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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.

[1] These authors contributed equally to this work.

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.

Keywords: *Hedyotis diffusa* willd; ovarian cancer; apoptosis; migration; bioactive phytochemicals

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, antioxidative, 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; 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 anti-tumor effects on ovarian cancer cells and further investigate the detailed mechanisms underlying this process. Moreover, we analyzed their content 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 *hedyotis 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 hedyotis diffusa Willd *extract*

The drying herb of *hedyotis diffusa* (20g) was extracted 2 times in a reflux extraction device with 300ml 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 (1.68g) was obtained. Accurately weighed extraction powder (0.1g) was transferred into a 100 mL Teflon-lined extraction vessel and methanol was added to the scale. The solution was filtered through 0.22μm 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 (5μm, 4.6mm×150mm) and a gradientsystem 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 wasused: linear gradient from 35% B to 70% in 0-15 min, 95% B for 18-20 min, return toinitial 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 negativeproduct 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 200μl culture medium containing different concentrations (0, 50, 100, 200, 300, 400, 600, 800 μg/ml) of HDW extracts were added to each well. After 24h HDW treatment, the MTT solution (5mg/ml) was added to each well and incubated for 4 h. Finally, the MTT solution was removed and replaced by 150μl DMSO each well to dissolve the formazan crystals and mixed for 10min. 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 24h, 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 24h. The cells were collected and washed with PBS three times. Then the samples were stained with Annexin V-FITC and PI for 5min 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 200μl 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 24h. Three randomly fields along the scraped line in each well were photographed at 0h and 24h. The average scraped width per well was measured and subtracted from the measurement prior to treatment. The distance of cells migration for each experimental condition was compared with that of the control group and quantified with computer-assisted microscope at 100× magnification.

2.9. Transwell chamber assay

Cell migration was also detected by the transwell chamber assay. 1×10^5 A2780 cells were seeded to the upper chamber with serum-free DMEM medium containing different concentrations (0, 50, 100 μg/ml) of HDW extracts and 500μl complete DMEM medium containing relative concentrations of HDW extracts was added the lower chamber as a chemoattractant. After 24h incubation, the cells on the upper surface of membrane were removed with cotton swabs and the cells on the underside of membrane were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet for 20min at room temperature. The migrated cells of random six fields of each Transwell membrane were photographed and counted under a computer-assisted microscope at 100× magnification field.

2.10. Western blot analysis

The total protein was extracted after 48h incubation with indicated concentrations of drug. The total cell lysates were separated by 10% SDS-PAGE and then transferred to nitrocellulose membrane by semi-dry apparatus for 35 min. The membranes was blocked with 5% non-fat milk for 1.5h and then incubated with primary antibodies against caspase-3/9, β-actin, Bcl-2 as well as MMP-2/9 respectively overnight at 4°C. The next day, the membranes were incubated with secondary antibody for 1h after washed with TBST for three times. Then, the protein bands were visualized by ECL kit after TBST washing as previously described.

2.11. Data analysis

All experiences were performed in triplicate. Data were presented as mean ±SD,

and were analyzed by one-way ANOVA test using the statistical software package SPSS 15.0. The value of $p<0.05$ was accepted statistically significant.

3. Results

3.1.Phytochemical screening

From the chromatogram obtained by HPLC-Q-TOF-MS (in positive and negative modes) (Fig. 1), 14 compounds were identified based on retention times, high-resolution mass spectrum data (MS and MS/MS), and comparisons with authentic samples and published data (Table. 1), in which quercetin-3-O-sambubioside was identified based on the fragment ions at m/z 595.1357 [M–H] and 597.1491 [M+H], similarly identified Quercetin at m/z 303.0483 [M+H] and Quercetin glucosideat m/z 465.1001 [M+H]. The presence of a sambubioside unit was indicated by the loss of 294 Da from the base peak at m/z 597.1491 in Fig. S1. Mass spectra data of the fragment ions was compatible with previous literature (Li et al., 2015). Likewise, the mass spectrum of other compounds (Fig. S2-S14) were shown.

3.2.The effect of HDW on the viability of ovarian cancer

The effects of HDW on the viability of ovarian cancer and normal ovarian cells were detected by MTT assay and cell morphology microscopy. Ovarian cancer A2780 cells and normal ovarian IOSE80 cells were treated with various concentrations of

HDW extracts for 24h. Ovarian cancer cells following HDW extracts treatment demonstrated a significant decrease in cell viability in a dose and time dependent manner ($p<0.05$), while same concentrations did not significantly affect the viability of normal ovarian IOSE80 cells (Fig. 2A). In addition, ovarian cancer cells treated with HDW extracts start to shrink, became round and dose-dependently detached from the flasks compared to non-treated cells. In contrast, we did not observe such morphologic changes in normal ovarian cells following HDW treatment (Fig. 2B). These data all suggested that HDW extracts inhibit viability of ovarian cancer cells.

No.	Rt (min)	Compound	Product ions
1	2.64	quercetin-3-O-sambubioside	595.1357 $[M-H]^-$, MS/MS: 549.1630, 179.0565, 163.0408; 597.1491 $[M+H]^+$, MS/MS: 465.1001, 303.0483, 175.0391
2	2.76	Quercetin 3-O-[2″-O-(6‴-O-feruloyl)-β-D-glucopyranosyl]-β-D-glucopyranoside	803.2010 $[M+H]^+$, MS/MS: 339.1068, 303.0489, 177.0537
3	3.79	Kaempferol 3-O-(2-O-β-D-glucopyranosyl)-β-D-galactopyranoside	609.1482 $[M-H]^-$, MS/MS: 300.0285; 611.1586 $[M+H]^+$, MS/MS: 465.1020, 303.0489, 287.0538
4	3.79	luteilin-7-O-β-D-glucopyranside	447.0917 $[M-H]^-$, MS/MS: 285.0394, 174.9553, 130.9659
5	3.79	Quercretin-3-O-β-D-glucopyranside	463.0858 $[M-H]^-$, MS/MS: 300.0258, 271.0226, 255.0271
6	3.81	rutin	609.1447 $[M-H]^-$, MS/MS: 300.0267, 151.0027;
7	5.75	scutellarein	285.0384 $[M-H]^-$, MS/MS: 163.8851, 137.0229, 117.0337
8	7.02	quercetin	301.0332 $[M-H]^-$, MS/MS: 178.9968, 151.0024, 121.0285, 107.0134; 303.0506 $[M+H]^+$, MS/MS: 229.0494, 153.0179, 137.0234
9	8.59	apigenin	269.0432$[M-H]^-$, MS/MS: 225.0534, 151.0024, 117.0338, 107.0131;
10	8.91	naringenin	271.0586 $[M-H]^-$, MS/MS: 177.0177, 151.0025, 119.0496, 107.0135, 93.0347
11	8.98	Kaemperol	285.0412 $[M-H]^-$, MS/MS: 239.0372, 185.0609; 287.0533 $[M+H]^+$, MS/MS: 241.0500, 153.0178
12	13.77	wogonin	283.0589 $[M-H]^-$, MS/MS: 268.0357, 163.0020, 110.0002;
13	13.82	chrysin	253.0477 $[M-H]^-$, MS/MS: 143.0487, 119.0493, 110.0392, 63.0261
14	14.78	2-hydroxy-6-methylanthrauinone	237.0556 $[M-H]^-$, MS/MS: 209.0607; 239.0695 $[M+H]^+$, MS/MS: 211.0734, 183.0795

Table 1 Chromatographic MS and MS/MS data of compounds identified in the extract of HDW

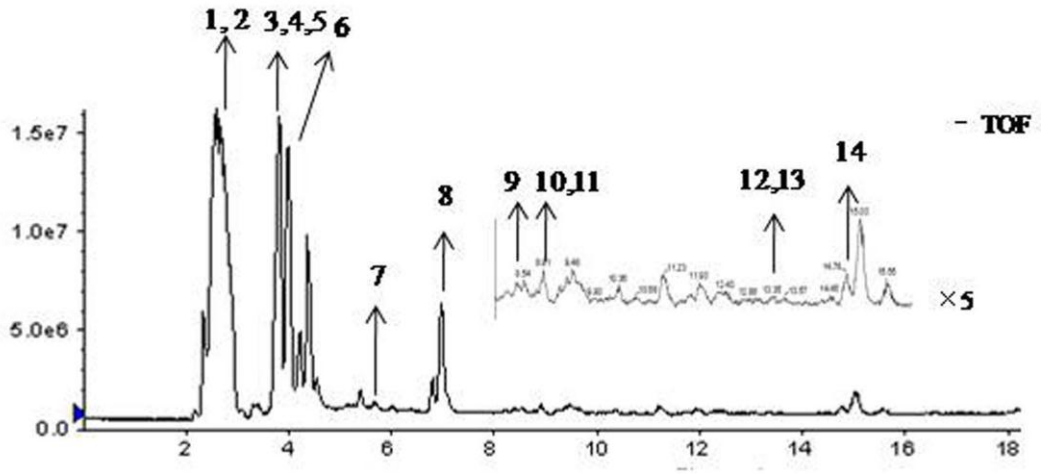

Fig. 1. Analytical HPLC-Q-TOF-MS chromatogram of HDW performed in negative modes.

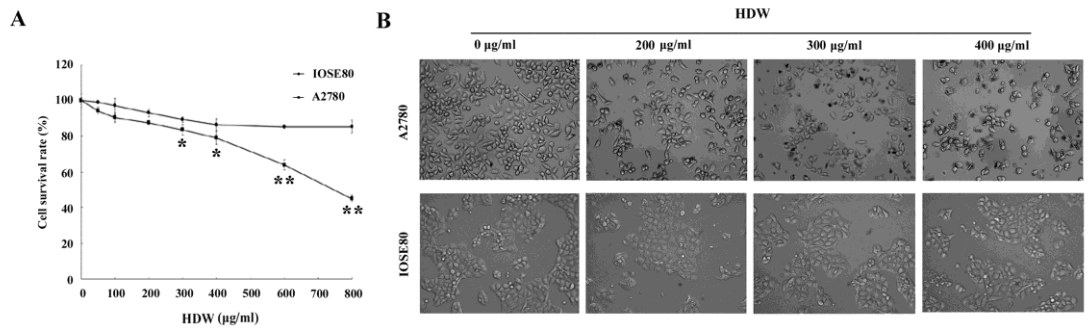

Fig. 2. Effect of HDW on the proliferation of A2780 cells. (A)The viability of A2780 cells and IOSE80 cells were determined by MTT assay after incubation with various concentrations of HDW (0, 50, 100, 200, 300, 400, 600, 800μg/ml) for 24h. The experiments were performed in triplicate with four replicated well at each dosage. *p<0.05, **p<0.01 compared to the control group. (B) A2780 and IOSE80 cells were treated with HDW (0, 200, 300, 400μg/ml) for 24h and cells were photographed with inverted contrast microscopy (magnification, 100×).

3.3.HDW induce ovarian cancer cells' apoptosis

The ovarian cancer cells were stained with DAPI (Fig. 3A). Nuclear staining of A2780 cells following HDW treatment was more intense than non-treated cells, suggesting HDW extracts trigger apoptosis of ovarian cancer cells. To further investigate whether HDW inhibit growth of ovarian cancer cells via apoptosis, the

Annexin V-FITC and propidium iodide (PI) staining were performed. The Annexin V-positive /PI-negative or Annexin V/PI double positive population indicates early or late apoptosis, respectively (Fig. 3B). Our data showed that HDW treatment significantly increased the percentage of early and late apoptosis cells in a dose-dependent manner (p<0.05) (Fig. 3C).

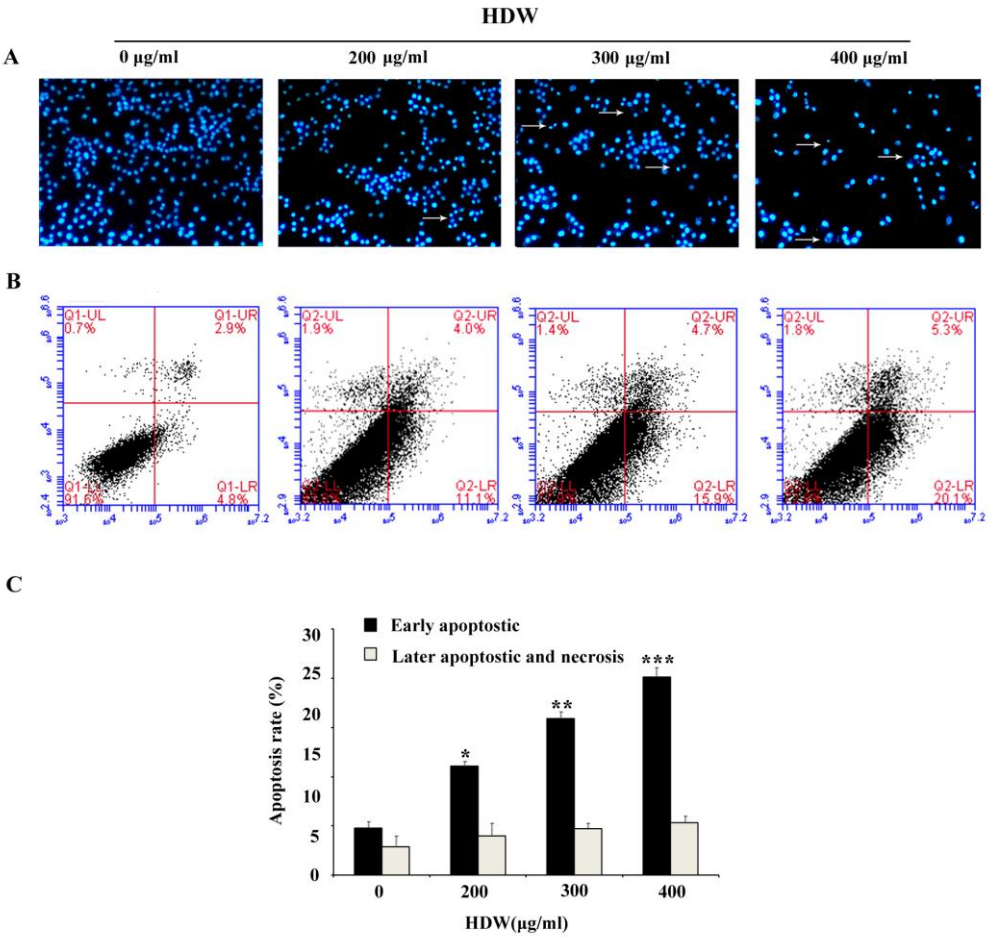

Fig. 3. HDW induce apoptosis of A2780 cells. The ovarian cancer cells were treated with HDW (0, 200, 300, 400μg/ml) for 24h. (A) The morphological changes of nuclei were examined by fluorescence microscopy using DAPI staining. The arrows indicate nuclear condensation and apoptotic bodies (magnification, 100×). （B）Cell apoptosis was determined with Annexin V-FITC/PI double staining and flow cytometry was applied to analyze. (C) Quantification of FACS analysis including early apoptosis and late apoptosis of each group is averaged with SD from three independent experiments.*p<0.05, **p<0.01, ***p<0.001 compared to the control group.

3.4.HDW upregulated apoptosis-related proteins and suppressed Bcl-2

Caspases are cytoplasmic, aspartate-specific cysteine proteases whose activation is very crucial for mitochondria-mediated apoptosis (Li et al., 1997). Our data indicated that HDW treatment could dramatically increase cleaved-caspase-3 and cleaved-caspase-9 expression levels in ovarian cancer cells (p<0.05) (Fig. 4A and 4C). Besides caspase members, the expression of anti-apoptotic protein Bcl-2 in ovarian cancer cells treated with HDW was also detected by western-blotting assay. We found that HDW extracts dramatically inhibit the expression of Bcl-2 (p<0.05), in accordance with the activation of caspases (Fig. 4B and 4D).

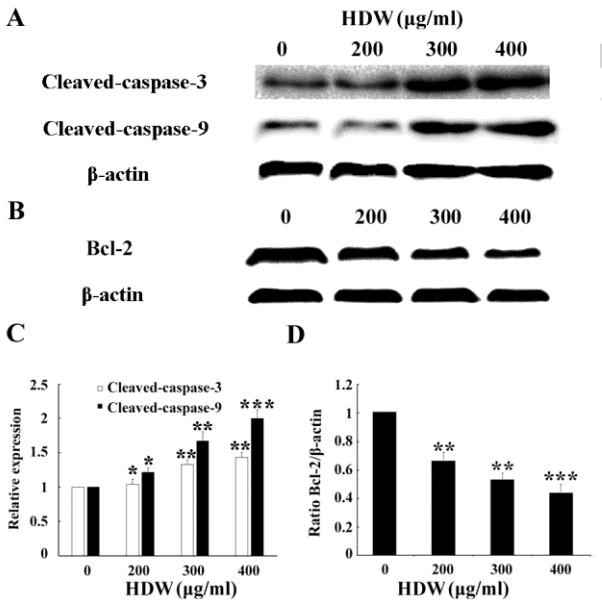

Fig. 4. Influence of HDW extract on caspase-3, caspase-9 and Bcl-2 activity in A2780 cells. Cells were treated with indicated doses of HDW for 48 h. (A) The expression of caspase-3 and caspase-9 in A2780 cells treated with varied concentrations of HDW were quantified by western blotting.β-actin was used as a loading control. The experiments were repeated at least three times. (B) Activation of Bcl-2 protein expression by HDW in A2780 cells. (C,D) Histograms show mean (±SD) level of caspase-3, caspase-9 and Bcl-2 from three independent experiments. The caspase-3, caspase-9 and Bcl-2 expression levels were expressed relative to β-actin and

standardized to non-treated control group. *p<0.05, **p<0.01, ***p<0.001 compared to the control group.

3.5. The effect of HDW on ovarian cancer migration

To investigate the anti-metastatic effect of HDW on ovarian cancer cells, wound healing assay was performed. HDW extracts dose-dependently decreased the movement of A2780 cells (Fig. 5A). The wound closure rates of HDW (50μg/ml and 100μg/ml) treated cells were 74.20% and 50.53% respectively compared to non-treated cells (p<0.05) (Fig. 5C). Moreover, the migration of A2780 cells into transwell chamber were significantly suppressed by HDW extracts in a dose-dependent manner (Fig. 5B). Treatment with 50μg/ml and 100μg/ml HDW inhibited cell migration by 38.71% and 63.71% respectively (Fig. 5D). Our data suggested that HDW extracts inhibited the migration of ovarian cancer cells.

3.6. The inhibition of the expression of MMP-2/9 by HDW

To further investigate the possible molecular mechanisms that HDW suppressed the migration of ovarian cancer cells, related protein MMP-2/9 level was examined by Western blotting. Our result showed that the expression of MMP-2/9 dose-dependently decreased after HDW treatment (Fig. 6). It suggested that the anti-metastatic effect of HDW on ovarian cancer was at least partially related to the inhibition of expression of MMP-2/9 protein.

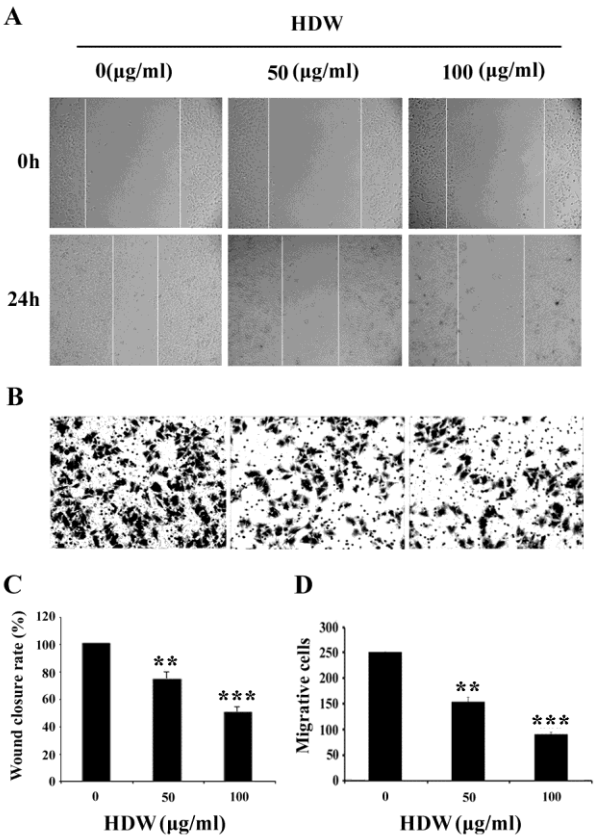

Fig. 5. Influence of HDW on the migration of A2780 cells. (A)Monolayers of A2780 cells were scratched with a pipette tip and treated with the indicated doses of HDW for 24 h. Representative photos under the microscope at 100× magnification field of the scratch are showed, before and after injury. (B) A2780 cells were treated with HDW (0, 50, 100μg/ml) for 24h. The photos represented cell migration into the underside of transwell membrane under microscope at 100×magnifcation field. (C) The migration of A2780 cells was quantified by measuring wound closure areas before and after injury. The migration of A2780 cells was quantified by measuring wound closure areas before and after injury. Data was representative of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ compared to the control group. (D) The cell number that crossed the transwell chamber of each field was counted and averaged. The experiments were repeated three times with three replicates each. $^{**}p<0.01$, $^{***}p<0.001$ compared to the control group.

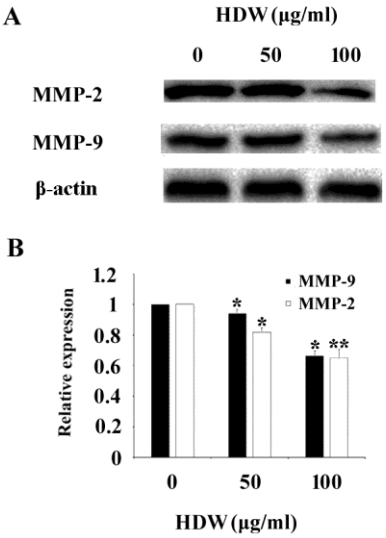

Fig. 6. HDW inhibit the expression of MMP-2/9 protein in A2780 cells. (A)Cells were treated with indicated doses of HDW (0, 50, 100 μg/ml)) for 48 h. MMP-2/9 protein in A2780 cells was detected by Western blot. (B) The MMP-2/9 expression levels were expressed relative to β-actin and standardized to non-treated control group. Data are presented as means ±SD of three experiments. *p<0.05, **p<0.01 compared to the control group.

4. Discussion

Some natural products derived from plants have demonstrated anti-cancer properties with fewer side effects compared to current anti-cancer treatments such as chemotherapy (Lin et al., 2015). *Hedyotis diffusa* Willd, a well-known traditional Chinese medicine, has been used to treat various kinds of cancers for long time (Lin et al., 2012). In the present study, we explored the effects of HDW on ovarian cancer and analyze the principal bioactive phytochemicals to develop novel effective anti-cancer drugs.

HDW has been reported to inhibit the growth of several kinds of cancers (Niu and Meng, 2013). It suppressed proliferation of human colorectal cancer, glioblastoma and leukemia cells (Lin et al., 2015). In this study, we observed that HDW significantly

inhibited the growth of ovarian cancer cells in a dose and time dependent manner. We also found the ovarian cancer cells treated with HDW become round and detached from the culture flask. All data suggested that HDW may prevent the proliferation of ovarian cancer.

Apoptosis plays very important roles in animal development and cellular homestasis (Danial and Korsmeyer, 2004; Yu et al., 2015). Deregulation of apoptosis is one of the hallmarks of cancer cells. Therefore, induction of apoptosis became the major target of most of anti-cancer agents. Our data indicated that ovarian cancer cells treated with HDW extracts displayed specific apoptotic morphological changes. In addition, the percentage of early and late apoptotic cells dramatically increased by HDW extracts. All these data suggested that HDW induce apoptosis in ovarian cancer cells.

Studies showed that mitochondrial proteins directly activate cellular apoptotic programs (Alenzi et al., 2010). Bcl-2 protein has been reports to involve in mitochondria-associate apoptotic pathway (Danial and Korsmeyer, 2004; Ji and Yu, 2015). Down regulation of Bcl-2 protein level could lead to loss of mitochondrial membrane potential and trigger a series of apoptotic events such as activation of caspase-9 and caspase-3 (Lin et al., 2011). In our study, HDW extracts significantly decrease Bcl-2 protein expression accompanied with upregulation of cleaved-caspase-9 and cleaved-caspase-3 levels in ovarian cancer cells. HDW extracts may induce apoptosis through mitochondria-associate apoptotic pathway.

Most phytochemicals detected in HDW by HPLC-Q-TOF MS analysis were

flavonoids and derivatives, which may mainly contributed to the anti-cancer effects of HDW. For example, quercetin has been demonstrated to be able to inhibit the proliferation and induce the apoptosis of various types of cancer cells, including colon, pancreatic, stomach, bladder and breast (Angst et al., 2013; Lee et al., 2016; Ma et al., 2006; Refolo et al., 2015; Ranganathan et al., 2015). In addition, kaempferol showed significant anti-proliferative activity on a panel of human cancer cell lines including human breast carcinoma, human stomach carcinoma, human cervical carcinoma and human lung carcinoma (Hung, 2004; Liao et al., 2016). Rutin also exhibited anticancer properties through cell cycle arrest and promoting apoptosis (Chen et al., 2013). Moreover, anthraquinone also possessed inhibitory effects on human breast cancer cells (Hong et al., 2014; Shi et al., 2008). It has been reported that methylanthraquinone from *Hedyotis diffusa* willd induces apoptosis via Ca^{2+}/calpain/caspase-4 pathway in MCF-7 cells (Liu et al., 2010). The presence of these phytochemicals in HDW may associate with its anti-cancer activity.

Migration is the necessary step for cancer cells. MMP-2 and MMP-9 belong to gelatin enzyme class of proteolytic enzymes. It plays a crucial role in tumor migration by mediating the degration of ECM in cancer cells (Pei et al., 2015). A previous study suggested that over expression of MMP-2 and MMP-9 was associated with metastasis of ovarian cancer (Du et al., 2015). HDW significantly inhibited migration of ovarian cancer cells. Moreover, we observed that MMP-2 and MMP-9 protein level was reduced by HDW in a dose-dependent manner. These findings indicated that HDW may suppress ovarian cancer migration through down-regulation of MMP-2 and

MMP-9 expression.

Taken together, our results demonstrated that HDW extracts inhibited the growth and migration of ovarian cancer cells. Moreover, the possible molecular mechanisms underlying this process were related to mitochondria-associate apoptosis and abnormal expression of MMP-2 and MMP-9. These findings contribute to the future development of HDW for the potential treatment of ovarian cancer.

Acknowledgment

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Graphical abstract