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ORIGINAL ARTICLE

Suppression of Cervical Cancer Cell Survival by Ursolic Acid Extracted from *Catalpa bungei* Leaves

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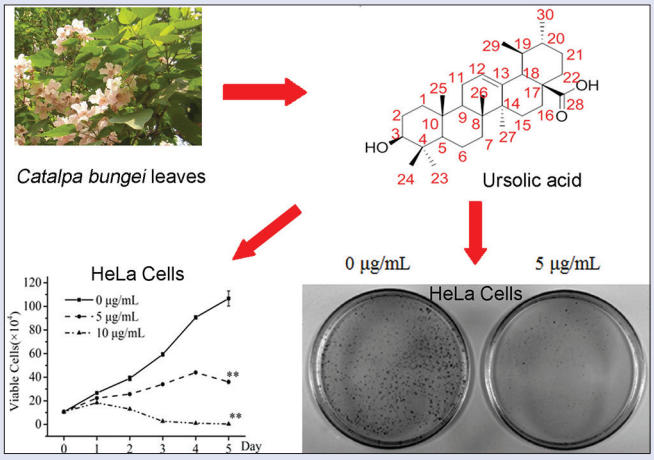
BSTRACT

Background: Previous phytochemical studies showed that extracts from leaves and seed oil of *Catalpa* plants showed antioxidant and antitumor activities. However, the active components and their roles and molecular mechanisms were still incompletely identified. **Objective:** We aimed to extract and identify the active fraction from *Catalpa bungei* leaves and investigate its underlying mechanism in suppressing cervical cancer cell survival. **Materials and Methods:** Extracts from *C. bungei* leaves with 80% methanol were separated into different fractions. The component with optimal inhibitory effect on HeLa cell growth was isolated, identified, and examined. **Results:** Extracts from *C. bungei* leaves with methanol were separated into petroleum ether, ethyl acetate (EA), *n*-butanol, and water fractions. The chemicals in EA fraction exhibited optimal inhibition effects, which were further separated into 29 fractions by the silica column chromatography. The compound with optimal antitumor activity was eventually determined to be ursolic acid (UA) based on the results of Sephadex column chromatography and ^{1}H nuclear magnetic resonance analysis. UA at 5.0 and 10.0 μg/mL substantially inhibited the growth and migration of HeLa cells. UA also retarded cell cycle at G0/G1 stage and promoted cell apoptosis through activating death receptor and mitochondria-associated pathways. **Conclusion:** UA isolated from *C. bungei* leaves could inhibit growth and migration and induce apoptosis in HeLa cells.

Key words: Apoptosis, *Catalpa bungei*, cervical cancer, ursolic acid

SUMMARY

- Our study aimed to extract and identify active components from *Catalpa bungei* leaves. Ursolic acid (UA) was fractioned and identified to have optimal anti-cervical cancer activity among all the chemicals. UA functioned to suppress cancer cell proliferation, migration, and cell cycle and promote cell apoptosis through activating death receptor and mitochondria-associated pathways, which may represent as a potential treatment strategy for cervical cancer.



Abbreviations used: UA: Ursolic acid; EA: Ethyl acetate; HPV: Human papillomavirus, PARP: Poly ADP-ribose polymeras; ROS: Reactive oxygen species; PBS: Phosphate buffered saline; SRB: Sulfonyl rhodamine B; DMSO: Dimethyl sulfoxide; TLC: Thin layer chromatography; NMR: Nuclear magnetic resonance; CAS: Chemical abstracts service.

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INTRODUCTION

Cervical cancer is a human papillomavirus (HPV)-related cancer that ranks among the most malignant tumors with high incidence and mortality secondary to breast cancer in gynecological cancers. The incidence of cervical cancer varies with age, race, geographical location, and economic conditions. It was estimated that the incidence of cervical cancer was 528,000 cases and there were 266,000 deaths in 2012 worldwide, which were higher in developing countries than that in developed countries.[1] Although the incidence and the mortality have been decreasing with HPV screening detections and new therapeutic approaches, there are still a significant number of patients with metastatic or recurrent disease.[2] For those patients not amenable to surgery or radiation therapies, palliative chemotherapy remains the standard of care. It is reported that chemotherapy regimens have limited activity in treating cervical cancers; therefore, novel pharmacologic strategies are needed.[3] Currently, it is well-acknowledged that natural compounds extracted from plants can effectively treat tumor with low side effects.[4] Therefore, active components from plant secondary metabolites may highlight a promising approach for the treatment of cervical cancers.

In China, abundant medicinal plant resources are available with long history of herbal medicine. Medicinal plant resources have been extensively studied in inhibiting the growth of multiple cancer cells. Chen and Xu studied plants in China and found that 72 plant species of 37 families had antitumor or potential antitumor effects.[5] The Catalpa plants of Bignoniaceae family consist of 13 species around the world and they are preferably distributed in America and Eastern Asia. It is

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documented that 5 species and 1 variant of Catalpa plants were cultivated in China, including *Crassula ovata* G. Don, *Clerodendrum bungei* C. A. Mey., *Castanopsis fargesii* Bur., variant of *Caragana tibetica* Forrest, *Chaenomeles speciosa* Ward., and *Cypripedium fargesii* f. duclouxii Dode, respectively.[6] Previous phytochemical studies showed that iridoids and naphthoquinones were the predominate constituents of *Catalpa* plants.[7] It was documented that extracts from leaves and seed oil of *Catalpa ovata* showed antioxidant and antitumor activities.[7,8] *C. bungei* is a deciduous arbor. Previous studies preferred to focus on the breeding and cultivation of *C. bungei*. Currently, whether extracts from *C. bungei* could suppress tumor growth, and the active fraction and underlying mechanism involved in this inhibition are currently unrevealed. Therefore, in this study, we extracted and identified the active fraction from *C. bungei* leaves and investigated the underlying mechanism of this active component in suppressing cervical cancer cell survival and migration. Our study may provide an experimental basis for determining antitumor active components from *C. bungei* and developing natural medicines for the treatment of cervical cancer.

MATERIALS AND METHODS

Materials

Leaves of *C. bungei* were collected from the experimental base of Forestry Science Institute (105°53'E, 34°32'N at the altitude of 1494 meters) in Xiaolong Mountain in Tianshui of Gansu Province in mid-October, 2013. The dried leaves were pulverized into powder and then passed through a 20-mesh sieve with the aperture diameter of 0.85 mm. HeLa cells were purchased from Basic Medical Institute of Chinese Academy of Medical Sciences (Beijing, China) and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C.

Extraction, isolation, and purification

The extraction and isolation of component fractions from *C. bungei* leaves are shown in Figure 1. The dried powder of leaves (1000 g) was mixed with 80% ethanol (3 × 10 L) at 80°C for 1 h, and the filtered solution was concentrated in rotary evaporator at 45°C. Then, the resultant 192.64 g extracts were mixed with 2 L distilled water, and the obtained suspension was separated into petroleum ether, ethyl acetate (EA), *n*-butanol, and water phase. Afterward, each extraction phase was concentrated and dried. Finally, we obtained 4 fractions: petroleum ether fraction (22.38 g), EA fraction (49.77 g), *n*-butanol fraction (54.69 g), and water fraction (53.55 g). The four fractions were then dissolved in dimethyl sulfoxide (DMSO) at different concentration and their inhibitory effects on HeLa cells were further evaluated.

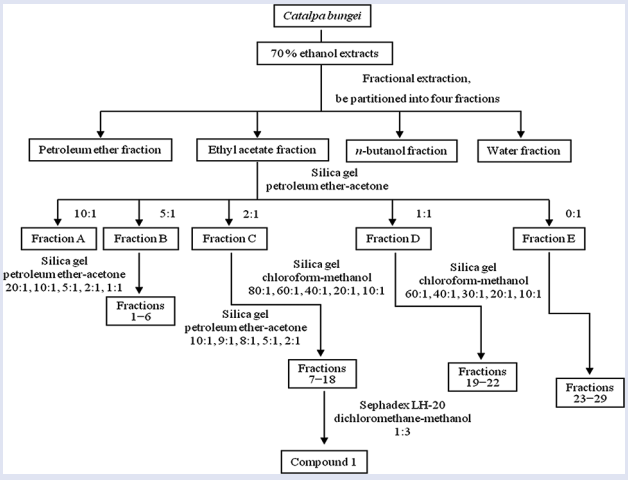

Figure 1: Extraction and isolation of active compounds from *Catalpa bungei* leaves

EA fraction dissolved in chloroform was separated using silica gel (100–200 mesh) column (6.5 cm × 3.0 m) through gradient elution with petroleum ether and acetone (10: 1, 5: 1, 2: 1, 1: 1, v/v). The obtained elute was pooled into 5 fractions (Fractions A, B, C, D, and E) through thin-layer chromatography (TLC). Fraction B was separated using silica gel (200–300 mesh) column (5.0 cm × 4.0 m) through gradient elution with petroleum ether and acetone (20: 1, 10: 1, 5: 1, 2: 1, 1: 1, v/v). The elute was pooled into 6 fractions (Fractions 1–6) through TLC. Fraction C was separated with silica gel (200–300 mesh) column (3.0 cm × 4.0 m) through gradient elution with petroleum ether and acetone (10: 1, 9: 1, 8: 1, 5: 1, 2: 1, v/v). The elute was pooled into 12 fractions (Fractions 7–18) through TLC. Fraction D was separated with silica gel (200–300 mesh) column (3.0 cm × 4.0 m) through gradient elution with chloroform and methanol (80: 1, 60: 1, 40: 1, 20: 1, 10: 1, v/v). The elute was pooled into 4 fractions (Fractions 19–22) through TLC. Fraction E was separated in silica gel (200–300 mesh) column (2.0 cm × 3.0 m) through gradient elution with chloroform and methanol (60: 1, 40: 1, 30: 1, 20: 1, 10: 1, v/v). The elute was pooled into 7 fractions (Fraction 23–29) through TLC. These 29 fractions were dissolved in DMSO to determine the inhibitory effects of the fractions on HeLa. Fraction 17 showed the maximum inhibitory effects on HeLa cell proliferation. Fraction 17 was purified by Sephadex LH-20 column chromatography (dichloromethane: methanol = 3:1) and compound 1 (85.5%) was obtained.

Cell proliferation assay

HeLa cells were grown in 24-well plate at a density of 5×10^5/well until the cells were adhere to the wall. Then HeLa cells were, respectively, treated with 5 μg/mL and 10 μg/mL ursolic acid (UA) with equal volume of DMSO as the negative control. The cells were stained with trypan blue (Sigma, Shanghai, China) after being treated with 0.25% trypsin. The cells were transferred to cell counting plate and counted under an inverted microscope for each 24 h within 5 days.

In addition, the effect of samples on HeLa cell growth was determined by sulfonyl rhodamine B (SRB) (Sigma, Shanghai, China) staining. The HeLa cells were incubated on a 96-well plate (2×10^3 cells/well) and cultured until cells adhered to the wall. The HeLa cells were treated with the samples in different concentrations with DMSO as the negative control. The medium was removed from well after 24 h treatment, and 100 μL of 10% trichloroacetic acid (w/v) was added into each well for 1 h at 4°C.

Wound healing assay

HeLa cells were incubated onto 6-cm cell culture dishes. Then, the culture medium was removed after cells were treated with the culture medium containing mitomycin (the volume ratio of mitomycin to medium = 1:1000) for 3 h. Subsequently, the wounds were created by scratching cell sheets with a sterile 200 μL pipette tip. The culture medium was replaced with refresh medium containing 5 μg/mL UA, and DMSO at the same volume was served as the negative control. The wound healing situation at the specific position on the scratched areas was observed, and images of wound healing were captured under an inverted microscope (Leica, Germany) with the 20 × objective lens every 24 h. The wound widths were measured and the relative wound widths were calculated.

Colony formation assay

The clonogenic assay was performed as described by Mahata *et al.*[4] HeLa cells (1×10^3 cells/dish) were incubated on a 6-cm culture dish

until the cells were adhered to the wall. Then, cells were treated with 5 μg/mL UA. When the colonies were visible after 2–3 weeks, the incubation was terminated and the supernatant was discarded. Then, the cells were washed with phosphate-buffered saline (PBS) buffer for twice. 10% paraformaldehyde was added to fix the cells for 15 min. The fixed cells were stained 0.1% crystal violet for 20 min, slowly washed with distilled water, and then air-dried. ChemiDoc XRS + imaging system (Bio-Rad, CA, USA) was used to capture the images of stained cell colonies. Surviving fraction was calculated as a ratio of the number of colonies to the number of cells plated (plating efficiency) divided by the same ratio calculated for the nontreated group.

Cell cycle determination

HeLa cells were incubated onto 6-well plates (5×10^5 cells/well). Cells were collected after being treated with UA (5 and 10 μg/mL) for 24 h. Then, 75% ethanol was added to fix cells overnight at 4°C and the cells were washed with PBS before staining. Subsequently, 400 μL of Propidium iodide (PI) staining solution was added and mixed well after the cells were treated with 100 μL of RNase A for 30 min in water bath at 37°C. Cell cycle was analyzed by flow cytometry (PARTEC, Germany) after they were mixed with the mixture solution for 30 min at 4°C in the dark to avoid lighting. The data were analyzed by ModFit LT 3.3 software (BD Bio-sciences, San Jose, CA, USA).

Hoechst 33258 staining assay

HeLa cells were incubated on 6-well plates at a density of 8×10^4 cells/well. Then, cells were treated with UA (5, 10, and 20 μg/mL) for 24 h and suspended in PBS buffer before being fixed with 4% formaldehyde solution for 10 min. Subsequently, the cells were re-suspended in PBS buffer and then stained with 100 μL of Hoechst 33,258 working solution for 10 min at room temperature. Hoechst 33258 (Kaiji Biological Scientific and Technological Development Co., Ltd. Nanjing, China) stock solution was diluted by 10 times with distilled water to obtain working solution. Then, the cells were suspended in PBS buffer twice. Images were captured under an inverted fluorescence microscope (BX 51, Olympus Optical Co., Ltd., Tokyo, Japan).

Apoptosis assay

HeLa cells were cultured in 6-well plate until cells were attached to the wall (5×10^5 cells/well). Then, the cells were treated with UA (5 and 10 μg/mL) for 24 h. After the cells were collected, 500 μL of dyeing solution buffer, 5 μL Annexin V-FITC, and 5 μL PI were, respectively, added for treatment for 15 min at room temperature. Subsequently, the apoptotic cells were counted using flow cytometry. The results were analyzed using FlowJO 7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

Western blotting

HeLa cells were treated with UA (5, 10, and 20 μg/mL) or equivalent DMSO for 24 h. After treatment, the cells were collected and lysed with RIPA lysis buffer containing 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. Then, the protein concentration was measured using BCA kits. Then, SDS-PAGE (10%–15%) was performed with equal amount of protein from each treatment. Then, the separated proteins were transferred to NC membrane, and incubated with appropriate primary antibodies against β-actin (CW0096A), Caspase-3 (AC031), Caspase-8 (AC056), Caspase-9 (AC062), poly (ADP-ribose) polymerase (PARP) (AP102), Cytochrome C (AC909), Bax (AB026), and Bcl-2 (AB112) (Beyotime Biotechnology, Shanghai, China) followed by the incubation with goat anti-mouse or anti-rabbit secondary antibodies conjugated to Horseradish peroxidase (CWBIO, Beijing, China), respectively. Protein blots were detected with ECL solution and ChemiDoc XRS + imaging system (Bio-Rad, USA). β-actin was served as protein standard control.

Statistical analysis

Each experiment was repeated at least for three times. Data were expressed as means ± standard deviation. ANOVA statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software and the followed least significant difference method was used to compare between two groups. $P < 0.05$ was considered statistically significant. IC_{50} was calculated with GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Inhibitory effects of extracts from *Catalpa bungei* leaves on HeLa cell proliferation

The effects of the components that were extracted from *C. bungei* leaves and distributed in petroleum ether fraction, EA fraction, *n*-butanol fraction, and water fraction on HeLa cell proliferation were measured using SRB staining. The results showed that all the four fractions from *C. bungei* leaves at different concentrations (50, 100, 150, 200, and 250 μg/mL) had inhibitory effects on HeLa cell growth. Besides, the inhibitory effect elevated with the increase of the concentrations of four fractions in a dose-dependent manner. Among the four fractions, EA fraction exhibited the optimal inhibitory effect on HeLa cell proliferation, followed by petroleum ether, *n*-butanol, and water fractions [Figure 2]. Therefore, the mixed chemical components dissolved in EA were further isolated through silica gel column chromatography, and 29 subfractions were obtained. Then, the inhibitory effect of these subfractions on the proliferation of HeLa cell was determined using SRB staining. The results showed that HeLa cells were more sensitive to the toxic effect of Fraction C (subfractions 7–18) than other fractions as reflected by the substantially decreased IC_{50} values. In addition, subfraction 17 exhibited optimal killing effect of cancer cells among the chemicals of Fraction C with IC_{50} value of 11.02 μg/mL [Figure 3].

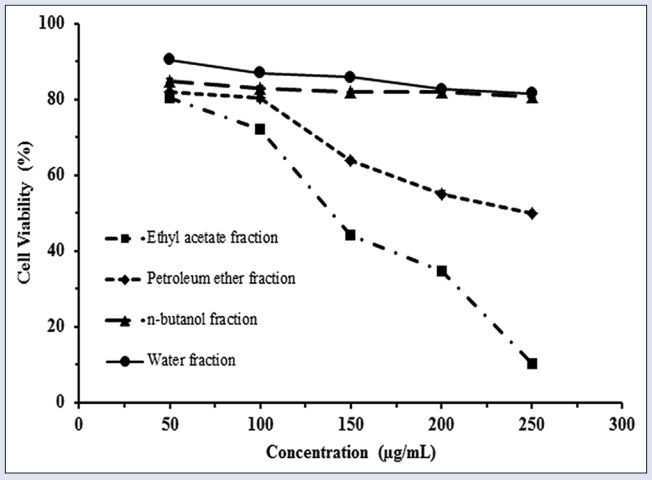

Figure 2: Inhibition of the proliferation of HeLa cells by the components in four fractions from *Catalpa bungei*. HeLa cells were treated with the four fractions (50–250 μg/mL) for 24 h. Cell viability was then determined using the sulfonyl rhodamine B assay. The experiment was repeated for triplicate. Data were expressed as means ± standard deviation

Isolation and identification of the active component in subfraction 17

Furthermore, subfraction 17 was subjected to Sephadex LH-20 gel chromatography, and the predominate component, named as compound 1, accounting for 85.5%, was isolated and purified. The structure of the isolated compound 1 was identified. The spectral data were provided as follow.

Compound 1: Chemical abstracts service (CAS): 77–52-1; ^{1}H nuclear magnetic resonance (NMR) (500 MHz, DMSO) δ: 11.92 (s, 1H,-COOH), 5.12 (t, J = 3.5 Hz, 1H, H-12), 4.27 (d, J = 5.0 Hz, 1H, H-3), 2.97–3.00 (m, 1H, H-18), 2.09 (d, J = 11.0 Hz, 1H, H-15), 1.89–1.93 (m, 1H, H-22), 1.79–1.86 (m, 3H, H-6, 16, 22), 1.42–1.59 (m, 10H, H-1, 6, 7, 9, 15, 16, 21), 1.24–1.31 (m, 4H, H-2, 19, 20), 0.98–1.04 (m, 4H, H-11, 27), 0.89–0.91 (m, 7H, H-11, 23, 29), 0.86 (s, 3H, H-26), 0.80 (d, J = 6.0 Hz, 3H, H-30), 0.74 (s, 3H, H-24), 0.66–0.67 (m, 4H, H-5, 25); ^{13}C NMR (125 MHz, DMSO) δ: 178.1 (C-28), 138.0 (C-13), 124.4 (C-12), 76.6 (C-3), 54.6 (C-5), 52.2 (C-18), 46.8 (C-17), 46.6 (C-9), 41.5 (C-14), 38.9 (C-20), 38.3 (C-19), 38.2 (C-4), 38.1 (C-1), 38.0 (C-8), 36.3 (C-22), 36.1 (C-10), 32.5 (C-7), 30.0 (C-21), 28.1 (C-23), 27.4 (C-2), 26.8 (C-15), 23.6 (C-16), 23.1 (C-27), 22.7 (C-11), 20.9 (C-30), 17.8 (C-6), 16.8 (C-29), 16.7 (C-24), 15.9 (C-26), 15.0 (C-25); electrospray ionization mass spectrometry m/z: 455.54 ([M-H]+, 100). The compound was identified as UA based on the spectral data [Figure 4a]. Effect of UA on HeLa cell proliferation was examined and IC_{50} value was calculated based on the inhibitory effects of UA treatment at concentration ranging from 5 to 25 μg·mL^{-1} for 24 h. As shown in Figure 4b, the inhibition efficiency of UA on HeLa cell proliferation enhanced with the increase of its concentration with the IC_{50} value at 9.5 μg/mL.

Ursolic acid inhibits growth, migration, and cell cycle of HeLa cells

The effect of UA on HeLa cell shape was observed under optical microscope inspection. It was demonstrated that 5 μg/mL UA did not induce substantial morphological change of the cells. While after HeLa cells were treated with 10 μg/mL UA for 24 h, more cells became round and were detached from culture plate in comparison with the control [Figure 5a]. Effect of UA on growth of HeLa cells was examined using cell counting method. Both 5 and 10 μg/mL UA showed inhibitory effects on cell growth on day 1, which had further decrease from day 2 to day 5. We also noticed that the inhibitory effect of 10 μg/mL UA on the growth of HeLa cells was obviously higher than that of 5 μg/mL UA [Figure 5b]. Moreover, we determined the effect of UA on migration of HeLa cells by wound healing assay. It was showed that the relative value of scratch width for HeLa cells untreated by UA was 0.20 after treatment for about 5 days and scratch was healed soon. However, the relative scratch width after treatment for about 5 days was 0.47 and scratch was recovered slowly after the treatment with 5 μg/mL UA compared with control group [Figure 5c and d].

We further examined the activity of UA on single cell clone formation. After treatment with 5 μg/mL UA, the single cell clone was small and the amount of single cell clone was relatively less when compared with that in the control group [Figure 5e]. Cell cycle distribution was investigated by flow cytometry after HeLa cell was treated with various concentrations of UA (0, 5, and 10 μg/mL) for 24 h. As shown in Figure 5f-5g, the proportion of cells at G0/G1 phase increased significantly (66.07%, 71.84%, and 75.92%) against the increase of ursolic concentration, indicating that HeLa cells cycle was obviously blocked by UA at G0/G1 phase.

Ursolic acid induced apoptosis of HeLa cells involved death receptor and mitochondria-associated pathways

The apoptosis of HeLa cells induced by various concentrations (0, 5, and 10 μg/mL) of UA was determined by Annexin V-FITC/PI double staining. It was demonstrated that ratio of apoptotic cells significantly increased (10.35 ± 1.32%) after treatment with 5 μg/mL UA for 24 h compared to the control group (1.62 ± 0.18%) (P < 0.01), which had a further increase to 21.3 ± 1.9% when exposed to 10 μg/mL UA [Figure 6a]. In addition, the cells were stained with Hoechst 33258, and then, morphological changes of cells in the absence of UA were observed under the fluorescence microscope. After treatment with 5 μg/mL UA for 24 h, the nuclear morphology of HeLa cell showed no significant change. However, when the concentration of UA reached 10 or 20 μg/mL, nucleus of HeLa cells were dense and pyknosis in fluorescence intensity [Figure 6b].

Moreover, we further evaluated whether death receptor and mitochondria-associated pathways were involved in UA-induced cell apoptosis. Protein expression levels of PARP, Caspases-3/8/9, Bcl-2, Bax, and Cytochrome C were detected by western blot [Figure 6c]. It was showed that UA promoted the expression of cleaved Caspase-3/8/9

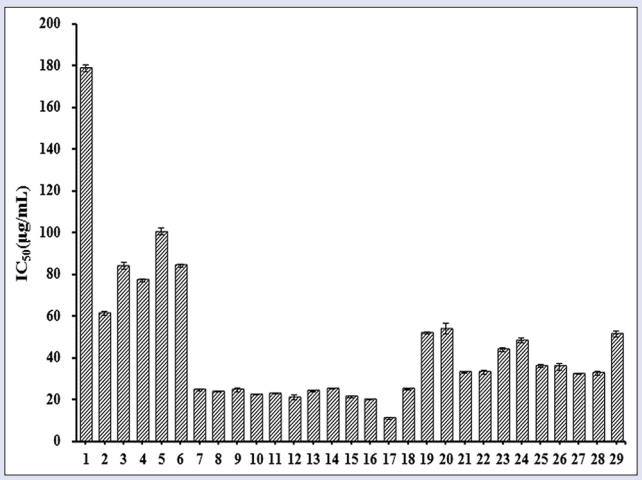

Figure 3: Inhibition of the proliferation of HeLa cells by 29 fractions of ethyl acetate phase. HeLa cells were treated with the 29 fractions of ethyl acetate fraction for 24 h. Cell viability was then determined using the sulfonyl rhodamine B assay. This experiment was repeated for three times. The data were represented as means ± standard deviation

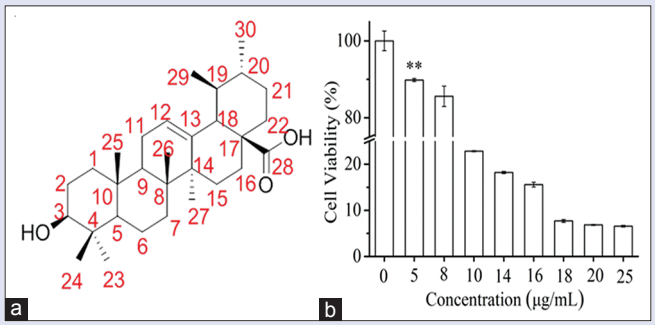

Figure 4: Inhibition of the proliferation of HeLa cells by ursolic acid. (a) Chemical structure of ursolic acid. The structure identification of the isolated compound was performed with MS, ^{1}H nuclear magnetic resonance, and ^{13}C nuclear magnetic resonance analyses. (b) HeLa cells were treated with various concentrations of ursolic acid for 24 h. Cell viability was then determined using the sulfonyl rhodamine B assay. This experiment was repeated three times. The asterisks indicated P < 0.05

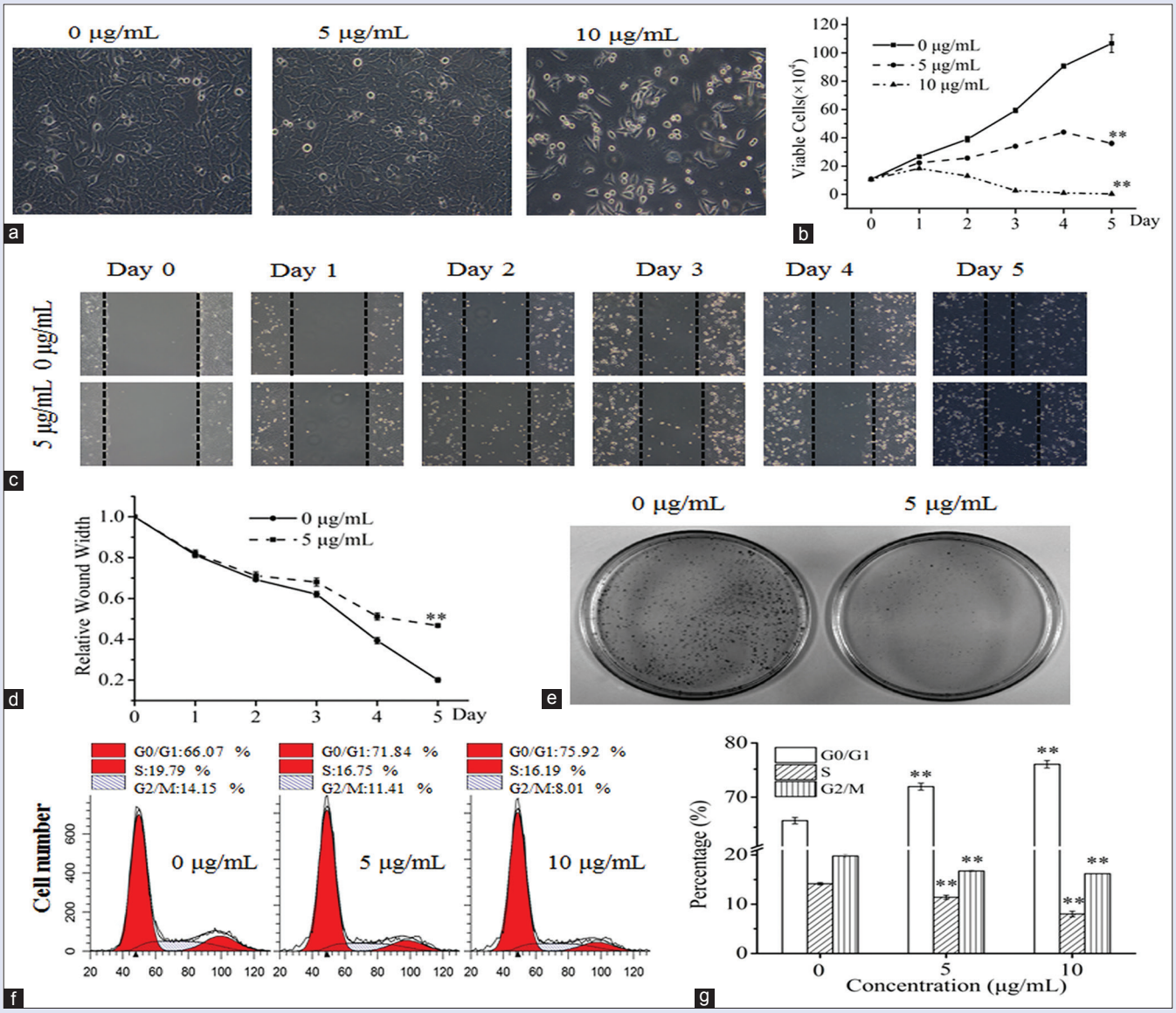

Figure 5: Effects of ursolic acid on the growth, migration, survival, and cell cycle of HeLa cells. (a) Morphological change of ursolic acid treated HeLa cells. (b) Effect of ursolic acid on the growth of HeLa cells. HeLa cells were treated of ursolic acid (5, 10 μg/mL), viable cells were counted every 24 h for 6 days. (c and d) Effect of ursolic acid on the migration of HeLa cells. (e) Effect of ursolic acid on colony formation of HeLa cells. (f) Cell cycle analysis of HeLa cells by Flow cytometry. $^{**}P < 0.01$ versus control. (g) Analysis of the effect of different dose of ursolic acid on cell cycle progression of HeLa cells

and PARP, as well as Bax and Cytochrome C, while downregulated the expression of Bcl-2.

DISCUSSION

C. bungei (Bignoniaceae) is a deciduous tree mainly distributed in central to southwestern China.[9] It is showed that *C. bungei* -derived chemicals have potential to be used as therapeutics in human diseases. However, less is known about the bioactive extracts from *C. bungei* in cancer treatment. In the current study, we found that the compound UA isolated from EA fraction exhibited optimal inhibition on the activity of cervical cancer cells, which may represent as a new strategy for the treatment of cervical cancer.

Currently, various plant-derived natural chemicals were used as herbal medicine. For example, the extracts from leaves of *Psidium guajava Linn.* of Myrtaceae can induce apoptosis of gastric cancer cells.[10] The extracts of *Alectoria virens Tayl.* may induce apoptosis of HT-29 cells, and the extracts from the roots of *Alpinia pricei* and *Ginkgo biloba* may induce apoptosis of oral cancer cells.[11-13] The extracts of *Toona sinensis* can induce apoptosis of human promyelocytic leukemia cells.[14] Xu *et al.* described the antioxidative activities of the compounds extracted from Catalpa plant leaves. The two flavonoid compounds isolated based on EA fraction, including luteolin and apigenin, were considered as predominate antioxidants.[6] Moreover, Oh *et al.* isolated 3 compounds from the EA soluble fraction of the methanolic extract of the leaves of *C. ovata* and showed that these compounds contributed to the regulation of T-cell-mediated immune responses and proliferation of leukemic cells.[7] In this study, we showed that UA isolated from leaves of *C. bungei* by bioassay-guided fractionation inhibited growth, migration, and survival of HeLa cells in a dose-dependent manner.

UA (3β-hydroxy-urs-12-en-28-oic acid) is a natural pentacyclic triterpenoid that is present in many plants, including medicinal herbs

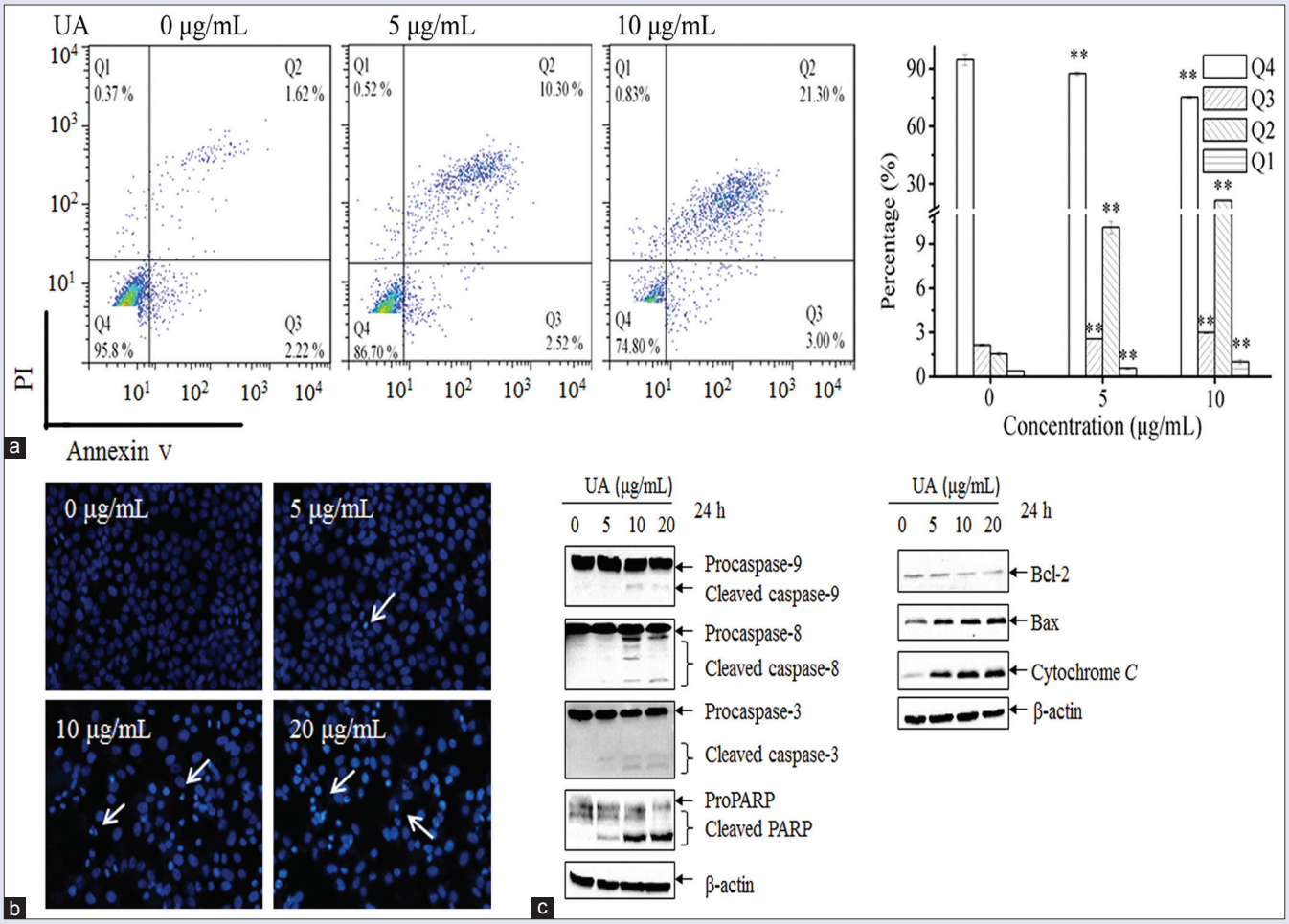

Figure 6: Ursolic acid induced apoptosis in HeLa cell *in vitro*. (a) Apoptosis analysis of HeLa cells by flow cytometry. (b) The morphologic change of HeLa cells treated with ursolic acid using Hoechst 33258 staining. Images were captured through fluorescence microscope (400×). (c) Western blot analysis of the expression of cell apoptosis-related proteins. HeLa cells were treated with indicated doses of ursolic acid for 24 h, the procaspase-8/9/3, poly (ADP-ribose) polymerase, and their cleaved products and Bcl-2, Bax, and Cytochrome C were indicated

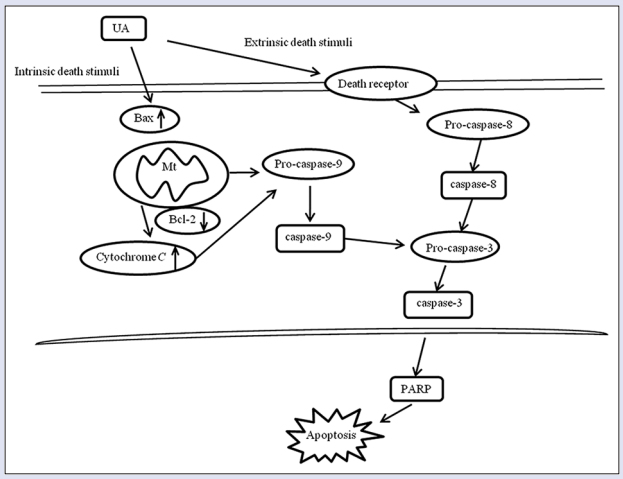

Figure 7: Schematic diagram of hypothetic mechanisms underlying ursolic acid-induced apoptosis of HeLa cells

and foods.[15] In our study, we further investigated the underlying mechanism of UA in inducing cell apoptosis. The data showed that both death receptor and mitochondria-associated pathways were involved in UA-induced cell apoptosis. As shown in Figure 7, UA induced apoptosis of HeLa cells through the intrinsic pathway involving the downregulation of antiapoptotic protein Bcl-2, upregulation of proapoptotic protein Bax, the increase in mitochondria permeability, as well as releasing Cytochrome *C* into cytoplasmic matrix for the subsequent activation of Caspase-9, the digestion of Caspase-3 and Caspase-9 restriction enzyme, and the hydrolysis of PARP. These results were consistent with what was reported by Li *et al.*[16] Besides, the results also indicated that apoptosis of HeLa cells was induced by the endogenous pathway too. With activated Caspase-8, inactive Caspase-3 precursor was hydrolyzed into two fragments after HeLa cells were treated with UA. Then, activated Caspase-3 hydrolyzed PARP and induced apoptosis of HeLa cells. Byun *et al.* elaborated that the natural triterpenoid pristimerin induced mitochondrial cell death through reactive oxygen species (ROS)-dependent activation of both Bax and PARP-1 in human cervical cancer cells and that Jun N-terminal kinase was involved in ROS-dependent Bax activation.[17]

CONCLUSION

Collectively, our study showed that the extracts from *C. bungei* leaves had active components for anti-cervical cancer. UA isolated from

C. bungei leaves could inhibit growth, migration, and survival of HeLa cells. UA induced apoptosis of HeLa cells in death receptor and mitochondria- dependent ways. These data indicated that UA might be a potential medicine for the treatment of cervical cancer.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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