H-9/H-12, H-12/H-17, H$_2$-19/H-8, which supported the α-configuration of the methoxyl group and H-12. Accordingly, **6** was assigned as 12β,14β-dihydroxy-3β,19-epoxy-3α-methoxy-5α-card-20(22)-enolide.

Compound **13**[16] was isolated as a white amorphous powder, and its molecular formula was unequivocally determined as

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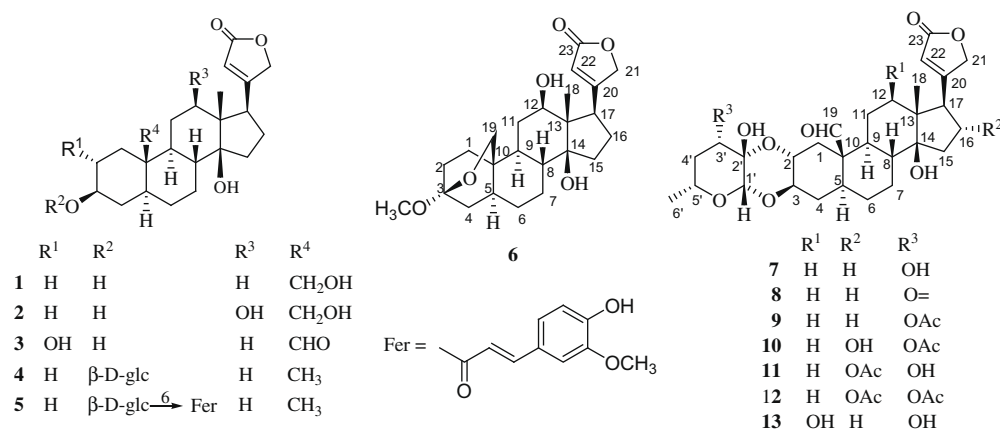*E-mail address:* haiyangliu@mail.kib.ac.cn (H.-Y. Liu).

Figure 1. Chemical structures of compounds **1–13**.

Table 1
^{1}H and ^{13}C NMR spectral data of **6** and **13** in C_5D_5N (δ in ppm, J in Hz)

Position	6		13	
	^{13}C	^{1}H	^{13}C	^{1}H
1	32.0 (t)	1.25 (m)	36.5 (t)	1.17 (m)
		2.16 (m)		2.52 (dd, 4.5, 12.5)
2	29.9 (t)	1.70 (m)	69.4 (d)	4.44 (m)
		2.09 (m)		
3	98.2 (s)		72.4 (d)	4.32 (ddd, 5.0, 10.0, 10.0)
4	38.6 (t)	1.61 (m)	32.9 (t)	1.59 (m)
		1.95 (m)		1.74 (m)
5	39.5 (d)	1.51 (m)	42.4 (d)	1.84 (m)
6	27.7 (t)	1.08 (m)	28.0 (t)	1.46 (m)
		2.34 (dd, 3.0, 13.0)		2.46 (m)
7	31.4 (t)	1.68 (m)	28.0 (t)	1.46 (m)
		2.02 (m)		2.46 (m)
8	41.9 (d)	1.69 (m)	43.6 (d)	1.36 (m)
9	42.1 (d)	1.21 (m)	45.9 (d)	1.41 (m)
10	34.0 (s)		52.6 (s)	
11	31.3 (t)	2.01 (m)	31.2 (t)	1.68 (m)
		1.46 (d, 11.5)		2.10 (m)
12	74.3 (d)	3.64 (dd, 3.5, 11.5)	73.8 (d)	3.64 (dd, 5.0, 9.0)
13	56.5 (s)		56.6 (s)	
14	84.9 (s)		84.7 (s)	
15	33.2 (t)	1.89 (m)	33.9 (t)	1.92 (m)
		2.13 (m)		2.08 (m)
16	27.6 (t)	1.92 (m)	27.6 (t)	1.90 (m)
		2.10 (m)		2.10 (m)
17	46.5 (d)	3.71 (t, 8.0)	46.4 (d)	3.70 (m)
18	10.1 (q)	1.21 (s)	9.9 (q)	1.13 (s)
19	67.0 (t)	3.96 (d, 8.8)	207.8 (d)	10.01 (s)
		4.27 (dd, 2.5, 8.8)		
20	176.4 (s)		176.3 (s)	
21	74.0 (t)	5.11 (dd, 1.5, 18.0)	73.9 (t)	5.09 (brs)
		5.24 (dd, 1.5, 18.0)		5.21 (s)
22	117.4 (d)	6.24 (s)	117.6 (d)	6.20 (s)
23	174.6 (s)		174.6 (s)	
OMe	49.1 (q)	3.33 (s)		
1′			97.3 (d)	5.01 (s)
2′			92.8 (s)	
3′			73.8 (d)	3.72 (m)
4′			39.9 (t)	1.99 (m)
				2.08 (m)
5′			68.4 (d)	4.11 (m)
6′			21.6 (q)	1.34 (d, 6.0)

All ^{1}H and ^{13}C NMR were obtained on DRX-500 MHz spectrometers.

$C_{29}H_{40}O_{10}$ by HR-ESI-MS (m/z 547.2550 [M−H]$^-$). Both the ^{1}H and ^{13}C NMR spectra of **13** (Table 1) showed the typical signals for a doubly linked cardenolide glycoside including a butenolactone ring [δ_H 6.20 (s), 5.21 (s), and 5.09 (br s); δ_C 176.3 (s), 174.6 (s), 117.6 (d), and 73.9 (t)], an anomeric singal [δ_H 5.01 (s); δ_C 97.3 (d)], as

well as an aldehyde group [δ_H 10.01 (s); δ_C 207.8 (s)] and a methyl [δ_H 1.13 (s); δ_C 9.9 (q)]. Comparison of the NMR spectrum of **13** and **7**[11] indicated that their structures were quite similar except that a methylene in **7** was replaced by an oxymethine in **13**, which was confirmed by the mass difference of m/z = 16. The chemical shift
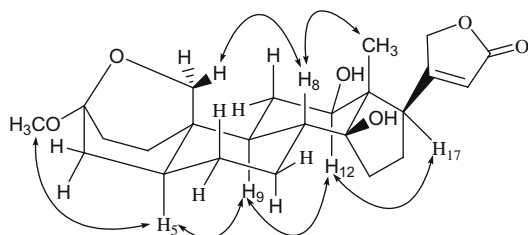
Figure 2. Key ROESY correlations of **6**.

of C-11 (δ_C 31.2 for **13**, δ_C 22.2 for **7**) and C-13 (δ_C 56.6 for **13**, δ_C 49.8 for **7**) were moved downfield dramatically. These features implied the hydroxyl group was linked at C-12, which was further confirmed by the HMBC correlations (Fig. 3) of H-12 (δ_H 3.64) with C-11 (δ_C 21.2), C-13 (δ_C 56.6), C-14 (δ_C 84.7), C-17 (δ_C 46.4), and C-18 (δ_C 9.9). Comparable coupling constants and patterns of the corresponding proton signals in **7** and **13** suggested that both had an identical relative configuration, as shown. The β-configuration of the hydroxyl group attached to C-12 was deduced from the ROESY correlations (Fig. 3) between H-12 (δ_H 3.64) and H-9 (δ_H 1.41) and H-17 (δ_H 3.70). On the basis of above evidence, **13** was tentatively elucidated as 12β-hydroxycalotropin.

The cytotoxic activities of all compounds **1**–**13** were evaluated against HepG2 and Raji cell lines using the improved MTT method. The cytotoxicity data and IC$_{50}$ values in μM were shown in Table 2. As evident from cytotoxic activity results, compounds **7**, **8**, **9**, **11**, **12**, and **13** showed stronger cytotoxicity on HepG2 cell line than that of the positive control *cis*-platinum (DDP) while compounds **1**, **4**, **7**, **8**, **9**, **10**, and **13** had stronger cytotoxicity on Raji cell line than that of the positive control DDP. Among them, asclepin (**9**) exhibited the most potent activity, with equal IC$_{50}$ values of 0.02 μM against HepG2 and Raji cell lines. The new compound **13** showed significant cytotoxicity on the two tumor cell lines with IC$_{50}$ values of 0.69 and 1.46 μM, respectively. However, the new cardenolide (**6**) displayed weak inhibitory activity against the two tumor cell lines with IC$_{50}$ values of 89.93 and 41.14 μM, respectively. As indicated by the IC$_{50}$ values, the doubly linked cardenolide glycosides (**7**-**13**) had stronger cytotoxicity ranging from 0.02 μM to 3.03 μM against HepG2 cell line than those of the cardenolides (**1**-**3**, **6**) and cardenolide glycosides (**4**, **5**). As for the doubly linked cardenolide glycosides (**7**–**13**), the appearance of the hydroxyl group at C-12 or C-16 and the acetoxyl group at C-16 in compounds **10**–**13** are dramatically less active than the compounds **7**–**9**, lacking this structural feature, against the two cell lines. In addition, the presence of an acetoxyl group at C-3′ in compound **9** as compared with **7** and **8**, or in compound **12** as compared with **11**, may have contributed to their potent cytotoxic activities against HepG2 cell line as demonstrated in this assay.

Table 2
IC$_{50}$ values of compounds **1**–**13** against tumor cell lines

Compounds	IC$_{50}$[a] (μM)	
	HepG2	Raji
1	6.70	0.14
2	5.17	14.56
3	56.61	24.28
4	8.53	0.75
5	>100	41.14
6	89.93	64.67
7	0.04	0.02
8	0.38	0.02
9	0.02	0.02
10	3.03	0.05
11	1.34	3.54
12	0.76	3.21
13	0.69	1.46
DDP[b]	2.57	2.10

[a] Each data point was the average value of two experimental measurements.
[b] DDP, an anti-tumor agent used as positive control.

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8. The dried powder of the aerial parts of *A. curassavica* (5 kg) was extracted with 75% ethanol under reflux (25 L × 3, each 3 h). After removal of the solvent under vacuum, the resulting residue was partitioned between H$_2$O and EtOAc. The EtOAc extract (110 g) was separated into fifteen fractions (fraction 1 to fraction 15) through MPLC (70 × 460 mm, silica gel, 200–300 mesh, 800 g) by elution with a gradient mixture of petroleum ether (PE)/EtOAc (1:0; 10:1; 5:1; 3:1; 1.5:1; 1:1; 0:1, v/v; each 10 L) and EtOAc/MeOH (6:1; 4:1; 0:1, v/v; each 10 L). Fraction 9 (23 g, PE/EtOAc 1.5:1) was subjected to MPLC on silica gel (36 × 460 mm, silica gel H, 400 g) with CHCl$_3$/MeOH (30:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1, each 5 L) to give ten subfractions (9.1–9.10). Subfraction 9.5 (1.0 g) gave **12** (58 mg) after repeated MPLC (26 × 230 mm, silica gel H, 75 g, CHCl$_3$/acetone, 4:1, 2 L). Crystalline material (from CHCl$_3$/MeOH, 2.0 g) which obtained from subfraction 9.6 (6.1 g) was chromatographed on silica gel (26 × 4600 mm, silica gel H, 150 g) with CHCl$_3$/acetone (3:1, 5 L) to give **8** (157 mg), **9** (87 mg), and **11** (90 mg). Subfraction 9.7 (3.1 g) was purified using repeated MPLC (26 × 460 mm, silica gel H, 150 g, CHCl$_3$/MeOH, 25:1; 19:1, each 6.5 L) and semi-prep HPLC C18 column (Zorbax SB-C18, 9.4 × 250 mm, MeOH/H$_2$O 50:50) to yielded **5** (34 mg), **7** (213 mg), and **10** (63 mg). Fraction 10 (4.0 g, PE/EtOAc 1:1) was subjected to MPLC (36 × 460 mm, silica gel H,

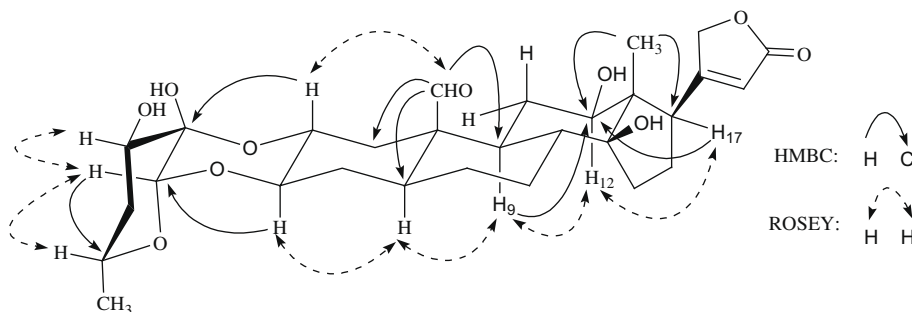**Figure 3.** Key HMBC and ROESY correlations of **13**.

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350 g, CHCl$_3$/MeOH 30:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1, each 1.5 L) to give 12 subfractions (10.1–10.12). Subfraction 10.4 (1.1 g) afforded **6** (12 mg) and **13** (24 mg) after repeated MPLC (26 × 230 mm, silica gel H, 75 g, CHCl$_3$/MeOH 30:1, 20:1, each 2 L), and then semi-prep HPLC (Zorbax SB-C18, 9.4 × 250 mm, MeOH/H$_2$O 60:40). Subfraction 10.6 (2.0 g) was separated on Sephadex LH-20 (20 × 1500 mm) (CHCl$_3$/MeOH, 1:1, 0.5 L) followed by MPLC (26 × 460 mm, silica gel H, 150 g, CHCl$_3$/MeOH 20:1, 12:1, each 3 L) to give 10 subfraction (10.6.1–10.6.10). Subfraction 10.6.5 (0.3 g) yielded **1** (80 mg) and **4** (55 mg) after reverse-phase MPLC (15 × 460 mm, C-18 silica gel, 40–63 μm, 50 g, MeOH/H$_2$O 40:60, 3 L). Subfraction 10.6.7 (0.2 g) and 10.6.8 (0.15 g) was recrystallised from CHCl$_3$/MeOH (3:1) to afford **3** (78 mg). Subfraction 10.10 (0.25 g) was purified using repeated MPLC (15 × 460 mm, gel H, 50 g, CHCl$_3$/MeOH, 20:1, 12:1, each 1.5 L) to afford **2** (60 mg).

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15. *12β,14β-Dihydroxy-3β,19-epoxy-3α-methoxy-5α-card-20(22)-enolide* (**6**): white amorphous powder; $[\alpha]_D^{20}$: +29.0 (*c* 0.01, C$_5$H$_5$N); UV (C$_5$H$_5$N): λ_{max} (log ε) = 220 nm (3.15); IR (KBr) ν_{max}: 3426, 2945, 2871, 1727, 1629, 1460, 1442, 1172, 1120, 1026 cm^{-1}; EI-MS (70 eV) *m/z*: 418 [M]$^+$(4), 400 [M−H$_2$O]$^+$(8), 326 (17), 241 (27), 147 (46), 135 (48), 111 (60); positive HR-ESI-MS *m/z*: 419.2436 [M+H]$^+$(calcd for C$_{24}$H$_{35}$O$_6$: 419.2433); ^{1}H and ^{13}C NMR (C$_5$D$_5$N$_5$) data, see Table 1.
16. *12β-Hydroxycalotropin* (**13**): white amorphous powder; $[\alpha]_D^{20}$ 0.0 (*c* 0.03, C$_5$H$_5$N); UV (C$_5$H$_5$N): λ_{max} (log ε) = 219 nm (3.15); IR (KBr) ν_{max}: 3419, 2933, 1702, 1629, 1061 cm^{-1}; negative FAB-MS *m/z*: 547 [M−H]$^-$ (100), 419 [M−H−128]$^-$ (35); negative HR-ESI-MS *m/z*: 547.2550 [M−H]$^-$ (calcd for C$_{29}$H$_{39}$O$_{10}$: 547.2543); ^{1}H and ^{13}C NMR (C$_5$D$_5$N$_5$) data, see Table 1.