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The anti-tumor effect and bioactive phytochemicals of *Hedyotis diffusa* willd on ovarian cancer cells

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ABSTRACT

Ethnopharmacological relevance: *Hedyotis diffusa* willd (HDW) is a widely used medicinal herb in China. It processed various medicinal properties including antioxidative, anti-inflamatory and anti-cancer effects. This study aimed to investigate the anti-tumor effects of HDW on ovarian cancer cells and the underlying mechanisms as well as identify the bioactive compounds.

Materials and methods: Effects of HDW on the viability of ovarian cancer A2780 cells were detected by MTT assay. Apoptosis was detected by cell morphologic observation through DAPI staining and flow cytometry analysis. The migration of ovarian cancer cells which exposed to HDW were detected by wound healing and transwell assays. The protein levels of caspase 3/9, Bcl-2 and MMP-2/9 in human ovarian cancer cells treated with HDW were assessed by western blotting analysis. The potential bioactive compounds were characterized by HPLC-Q-TOF-MS.

Results: HDW significantly inhibited the growth of A2780 ovarian cancer cells and induced apoptosis. The induction of apoptosis by HDW was associated with down-regulation of anti-apoptotic protein Bcl-2 and the activation of caspase 3/9. Wound healing and transwell chamber assays indicated HDW suppressed the migration of ovarian cancer cells. HDW dramatically decreased MMP-2/9 expression. A HPLC-Q-TOF-MS analysis of HDW indicated the presence of 13 flavonoids compounds and one anthraquinone compound, which may contribute to the anticancer activity of the HDW.

Conclusions: HDW effectively restricted the growth of ovarian cancer cells and induced apoptosis through the mitochondria-associated apoptotic pathway. Furthermore, HDW suppressed the migration of ovarian cancer cells through down-regulation of MMP-2 and MMP-9 expression. These results showed that HDW hold potential therapeutic effect for ovarian cancer patients.

1. Introduction

Ovarian malignant tumor is one of the most common gynecological malignancies (Khandakar et al., 2015; Teixeira et al., 2015). Due to lack of effective screening strategies, most of ovarian cancer patients have been diagnosed at late stage and the 5-year survival rate is only 20–30% (Chien et al., 2015; Khandakar et al., 2015; Zhao et al., 2015). Over the past few years, the first-line clinical treatments for ovarian cancer patients are cytoreductive surgery and combined chemotherapy (Khandakar et al., 2015; Srivastava et al., 2015). However, the re-emergence of ovarian cancer because of drug resistance results in a poor overall survival rate (Khandakar et al., 2015). Therefore, development of novel effective and less toxic drugs is urgent for ovarian cancer patients.

Many species of herbal medicines show an important role in the development of new medicine (Fang et al., 2012; Wang et al., 2012). *Hedyotis diffusa* willd (HDW) is a traditional Chinese herbal medicine and widely distributed throughout Northeast Asia (Cai et al., 2012). It has been reported to possess anti-cancer, anti-oxidative, anti-inflamatory and neuroprotective effects (Li et al., 2015; Lin et al., 2015; Ye et al., 2015). Combination with *Scutellaria barbata*, HDW is used to treat various types of cancer in China for many years (Yeh et al., 2014). Previous studies showed that HDW extracts inhibited the growth of colon cancer and colorectal cancer via induction of cancer cell apoptosis and the inhibition of tumor angiogenesis (Cai et al., 2012; Li et al., 2015; Lin et al., 2010; J. Lin et al., 2011). However, the effects of HDW on ovarian cancer and the underlying molecular mechanisms are still unclear.

In this study, we explored whether HDW extracts could exert

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anti-tumor effects on ovarian cancer cells and further investigate the detailed mechanisms underlying this process. Moreover, we analyzed their contents by using HPLC-Q-TOF-MS to identify the principal bioactive phytochemicals. The research findings may provide a basis for development of HDW as an effective treatment for ovarian cancer patients.

2. Materials and methods

2.1. Regents and materials

Dulbecco's modified Eagle's medium (DMEM), double-antibody and pancreatic enzyme were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from TransGen company (Beijing, China). Transwells were purchased from BD Biosciences (San Jose, USA). DMSO, crystal violet and methanol were from Romeo reagent company (Tianjin, China). Antibodies against caspase-3, caspase-9, Bcl-2, MMP-2, MMP-9 and β-actin were obtained from Ptoteintech Group (Chicago, Illinois, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science, Inc. (Chicago, Illinois, USA). The Annexin V-conjugated FITC apoptosis detection kit was purchased from NanJing KeyGen Biotech Co., Ltd (Nanjing, Jiangsu province, China). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) and DAPI (2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride) were obtained from Sigma Chemical Co. (St. Louis, MO). Actonitrile and methanol (HPLC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water for HPLC analysis was purified by a Milli-Q system (Millipore, Milford, MA, USA). The dry grass of *H. diffusa* was obtained from Dalian Metro pharmaceutical (Dalian, Liaoning province, China), and authenticated by prof. Lin Zhang (Dalian Medical University). Voucher specimens were deposited at the laboratory of authors. All the standards were purchased from Chengdu Pufeide Biological Technology Co., Ltd. (Sichuan, China). The purity of standard compounds was higher than 97%, which was confirmed by UHPLC-DAD analysis.

2.2. Preparation of H. diffusa Willd extract

The drying herb of *H. diffusa* (20 g) was extracted 2 times in a reflux extraction device with 300 ml alcohol each time. After the extracting solutions were mixed and filtered, a third volume of water on the rotary evaporation apparatus was added, and then concentrated in the 60 °C water bath until no alcohol taste. The frozen concentrate solution from −80 °C refrigerator (Thermo Company) was put in freeze drier (Beijing, China) to carry on the freeze drying. Finally the crude extracts of the herb (1.68 g) was obtained. Accurately weighed extraction powder (0.1 g) was transferred into a 100 ml Teflon-lined extraction vessel and methanol was added to the scale. The solution was filtered through 0.22 μm filter before sample injection. The filtrate was injected to HPLC-Q-TOF-MS for analysis.

2.3. Cell culture

The ovarian cancer A2780 cell line was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco modified Eagle medium supplemented with 10% FBS at 37 °C in 5% CO_2. Cells were digested with 0.25% trypsin and treated with different concentrations of HDW with appropriate corresponding controls.

2.4. Chromatography and Q-TOF-MS conditions

LC–MS analysis was performed on a Shimadzu HPLC 20ADXR LC system in-line with an AB-Sciex 5600 Triple TOF mass spectrometer. The autosampler temperature was set at 4 °C, and the injection volume was set at 5 μl. LC was performed at 40 °C using a Kromasil 100-5-C18 (5 μm, 4.6 mm × 150 mm) and a gradient system with the mobile phase consisting of solvent A (water contained 0.1% formic acid) and solvent B (acetonitrile contained 0.1% formic acid) at a flow rate of 450 μl/min. The following gradient program was used: linear gradient from 35% B to 70% in 0–15 min, 95% B for 18–20 min, return to initial conditions in 21 min, and equilibrate for 4 min before the next sample injection. MS experiments were performed using an AB-Sciex 5600 Triple TOF mass spectrometer in positive and negative product ion mode with a source temperature of 550 °C. For our instrument, the declustering potential was set to 65 and −65 for positive and negative respectively, and the collision energy was set to 25 and −30 for positive and negative respectively.

2.5. MTT assay

Cell viability was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltet-razolium bromide (MTT) colorimetric assay. The logarithmic phase of A2780 and IOSE80 cells were seeded into 96-well plates in the density of 1×10^4/well and incubated overnight. Then 200 μl culture medium containing different concentrations (0, 50, 100, 200, 300, 400, 600, 800 μg/ml) of HDW extracts were added to each well. After 24 h HDW treatment, the MTT solution (5 mg/ml) was added to each well and incubated for 4 h. Finally, the MTT solution was removed and replaced by 150 μl DMSO each well to dissolve the formazan crystals and mixed for 10 min. Absorbance of the solution was determined by a Multiskan Ascent plate reader at 540 nm wavelength. The experiment was repeated three times with four replicates in each repeat.

2.6. DAPI staining assay

DAPI staining was used to assess nuclei morphology of cells. The logarithmic phase of A2780 cells were seeded into 6-well plates in the density of 4×10^4/well and cultured overnight. After treatment with different concentrations (0, 200, 300, 400 μg/ml) of HDW extracts for 24 h, the cells were stained with DAPI after fixing with 3.7% formaldehyde. The samples were then washed with PBS and detected by fluorescence microscopy.

2.7. Flow cytometry assay

Annexin V-FITC and propidium iodide (PI) staining was used to detect apoptotic cells. A2780 cells were treated with different concentrations (0, 200, 300, 400 μg/ml) of HDW extracts for 24 h. The cells were collected and washed with PBS three times. Then the samples were stained with Annexin V-FITC and PI for 5 min in the dark. Finally, early or late apoptosis were detected by a FACS/Calibur flow cytometer (Becton Dickin-son, Franklin Lakes, NJ, USA).

2.8. Wound healing assay

The migration ability of A2780 cells was assessed by wound healing assay. A2780 cells were seeded into 6-well plates with the density of 5×10^4/ml and 500ul each well. Upon 70–80% of the well was covered, the cell monolayer was scratched using a 200 μl sterile pipette tip. Cells were washed twice with PBS to remove detached cells and then treated with different concentrations (0, 50, 100 μg/ml) of HDW extracts for 24 h. Three randomly fields