The anticancer effect and mechanism of α-hederin on breast cancer cells

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Abstract. Natural plant products occupy a very important position in the area of cancer chemotherapy. Many triterpenoid saponins have been proved as potential agents for chemoprevention and therapy of breast cancer. α-hederin, a monodesmosidic triterpenoid saponin distributed in *Hedera* or *Nigella* species, displays many biological activities. It is increasingly investigated for its promising anticancer potential since it has been shown to have cytotoxicity against several types of cancer cells. However, studies of α-hederin on breast cancer are limited, most of which focus on biological activity, while the mechanisms have not been widely reported yet. Previously, we purified and identified α-hederin from *Clematis ganpiniana*, a herb used in traditional Chinese medicine with antitumor action. In the present study, α-hederin showed strong inhibitory activity on the growth of breast cancer cells and induced apoptosis in these cells. α-hederin induced depolarization of mitochondrial membrane potential which released Apaf-1 and cytochrome *c* from the intermembrane space into the cytosol, where they promoted caspase-3 and caspase-9 activation. This is the first report on the growth inhibition and pro-apoptotic effects of α-hederin on breast cancer cells and the relative apoptosis pathways. It implied that triterpenoid saponin α-hederin could be a promising candidate for chemotherapy of breast cancer.

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Introduction

Breast cancer is the most frequently diagnosed and the leading cause of cancer related death in women (1). A variety of drugs have been developed to treat breast cancer, however, drug resistance often occurs, and unexpected side-effects are common (2). Thus, novel chemotherapies that overcome drug resistance and improve patient outcomes are urgently needed. Using compounds from natural plants as potential cancer preventive and/or therapeutic agents has become a fascinating strategy (3,4). Identification and investigation of active components from natural plants are important for assessing their potential for clinical use. A large number of components purified from herbs have been used to treat various cancers including breast cancer. For example, paclitaxel (Taxol), a natural chemotherapeutic drug isolated from the bark of the pacific yew, is currently used widely for treating breast cancer (5). Therefore, development of new therapeutic agents from natural source has great promise for breast cancer treatment.

We have purified and identified four kinds of triterpenoids derivatives from *Clematis ganpiniana*. They showed cytotoxicity against breast cancer cells (6). One of them was α-hederin, which belonged to triterpenoid saponins. Triterpenoid saponins are an important class of natural products and distributed widely in the plant kingdom (7,8). Several excellent studies provided an overview of the triterpenoids as potential agents for chemoprevention and therapy of breast cancer (9,10).

α-hederin, a monodesmosidic triterpenoid saponin distributed in *Hedera* or *Nigella* species displays many biological activities such as anti-viral activity (11); anti-inflammatory activity (12); anti-oxidant activity (13); anti-leishmanial activity (14) and anti-spasmodic activity (15). Moreover, α-hederin is increasingly investigated for its promising anticancer potential since it revealed cytotoxicity against various cancer cell lines such as lung carcinoma, larynx epidermoid carcinoma, colon adenocarcinoma and pancreas carcinoma (16-20) and *in vivo* tumors (21-23). It has been suggested that α-hederin exerted its cytotoxic activity by promoting apoptosis and/or membrane alterations (24,25), however, the molecular and cellular mechanisms are far from being fully elucidated. Moreover, reports on

the anti-breast cancer acivity of α-hederin are scarce, most of which focus on biological activity, while the mechanisms have not been widely reported yet.

α-hederin have been previously reported to inhibit growth and induce apoptosis of breast cancer cells (6), however, further effects and mechanisms of α-hederin on breast cancer is currently unavailable. In this study, we evaluated effects of α-hederin on growth and apoptosis of various human breast cancer cell lines, and explored the underlying mechanisms.

Materials and methods

Drug preparations. Protocols of the collection, storage, extraction of the plant material of *Clematis ganpiniana*, the methods of the purification and analysis of the α-hederin were described in a previous study (6).

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA, USA) and incubated in a humidified atmosphere of 5% CO_2 in air at 37˚C and fed with the culture medium of high glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution. For routine passages, cultures were split 1:3 when they reached 80-90% confluence generally every 2-3 days. All experiments were performed on exponentially growing cells. MCF-7 and MDA-MB-231 cells are widely used in studies on human breast cancer. In this study, we used these two cell lines to evaluate the growth inhibition and explore the underlying molecular mechanisms of α-hederin.

MTT assay. The MTT assay was used to measure the inhibition of growth by α-hederin in breast cancer cell lines. Briefly, $5x10^3$ cells were seeded into a 96-well plate in triplicate and 8 h later α-hederin was added into the wells at the indicated final concentrations (0.08, 0.4, 2 and 10 μg/ml), while cells cultured in medium with 0.05% DMSO as a negative control. After incubation with α-hederin for 12, 24 and 48 h, the medium in each well was replaced with 20 μl of MTT at 5 mg/ml final concentration, and 4 h later 150 μl DMSO/well was added to dissolve the formed violet formazan crystals within metabolically viable cells. The plates were incubated at room temperature for 15 min and then read at 490 nm with a microplate reader (Tecan, Grödig, Austria). The percentage of growth inhibition was calculated as (OD of the control - OD of the experiment samples)/OD of the control x 100.

Apoptosis analysis by flow cytometry. After exposure to 2 μg/ml α-hederin for 6, 12 and 24 h, breast cancer cells were washed twice with PBS at 4˚C, resuspended in stain containing Annexin V-FITC and propidium iodide (PI) for 15 min incubation on ice, and analyzed with FACSAria flow cytometer (Becton-Dickinson, San Jose, CA, USA) using FACSDiva software. Approximately 10^5 cells were analyzed for each treatment.

Measurement of the mitochondrial membrane potential (ΔΨm) with JC-1. The mitochondrial membrane potential was measured according to the manufacturer's instruction with JC-1. After exposure to 2 μg/ml α-hederin for 6, 12 and 24 h, cells were washed twice with PBS, incubated in the working solution of 2 μg/ml JC-1 for 30 min at 37˚C in 5% CO_2 atmosphere, and observed with Zeiss LSM 5 Live confocal microscope (Carl Zeiss, Jena, Germany). The fluorescence was measured at an excitation:emission of 485/538 for green monomers and at an excitation:emission of 485/590 for red aggregates. Valinomycin was used at a concentration of 0.1 μM as a positive control for depolarization of the ΔΨm.

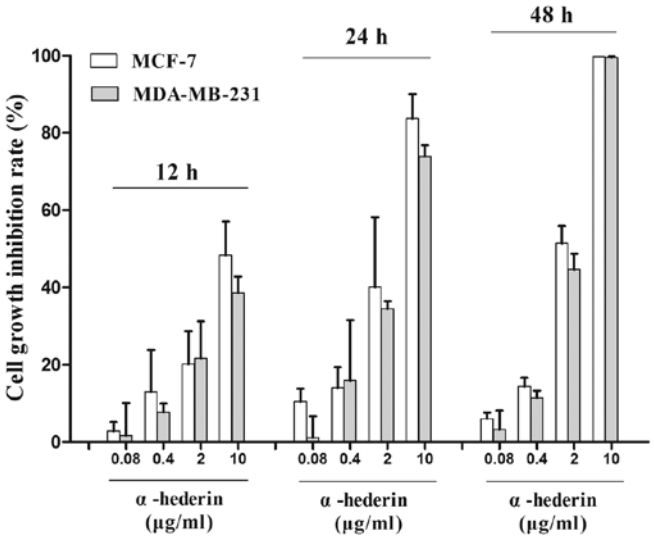

Figure 1. α-hederin inhibits growth of breast cancer cells. MTT assay of MCF-7 and MDA-MB-231 cells treated with various concentrations of α-hederin for 12, 24 and 48 h. Data are mean ± standard error of mean (SEM) of three independent experiments.

Measurement of cellular caspase-3 and -9 activity. Caspase-3 and -9 activity was quantified by measuring cleavage of the colorimetric peptides RED-DEVD-FMK and RED-LEHD-FMK, respectively (BioVision, Palo Alto, CA, USA). Briefly, at the end of designated treatment (24 h of exposure to 2 μg/ml α-hederin), equal number of control or treated cells were incubated with RED-DEVD-FMK and RED-LEHD-FMK, respectively (2 μg/ml) for 20 min at 37˚C in 5% CO_2 atmosphere, then washed twice by PBS and analyzed with FACSAria flow cytometer. For the caspase inhibition study, the cells were pre-incubated with inhibitors: z-DEVD-FMK and z-LEHD-FMK (respectively, caspase-3 and -9 inhibitors) 1 h before α-hederin treatment.

Western blot analysis. MCF-7 and MDA-MB-231 cells were seeded at $1x10^6$ cells in 100-mm^2 dishes. Cells were treated in complete medium with α-hederin for 6, 12 and 24 h. After treatment, adherent cells were gently scraped from the plates into the medium containing floating cells to obtain all the cells. Cells were then centrifuged, washed in PBS, lysed in ice-cold lysis buffer containing phosphatase inhibitor cocktail and protease inhibitor cocktail (Boehringer, Mannheim, Germany) to obtain total protein. Protein concentrations were determined using the Bradford method.

Apaf-1 and cytochrome *c* in mitochondrial fraction were analyzed by isolation of mitochondrial protein using the Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology,

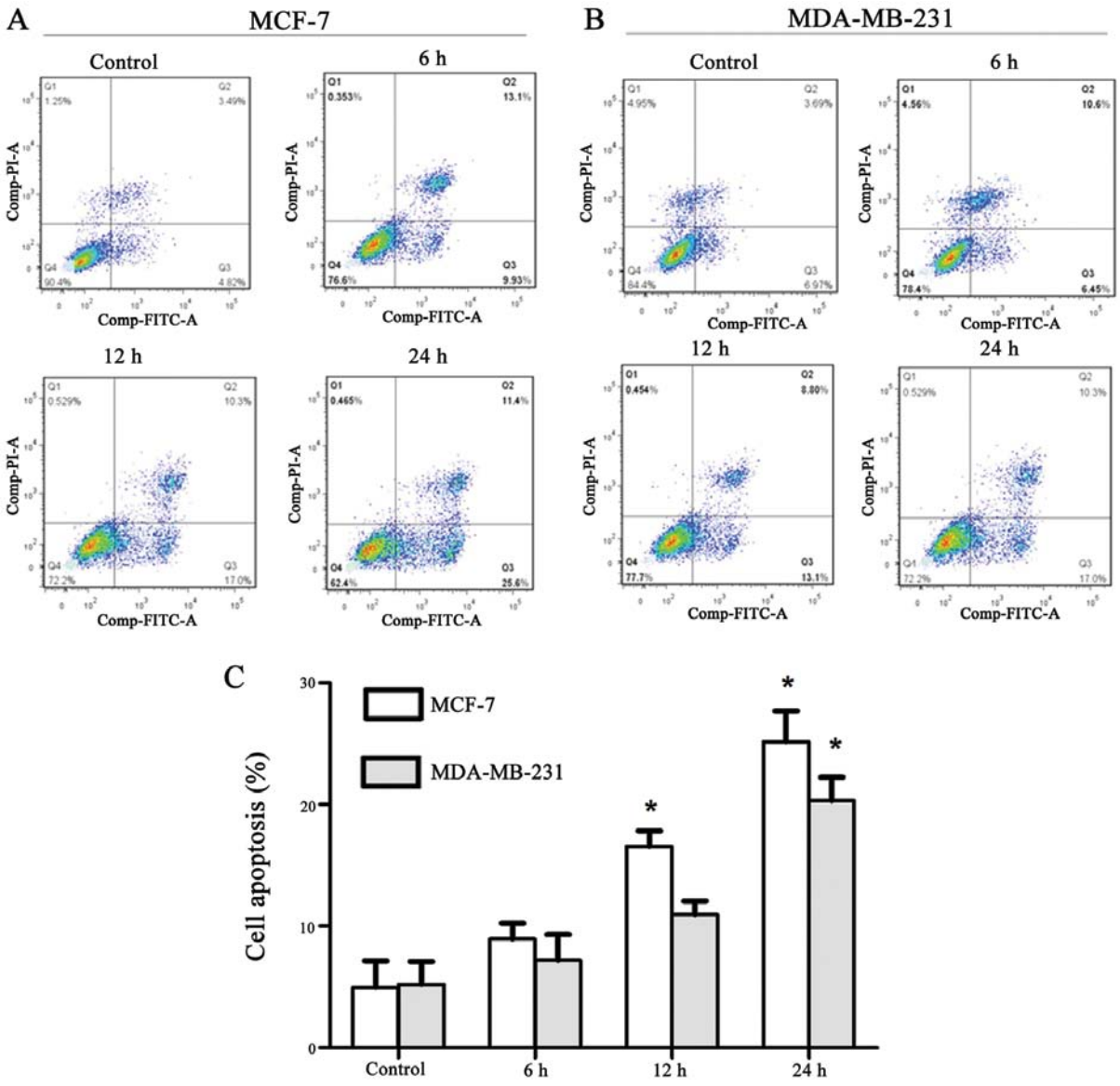

Figure 2. α-hederin induced apoptosis in breast cancer cells. (A and B) The apoptosis rate of MCF-7 and MDA-MB-231 cells measured by flow cytometry. α-hederin induced early apoptosis in MCF-7 and MDA-MB-231 cells. (C) Early apoptosis rate of MCF-7 and MDA-MB-231 cells treated with α-hederin of three independent experiments are shown in column statistics. Data are mean ± SEM of three independent experiments. *p<0.05 vs. α-hederin-untreated group.

Beijing, China). Briefly, after exposure, MCF-7 and MDA-MB-231 cells were harvested and centrifuged at 800 x g at 4˚C for 10 min. The pellets were added with 20 mM N-2-hydroxyethylpiperazine-N0-20-ethanesulfonic acid (HEPES) buffer containing protease inhibitor cocktail and disrupted with a glass tissue grinder. Homogenates were centrifuged at 800 x g at 4˚C for 10 min, and the resulting supernatants were transferred to 0.5 ml conical tubes, and further centrifuged at 10,000 x g at 4˚C for 20 min. The final pellets, containing the mitochondrial fraction, were analyzed for protein content using the Bradford method.

Cell lysates were electrophoresed through 10-12% SDS-PAGE gel, and transferred to PVDF membranes, which were activated in methanol. The blots were probed or reprobed with antibodies. GAPDH was used to normalize for protein loading. The membranes were probed using ECL and autoradiographed. The intensity of the bands was determined using densitometric analysis. The primary antibodies used were purified mouse anti-human apoptotic protease activating factor-1 (Apaf-1) and cytochrome *c*, purchased from BD Bioscience. β-actin was from Sigma. Anti-mouse secondary antibodies were from Cell Signaling Technology. The antibodies were diluted according to the manufacturer's instructions.

Statistical analysis. The data were analyzed using the SPSS 20.0 software. For all the measurements, oneway ANOVA followed by Bonferroni test was used to assess the statistical significance of difference between control and groups-treated. A statistically significant difference was considered at the level of $p<0.05$.

Results

α-hederin inhibits the growth of breast cancer cells. In this study, two breast cancer cell lines MCF-7, MDA-MB-231, were

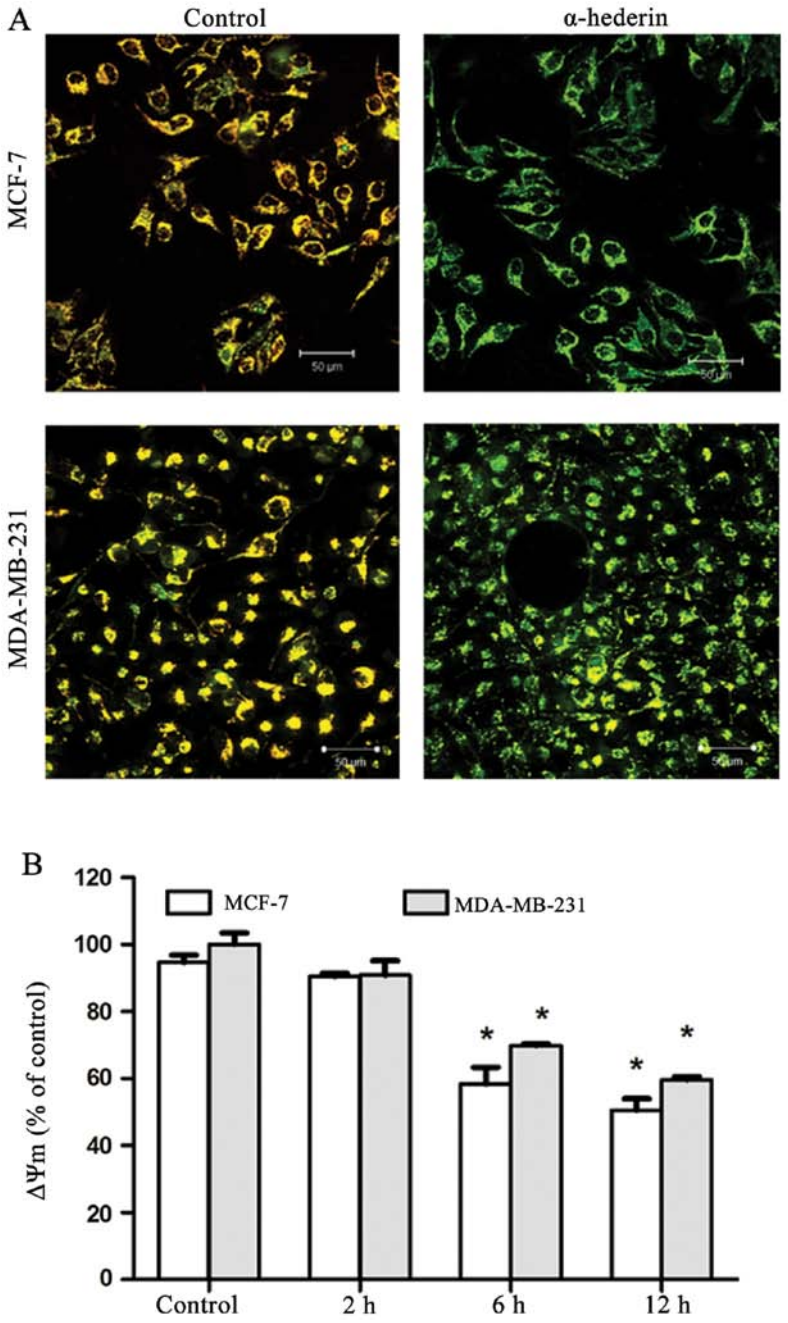

Figure 3. α-hederin reduced the mitochondrial membrane potential of MCF-7 and MDA-MB-231 cells. (A) Effects of α-hederin on the mitochondrial depolarization in MCF-7 and MDA-MB-231 cells. After the application of DRβ-H, JC-1 fluorescence shifted from red-orange to greenish yellow, which indicated the depolarization of mitochondrial membrane potential. (B) Quantification of mitochondrial membrane potential ΔΨm (% of control) was expressed as a ratio of J-aggregate to JC-1 monomer (red: green) fluorescence intensity. ΔΨm (% of control) of cells treated with α-hederin for 2, 6 and 12 h of three independent experiments are shown in column statistics. $^{*}p<0.05$ vs. α-hederin-untreated group.

used. The inhibitory rate of growth was determined by MTT assay. α-hederin showed inhibition in the two breast cancer cell lines which were statistically significant compared to the negative control ($p<0.05$) (Fig. 1).

α-hederin induces apoptosis in breast cancer cells. The apoptosis rate was measured by flow cytometry. MCF-7 and MDA-MB-231 treated with 2 μg/ml α-hederin for indicated times (6, 12 and 24 h) were first double-stained with Annexin V and PI, and then analyzed by flow cytometry. In cells treated with α-hederin, we detected a major increase in the Annexin V^+/PI^- fraction (regarded as early apoptotic) subpopulations. After incubated with 2 μg/ml α-hederin for 24 h, early apoptosis rate of MCF-7 and MDA-MB-231 cells were significantly increased up to 25.6 and 17.0%, respectively (Fig. 2A and B). Early apoptosis rate of cells treated with α-hederin of three independent experiments are shown in column statistics (Fig. 2C).

α-hederin affects the mitochondrial membrane potential (ΔΨm) of breast cancer cells. MCF-7 and MDA-MB-231 cells

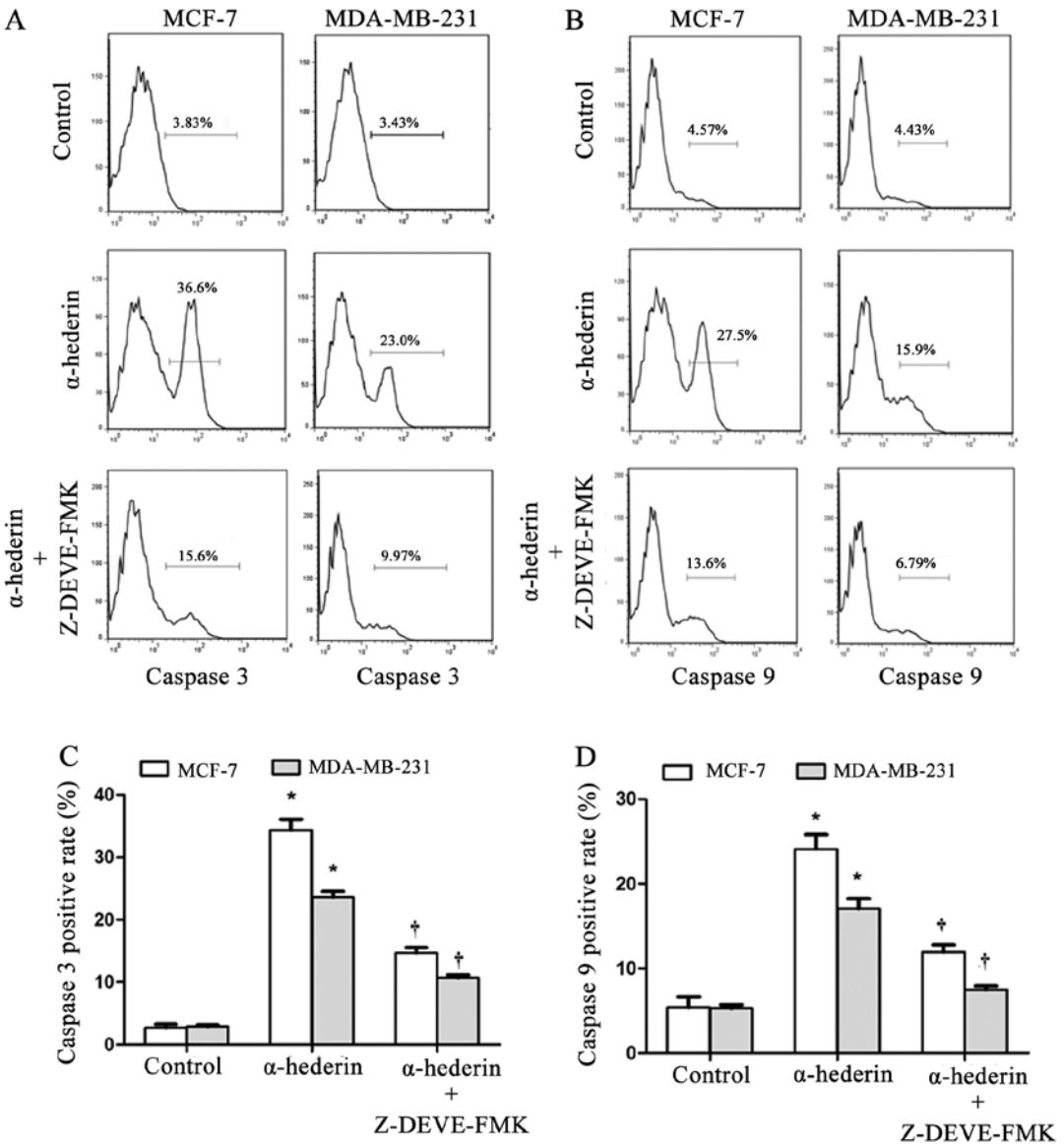

Figure 4. α-hederin increases the activity of caspase-3 and caspase-9 of MCF-7 and MDA-MB-231 cells. (A and B) Effect of α-hederin on caspase-3 and caspase-9 activation. α-hederin increased the activity of caspase-3 and caspase-9 in both MCF-7 and MDA-MB-231 cells. This activation could be reversed by the caspase inhibitors respectively. (C and D) Caspase-3, and caspase-9 positive rate of cells treated with α-hederin with/without caspase inhibitors of three independent experiments are shown in column statistics. *p<0.05 vs. α-hederin-untreated group. †p<0.05 vs. α-hederin -treated alone group.

were treated with 2 μg/ml α-hederin for 6, 12 and 24 h, and then mitochondrial membrane potential was measured. After the application of α-hederin, JC-1 fluorescence shifted from red-orange to greenish yellow, which indicated the depolarization of mitochondrial membrane potential (Fig. 3A). Mitochondrial membrane potential ΔΨm of cells treated with α-hederin of three independent experiments are shown in column statistics (Fig. 3B).

α-hederin regulates caspase-3 and caspase-9 activation. After exposure to α-hederin (2 μg/ml) for 24 h, activity of caspase-3 and caspase-9 was increased in both MCF-7 and MDA-MB-231 cells. This activation could be reversed by the caspase inhibitors (Fig. 4A and B). Caspase-3, and caspase-9 positive rate of cells treated with α-hederin with/without caspase inhibitors of three independent experiments are shown in column statistics (Fig. 4C and D).

α-hederin regulates the Apaf-1 and cytochrome c release. MCF-7 and MDA-MB-231 cells were treated for 2, 6, 12 or 24 h with α-hederin (2 μg/ml) and both mitochondrial Apaf-1 and cytochrome *c* level were detected by western blot analysis. DRβ-H decreased both mitochondrial Apaf-1 and cytochrome *c* expressions in a time-dependent manner (Fig. 5A). Expressions of mitochondrial Apaf-1 and cytochrome *c* of cells treated with α-hederin of three independent experiments are shown in column statistics (Fig. 5B and C).

Discussion

Our data indicate that α-hederin from *Clematis ganpiniana* had strong inhibitory activity on different breast cancer cells. α-hederin was found to induce apoptosis in both the ER^+ human breast cancer cell line MCF-7 and ER^- breast

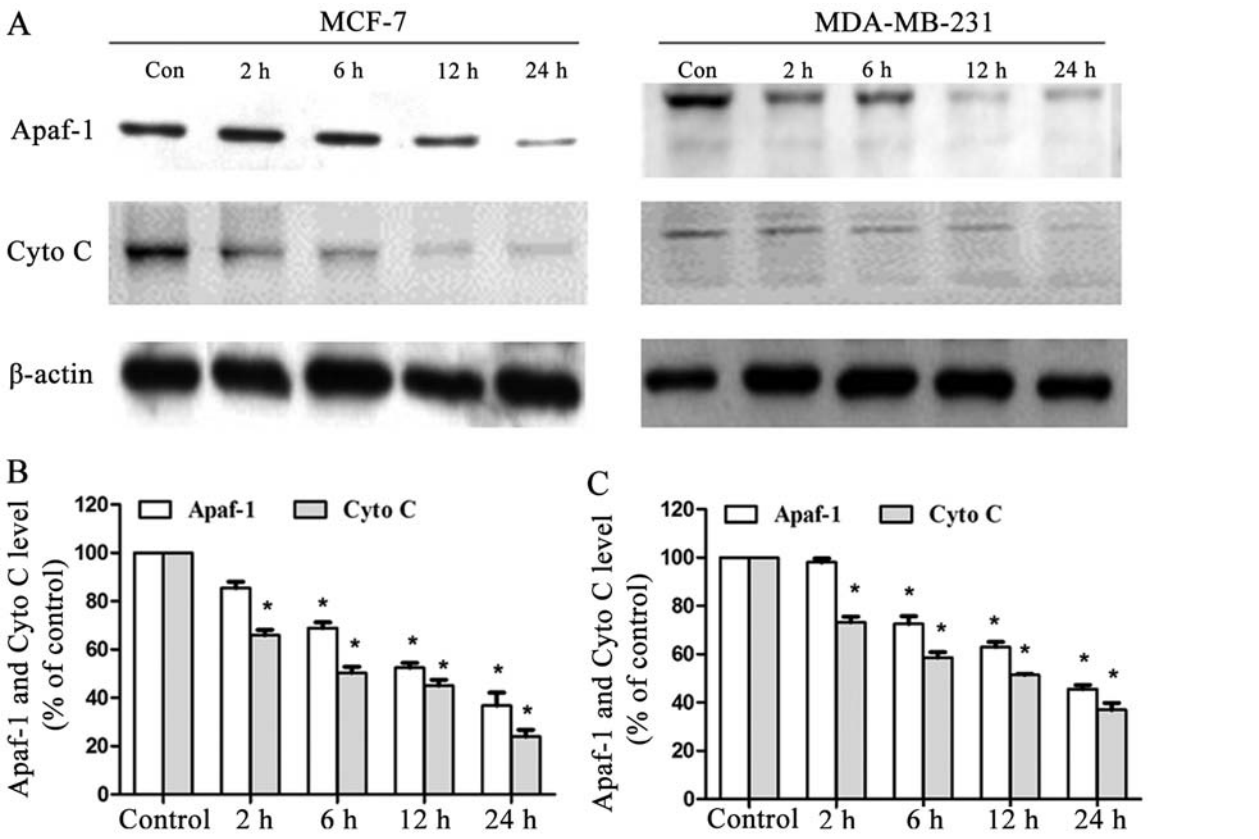

Figure 5. α-hederin decreased mitochondrial Apaf-1 and cytochrome *c* expressions of MCF-7 and MDA-MB-231 cells. (A) Effect of α-hederin on Apaf-1 and cytochrome *c* release. Cyto C, cytochrome *c*. α-hederin decreased the expression of both mitochondrial Apaf-1 and cytochrome *c* in a time-dependent manner. (B and C) Expression of mitochondrial Apaf-1 and Cytochrome C of cells treated with α-hederin of three independent experiments are shown in column statistics. *$p<0.05$ vs. α-hederin-untreated group.

cancer cell line MDA-MB-231. Disruption of mitochondrial membrane potential, release of Apaf-1 and cytochrome *c*, and subsequent activation of caspase-9 and caspase-3 was detected in α-hederin-treated cells.

The abstract of *Clematis ganpiniana* was traditionally used as a diuretic agent and an anti-inflammatory remedy by the Naxi people in China. α-hederin extracted from *Clematis ganpiniana* showed cytotoxicity on breast cancer cells. Importantly, α-hederin was found to induce apoptosis in various breast cancer cells. Apoptosis is required for proper tissue homeostasis. Defects in apoptosis signaling pathways contribute to carcinogenesis and chemoresistance. Most cancer therapeutic approaches inhibit tumors by triggering cancer cell apoptosis (26).

JC-1 staining was used to detect the membrane potential of mitochondria. The membrane potential of mitochondria in breast cancer cells was greatly reduced by α-hederin. Apaf-1 and cytochrome *c* were released from the mitochondria to the cytoplasm. In α-hederin-induced apoptosis, caspase-3 and caspase-9 were involved. The activation of caspase family members is a critical component of the apoptotic machinery. The caspases generally consist of the upstream initiator caspases, such as caspase-2, -8, -9 and -10, and the downstream effect of caspases, such as caspase-3, -6 and -7 (27). The results suggested that the caspase-dependent pathway mediated α-hederin-induced apoptosis in breast cancer cells through the mitochondrial pathway.

Mitochondria play a central role in cancer survival and are one of the main targets for developing anticancer drugs (28). Both the extrinsic and the intrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization (29). Mitochondrial apoptotic pathway was reported widely for the actions of triterpenoid saponins in other human cancers including liver cancer (30-32), gastric cancer (33), esophageal cancer (34), and colorectal cancer (35). It was reported that α-hederin from *Nigella sativa* induced apoptosis via mitochondrial perturbations in murine leukemia P388 cells (24). We first reported mitochondrial apoptotic activity of α-hederin in breast cancer cells.

In conclusion, we showed α-hederin effectively inhibited the growth and induced apoptosis of breast cancer cells. α-hederin reduced the mitochondrial membrane potential and decreased mitochondrial Apaf-1 and cytochrome *c* expressions of breast cancer cells. Moreover, α-hederin increased the activity of caspase-3 and caspase-9 remarkably in breast cancer cells. Consistent with these results, α-hederin induced mitochondria-mediated apoptosis of MCF-7 and MDA-MB-231 cells. This is the first report on both chemotherapeutic effects and the mechanism of α-hederin on human breast cancer cells, which may provide a potential option for the drug development and treatment of breast cancer. Oriental medicinal herbs are rich sources of potential cancer chemopreventive and therapeutic agents. Rigorous and systematic pre-clinical evaluations *in vitro* was exemplified in the

current study to transform traditional herbal practices into evidence-based medicine.

Acknowledgements

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