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A Potential Anti-cancer Compound Separated from the Chloroform Extract of the Chinese Medicine Formula Shenqi San*

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Summary: This study examined anti-cancer compounds present in the chloroform extract of the Chinese medicine formula Shenqi San (CE-SS). Silica gel column chromatography, Sephadex LH-20, octadecylsilyl (ODS) column chromatography, and high performance liquid chromatography (HPLC) were used to separate the compounds from CE-SS. The structural formulas of the separated compounds were determined using 1D ^{1}H and ^{13}C experiments as well as high resolution electrospray ionization mass spectroscopy (HRESIMS). The corresponding results were compared with the reported literature data. A total of six compounds were separated and their structures were identified on the basis of corresponding spectroscopic and physico-chemical properties. They were Saikogenin F (Ⅰ), Prosaikogenin D (Ⅱ), Prosaikogenin F (Ⅲ), β-sitosterol (Ⅳ), 3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-O-β-D-glucopyranoside (Ⅴ), and methyl ursolic acid (Ⅵ). The separated compounds were evaluated *in vitro* for their inhibitory ability against the proliferation of A549 cells via MTT assay. Apoptosis was investigated using Annexin V-FITC/propidium iodide (PI) by flow cytometry. Apoptosis-associated proteins were examined by Western blotting. All the compounds were observed to have inhibitory activities against the proliferation of A549 cells to different degrees. Flow cytometry showed that compound Ⅴ increased the proportion of apoptotic A549 cells in a dose-dependent manner. Western blotting showed that compound Ⅴ increased the expression of Bax, cleaved-caspase-3, cleaved-caspase-9 and cleaved-poly ADP-ribose polymerase (PARP), and decreased the expression of Bcl-2. These results indicated that compound Ⅴ featured a significant inhibitory effect on A549 cells when compared with other compounds, and it may be considered a potential drug against cancers.
Key words: Shenqi San; separation; identification; A549; apoptosis

Lung cancer represents the most common cancer and the leading cause of all cancer deaths. More than 85% of lung cancer cases are non-small-cell lung cancer (NSCLC), for which the predicted 5-year survival rate is only 15.9%[1, 2]. Unfortunately, this figure has been only marginally improved over the past few decades[3]. One of the most important causes for the extremely high mortality rate associated with lung cancer is drug resistance that can frequently be observed during chemotherapy.

The Chinese medicine formula Shenqi San (SS) has been used in anticancer therapy for many years. Our previous studies showed that the chloroform extract of SS (CE-SS) exhibited a significant anticancer effect on human NSCLC A549 cells, which was accompanied with a significant analgesic effect in mice[4]. Additionally, CE-SS could promote the apoptosis of A549 cells and eight compounds had been isolated from CE-SS[5]. In this study, six other compounds were separated from CE-SS and their anti-proliferative effects on A549 cells were evaluated. Meanwhile, the anti-tumor mechanism of compound Ⅴ was investigated.

1 MATERIALS AND METHODS

1.1 General Experimental Procedures

For nuclear magnetic resonance (NMR) experiments, a Bruker DRX-600 NMR spectrometer (Bruker,

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Rheinstetten, Germany) was used. Using the UXNMR software package, chemical shifts were expressed as δ (parts per million), referring to the solvent peaks δ_H 3.34 and δ_C 49.0. With CD_3OD as a solvent, coupling constants (J) were shown in Hertz. ^1H and ^{13}C NMR experiments were carried out using conventional pulse sequences as described in the literature[6]. Exact mass numbers were obtained using high resolution electrospray ionization mass spectroscopy (HRESIMS) by a micrOTOF-Q triple-quadrupole orthogonal time-of-flight (TOF) instrument (Bruker, USA), equipped with an electrospray ionization source in a TOF mode at 10 000 resolving power.

1.2 Cell Culture

A549 cells were obtained from Wuhan University (China). The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U of penicillin G and 100 μg of streptomycin per mL. Cells were incubated at 37°C under 5% CO_2 in a humidified atmosphere.

1.3 Separation and Purification of CE-SS

CE-SS was prepared according to a pre-study[5]. It (17 g) was reconstituted and subjected to chromatography on silica (100–200 mesh size) and eluted with $CHCl_3$-MeOH to yield six fractions (1–6).

Fraction 6 (4.1 g) was then dissolved in a mixture of $CHCl_3$-MeOH (2:1) and subjected to column chromatography on the silica gel. The eluent consisted of a mixture of $CHCl_3$-MeOH (100:0, 20:1, 15:1, 10:1, 5:1, 1:1, 0:100, V/V) and three fractions (6a–6c) were obtained. Fraction 6a was then subjected to preparative high performance liquid chromatography (HPLC) on an octadecylsilyl (ODS) column to provide compound Ⅰ. Then, fraction 6a was further subjected to chromatography on a Sephadex-LH20 column to yield four additional fractions (6d–6g). Fractions 6e and 6g were then subjected to preparative HPLC on an ODS column and eluted with ACN-H_2O to provide compounds Ⅱ, Ⅲ and Ⅳ, respectively.

The above fraction 4 (2.3 g) was dissolved in a mixture of $CHCl_3$-MeOH (2:1) and subjected to column chromatography on the silica. The fraction was then eluted with a mixture of petroleum ether (PET)-ACTN (100:0, 1:2, 1:4, 1:6, 1:8, 1:10, 0:100, V/V) to yield four fractions (4a–4d). Fraction 4a was further subjected to chromatography on a Sephadex-LH20 column to provide compound Ⅴ. Fraction 4c was then subjected to chromatography on a silica gel column and eluted with PET-ACTN to provide compound Ⅵ.

1.4 Cell Proliferation Assay

A549 cells (5×10^4/mL) were seeded into 96-well plates and treated with various concentrations (1, 2, 4, 6 and 8 μg/mL) of different compounds from CE-SS at 37°C for 24 h. A total of 10 μL MTT (Merck, DA, Germany) was added to each well and incubated for 4 h at 37°C. DMSO (150 μL) was then added to each well for 10 min at 37°C. Agitation and absorbance were measured at 570 nm.

1.5 Flow Cytometry

A549 cells were seeded in six-well plates (2.0×10^5 cells per well). After 12 h, the cells were treated with various concentrations (1, 2, 4 and 6 μg/mL) of compound Ⅴ for 6 h. The cells were harvested and washed twice with phosphate-buffered saline (PBS). Cells were then stained with 10 μL Annexin V-FITC and 5 μL PI for another 15 min using an Annexin V-FITC/PI kit (KeyGEN Biotech, NJ, China) according to the manufacturer's instructions. After treatment, the cells were analyzed using a FACScan flow cytometer with Cell Quest Software (FACSCalibur, Becton Dickinson, USA).

1.6 Western Blotting

A549 cells were seeded in six-well-plates (2.0×10^5 cells per well). After 12 h, the cells were treated with various concentrations of compound Ⅴ for 6 h. The cells were then lysed, and protease inhibitor cocktail was added to obtain the whole protein content for quantification. The lysates were boiled for 5 min in protein loading buffer. The supernatants were collected and proteins (40 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% gel). The separated proteins were then transferred to polyvinylidene fluoride membranes (Millipore, USA) and blocked in 5% non-fat milk for 2 h at room temperature. Membranes were then incubated overnight at 4°C with the following primary antibodies diluted in the blocking buffer according to the manufacturer's instructions: anti-cleaved-caspase-3, anti-cleaved-caspase-8, anti-cleaved-caspase-9, anti-cleaved-PARP, anti-Bax, anti-Bcl-2 and anti-β-actin (Cell Signaling Technology, USA). After washing three times with tris-buffered saline (TBS) containing 0.5% (v/v) Tween-20, the membranes were incubated for 2 h at room temperature with HRP-conjugated secondary antibodies diluted in the blocking buffer according to the manufacturer's instructions. After washing three times with TBS, the blots were developed using an ECL detection reagent (Bio-Rad, USA) and analyzed using the FluorChem FC3 system (ProteinSimple, USA).

1.7 Statistical Analysis

Data were statistically analyzed using the SPSS22.0 software package. Measurement data were expressed as mean ± standard deviation (SD). The difference among the samples was compared with one-way analysis of variance and a P value of < 0.05 was considered statistically significant.

2 RESULTS

2.1 Chemical Composition Analysis of the Compounds Extracted from CE-SS

The HRESIMS results of the compounds extracted

from CE-SS are shown in table 1. The corresponding compound structures are shown in fig. 1.

Compound Ⅰ was characterized as follows: ^{1}H NMR (600 MHz, CD3OD) δ (ppm): 0.75, 0.91, 0.93, 0.98, 1.05, 1.11 (3H, s), 1.93 (1H, s, H-9), 3.08 (1H, d, *J*=7.2 Hz, H-28), 3.93 (1H, d, *J*=6.6 Hz, H-28), 4.20 (1H, dd, *J*=10.7, 6.0 Hz, H-16), 5.41 (1H, dd, *J*=10.5, 3.2 Hz, H-12), 5.96 (1H, d, *J*=10.7 Hz, H-11); 13C NMR (150 MHz, CD3OD) δ (ppm): 38.51 (C-1), 27.31 (C-2), 73.45 (C-3), 43.49 (C-4), 49.57 (C-5), 18.43 (C-6), 32.31 (C-7), 42.93 (C-8), 53.94 (C-9), 37.23 (C-10), 132.69 (C-11), 130.57 (C-12), 85.67 (C-13), 46.58 (C-14), 35.96 (C-15), 65.34 (C-16), 47.56 (C-17), 53.06 (C-18), 37.47 (C-19), 32.08 (C-20), 35.21 (C-21), 26.08 (C-22), 66.73 (C-23), 12.13 (C-24), 18.75 (C-25), 20.16 (C-26), 21.18 (C-27), 73.39 (C-28), 33.94 (C-29), 24.06 (C-30).

Compound Ⅱ was characterized as follows: ^{1}H NMR (600 MHz, CD3OD) δ (ppm): 6.45 (1H, dd, J = 10.6, 2.8 Hz, H-11), 5.58 (1H, d, *J*=10.7Hz, H-12), 4.08 (1H, t, *J*=3.1 Hz, H-16), 3.67 (1H, m, H-3), 2.03 (1H, s, H-9), 1.28 (3H, s, H-27), 0.99 (3H, s, H-30), 0.98 (3H, s, H-25), 0.89 (3H, s, H-29), 0.77 (3H, s, H-26), 0.75 (3H, s, H-24), 1.26 (1H, m, H-5), 3.72 (1H, d, *J*=11.6 Hz, H-28a), 3.65 (1H, d, *J*=11.5 Hz, H-23a), 3.29 (1H, m, H-23b), 3.26 (1H, d, *J*=11.9 Hz, H-28b), 4.34 (d, *J*=7.1 Hz, H-1′), 3.63 (1H, m, H-5′), 3.44 (1H, m, H-4′), 3.49 (1H, m, H-2′), 3.43 (1H, m, H-3′), 1.25 (3H, s, H-6′); ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 39.60 (C-1), 26.75 (C-2), 83.65 (C-3), 44.55 (C-4), 48.63 (C-5), 19.54 (C-6), 33.41 (C-7), 42.38 (C-8), 55.35 (C-9), 37.78 (C-10), 127.56 (C-11), 127.15 (C-12), 137.75 (C-13), 42.88 (C-14), 32.49 (C-15), 69.53 (C-16), 45.87 (C-17), 133.15 (C-18), 39.85 (C-19), 33.68 (C-20), 36.27 (C-21), 24.99 (C-22), 65.20 (C-23), 12.53(C-24), 19.41 (C-25), 18.05 (C-26), 22.57 (C-27), 65.49 (C-28), 22.75 (C-29), 33.37 (C-30), 106.64 (C-1′), 73.61 (C-2′), 75.89 (C-3′), 73.46 (C-4′), 72.27 (C-5′), 17.42 (C-6′).

The data of compound Ⅲ were shown as follows: ^{1}H NMR (600 MHz, CD3OD) δ (ppm): 5.99 (1H, d, *J*=10.6 Hz, H-11), 5.37 (1H, dd, *J*=10.4, 3.1Hz, H-12), 4.33 (1H, d, *J*=7.5 Hz, H-1′), 4.22 (1H, q, *J*=10.7, 6.3 Hz, H-16), 3.90 (1H, d, *J*=7.3 Hz, H-28a), 3.66 (1H, m, H-23a), 3.64 (1H, m, H-3), 3.62 (1H, d, *J*=11.2 Hz, H-4′), 3.43 (1H, m, H-2′), 3.49 (1H, m, H-3′), 3.32 (1H, m, H-23b), 3.05 (1H, d, *J*=7.2 Hz, H-28b), 1.95 (1H, m, H-9), 1.26 (3H, d, *J*=6.8 Hz, H-6′), 1.22 (1H, m, H-5), 1.15 (3H, s, H-26), 1.04 (3H, s, H-27), 1.01 (3H, s, H-29), 0.97 (3H, s , H-25), 0.92 (3H, s, H-30), 0.69 (3H, s, H-24); ^{13}C NMR (150 MHz, CD3OD) δ (ppm): 39.22 (C-1), 28.08 (C-2), 82.92 (C-3), 44.07

Table 1 HRESIMS data of compounds found in CE-SS

No.	Compound	Molecular formula	[M-1]$^{-}$m/z	Calcd.
Ⅰ	Saikogenin F	$C_{30}H_{48}O_4$	495.50	472.33
Ⅱ	Prosaikogenin D	$C_{36}H_{38}O_9$	633.55	634.84
Ⅲ	Prosaikogenin F	$C_{36}H_{58}O_8$	617.66	618.84
Ⅳ	β-sitosterol	$C_{29}H_{50}O$	413.60	414.71
Ⅴ	3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-O-β-D-glucopyranoside	$C_{36}H_{58}O_9$	680.33	681.40
Ⅵ	Methyl ursolic acid	$C_{31}H_{50}O_3$	469.78	470.73

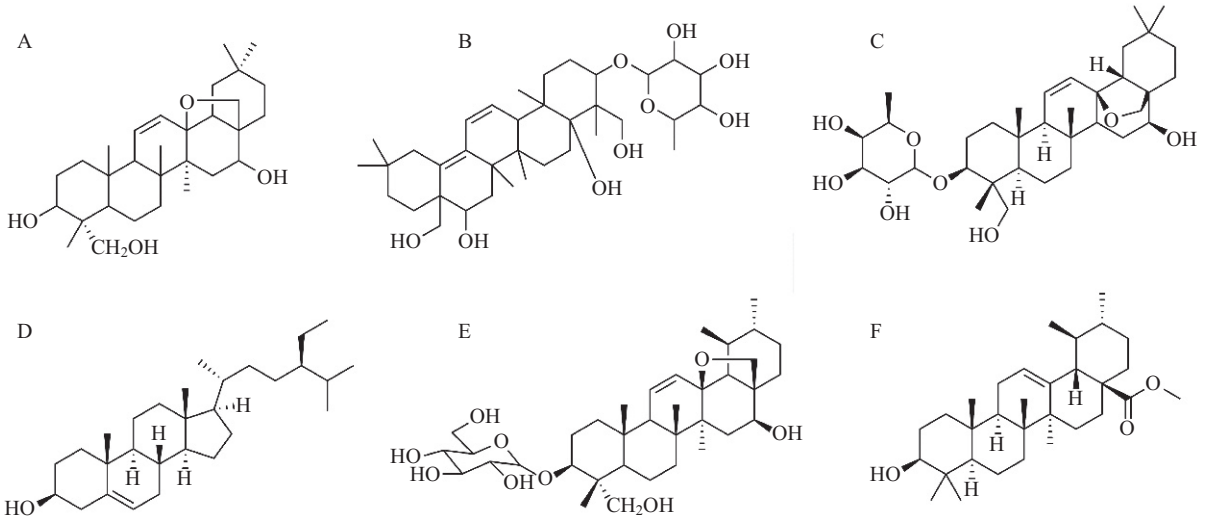

Fig. 1 Structures of compounds (Ⅰ–Ⅵ)
A: Saikogenin F; B: Prosaikogenin D, C: Prosaikogenin F; D: β-sitosterol; E: 3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-O-β-D-glucopyranoside; F: Methyl ursolic acid

(C-4), 47.92 (C-5), 18.16 (C-6), 32.09 (C-7), 42.96 (C-8), 53.98 (C-9), 37.04 (C-10), 134.23 (C-11), 130.53 (C-12), 85.69 (C-13), 46.49 (C-14), 35.97 (C-15), 64.53 (C-16), 47.56 (C-17), 53.06 (C-18), 38.51 (C-19), 32.31 (C-20), 35.21 (C-21), 26.20 (C-22), 65.35 (C-23), 12.76(C-24), 18.90 (C-25), 20.20 (C-26), 21.20 (C-27), 72.91 (C-28), 33.94 (C-29), 24.06 (C-30), 106.14(C-1′), 73.39 (C-2′), 75.37 (C-3′), 73.10 (C-4′), 71.74 (C-5′), 16.92 (C-6′).

^{1}H NMR (CDCl3, 600 MHz) δ (ppm) of compound Ⅳ was described as follows: 5.40 (1H, d, H-6), 3.49(1H, m, H-3), 1.01 (3H, s, C-19), 0.93 (3H, d, *J*=7. 0 Hz, C-29), 0.80~0.85 (9H, C-21, 26, 27), 0.66 (3H, s, C-18). ^{13}C NMR (150 MHz, CDCl3) δ (ppm) of compound Ⅳ showed 37.27 (C-1), 31.68 (C-2), 71.82 (C-3), 45.85 (C-4), 140.77 (C-5), 121.73 (C-6), 33.96 (C-7), 11.89 (C-8), 50.14 (C-9), 36.52 (C-10), 21.09 (C-11), 39.79 (C-12), 42.23 (C-13), 56.78 (C-14), 24.32 (C-15), 28.27 (C-16), 55.97 (C-17), 11.89 (C-18), 19.42 (C-19), 36.16 (C-20), 18.72 (C-21), 31.90 (C-22), 18.91 (C-23), 29.16 (C-24), 28.94 (C-25), 19.84 (C-26), 23.03 (C-27), 26.03 (C-28), 11.89 (C-29).

NMR data of compound Ⅴ were as follows: ^{1}H NMR (CD3OD, 600 MHz) δ (ppm): 5.84 (1H, d, *J*=10.1 Hz, H-12), 5.52 (1H, dd, *J*=2.8, 10.1 Hz,H-11), 4.20 (1H, dd, *J*=5.2, 10.1 Hz, H-16), 3.88 (1H, d, *J*=7.7 Hz,H-28a), 3.70 (1H, m, H-23a), 3.65 (1H, dd, *J*=5.0, 11.9 Hz, H-3), 4.40 (1H, d, *J*=7.6 Hz, H-1), 3.85 (1H, dd, *J*=2.5, 12.0 Hz, H-6a), 3.66 (1H, dd, *J*=4.0, 12.0 Hz, H-6b), 3.30 (1H, t, *J*=9.0 Hz, H-3), 3.25 (1H, t, *J*=9.0 Hz, H-4), 3.22 (1H, m, H-5), 3.15 (1H, dd, *J*=7.6, 9.0 Hz, H-2); ^{13}C NMR (CD_3OD, 150 MHz) δ (ppm): 36.82 (C-1), 24.39 (C-2), 82.80 (C-3), 39.00 (C-4), 44.07 (C-5), 16.72 (C-6), 31.39 (C-7), 38.92 (C-8), 45.87 (C-9), 32.26 (C-10), 133.83 (C-11), 131.19 (C-12), 86.55 (C-13), 42.36 (C-14), 31.85 (C-15), 64.35 (C-16), 43.87 (C-17), 51.87 (C-18), 35.25 (C-19), 37.12 (C-20), 26.01 (C-21), 33.72 (C-22), 53.71 (C-23), 12.58 (C-24), 16.72 (C-25), 19.50 (C-26), 17.96 (C-27), 72.70 (C-28), 18.30 (C-29), 18.76 (C-30), 105.95 (C-1′), 78.24 (C-2′), 77.78 (C-3′), 75.17 (C-4′), 72.90 (C-5′), 64.35 (C-6′).

NMR data of compound Ⅵ were noted as follows: ^{1}H NMR(600MHz, C5D5N) δ (ppm): 0.86 (3H, s ,H-24), 0.94 (3H, s, H-26), 1 .01 (3H, s, H-16), 1.02 (3H, s, H-30), 1.06 (3H, s, H-25), 1.23 (6H, s, H-4), 2.63 (1H, d, *J*=11.2 Hz, H-18), 3.35 (1H, dd, *J*=10.2 , 6.0Hz, H-3), 3.53 (3H, s, H-31); ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 39.42 (C-1), 26.20 (C-2), 78.18 (C-3), 39.11 (C-4), 55.86 (C-5), 18.82 (C-6), 33.62 (C-7), 39.45 (C-8), 48.09 (C-9), 37.50 (C-10), 23.67 (C-11), 125.70 (C-12), 139.31 (C-13), 42.55 (C-14), 28.15 (C-15), 24.95 (C-16), 48.09 (C-17), 53.60 (C-18), 39.53 (C-19), 39.12 (C-20), 31.11 (C-21), 37.32 (C-22), 28.72 (C-23), 15.71 (C-24), 16.61 (C-25), 17.55 (C-26), 23.67 (C-27), 179.96 (C-28), 17.50 (C-29), 21.45 (C-30), 51.50 (C-31).

With comparing with the reported data[7], compound Ⅰ was found to be saikogenin F, compound Ⅱ to be prosaikogenin D, compound Ⅲ to be prosaikogenin F, compound Ⅳ to be β-sitosterol, compound Ⅴ to be 3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-O-β-D-glucopyranoside, and compound Ⅵ to be methyl ursolic acid.

2.2 Isolated Compounds from CE-SS Suppress the Proliferation of A549 Cells

The MTT assay (fig. 2) showed that compounds Ⅰ, Ⅱ, Ⅲ, Ⅴ, and Ⅵ except compound Ⅳ featured different inhibitory effects on A549 cells. The half-maximal inhibitory concentration (IC_{50}) values of compounds Ⅰ–Ⅵ are shown in table 2. Among them, compound Ⅴ had the best inhibitory effect and exhibited antiproliferation activity with an IC_{50} value of 4.9 μg/mL.

Saikogenin F inhibited the proliferation of A549 cells with inhibition rates of 18.81%, 47.64% and 86.01% at concentrations of 50, 70 and 90 μg/mL,

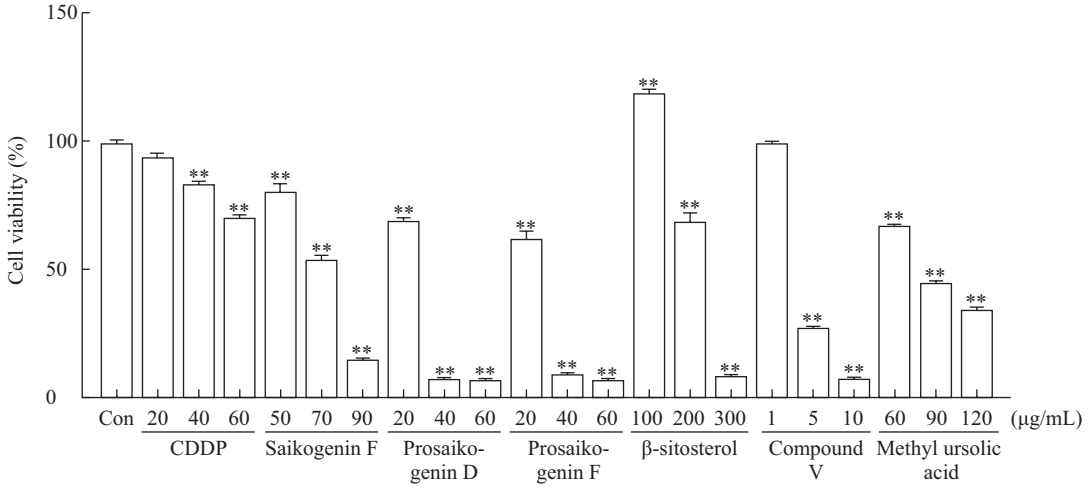

Fig. 2 Inhibitory effects of different concentrations of compounds from CE-SS on the proliferation of A549 cells
$^{**}P$<0.01 *vs.* control (Con)

Table 2 IC_{50} values of compounds found in CE-SS (mean±SD, *n*=3)

No.	Compound	IC_{50} (μg·mL^{-1})
Ⅰ	Saikogenin F	65.4±2.25#
Ⅱ	Prosaikogenin D	57.2±1.90#
Ⅲ	Prosaikogenin F	58.9±5.70#
Ⅳ	β-sitosterol	222.5±2.77#
Ⅴ	3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-O-β-D-glucopyranoside	4.9±1.21
Ⅵ	Methyl ursolic acid	88.4±6.35#

#*P*<0.05 *vs.* compound V

respectively. The inhibition rates of Prosaikogenin D at concentrations of 20, 40 and 60 μg/mL were 33.29%, 94.02% and 94.30%, respectively. Meanwhile, Prosaikogenin F inhibited A549 proliferation with inhibition rates of 40.53%, 92.62% and 93.07% at concentrations of 20, 40 and 60 μg/mL, respectively. The compound β-sitosterol inhibited A549 proliferation with inhibition rates of 19.75%, 29.51% and 92.70% at concentrations of 100, 200 and 300 μg/mL, respectively. Furthermore, compound Ⅴ inhibited A549 proliferation with inhibition rates of 0.91%, 72.83% and 93.93% at concentrations of 1, 5 and 10 μg/mL, respectively. Methyl ursolic acid inhibited A549 proliferation with inhibition rates of 35.11%, 55.28% and 66.27% at concentrations of 60, 90 and 120 μg/mL, respectively. These results showed that these compounds could dose-dependently inhibit the proliferation of A549 cells. Compared with the control, compounds Ⅰ–Ⅵ exhibited significant inhibitory activities at various concentrations (*P*<0.05). According to its IC_{50} value and compared with compounds Ⅰ, Ⅱ, Ⅲ, Ⅴ, Ⅵ, compound Ⅴ exhibited a significant inhibitory activity (*P*<0.05).

2.3 Compound Ⅴ Decreases the Proliferation of A549 Cells

As shown in fig. 3, as the concentrations of compound Ⅴ increased, cell viability was significantly decreased in A549 cells compared with that in the control (*P*<0.05 and *P*<0.01). The IC_{50} of compound Ⅴ for A549 cells was 4.9 μg/mL. Cell viability of A549 treated for 24 h with 1, 2, 4, 6 and 8 μg/mL compound Ⅴ was 83.13%, 76.23%, 70.04%, 30.47% and 27.35%, respectively. Thus, the A549 cell viability was decreased in a dose-dependent manner.

2.4 Compound Ⅴ Induces A549 Cell Apoptosis

Flow cytometry showed that the proportions of apoptotic A549 cells following treatment with 1, 2, 4 and 6 μg/mL compound V for 6 h were 10.41%, 13.95%, 20.83% and 31.47%, respectively (fig. 4A and 4B). Compared to the untreated control group, the proportions of apoptotic A549 cells were significantly increased (*P*<0.05 and *P*<0.01) after treatment with 4 μg/mL compound Ⅴ. These results indicated that compound Ⅴ induced A549 cell apoptosis in a dose-dependent manner. In addition, the levels of cleaved-caspase-3, cleaved-caspase-9 and cleaved-PARP were significantly increased after treatment with compound Ⅴ for 6 h when compared to those in the control group (fig. 4C and 4D).

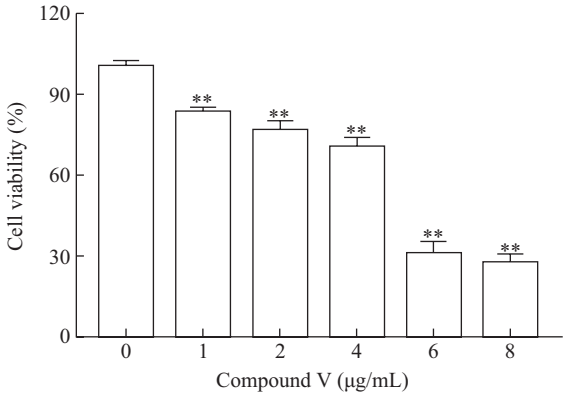

Fig. 3 Effect of compound Ⅴ on the viability of A549 cells
After treatment with compound Ⅴ for 24 h, the viability of A549 cells was measured by MTT assay. **P<0.01 *vs.* control

Next, the involvement of the mitochondrial pathway was investigated in the mechanism by which compound Ⅴ induced the apoptosis of A549 cells. Our result showed that Bax was significantly upregulated, while Bcl-2 was significantly downregulated after treatment with compound Ⅴ. These results indicated that compound Ⅴ induced the apoptosis of A549 cells via the mitochondrial pathway.

3 DISCUSSION

In the present study, synergistic separation methods such as silica gel column chromatography, gel column chromatography, as well as thin-layer chromatography were used to separate and identify six compounds from CE-SS. As published in the literature, five of these compounds have already been known. ^{1}H and ^{13}C NMR data of compound Ⅴ were consistent with those previously reported[8]. The free radical scavenging and anti-microbial activities have already been demonstrated as previously reported[8]. Simultaneously, the inhibitory activity of the six compounds on A549 cells was examined, and it was found that compound Ⅴ

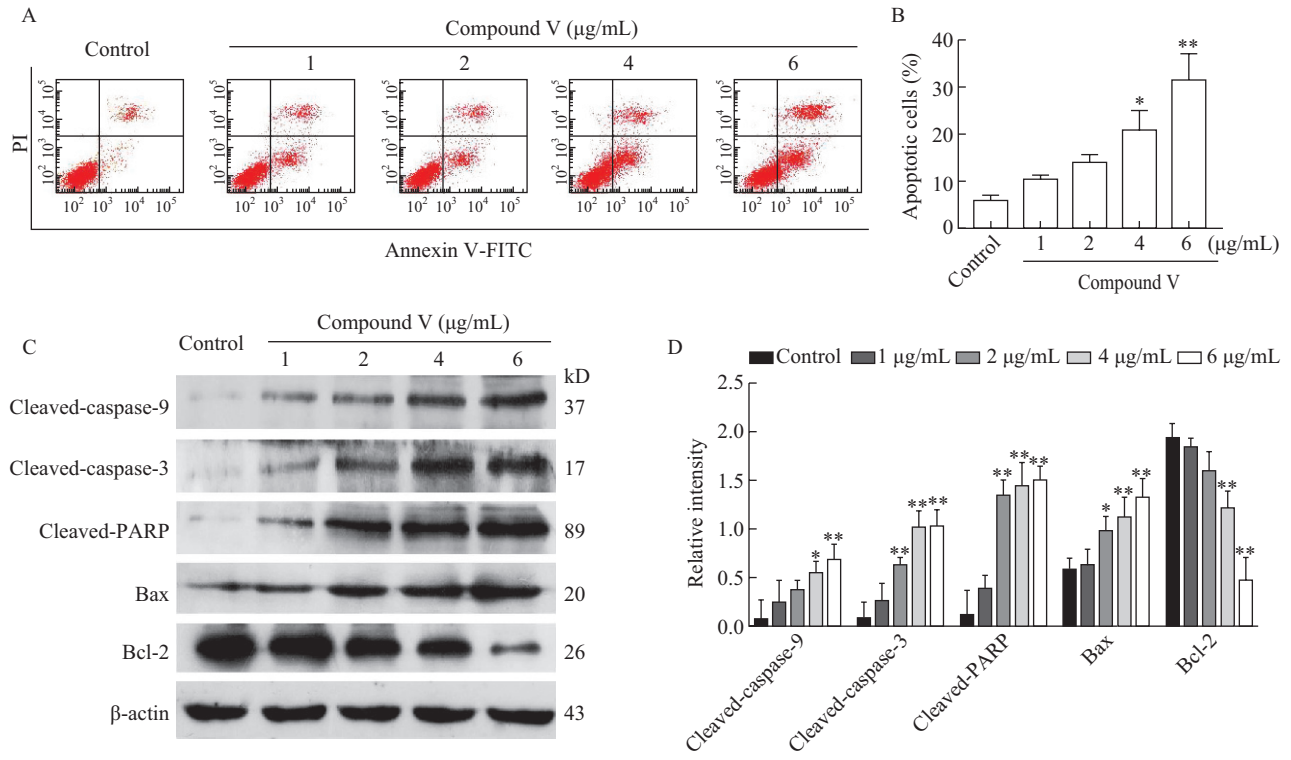

Fig. 4 Apoptosis of A549 cells induced by compound V

A: Flow cytometric analysis of the effect of different concentrations of compound V on the apoptosis of A549 cells; B: Compound V increases the proportion of apoptotic cells in a dose-dependent manner; C, D: Compound V increases the expression of Bax, cleaved-caspases-3, cleaved-caspases-9, cleaved-PARP and decreases the expression of Bcl-2 in A549 cells. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* control

exhibited an improved effect on A549 cell proliferation when compared with the other compounds. Our results suggested that compound V may be used as a potential anticancer drug for NSCLC.

A variety of Chinese herbal formulas have been used in clinical practice for the treatment of cancer for many years. They are found to have anti-inflammatory, anti-oxidative, anti-bacterial, and anti-cancer effects[9–13]. However, their active compounds and pharmacological mechanisms are not fully understood. Apoptosis describes a natural way of removing aged or damaged cells from the organism. Various anticancer therapies trigger the apoptosis and cell death networks to eliminate malignant cells[14]. Nowadays, an increasing number of Chinese herbal extracts have been reported to induce cancer cell apoptosis[15–18]. Our previous studies confirmed that CE-SS inhibited the growth of A549 cells by inducing apoptosis. Therefore, in this study, we investigated the ability of compound V to induce apoptosis in A549 cells. Compound V was demonstrated to promote the apoptosis of A549 cells. Bcl-2 and Bax are commonly used to monitor the apoptosis of tumor cells and evaluate the effect of cancer drugs[19]. Bax, an important homolog of Bcl-2, is a promoter of apoptosis. It has been proposed that the sensitivity of cells against apoptosis stimuli is closely related to the ratio of Bcl-2/Bax and other Bcl-2 homologues, with a relative excess of Bcl-2 mediating a protective effect. In contrast, when Bax is in excess and Bax homodimers dominate, cells are susceptible to apoptosis[20, 21]. Caspases (cysteine-aspartic proteases) are proteolytic enzymes largely known for their role in controlling cell death and inflammation. The apoptotic caspases (caspase-3, -6, -7, -8, and -9) play a role in the regulation of programmed cell death[22, 23]. Apoptosis is mediated by two convergent pathways: the intrinsic and extrinsic pathways[24]. The mitochondria-mediated (intrinsic) apoptotic pathway is initiated by caspase-9, which activates the execution molecule caspase-3. Poly ADP-ribose polymerase (PARP) acts as an important indicator of caspase-3 activation and also as a vital biomarker of cell apoptosis[25, 26]. Our results showed that compound V could increase the levels of Bax, cleaved-caspase-3, cleaved-caspase-9, cleaved-PARP and decrease the levels of Bcl-2, which suggested that compound V inhibited A549 cell proliferation by inducing apoptosis.

In conclusion, our study found that among the six compounds from CE-SS, compound V showed a strong anti-proliferation effect on A549 cells. It could induce the apoptosis of A549 cells, and the IC_{50} of compound V for A549 cells was 4.9 μg/mL. Further studies on the

autophagy and related signaling pathways of compound Ⅴ will be performed in the future.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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