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ScienceDirect
Chinese Journal of Natural Medicines 2022, **20**(3): 202-209
doi: 10.1016/S1875-5364(21)60098-5
Chinese Journal of Natural Medicines

•Research article•

Two cardenolide glycosides from the seed fairs of *Asclepias curassavica* and their cytotoxic activities

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Available online 20 Mar., 2022

[ABSTRACT] Two cardenolide glycosides, corotoxigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside] (**1**) and coroglaucigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside] (**2**), were isolated from the seed fairs of *Asclepias curassavica*. The structures of **1**−**2** were determined based on the combination of the analysis of their MS, NMR spectroscopic data and acid hydrolysis. The inhibitory effects of compounds **1** and **2** on human colorectal carcinoma cells (HCT116), non-small cell lung carcinoma cells (A549) and hepatic cancer cells (SMMC-7721) were evaluated. The results showed that both compounds **1** and **2** significantly inhibited the viability, proliferation, and migration of A549, HCT116 and SMMC-7721 cells, suggesting that compounds **1** and **2** can be applied in the treatment of lung, colon and liver cancers in clinical practice. This study may not only provide a scientific basis for clarifying the active ingredients in *A. curassavica*, but also help to understand its antitumor activity, which can promote the application of *A. curassavica* in clinical treatment of various cancers.

[KEY WORDS] *Asclepias curassavica*; Cardenolide glycosides; Structure identification; Cytotoxic activity
[CLC Number] R284.1; R965 **[Document code]** A **[Article ID]** 2095-6975(2022)03-0202-08

Introduction

Asclepias curassavica L. (the milkweed family Asclepiadaceae) is a common ornamental garden plant native to the American tropics [1]. Currently, it is cultivated as a source of food for butterflies worldwide [2]. Previous phytochemical studies of *A. curassavica* have led to the identification of numerous cardenolides and pregnane glycosides [3]. Monarch butterflies (*Danaus plexippus* L.) and other sucking insects store these cardenolides in the adult body by feeding on the *Asclepias* genus, including *A. curassavica*, as a defense substance for protecting against vertebrate predators [4]. From an ecological point of view, this host-guest-predator relationship has been well established.

Notably, this plant is also used for the treatment of cancer in traditional medical practice. A series of cardenolides and their glycosides from this herb showed strong cytotoxic activity against A549, MCF-7 and MDA-MB-231, and HepG2 cells [5-8]. In our previous research in *A. curassavica*, cardenolide lactates and dioxane double linked cardenolide glycosides were identified and displayed inhibitory activity against DU145 cells [5]. Interestingly, the flat seeds have silky hairs which allow the seeds to float on air currents in order to spread further afield. However, its active ingredients have never been reported. Herein, our continuing study of the bioactive substances from silky hairs on the seeds of *A. curassavica*, has led to the isolation and identification of two new cardenolide glycosides (**1**−**2**) (Fig. 1). Here, the isolation and structural analysis of compounds **1**−**2** are discussed.

In recent years, lung cancer remains the leading cause of

[Received on] 07-Apr.-2021
[Research funding] This work was supported by the Science and Technology Program of Guangzhou, China (No. 201804010083), the National Natural Science Foundation of China (Nos. 81720108033 and 81930114), Guangdong Key Laboratory for Translational Cancer Research of Chinese Medicine (No. 2018B030322011), the Project of Guangzhou University of Chinese Medicine (No. QNYC20190103) and the Project of Traditional Chinese Medicine Bureau of Guangdong Province (No. 20211111).
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These authors have no conflict of interest to declare.

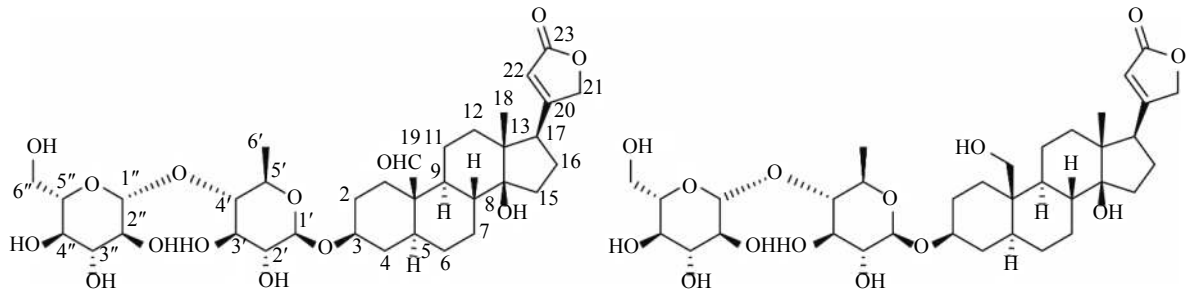

Fig. 1 Structures of 1–2 from the ethanol extract of the seed fairs of *Asclepias curassavica*

cancer related deaths worldwide due to its high incidence, considerable mortality, and poor prognosis [9-10]. Colorectal cancer is one of the most common gastrointestinal malignancies with high morbidity, recurrence and mortality [9-10]. Liver cancer is also one of the most common cancers and one of the most frequent cause of death from cancer [9-10]. It is of great significance to discover more efficient agents and components to treat these cancers. In the curret study, the effects of compounds 1 and 2 on the viability, proliferation and migration of human colorectal carcinoma cells (HCT116), non-small cell lung carcinoma cells (A549) and hepatic cancer cells (SMMC-7721) were evaluated. This study may not only provide a scientific basis for clarifying the active ingredient in *A. curassavica* L. but also help to understand the anti-tumor activity of *A. curassavica*, which can promote the application of *A. curassavica* in cancer treatment.

Results and Discussion

Structural identification of new compounds 1 and 2

Compound **1** was isolated as white powder. The molecular formula was deduced as $C_{35}H_{52}O_{14}$ (10 indices of hydrogen deficiency) based on the deprotonated molecule at *m/z* 695.3338 $[M-H]^-$ (Calcd. for $C_{35}H_{51}O_{14}$, 695.3342) and ^{13}C NMR data (Table 1). The IR data at 1703 cm^{-1} revealed the presence of carbonyl groups, while the UV absorption at 216 nm suggested the presence of an *α*, *β*-unsaturated carbonyl group. The ^1H NMR resonances at δ_H 4.99 and 4.94 (both as dd, J = 18.4 and 1.5 Hz, H_2-21) and δ_H 5.89 (brs, H-22), and ^{13}C NMR resonances at δ_C 177.2, 178.2, 117.9, and 75.3 indicated the presence of an *α*, *β*-unsaturated-*γ*-lactone moiety in **1**. A singlet proton at δ_H 9.99 (s, H-19) and a methyl signal at δ_H 0.81 (s, H_3-18) indicated the presence of corotoxigenin. In addition, the anomeric regions in the ^1H and ^{13}C NMR spectra of **1** showed two anomeric protons at δ_H 4.71 (d, J = 8.0 Hz) and 4.38 (d, J = 8.0 Hz), corresponding to anomeric carbon signals at δ_C 99.7 and 105.9, respectively. The ^{13}C NMR and HSQC spectrum of **1** displayed 35 carbon signals, of which 23 were assigned to the cardenolide aglycone and twelve to two sugar portions. Detailed analysis of the ^1H, ^{13}C, HSQC and COSY NMR spectra of **1** revealed two sugar spin systems: H-1′ (δ_H 4.38)/H-2′ (δ_H 3.32)/H-3′ (δ_H 3.27)/H-4′ (δ_H 3.28)/H-5′ (δ_H 3.83)/H_3-6′ (δ_H 1.27), and H-1″ (δ_H 4.71)/H-2″ (δ_H 3.30)/H-3″ (δ_H 3.21)/H-4″ (δ_H 4.30)/H-5″ (δ_H 3.30)/H_2-6″ (δ_H 3.82, 3.67) (Fig. 2). These two sugars was determined to be 6-deoxy-*β*-glucopyranose and *β*-glucopyranose due to the large coupling constant of their anomeric proton signals [δ_H 4.38 (d, J = 8.0 Hz)]; [δ_H 4.71 (d, J = 8.0 Hz)], and NOESY correlations (H-1′/H-3′, H-5′; H-3′/H-5′, and H-2′/H-4′); (H-1″/H-3″, H-5″; H-3″/H-5″, and H-2″/H-4″). In the HMBC spectrum, the anomeric protons at positions H-1′ and H-1" showed J correlations with C-3 and C-4′, respectively. Hence, the two sugars were linked from the 4′-position of the first sugar to the 1″-position of the second, and the H-4′ and H-1″ protons were oriented *cis* to each other. Furthermore, the 6-deoxy-*β*-glucopyranose residue was linked to 3-OH of the corotoxigenin. In addition, the absolute configuration of the sugars were assigned after acid hydrolysis of **1** with 5% HCl at 70 °C for 5 h to give 6-deoxy-*β*-D-glucopyranose [11] showing $[\alpha]_D^{26}$ +40.2 (*c* 0.04, H_2O), and *β*-D-glucopyranose [12] showing $[\alpha]_D^{26}$ +57.4 (*c* 0.04, H_2O). The structure of compound **1** was thus elucidated as corotoxigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside].

Compound **2**, white powder, has the molecular formula of $C_{35}H_{53}O_{14}$ according to HR-ESI-MS analysis ($[M-H]^-$ *m/z* 697.3453, Calcd. 697.3435), indicating 9 degrees of unsaturation. The ^1H NMR resonances at δ_H 4.99 and 4.94 (both as dd, J = 18.4 and 1.5 Hz, H_2-21) and δ_H 5.89 (brs, H-22), and ^{13}C NMR resonances at δ_C 177.3, 178.6, 117.9, and 75.4 indicated the presence of an *α*, *β*-unsaturated-*γ*-lactone moiety in **2**. Hence, compound **2** highly resembled those of **1**. The main difference was that the aldehyde group located at C-10 in **1** was replaced by a methylol group in **2**, which were confirmed by the HMBC correlations of H_2-19/C-5 (δ_C 59.9) for **2** in Fig. 2. In addition, the sugars in **2** were also 6-deoxy-*β*-D-glucopyranose and *β*-D-glucopyranose, which was assigned by the same acid hydrolysis method as **1**. Thus, the structure of **2** was elucidated as coroglaucigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside].

Compounds 1 and 2 significantly inhibited the viability of cancer cells

The inhibitory effects of compounds **1** and **2** on tumor proliferation were detected *in vitro* using human colorectal carcinoma cells HCT116, non-small cell lung carcinoma A549 cells and hepatic cancer cells SMMC-7721. MTT assay showed that both compounds **1** and **2** exhibited obvious

Table 1 NMR data of 1–2 in CD_3OD (400 MHz for ^1H; 100 MHz for ^{13}C)

No.	1		2	
	δ_H	δ_C	δ_H	δ_C
1	1.07, m	37.0	1.07, m	35.8
	1.89, m		1.89, m	
2	1.25, m	29.6	1.25, m	29.4
	1.36, m		1.36, m	
3	3.67, m	78.8	3.67, m	79.4
4	1.43, m	32.7	1.43, m	32.7
	1.60, m		1.60, m	
5	1.52, m	52.8	1.52, m	52.1
6	1.81, m	28.6	1.81, m	28.7
	2.05, m		2.05, m	
7	1.13, m	27.9	1.13, m	28.1
	1.83, m		1.83, m	
8	1.60, m	43.8	1.60, m	43.0
9	1.01, m	44.1	1.01, m	45.8
10		52.8		41.4
11	1.53, m	22.9	1.53, m	24.0
	1.53, m		1.53, m	
12	1.49, m	40.4	1.49, m	40.5
	1.49, m		1.49, m	
13		50.7		51.1
14		85.8		86.5
15	1.71, m	32.1	1.71, m	33.4
	2.13, m		2.13, m	
16	1.84, m	31.6	1.84, m	30.8
	2.15, m		2.15, m	
17	2.81, m	51.8	2.81, m	51.4
18	0.81, s	16.2	0.92, s	16.5
19	9.99, s	210.5	3.84, m	59.9
			3.74, m	
20		178.2		178.6
21	4.94, dd (18.4, 1.5) (18.4, 1.5)	75.3	4.94, dd (18.4, 1.5) (18.4, 1.5)	75.4
	4.99, dd (18.4, 1.5) (18.4, 1.5)		4.99, dd (18.4, 1.5) (18.4, 1.5)	
22	5.89, brs	117.9	5.89, brs	117.7
23	1.07, m	177.2	1.07, m	177.3
1′	4.38, d (8.0)	105.9	4.38, d (8.0)	105.9
2′	3.32, m	71.1	3.32, m	71.1
3′	3.27,m	72.3	3.27,m	72.0
4′	3.27, m	83.8	3.27, m	83.9
5′	3.83, m	69.4	3.83, m	69.4
6′	1.27, d (6.2)	18.1	1.27, d (6.2)	18.2
1″	4.71, d (8.0)	99.7	4.71, d (8.0)	99.6
2″	3.30, m	77.8	3.30, m	77.9
3″	3.21, m	75.0	3.21, m	75.1
4″	4.30, t, (2.8)	77.7	4.30, t, (2.8)	77.7
5″	3.30, m	72.3	3.30, m	72.3
6″	3.82, m	62.3	3.82, m	62.3
	3.69, m		3.69, m	

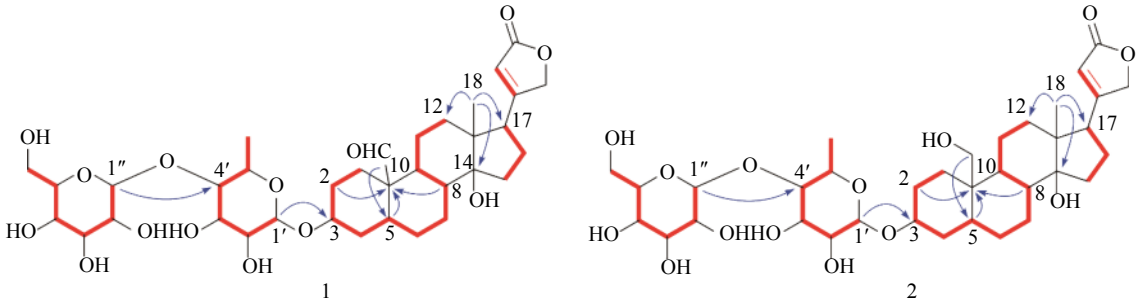

Fig. 2 key ^{1}H–^{1}H COSY (red bold lines) and HMBC (blue arrows) correlations of 1 and 2

inhibitory effects on the three types of cells in a dose dependent manner (Figs. 3A–3F, $P < 0.001$). The IC_{50} values of compound **1** towards A549, HCT116 and SMMC-7721 cells were 0.255, 7.922 and 21.820 μmol·L^{-1}, respectively. The IC_{50} values of compound **2** towards A549, HCT116 and SMMC-7721 cells were 0.4055, 27.750 and 7.891 μmol·L^{-1}, respectively. 5-Fluorouracil, cisplatin and sorafenib, which are the commonly used anti-tumor drugs were used as positive controls, which effectively suppressed the viability of A549, HCT116 and SMMC-7721 cells, respectively (Figs. 3G–3I, $P < 0.001$). These results suggested that both compounds **1** and **2** produced more toxicity towards A549 cells, indicating that compounds **1** and **2** may be used more suitable in the treatment of lung cancer.

*Compounds **1** and **2** significantly inhibited the proliferation of cancer cells*

EdU assay was used to further evaluate the inhibitory effects of compounds **1** and **2** on the proliferation of cancer

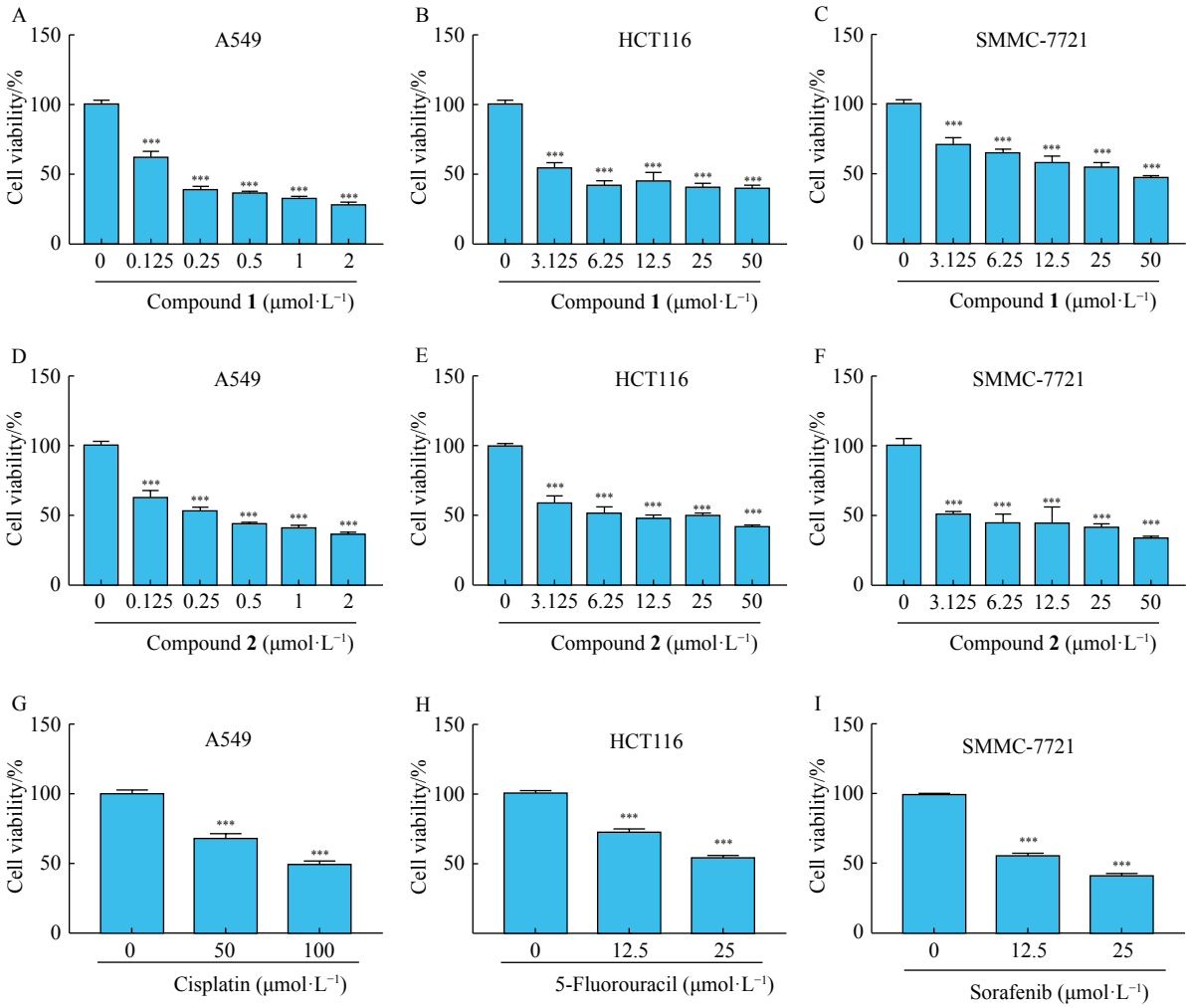

Fig. 3 Effects of compounds 1 and 2 on the viability of A549, HCT116 and SMMC-7721 cells

cells. Compared with the control group, the number of EdU-positive cells in the treated groups were significantly reduced in a dose dependent manner (Fig. 4), indicating that both compounds **1** and **2** significantly inhibited the proliferation of A549, HCT116 and SMMC-7721 cells. 5-Fluorouracil, cisplatin and sorafenib, which were used as positive controls, effectively reduced the proliferation of A549, HCT116 and SMMC-7721 cells, respectively (Fig. 4). Meanwhile, the number of cells stained by Hochest33342 was also significantly reduced, which suggested that both compounds **1** and **2** promoted the death of tumor cells while inhibiting tumor cell proliferation, and further demonstrated their potent inhibitory activity against A549, HCT116 and SMMC-7721 cells.

*Compounds **1** and **2** significantly inhibited the migration of cancer cells*

Wound healing assay was used to evaluate the migration ability of cancer cells. As shown in Fig. 5, both compounds **1** and **2** exhibited significantly inhibitory effects on the migration of three cancer cell lines (Fig. 5, $P < 0.001$). Compared with the control group, both compounds completely inhibited the cell migration at high doses (12.5 μmol·L^{-1} for HCT116 and SMMC-7721 cells, and 0.5 μmol·L^{-1} for A549 cells respectively). 5-Fluorouracil, cisplatin and sorafenib, which were used as positive controls, effectively reduced the migration of A549, HCT116 and SMMC-7721 cells, respectively (Fig. 5, $P < 0.001$). Transwell migration assay further demonstrated that both compounds **1** and **2** significantly inhibited cell migration. (Fig. 6, $P < 0.001$). Tumor migration is closely related to the progression of various cancers [13]. Inhibition of tumor migration is a key criterion in determining whether a compound can be an effective tumor chemopreventive agent [14]. Hence, utilizing food-derived or plant-derived interventions with broad effectiveness to prevent tumor migration is a promising approach for overall survival improvement. These data further suggested that both compounds **1** and **2** can be applied for the treatment of lung, colon and liver cancers due to their potent inhibitory effects on the migration of cancer cells.

Experimental

General experimental procedures

Optical rotation values were measured on an Anton Paar MCP 500 (Anton Paar, France) at room temperature. The UV absorptions were measured by a Shimadu Europe UV-2600 spectrometer (Shimadu, Japan), while the IR spectra were recorded with a Shimadu IRAffinity-1 (Shimadu, Japan). The NMR spectra were obtained on a Bruker Avance III HD 400 MHz digital NMR spectrometer (Bruker BioSpin Group,

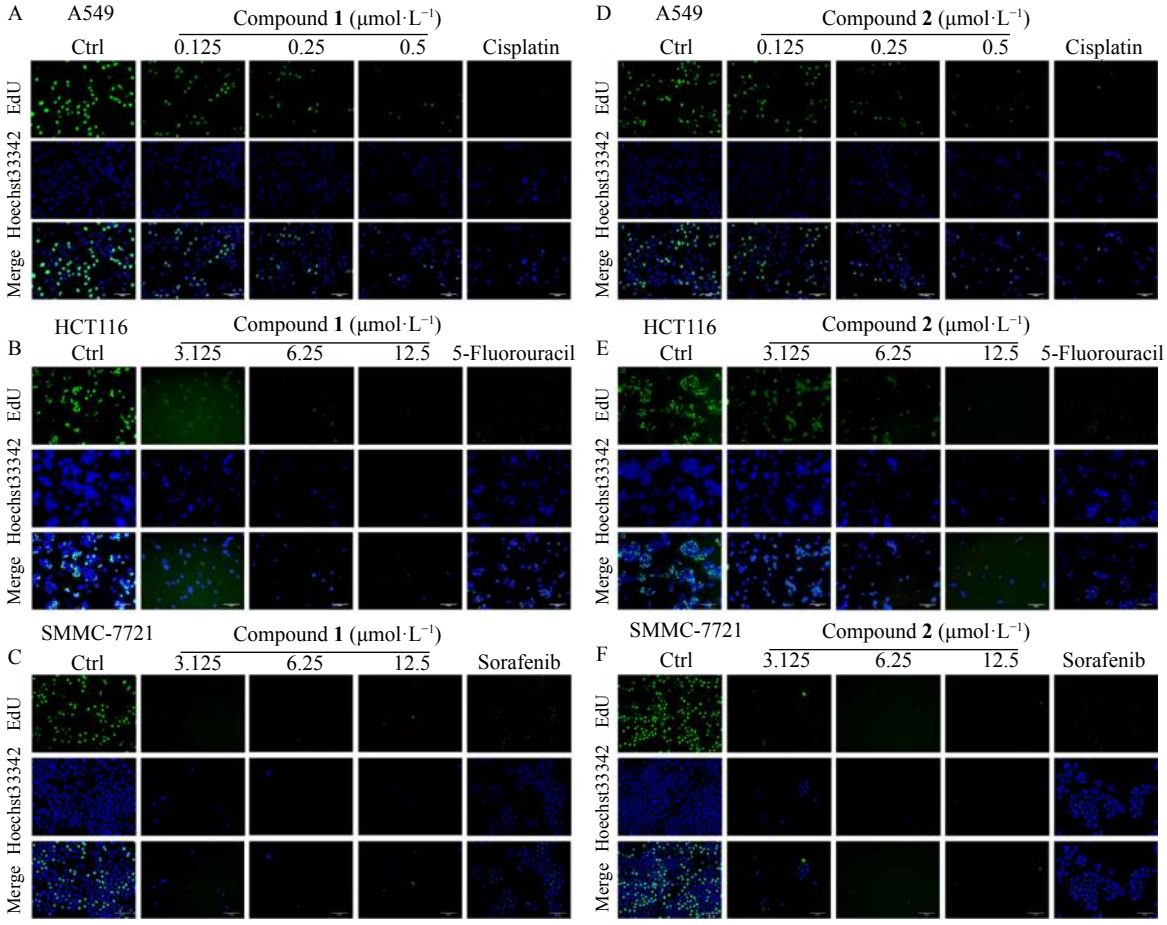

Fig. 4 Effects of compounds 1 and 2 on the proliferation of A549, HCT116 and SMMC-7721 cells

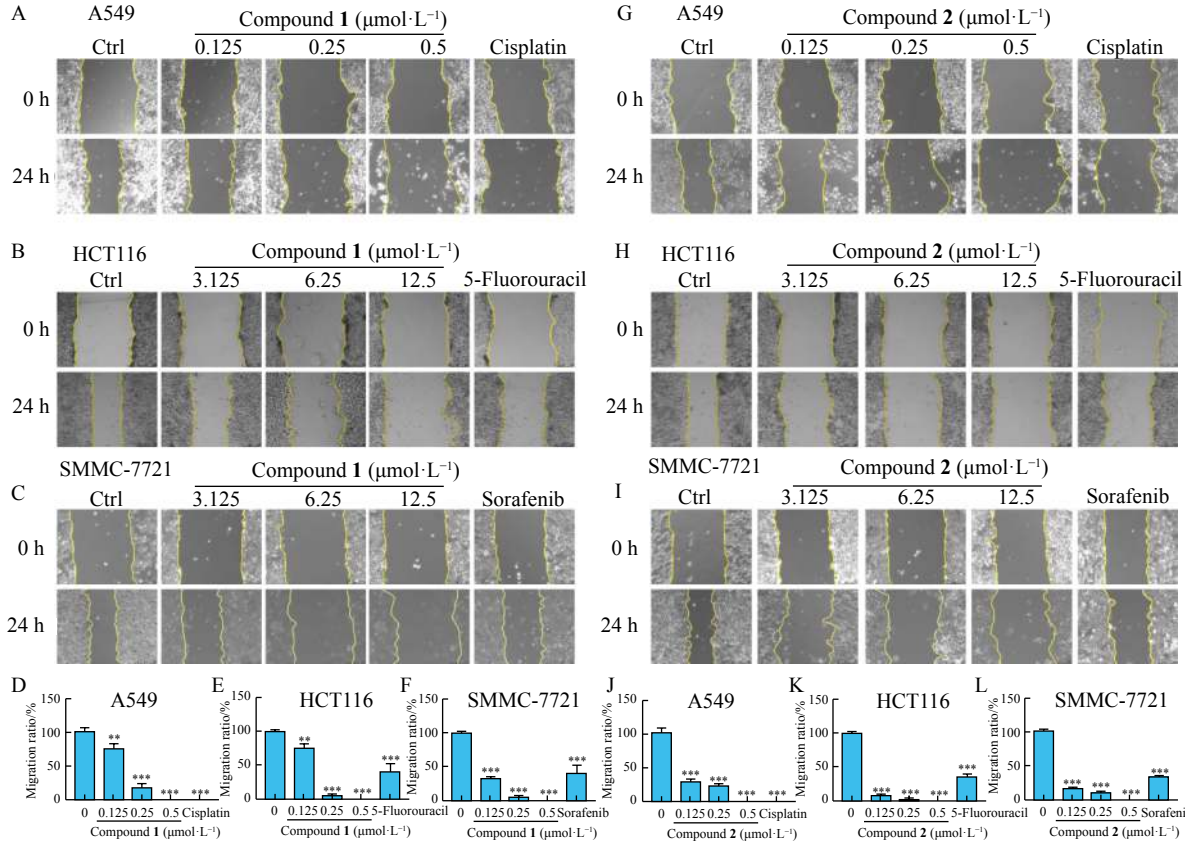

Fig. 5 Effects of compounds 1 and 2 on the migration (wound-healing assay) of A549, HCT116 and SMMC-7721 cells

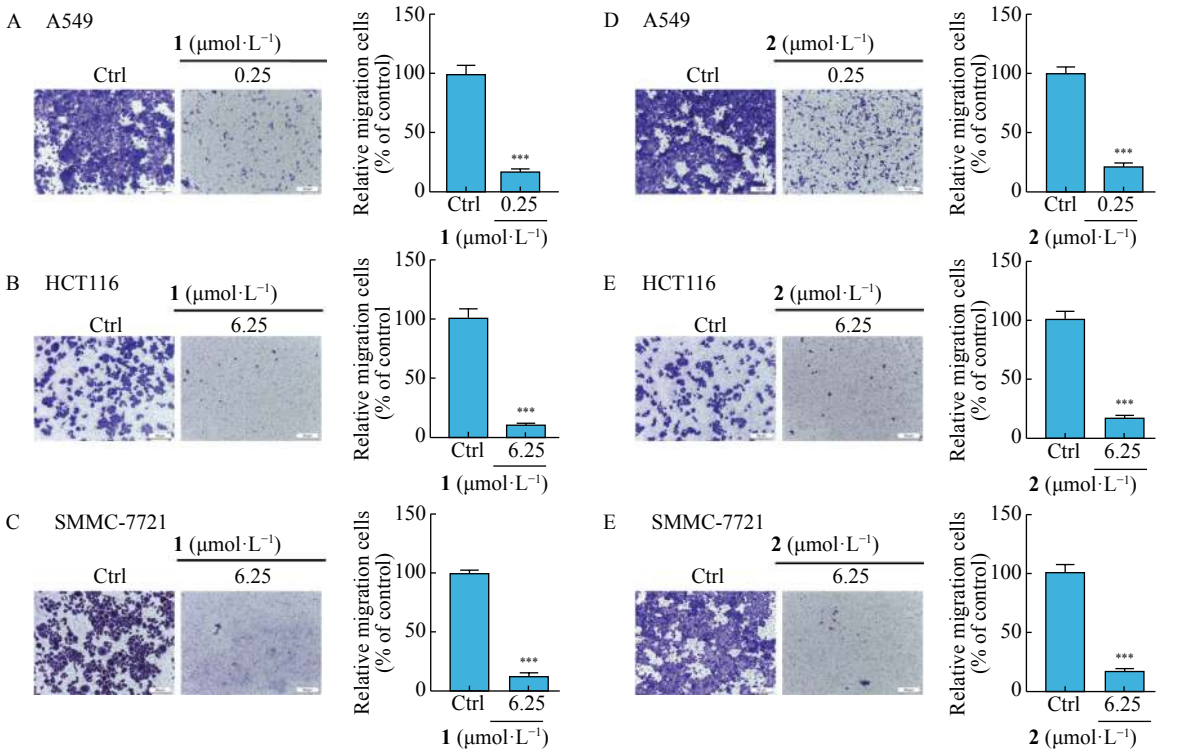

Fig. 6 Effects of compounds 1 and 2 on the migration (transwell migration assay) of A549, HCT116 and SMMC-7721 cells

Switzerland), in the solvents indicated and referenced to residual signals (δ_H 3.30 and δ_C 49.0 ppm for CD_3OD) in deuterated solvents. High-resolution electrospray mass spectroscopy was performed on an Agilent Q-TOF 6540 (Agilent Technologies, USA). Column chromatography (CC) was performed on ODS (50 μm YMC). Analytical HPLC was performed on an Agilent HPLC system equipped with a G1311A pump, a G1314A VWD detector, a G1316A column compartment, and a G1313A autosampler (Dionex, USA) using COSMOSIL Packed Column CHIRAL 5A (5 μm, 250 mm × 4.6 mm). Preparative HPLC (Shimadzu, Japan) was used together with an SPD-20A UV detector, a LC-20AT binary pump and a Cosmosil C_{18} column (5 μm, 20 mm × 250 mm).

Plant material, chemicals and reagents

The seed hairs of *A.curassavica* were collected from Zhongshan, Guangdong Province, China, in August 2018, and authenticated by Prof. CHEN Xin-Lian (School of Pharmaceutical Sciences, Sun Yat-Sen University). A specimen (No. 2018081007) was deposited at the International Institute for Traditional Chinese Medicine, Guangzhou University of Traditional Chinese Medicine, Guangzhou, China. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were bought from Alfa Aesar (Ward Hill, MA, United States). The BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 was purchased from Beyotime Biotechnology (Haimen, China). Cell culture reagents were purchased from Invitrogen (Gibco, Waltham, MA, United States). 5-Fluorouracil, cisplatin and sorafenib were bought from MedChem Express (Monmouth Junction, NJ, USA). Transwell chambers with an 8-μm pore polycarbonate membrane were bought from Corning Costar (Beijing, China).

Extraction and isolation

Dried seed hairs of *A. curassavica* (5 kg) were extracted with 80% (*V*/*V*) EtOH at 85 °C. After evaporation of the solvent under reduced pressures, the residue was suspended in H_2O and extracted with EtOAc and *n*-BuOH successively. The residue of the *n*-BuOH layer (11g) was loaded on the ODS column eluted with a MeOH/H_2O gradient (2 ∶ 8→ 10 ∶ 0) to give seven fractions (Fr. 1−Fr. 7). Fr. 6 (92 mg) was applied to Sephadex LH-20 CC (MeOH−H_2O, 1 ∶ 1) to give five fractions (Fr. 1A−Fr. 1E). Fr. 1D was purified by preparative HPLC and eluted with CH_3CN/H_2O (1 ∶ 1) to afford compound **1** (16 mg) and compound **2** (11 mg).

Corotoxigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside] (1): white powder (CH_3OH), $[\alpha]_D^{26}$ −49 (*c* 0.10, CH_3OH); IR (KBr) ν_{max}: 3476, 2931, 2861, 2524, 1703, 1030 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) = 216 (7.67); HR-ESI-MS *m*/*z* 695.3338 $[M - H]^-$ (Calcd. for $C_{35}H_{51}O_{14}$, 695.3342).

Coroglaucigenin 3-*O*-[*β*-D-glucopyranosyl-(1→4)-6-deoxy-*β*-D-glucopyranoside] (2): white powder (CH_3OH), $[\alpha]_D^{26}$ −49 (*c* 0.10, CH_3OH); IR (KBr) ν_{max}: 3380, 2929, 2865, 1734, 1078, 1020 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) = 218 (14.6); HR-ESI-MS *m*/*z* 697.3453 $[M - H]^-$ (Calcd. for $C_{35}H_{53}O_{14}$, 697.3435).

*Acid hydrolysis of **1** and **2***

A mixture of compound **1** (2.2 mg) and 5% HCl (5 mL) was heated at 70 °C for 5 h, and then allowed to cool to room temperature. The reaction mixture was then extracted with EtOAc. The aqueous phase was dried to afford a crude sugar fraction. *β*-D-glucopyranose (R_f 0.1 (CH_2Cl_2−MeOH, 90 ∶ 10) and 6-deoxy-*β*-glucopyranose (R_f 0.25 (CH_2Cl_2−MeOH, 90 ∶ 10) were isolated from this sugar fraction by preparative HPLC (RP-18, 85% CH_3OH). The absolute configurations of *β*-glucopyranose and 6-deoxy-*β*-glucopyranose were established by comparing the experimental and reported rotation values. Compound **2** was hydrolyzed using the same method mentioned above.

Cell culture

Human colorectal carcinoma cells HCT116, non-small cell lung carcinoma A549 cells and hepatic cancer cells SMMC-7721 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT-116 and SMMC-7721 cells were cultured in RPMI-1640 with 10% FBS. A549 cells were cultured in DMEM medium with 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

MTT assay

MTT assay was used to evaluate cell viability. A549, HCT116 and SMMC-7721 cells were seeded in 96-well plates with 5000 cells/well overnight, to which different concentrations of compounds **1** or **2** were added, respectively. 5-Fluorouracil, cisplatin and sorafenib were used as positive controls for inhibiting the growth of A549, HCT116 and SMMC-7721 cells, respectively. After incubation for 24 h, 0.5 $mg \cdot mL^{-1}$ MTT solution was added to each well and the cells were incubated at 37 °C for additional 4 h. Then, 150 μL DMSO was used to dissolve the formazan in each well after the medium was removed. Then the absorbance was measured by a Victor X3 microplate reader (PerkinElmer, Waltham, MA, USA) at 490 mm.

5-Ethynyl-2'-deoxyuridine (EdU) assay

5-Ethynyl-2'-deoxyuridine (EdU) assay was used to measure the ability of cell proliferation. HCT116, A549 and SMMC-7721 cells were seeded in 96-well plates with 5000 cells/well, and treated with different concentrations of compounds **1** or **2**, respectively. 5-Fluorouracil, cisplatin and sorafenib were used as positive controls for inhibiting the proliferation of A549, HCT116 and SMMC-7721 cells, respectively. After incubation for 24 h, the cells were incubated with EdU labeling medium (50 $mmol \cdot L^{-1}$) at 37 °C for 2 h. Then the medium was removed, 4% paraformaldehyde was used to fix the cells for 15 min, and 0.3% Trison-100X was used to permeabilize cells for 10 min. Finally, the cells were incubated with Click Additive solution for 30 min in the dark, and stained with Hoechst 33342 solution for 10 min. The images were captured under a Leica3000B fluorescence microscope (Leica, Germany).

Wound healing and transwell migration assays

Wound healing assay was used to evaluate the ability of cell migration. HCT116, A549, and SMMC-7721 cells were

seeded in 6-well plates with 8×10^5 cells/well. After growth to 70%−80% confluence, the cells were scratched in the center of the well, and the micrographs of the scratch lines were taken under a Leica3000B microscope (Leica, Germany). Then, the cells were treated with different concentrations of compounds **1** or **2** for 24h before being photographed again. 5-Fluorouracil, cisplatin and sorafenib were used as positive controls for inhibiting the migration of A549, HCT116 and SMMC-7721 cells, respectively. The initial and final wound sizes were measured using Image J software and the migration ratio was calculated.

Transwell assay was used to further evaluate the ability of cell migration. HCT116, SMMC-7221 and A549 cells were seeded in serum-free media at a density of 3×10^4 cells in the upper chamber of the transwell, and treated with different doses of compounds **1** or **2** (6.25 μmol·L^{-1} for HCT116 and SMMC-7221 cells, and 0.25 μmol·L^{-1} for A549 cells), respectively. Meanwhile, medium containing 20% FBS was added to the lower chamber of the transwell as a chemoattractant. After co-cultured for 24 h, the migrated cells were photographed by a Leica3000B fluorescence microscope (Leica, Germany), and the cells were counted by Image J software.

Data analysis

Results are expressed as mean ± standard deviation (SD). The statistical significance of data was analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) by SPSS 19.0 software. Values of $P < 0.05$ was considered to be statistically significant. The half inhibitory concentration (IC50) was calculated using the GraphPad Prism 7.

Supplementary Materials

Supplementray information can be acquired by e-mail to corresponding author.

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Cite this article as: JI Ai-Jia, MA Qing, KONG Mu-Yan, LI Le-Yan, CHEN Xin-Lian, LIU Zhong-Qiu, WU Jin-Jun, ZHANG Rong-Rong. Two cardenolide glycosides from the seed fairs of *Asclepias curassavica* and their cytotoxic activities [J]. *Chin J Nat Med*, 2022, **20**(3): 202-209.