

Dehydrocostus lactone suppresses cell growth and induces apoptosis in recombinant human papilloma virus-18 HaCaT cells via the PI3K/Akt signaling pathway

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Abstract. Dehydrocostus lactone is considered to be the major cholagogic ingredient of the *Costus* genus of plants. It exhibits strong cholagogic effects, and also exerts antimicrobial and antineoplastic activity. The present study aimed to investigate the effects of dehydrocostus lactone on the cell growth and apoptosis of recombinant human papilloma virus (HPV)-18 HaCaT cells. The HPV-18 genome was transfected into HaCaT cells, which were subsequently used for analysis. The results demonstrated that dehydrocostus lactone reduced the cell proliferation and induced apoptosis of HPV-18 HaCaT cells, as determined by MTT and N-acetyl-Asp-Glu-Val-Asp p-nitroanilide assays, respectively. Furthermore, caspase-3/9 activity was determined using a caspase-3/9 activity kit and western blotting was performed to investigate the expression of certain proteins. The results demonstrated that caspase-3/9 activities, and the protein expression of Bcl-2-associated X and p53, in HPV-18 HaCaT cells were significantly increased, while cyclin D1 protein expression was suppressed by dehydrocostus lactone. Additionally, dehydrocostus lactone significantly upregulated the protein expression of phosphatase and tensin homolog and inhibited the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in HPV-18 HaCaT cells. Therefore, the results of the present study indicate that dehydrocostus lactone may suppress cell growth and induce apoptosis in recombinant HPV-18 HaCaT cells via the PI3K/Akt signaling pathway, and may be a represent a novel potential therapeutic agent for the treatment of condyloma acuminatum.

Introduction

Condyloma acuminatum (CA) is a common sexually transmitted disease that is caused by infection with human papilloma virus (HPV) (1), and its incidence is increasing annually. Clinically, it presents as cauliflower-like or papillary growths in the genital area. Although it is a benign hyperplasia, certain cases may transform into malignant tumors (1). A minority of cases may develop large CA due to excessive hyperplasia in a short period of time. CA is very infectious (2), and has a negative impact on society and the family of patients as it adversely affects the physical and mental health of patients. High relapse rates are an issue following CA treatment (3), and it is extremely difficult to control the transmission and prevalence of the disease (3). Therefore, this disease has attracted attention in research.

Humans are the only natural host of HPV. Three types of squamous epithelial cells on human skin, mucosa and metaplasia are sensitive to HPV, which infects them via damaged squamous epithelial cells (4). Studies have reported that after human squamous epithelial cells are infected with HPV, cells exhibit abnormal proliferation and apoptosis (4,5). The specific mechanism underlying the development of CA abnormal growths following HPV infection has not previously been identified, however, increased cell division or reduced cell death following HPV infection may be implicated (6). Tumor necrosis factor-related apoptosis-inducing ligand led to the apoptosis of tumor cells, virus-infected cells and transformed cells (6). Caspase-3 is an established death protease (6) that is a key protein in the apoptosis pathway, and participates in cell apoptosis induced by a variety of factors (4).

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a typical signal transduction pathway that inhibits cell apoptosis and promotes cell survival (7). In addition, the pathway has a key role in the resistance of tumors to chemotherapy and radiotherapy, the genesis and proliferation of tumor cells, and the invasiveness and metastasis of tumor cells to other tissues (8). The PI3K/Akt signaling pathway is an essential pathway in cells. Specifically, it has an important role in the genesis and development of CA cells (8). Studies have demonstrated that the PI3K/Akt pathway induces CA through various mechanisms: The pathway inhibits the

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expression of protein p53, a tumor suppressor gene, in the cell nucleus by promoting the anti-nuclear movement of Mdm2 proto-oncogene; therefore, excessive activation of the PI3K signaling pathway leads to uncontrollable proliferation of CA cells. Additionally, the pathway inhibits the cell apoptosis process via the phosphorylation of various proteins, including Bcl-2-associated agonist of cell death, caspase-9 and other components of the apoptosis pathway, and also inhibits conformational changes of certain apoptosis proteins, such as Bcl-2-associated X (Bax) (8-10).

Dehydrocostus lactone, the structure of which is presented in Fig. 1, is extractable from dry *Costus* root. *Costus* originated from India, and was introduced and cultivated in the province of Yunnan in China (11,12). Dehydrocostus lactone is the major ingredient of *Costus* root essential oil (13). It has been demonstrated through modern pharmacological studies that *Costus* has certain effects on the digestive, respiratory and cardiovascular systems (14). Dehydrocostus lactone, as the primary component of *Costus*, has been reported to improve intestinal functions, promote gastric motility and choleresis, and exhibit antidiarrheic, antihypertensive and antibacterial effects (15). The aim of the present study was to investigate the effects of dehydrocostus lactone on the cell growth and apoptosis of recombinant HPV-18 HaCaT cells, and to determine whether these effects may occur via the PI3K/Akt signaling pathway.

Materials and methods

Cell lines and transfection of the HPV-18 genome into HaCaT cells. The HaCaT human epithelial cell line was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), propagated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37°C in an humidified atmosphere containing 95% air and 5% CO₂. A total of 200 ng of HPV-18 expression plasmid was purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and transfected into cells (1x10⁶ cell/well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 4 h, the medium was replaced with fresh DMEM medium supplemented with dehydrocostus lactone (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany). Following a 48 h incubation at 37°C, the Caspase-3 and caspase-9 expression levels were investigated via western blot analysis and following a 72 h incubation at 37°C, the MTT assay was performed.

MTT assay. Recombinant HPV-18 HaCaT cells (1x10⁶ cells/well) were seeded in 96-well plates and treated with either 2 µl of dimethyl sulfoxide (DMSO) or 2 µl of dehydrocostus lactone (2.5, 5 and 10 µg/ml) for 0, 24, 48 and 72 h at 37°C. Following treatment, MTT tetrazolium salt (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) was added for 4 h. The medium was then removed and 150 µl DMSO was added per well to dissolve the formazan crystals. Absorbance was measured using the SpectraMax 190 microplate reader (Molecular Devices, LLC., Sunnyvale, CA, USA) at 490 nm.

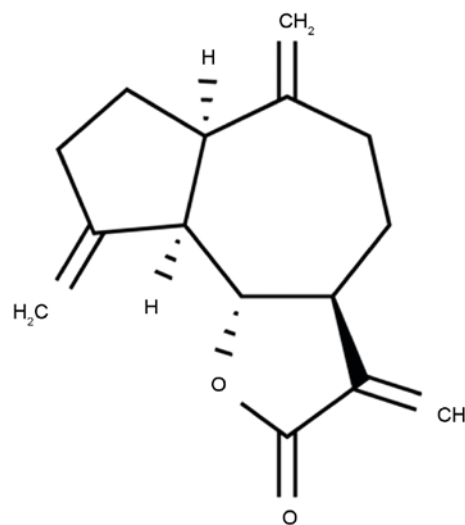


Figure 1. Chemical structure of dehydrocostus lactone.

Caspase-3 and caspase-9 activity levels. Recombinant HPV-18 HaCaT cells (1x10³ cell/well) were seeded in 6-well plates and treated with 2 µl of DMSO or dehydrocostus lactone (2.5, 5 and 10 µg/ml) for 48 h at 37°C. Cells were subsequently washed with PBS and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China). Cell extracts were clarified by centrifugation at 10,000 x g for 15 min at 4°C and protein concentration was measured by a BCA assay. Proteins (10 µg per sample) were incubated with N-acetyl-Asp-Glu-Val-Asp p-nitroanilide (also termed Ac-DEVD-pNA; Beyotime Institute of Biotechnology) for 2 h at 37°C. The activity levels of caspase-3 and caspase-9 were subsequently determined using the SpectraMax 190 microplate reader at 405 nm.

Apoptosis rate. Recombinant HPV-18 HaCaT cells (1x10³ cells/well) were seeded into 6-well plates and treated with 2 µl of DMSO or dehydrocostus lactone (2.5, 5 and 10 µg/ml) for 48 h at 37°C. Cells were subsequently washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were then stained with 5 µl of Annexin V-fluorescein isothiocyanate (cat. no. 556570; BD Biosciences, San Jose, CA, USA) and 5 µl of propidium iodide (cat. no. 556570; BD Biosciences) for 20 min in darkness at room temperature. Cell apoptosis rate was then detected using a flow cytometer (C6; BD Biosciences) and analyzed using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from HaCaT cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (500 ng) was reverse-transcribed using a First-Strand cDNA Synthesis kit (GeneCopoeia, Inc., Rockville, MD, USA) at 42°C for 2 min, 37°C for 30 min and 85°C for 5 sec. Following this, qPCR was performed using SYBR Green PCR Master Mix (cat. no. 303410; Takara Biotechnology Co., Ltd., Dalian, China) by a LightCycler® 2.0 apparatus (Roche Applied Science, Mannheim, Germany). The thermocycling conditions were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, annealing at 60°C for

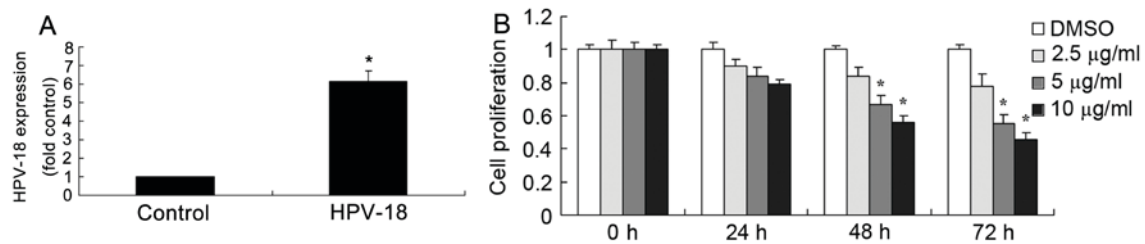


Figure 2. Dehydrocostus lactone reduced cell proliferation of HaCaT cells. (A) Determination of HPV-18 expression using quantitative-polymerase chain reaction. (B) An MTT assay was performed to determine the effect of treatment with 2.5, 5 and 10 $\mu\text{g/ml}$ dehydrocostus lactone for 0, 24, 48 and 72 h on the proliferation of recombinant human papilloma virus-18 HaCaT cells. DMSO was used as control treatment. * $P < 0.01$ vs. DMSO control group. DMSO, dimethyl sulfoxide.

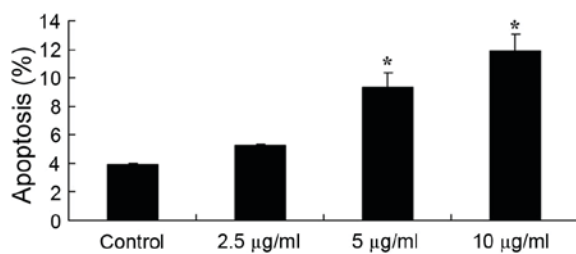


Figure 3. Dehydrocostus lactone induced apoptosis in HaCaT cells. The effects of 2.5, 5 and 10 $\mu\text{g/ml}$ dehydrocostus lactone treatment for 48 h on apoptosis levels in recombinant human papilloma virus-18 HaCaT cells were determined. DMSO was used as control treatment. * $P < 0.01$ vs. DMSO control group. DMSO, dimethyl sulfoxide.

intensity of each band was detected using BeyoECL Plus reagent (cat. no. P0018A; Beyotime Institute of Biotechnology, Nanjing, China) and quantified using ImageJ software 6.0 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean + standard deviation using SPSS v.20 (IBM Corp., Armonk, NY, USA). All experiments were performed in triplicate. One-way analysis of variance followed by Tukey post hoc test was performed to determine the significance of differences among the experimental groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Dehydrocostus lactone reduces the proliferation of recombinant HPV-18 HaCaT cells. The effect of dehydrocostus lactone on recombinant HPV-18 HaCaT cell proliferation was determined using an MTT assay. As revealed in Fig. 2A, transfection with HPV-18 expression plasmid significantly increased the expression of HPV-18 miRNA expression in HaCaT cells, compared with the negative control group. As demonstrated in Fig. 2B, treatment with dehydrocostus lactone reduced the proliferation of recombinant HPV-18 HaCaT cells in dose- and time-dependent manner, compared with DMSO-treated cells. In particular, 5 and 10 $\mu\text{g/ml}$ dehydrocostus lactone for 48 or 72 h significantly reduced the proliferation of recombinant HPV-18 HaCaT cells, compared with the DMSO control group at the same time points (Fig. 2B).

Dehydrocostus lactone induces apoptosis in HaCaT cells. Furthermore, the present study also investigated the effect of dehydrocostus lactone on apoptosis in recombinant HPV-18 HaCaT cells. The results demonstrated that the apoptosis rate of recombinant HPV-18 HaCaT cells was significantly increased following treatment for 48 h with 5 and 10 $\mu\text{g/ml}$ dehydrocostus lactone, compared with the DMSO control group (Fig. 3).

Dehydrocostus lactone promotes caspase-3/9 activity in HaCaT cells. The activities of caspase-3/9 in DMSO- and dehydrocostus lactone-treated recombinant HPV-18 HaCaT cells were also investigated in the present study. As demonstrated in Fig. 4, dehydrocostus lactone treatment for 48 h also led to significant increases in the caspase-3/9 activities of recombinant HPV-18 HaCaT cells at concentrations of 5 and 10 $\mu\text{g/ml}$, compared with the DMSO control group.

30 sec, and a final extension of 72°C for 30 sec. Primers used for amplification were as follows: HPV-18 forward, 5'-TAC CTGTGTCACAAGCCGTT-3' and reverse, 5'-CAGCAGTGT AAGCAACGACC-3'; GAPDH forward, 5'-ACAGCAACA GGGTGGTGGAC-3' and reverse, 5'-TTTGAGGGTGCACGA ACTT-3'. The data were analyzed using the $2^{-\Delta\Delta C_q}$ method (16).

Western blot analysis. Recombinant HPV-18 HaCaT cells (1×10^6 cell/well) were seeded in 6-well plates and treated with either 2 μl of DMSO or 2 μl of dehydrocostus lactone (2.5, 5 and 10 $\mu\text{g/ml}$) for 48 h at 37°C. Cells were washed with PBS and lysed using RIPA assay. Cell extracts were clarified by centrifugation at 10,000 \times g for 15 min at 4°C and protein concentration was measured by a BCA assay. Proteins (25 μg per sample) were analyzed on an 8-10% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (Merck KGaA). Membranes were subsequently blocked with 5% skim milk powder in TBS-0.1% Tween-20 for 1 h at 37°C and incubated with Bax (cat. no. sc-6236; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p53 (cat. no. sc-6243; 1:1,000; Santa Cruz Biotechnology, Inc.), cyclin D1 (cat. no. sc-717; 1:500; Santa Cruz Biotechnology, Inc.), phosphatase and tensin homology (PTEN; cat. no. sc-6817-R; 1:1,000; Santa Cruz Biotechnology, Inc.), PI3K (cat. no. sc-7174; 1:500; Santa Cruz Biotechnology, Inc.), Akt (cat. no. sc-8312; 1:500; Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Akt (cat. no. sc-16646-R; 1:500; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-25778; 1:2,000; Santa Cruz Biotechnology, Inc.) primary antibodies at 4°C overnight, which was followed by incubation with goat anti-rabbit immunoglobulin G-horseradish peroxidase (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. The

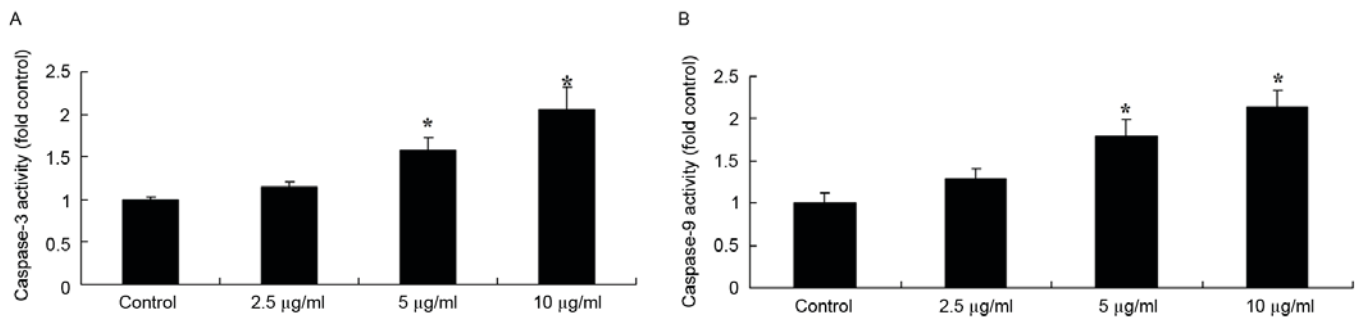


Figure 4. Dehydrocostus lactone increased the caspase-3/9 activities in HaCaT cells. Dehydrocostus lactone increased the activities of (A) caspase-3 and (B) caspase-9 in recombinant human papilloma virus-18 HaCaT cells. DMSO was used as control treatment, and 2.5, 5 and 10 µg/ml concentrations of dehydrocostus lactone were employed. *P<0.01 vs. DMSO control group. DMSO, dimethyl sulfoxide.

Dehydrocostus lactone promotes Bax and p53 protein expression, and suppresses the protein expression of cyclin D1 in HaCaT cells. To observe the effect of dehydrocostus lactone on Bax, p53 and cyclin D1 protein expression in recombinant HPV-18 HaCaT cells, Bax and p53 protein expression was determined by western blot analysis. Dehydrocostus lactone (5 and 10 µg/ml) treatment for 48 h significantly increased Bax and p53 protein expression, and suppressed cyclin D1 protein expression in recombinant HPV-18 HaCaT cells, compared with the DMSO control group (Fig. 5).

Dehydrocostus lactone increases PTEN protein expression and downregulates the PI3K/Akt signaling pathway in HaCaT cells. To determine the effect of dehydrocostus lactone on PTEN protein expression and the PI3K/Akt signaling pathway in recombinant HPV-18 HaCaT cells, western blot analysis was performed. The results in Fig. 6 demonstrate that dehydrocostus lactone (5 and 10 µg/ml) treatment for 48 h significantly increased PTEN protein expression, and suppressed PI3K and p-Akt levels in recombinant HPV-18 HaCaT cells, compared with the DMSO control group.

Discussion

A previous study on CA has indicated that human immune responses, particularly cellular immune responses, are the principal factor determining the outcome of CA (2). Patients with deficits in the cellular immune response, including patients with acquired immune deficiency syndrome, Hodgkin lymphoma, malignant lymphoma and chronic lymphocytic leukemia, are at an increased risk of being affected by HPV (6). Patients that are taking immunosuppressive drugs, particularly those following renal transplantation and heart transplants, are prone to broad and persistent warts (6). The present study demonstrated that dehydrocostus lactone significantly reduced the proliferation of recombinant HPV-18 HaCaT cells, which indicates that it may be useful for the prevention/treatment of CA.

CA is a type of benign proliferative disease of the skin membrane at the genitals, anus and perineum (17). It is a gynecological disease that is commonly observed in the clinic. The primary methods of its transmission between individuals are by sexual contact or through indirect contact, and mother-to-child vertical transmission (17). With the development of the economy, and increased international and domestic exchanges, CA has

become a sexually transmitted disease with one of the highest growth rates (18). A previous study demonstrated that the genesis and development of CA involves a complicated gene regulation process (18). Humans are the only natural host of HPV (6); following infection of human squamous epithelial cells by HPV, cells exhibit abnormal proliferation and apoptosis. The results of the present study demonstrated that dehydrocostus lactone significantly reduced proliferation, and increased apoptosis, in recombinant HPV-18 HaCaT cells. Furthermore, Sun *et al* (15) reported that dehydrocostus lactone suppressed the proliferation of colorectal carcinoma cells via downregulation of eukaryotic translation initiation factor 4E expression.

Apoptosis is a type of programmed cell death and abnormal apoptosis levels may lead to the excessive proliferation of cells (19). Clinically, a minority of patients with CA develop large CA due to excessive hyperplasia in a short period of time (20). Additionally, CA may develop and deteriorate further in certain patients, leading to the genesis of malignant tumors (20). The caspase-3 protein family and Bax have been demonstrated to enhance apoptosis (21). However, few studies have investigated their roles in hyperplastic diseases of the skin. Caspase-3 and Bax have important roles in apoptosis (21,22), therefore, the present study investigated the activity/expression of these proteins, and the results demonstrated that dehydrocostus lactone significantly increased caspase-3/9 activities and induced Bax protein expression in recombinant HPV-18 HaCaT cells.

A previous study demonstrated that the PI3K/Akt signaling pathway is closely associated with CA (23). Additionally, studies concerning CA have demonstrated that the PI3K/Akt signal transduction pathway is involved in resisting Fas regulation and the apoptotic process (23). Therefore, the activation of the PI3K/Akt signal transduction pathway may lead to the genesis of CA (23), and its mechanism of action may involve the abnormal expression of PTEN (24). A further study regarding CA also reported that PI3K/Akt signaling may increase the expression of growth factor receptors to promote mitosis (23). Therefore, the PI3K/Akt signaling pathway is closely associated with the genesis and development of CA (24), and indicators of PI3K/Akt signaling pathway activity in CA tissues may be used as an indicator to evaluate CA malignant grade (24).

The PI3K/Akt signaling pathway is an important signaling pathway in various biological processes. It is a signal transduction pathway that has one of the strongest associations with cell

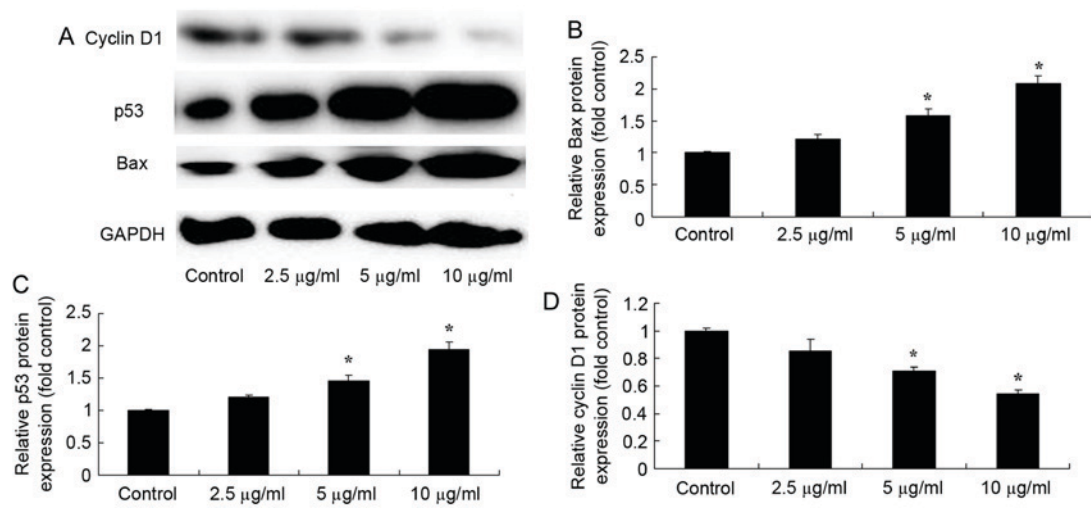


Figure 5. Dehydrocostus lactone promoted Bax and p53 protein expression, and suppressed cyclin D1 protein expression in HaCaT cells. (A) Representative western blot of Bax, p53 and cyclin D1 protein expression. Densitometric and statistical analysis of (B) Bax, (C) p53 and (D) cyclin D1 protein expression in recombinant human papilloma virus-18 HaCaT cells. DMSO was used as control treatment, and 2.5, 5 and 10 $\mu\text{g/ml}$ concentrations of dehydrocostus lactone were employed for 48 h. * $P < 0.01$ vs. DMSO control group. Bax, Bcl-2-associated X; DMSO, dimethyl sulfoxide.

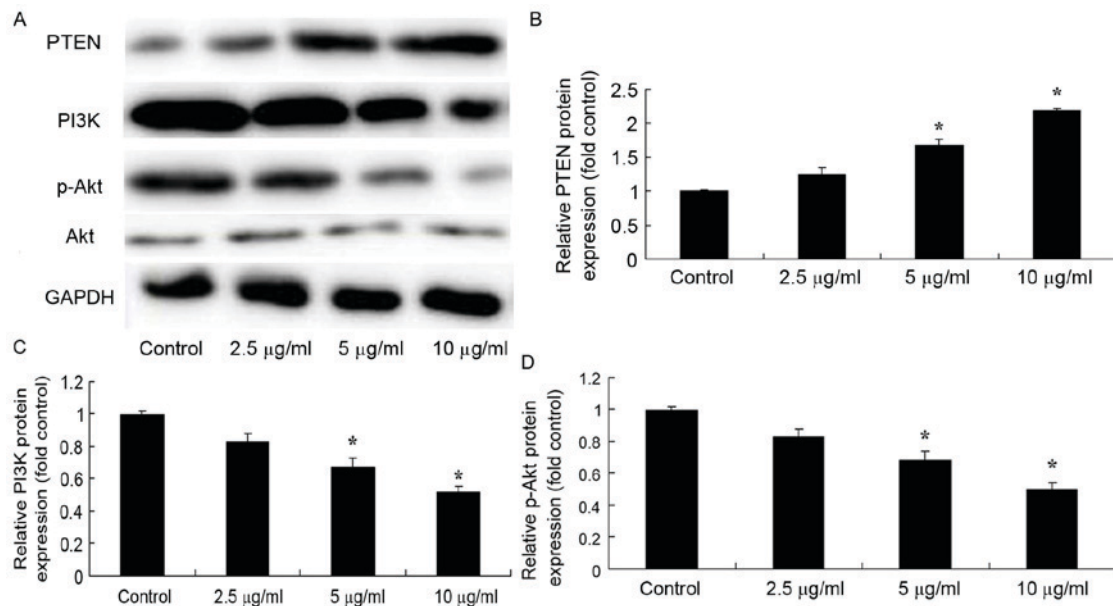


Figure 6. Dehydrocostus lactone upregulated PTEN protein expression and downregulated the PI3K/Akt signaling pathway in HaCaT cells. (A) Representative western blot of PTEN, PI3K, Akt and p-Akt protein levels. Densitometric and statistical analysis of (B) PTEN, (C) PI3K and (D) p-Akt protein levels in recombinant human papilloma virus-18 HaCaT cells. DMSO was used as control treatment, and 2.5, 5 and 10 $\mu\text{g/ml}$ concentrations of dehydrocostus lactone were employed for 48 h. * $P < 0.01$ vs. DMSO control group. PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol 3-kinase; p-, phosphorylated-; DMSO, dimethyl sulfoxide.

proliferation and apoptosis (23). A previous study demonstrated that the PI3K/Akt signaling pathway is abnormally activated in various tumor types (23). The activation of the signal transduction pathway inhibits cell apoptosis induced by various stimuli, and promotes cell cycle progression to promote cell survival and proliferation (25). In addition, this signaling pathway has important roles in angiopoiesis and tumor formation, and is implicated in the invasion and metastasis of tumors (26). The results of the present study demonstrated that dehydrocostus lactone significantly increased PTEN protein expression, and downregulated PI3K and p-AKT protein expression, in recombinant HPV-18 HaCaT cells. Furthermore, Jiang *et al* (27) demonstrated that

dehydrocostus lactone inhibited the proliferation and invasion of cervical cancer cells via the PI3K/Akt signaling pathway.

In conclusion, the results of the present study indicate that dehydrocostus lactone may suppress cell growth and induce apoptosis in recombinant HPV-18 HaCaT cells via the PI3K/Akt signaling pathway, and suggests that dehydrocostus lactone may exhibit anti-condyloma acuminatum effects. This novel target of dehydrocostus lactone may provide a potential prognostic marker and therapeutic target for CA patients. Future studies should investigate the effects of dehydrocostus lactone using animal models and investigate its effect on genital warts in a clinical setting.

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Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WL designed the study. YBM, YQM and TL performed the experiments. WL and YBM analysed the data. WL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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