Effect of *Hedyotis Diffusa Willd* extract on tumor angiogenesis

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Abstract. Inhibition of tumor angiogenesis has become an attractive target of anticancer chemotherapy. However, drug resistance and cytotoxicity against non-tumor associated endothelial cells limit the long-term use and the therapeutic effectiveness of angiogenesis inhibitors, thus increasing the necessity for the development of multi-target agents with minimal side effects. Traditional Chinese medicine (TCM) formulas, which have relatively fewer side effects and have been used clinically to treat various types of diseases, including cancer, for thousands of years, are considered to be multi-component and multi-target agents exerting their therapeutic function in a more holistic way. *Hedyotis Diffusa Willd* (EEHDW) has long been used as an important component in several TCM formulas to treat various types of cancer. Although recently we reported that EEHDW promotes cancer cell apoptosis via activation of the mitochondrial-dependent pathway, the precise mechanism of its tumoricidal activity still remains to be clarified. In the present study, we investigated the angiogenic effects of the ethanol extract of EEHDW. Cell cycle analysis was perfomed using flow cytometry. Cell viability was analyzed using MTT assay. We found that EEHDW inhibited angiogenesis *in vivo* in chick embryo chorioallantoic membrane (CAM). In addition, we observed that EEHDW dose- and time-dependently inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) by blocking the cell cycle G1 to S progression. Moreover, EEHDW inhibited the migration and tube formation of HUVECs. Furthermore, EEHDW treatment down-regulated the mRNA and protein expression levels of VEGF-A in HT-29 human colon carcinoma cells and HUVECs. Our findings suggest that inhibiting tumor angiogenesis is one of the mechanisms by which EEHDW is involved in cancer therapy.

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Introduction

Angiogenesis, a physiological process involving the growth of new blood vessels from the pre-existing vasculature, is crucial to wound healing, reproduction and embryonic development (1). However, deregulated angiogenesis plays a crucial role in the development of various diseases including cancer (2-5). Solid tumors consist of both tumor and stromal cells. The interactions between tumor cells and stromal cells create an organ-specific microenvironment that affects tumor growth and metastasis (6,7). Initially, tumor cells obtain oxygen and nutrients from nearby blood vessels by simple passive diffusion. However, when the tumor grows to a certain size, oxygen delivery by diffusion is no longer sufficient, causing tumor cells to induce the sprouting of new blood vessels from pre-existing vasculature, creating a blood supply system within the tumor that is essential for continued tumor growth as well as providing a pathway for hematogenous metastasis (8,9).

Induction of angiogenesis is mediated by a variety of molecules released by tumor cells (10-12). Vascular endothelial growth factor A (VEGF-A) is considered to be one of the strongest stimulators of angiogenesis (13-15). VEGF-A is highly expressed in a wide variety of human tumors, and has been associated with tumor progression, invasion and metastasis, and a poorer survival and prognosis in patients (16-18). When VEGF-A is secreted from tumor cells, it primarily binds to specific receptors located on vascular endothelial cells (EC) (15), including VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1 or KDR). VEGFR-2 is believed to be a significant mediator of the angiogenic effects of VEGF-A, whereas the function of VEGFR-1 is complex and remains to be elucidated (15,16,19). Binding of VEGF to VEGFR-2 triggers a tyrosine kinase signaling cascade that induces EC proliferation, migration, survival, sprouting and eventually tube formation (15,20).

Due to the key role of angiogenesis in the progression of solid tumors, inhibition of tumor angiogenesis has become a major focus of anticancer drug development. A variety of anti-angiogenic agents is currently in preclinical development, with some of these agents being used in clinical trials. However, the wide use of angiogenesis inhibitors leads to adverse events due to the intrinsic cytotoxicity of these inhibitors against

non-tumor-associated endothelial cells. The most commonly observed side effects are hypertension, bleeding, proteinuria and thrombosis (21-24). In addition, multiple signaling pathways are involved in tumor angiogenesis; inhibitors that affect a single pathway may be insufficient and probably lead to resistance (25). Consequently, multi-target agents with minimal side effects and toxicity are required.

Natural products, including traditional Chinese medicine (TCM), have received attention as they have relatively few side effects and have been used clinically for thousands of years as important alternative remedies for a variety of diseases (26-28). *Hedyotis Diffusa Willd* (EEHDW) is a medicinal herb widely distributed in northeast Asia. As a well known traditional Chinese folk medicine, it is used for heat-clearing, detoxification, promotion of blood circulation and removal of blood stasis (29). EEHDW has long been used as a significant component in several TCM formulas to treat various types of cancer (29,30). Recently, we reported that EEHDW is capable of promoting cancer cell apoptosis via the activation of the mitochondrial-dependent pathway (30). To further elucidate the mechanism of the tumoricidalactivity of EEHDW, we investigated its effect on angiogenesis *in vivo* and *in vitro*.

Materials and methods

Materials and reagents. Roswell Park Memorial Institute medium 1640 (RPMI-1640), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). SuperScript II reverse transcriptase was obtained from Promega (Madison, WI, USA). An *in vitro* angiogenesis assay kit was purchased from Millipore (Billerica, MA, USA). A human VEGF-A ELISA kit was obtained from Shanghai Xitang Biological Technology Ltd. (Shanghai, China). All other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO, USA).

Preparation of ethanol extract from Hedyotis Diffusa Willd. Authentic plant material was purchased from the Guo Yi Tang Chinese Herbal medicine store, Fujian, China. The original herb was collected in Fujian Province, China (July, 2009) and identified as *Hedyotis Diffusa Willd* (EEHDW) by Dr Wei Xu at the Department of Pharmacology, Fujian University of TCM, China. Voucher specimen (no. 09072201) was deposited at the Herbarium of the Department of Pharmacology, Fujian University of TCM. EEHDW was prepared as previously described (30). Stock solutions of EEHDW were prepared by dissolving the EEHDW powder in 40% DMSO to a concentration of 400 mg/ml, and stored at -20°C. The working concentrations of EEHDW were made by diluting the stock solution in the culture medium. The final concentrations of DMSO in the medium were <0.5%.

HPLC analysis. EEHDW was analyzed on an Agilent 1100 HPLC system (Santa Clara, CA, USA) using a C-18 column. Absorbance was measured at 355 nm (Fig. 1). The mobile phase consisted of acetonitrile:methanol:0.2% phosphoric acid at 10:45:60 at a flow rate of 1 ml/min with an injection volume of 10 μl. A sample containing quercetin and kaempferol was used as a control.

Cell culture. Human umbilical vein endothelial cells (HUVECs) and human colon carcinoma HT-29 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HUVECs or HT-29 cells were grown in RPMI-1640 or DMEM, respectively. RPMI-1640 and DMEM were supplemented with 10% (v/v) FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin. Cell lines were cultured at 37°C, 5% CO_2 in a humidified environment.

Evaluation of cell viability by MTT assay. Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. HUVECs were seeded into 96-well plates at a density of $1x10^4$ cells/well in 0.1 ml medium. The cells were treated with various concentrations of EEHDW for 24 h or with 3 mg/ml of EEHDW for different periods of time. At the end of the treatment, 10 μl MTT (5 mg/ml in phosphate buffered saline, PBS) were added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO. Absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELX800, USA).

Cell cycle analysis of HUVECs. The cell cycle analysis was carried out by flow cytometry using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson, CA, USA) and propidium iodide (PI) staining. Following treatment with the indicated concentrations of EEHDW for 24 h, HUVECs were harvested and adjusted to a concentration of $1x10^6$ cells/ml, and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS, and then incubated for 30 min with RNase (8 μg/ml) and PI (10 μg/ml). The fluorescent signal was detected through the FL2 channel and the proportion of DNA in different phases was analyzed using ModfitLT version 3.0 (Verity Software House, Topsham, ME, USA).

Migration assay on HUVECs. Migration of HUVECs was performed by the wound healing method. HUVECs were seeded into 12-well plates at a density of $2x10^5$ cells/well in 1 ml medium. After 24 h of incubation, cells were scraped away vertically in each well using a P100 pipette tip. Three randomly selected views along the scraped line were photographed on each well using a phase-contrast inverted microscope at a magnification of 100x. Cells were then treated with indicated concentrations of EEHDW for 24 h, and another set of images were captured by the same method. A reduction in the scraped area indicates a sign of migration.

Tube formation assay on HUVECs. The HUVEC tube formation was examined using the ECMatrix assay kit (Millipore), following the manufacturer's instructions. Briefly, confluent HUVECs were harvested and diluted ($1x10^4$ cells) in 50 μl of medium, containing the indicated concentrations of EEHDW. The harvested cells were then seeded into 1:1 ECMatrix gel (v/v) coated 96-well plates and incubated for 9 h at 37°C. The network-like structures were examined using phase-contrast inverted microscope. The images were captured at a magnification of x100. The level of the tube formation was quantified by calculating the length of tubes in three randomly chosen fields from each well.
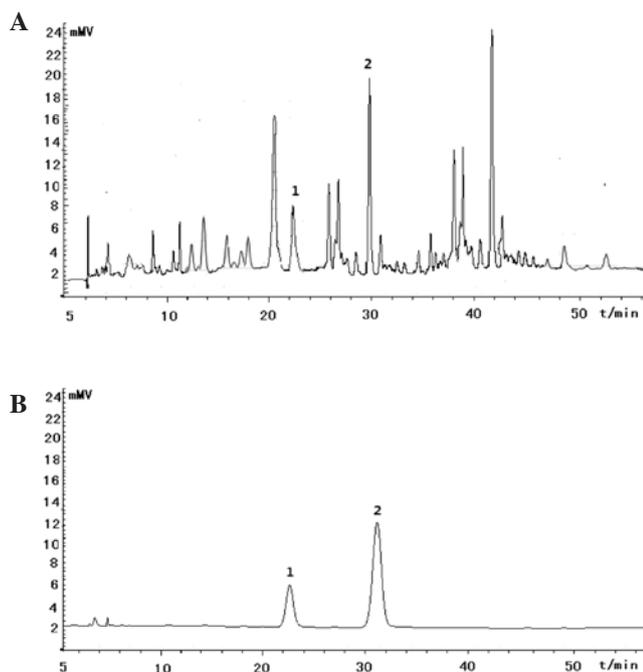
Figure 1. HPLC profiles of (A) *Hedyotis Diffusa Willd* (EEHDW) and (B) a control sample. The mobile phase consisted of consisted of acetonitrile: methanol:0.2% phosphoric acid at 10:45:60. The control sample comprised quercetin (peak 1) and kaempferol (peak 2).
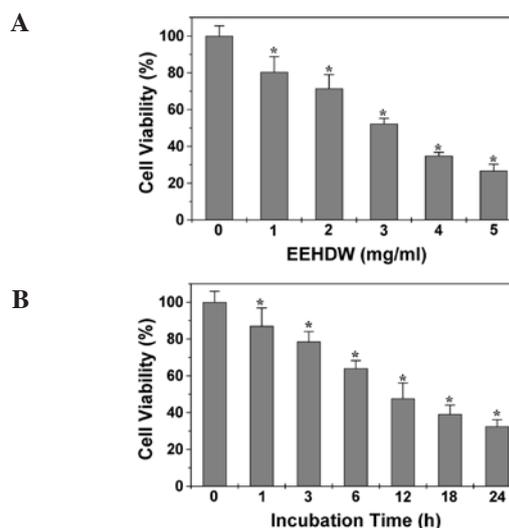

Figure 2. Effect of *Hedyotis Diffusa Willd* (EEHDW) on the viability of human umbilical vein endothelial cells (HUVECs). (A) Cells were treated with the indicated concentrations of EEHDW for 24 h. (B) Cells were treated with 3 mg/ml of EEHDW for the indicated time periods. Cell viability was determined by the MTT assay. The data were normalized to the viability of the control cells (100%, treated with 0.5% DMSO vehicle). Data are the means ± SD (error bars) from at least three independent experiments. *P<0.01, vs. the control cells.

Chick chorioallantoic membrane (CAM) assay. A CAM assay was performed to determine the *in vivo* antiangiogenic activity of EEHDW. Briefly, 10 μl of EEHDW (400 μg/μl) was loaded onto a 0.5 cm diameter Whatman filter paper. The filter was then applied to the CAM of a 7-day embryo. After incubation for 72 h at 37°C, angiogenesis around the filter was photographed with a digital camera. The number of blood vessels was quantified manually in a circular perimeter surrounding the implants, at a distance of 0.25 cm from the edge of the filter. Assays were performed twice, containing totally 10 eggs for each data point.

RNA extraction and RT-PCR analysis. HUVECs (2×10^5) or HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with the indicated concentrations of EEHDW for 24 h. Total RNA was isolated with TRIzol reagent. Oligo(dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of VEGF-A by PCR. GAPDH was used as an internal control.

Measurement of VEGF-A secretion by ELISA. The secretion level of VEGF-A was measured using an ELISA kit (Xitang) according to the manufacturer's instructions. The wells were coated with 100 μl capture antibody diluted in coating buffer. The plate was sealed and incubated overnight at 4°C. After three washes, the wells were blocked with 200 μl assay diluents at room temperature for 1 h, followed by another three washes. Then, 100 μl diluted VEGF-A standard and test samples were added and incubated for 2 h at room temperature. After repeated washes, the substrate was added and incubated for 20 min at room temperature, and the absorbance was measured at 450 nm.

Statistical analysis. Data are the means of three determinations. Data were analyzed using the SPSS package for Windows (version 11.5). Statistical analysis of the data was performed using the Student's t-test and ANOVA. Differences with P<0.05 were considered to be statistically significant.

Results

EEHDW inhibits the proliferation of HUVECs. Angiogenesis requires the proliferation of endothelial cells. Therefore, we first evaluated the effect of EEHDW on the growth of HUVECs. Cell viability was determined using a MTT assay after HUVECs were treated with various concentrations of EEHDW for 24 h or with 3 mg/ml of EEHDW for different periods of time. As shown in Fig. 2A and B, EEHDW treatment reduced cell viability in a dose- and time-dependent manner (P<0.01, vs. untreated control cells), suggesting that EEHDW inhibits the proliferation of HUVECs.

EEHDW blocks cell cycle progression of HUVECs. G1/S transition is one of the two main checkpoints used by cells to regulate cell cycle progression and thus cell proliferation. We therefore investigated the effect of EEHDW on the G1 to S progression in HUVECs via PI staining followed by FACS analysis. As shown in Fig. 3, the percentage proportion of S-phase cells following treatment with 0, 1, 3 and 5 mg/ml of EEHDW was 48.07, 35.48, 28.21 and 23.13%, respectively (P<0.01), indicating that EEHDW inhibits HUVEC proliferation by blocking the cell cycle G1 to S progression.

EEHDW inhibits HUVEC migration and tube formation. Typical physiological processes of angiogenesis include the migration and alignment of endothelial cells to form new lumen vessels. The effect of EEHDW on EC migration was determined
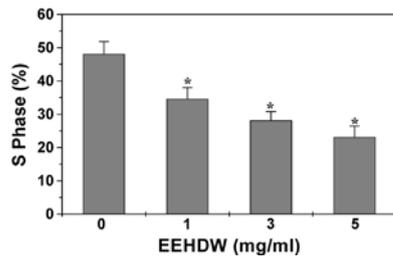
Figure 3. Effect of *Hedyotis Diffusa Willd (*EEHDW) on the cell cycle progression of human umbilical vein endothelial cells (HUVECs). Cells were treated with indicated concentrations of EEHDW for 24 h, stained with PI and analyzed by FACS. The proportion of DNA in the S-phase was calculated using ModfitLT version 3.0 software. Data shown are the means ± SD (error bars) from three independent experiments. *P<0.01, vs. the control cells.
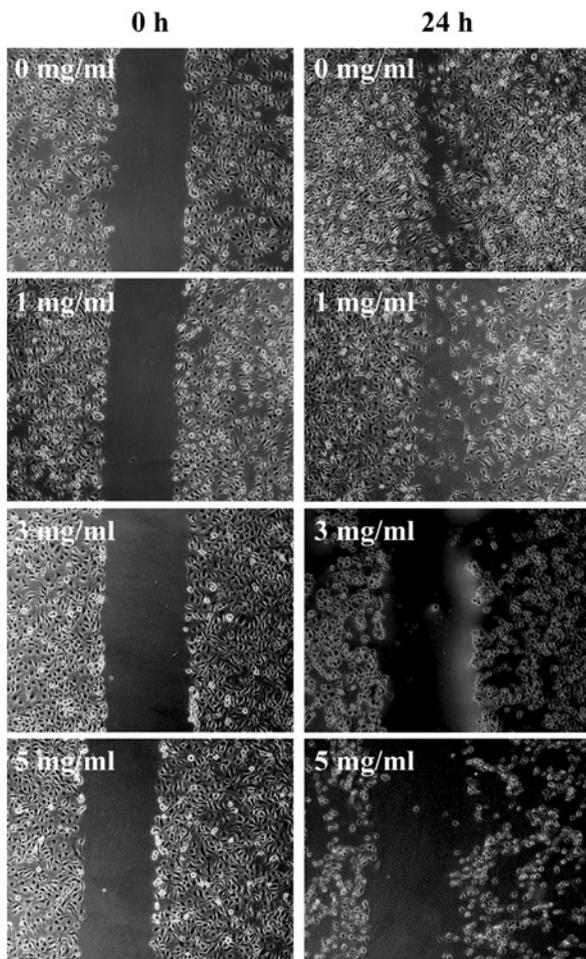

Figure 4. Effect of *Hedyotis Diffusa Willd (*EEHDW) on the migration of human umbilical vein endothelial cells (HUVECs). Following treatment with the indicated concentrations of EEHDW for 24 h, the migration pattern of HUVECs was observed using phase-contrast microscopy. Images were captured at a magnification of x100. Images are representative of three independent experiments.

Figure 5. Effect of *Hedyotis Diffusa Willd (*EEHDW) on human umbilical vein endothelial cell (HUVEC) tube formation. (A) HUVECs were harvested and diluted with medium containing various concentrations of EEHDW. The harvested cells were then seeded into 1:1 ECMatrix gel (v/v) coated plates and incubated for 9 h at 37˚C. The network-like structures were examined phase-contrast microscopy. Images were captured at a magnification of x100. Images are representative of three independent experiments. (B) The total length of capillary-like tubes was measured and normalized to the untreated control. *P<0.01, vs. the control cells.

using the wounding-healing method. As shown in Fig. 4, after post-wounding for 24 h, untreated HUVECs migrated into the clear area, whereas EEHDW treatment dose-dependently inhibited HUVEC migration. We evaluated the effect of EEHDW on EC capillary tube formation using the ECMatrix system, a solid 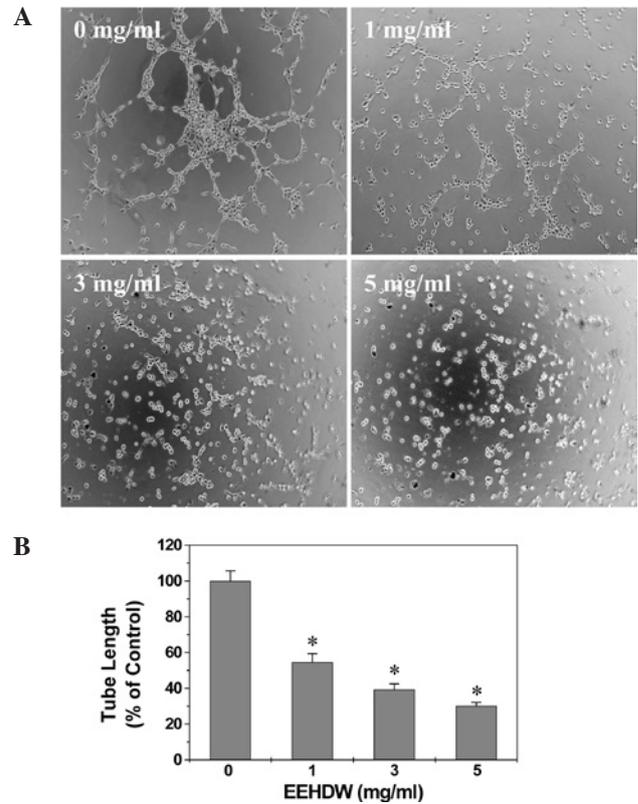gel containing mouse basement membrane proteins, in which cultured endothelial cells are able to rapidly align and form hollow tube-like structures. As shown in Fig. 5A and B, when untreated HUVECs were plated on ECMatrix gel, there was a clear formation of elongated tube-like structures. In contrast, EEHDW treatment significantly resulted in a decrease in capillary tube formation, in a dose-dependent manner.

EEHDW exhibits anti-angiogenic activity in an in vivo CAM model. The inhibitory effects of EEHDW on HUVEC proliferation, migration and tube formation are consistent with its possible antitumor action. Therefore, we tested the effect of EEHDW on *in vivo* angiogenesis using a chick CAM model. We observed that EEHDW treatment significantly reduced the total number of blood vessels in the chicken embryos, compared with the untreated control (Fig. 6), indicating that EEHDW is able to suppress angiogenesis *in vivo*.

EEHDW suppresses the expression of VEGF in both HT-29 cells and HUVECs. Tumor angiogenesis is highly regulated by growth factors such as VEGF-A which is secreted by tumor cells and ECs and functions via paracrine and autocrine signaling pathways. To further investigate the underlying mechanism of the anti-angiogenic activity of EEHDW, we examined the effect of EEHDW on the expression of VEGF-A in both HT-29 human colon carcinoma cells and HUVECs. The results of
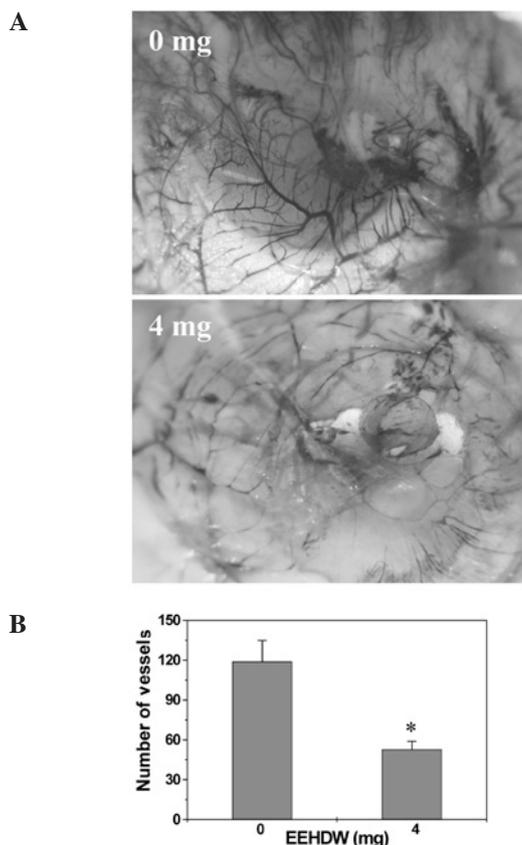
A

B

Figure 6. Effect of *Hedyotis Diffusa Willd (*EEHDW) on the angiogenesis of chick chorioallantoic membrane (CAM). (A) A 0.5 cm-diameter filter paper loaded with or without 4 mg of EEHDW was applied to the CAM and incubated at 37˚C for 72 h. Angiogenesis around the filter was photographed with a digital camera. The number of blood vessels was quantified manually in a circular perimeter surrounding the implants, at a distance of 0.25 cm from the edge of the filter. Assays were performed twice, containing 10 eggs in total for each data point. Images are representative photographs. (B) Quantification of CAM assay. *P<0.01, vs. the control cells.

the RT-PCR assay showed that EEHDW treatment profoundly reduced VEGF-A mRNA expression in both HT-29 cells and HUVECs, in a dose-dependent manner (Fig. 7A and B). Data from ELISA assay showed that the protein expression pattern of VEGF-A was similar to its mRNA level (Fig. 7C and D).

Discussion

Angiogenesis has become an attractive target for anticancer chemotherapy due to its essential role for the growth, progression and metastasis of solid tumors. A variety of anti-angiogenic agents is currently in the stage of preclinical development, with some of these agents being used in clinical trials. However, the administration of angiogenesis inhibitors usually causes cardiovascular complications, including impaired wound healing, bleeding, hypertension, proteinuria and thrombosis (21-24), due to the intrinsic cytotoxicity of these inhibitors against non-tumor associated endothelial cells. In addition, multiple signaling pathways are involved in tumor angiogenesis; inhibitors that affect a single pathway may be insufficient and probably lead to resistance (25). As a result, multi-target agents with minimal side effects and toxicity are required. Natural products, including TCM, have relatively fewer side effects and have been used clinically to treat various types of diseases including cancer for thousands of years (26-28). TCM formula is a complex combination of a number of natural products, each of which contains numerous chemical compounds. Therefore, TCM formulas are considered to be multi-component and multi-target agents exerting their therapeutic function in a more holistic way. Therefore, identifying naturally occurring agents is a promising approach for anticancer treatment.

EEHDW, an important traditional heat-clearing and detoxifying Chinese herb, has been used as a major component in several TCM formulas for the clinical treatment of various types
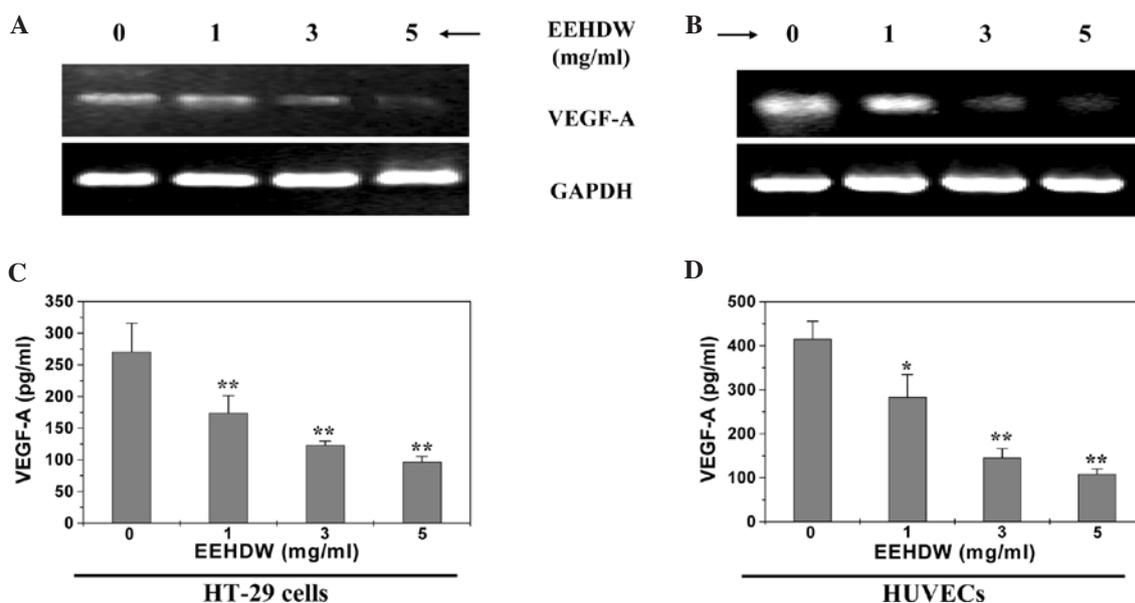

Figure 7. Effect of *Hedyotis Diffusa Willd (*EEHDWS) on the expression of VEGF-A in both HT-29 colon carcinoma cells and human umbilical vein endothelial cells (HUVECs). Cells were treated with the indicated concentrations of EEHDW for 24 h. The mRNA levels of VEGF-A were determined by RT-PCR in (A) HT-29 cells or (B) HUVECs. GAPDH was used as an internal control and data are representative of three independent experiments. The protein secretion levels of VEGF-A were determined by ELISA in (C) HT-29 cells or (D) HUVECs. Data are the means ± SD (error bars) from at least three independent experiments. *P<0.05, **P<0.01, vs. the control cells.

of cancer (29,30). Recently, we reported that EEHDW promotes cancer cell apoptosis via the activation of the mitochondrial-dependent pathway (30). However, the mechanism of its antitumor remains to determined. Therefore, before EEHDW can be developed as an anticancer agent, the molecular mechanism of its antitumor activity should be further elucidated.

We have reported for the first time that the ethanol extract of EEHDW is able to inhibit angiogenesis *in vivo* in CAM and *in vitro* in HUVECs. Angiogenesis typically consists of several features, including proliferation, migration and capillary tube formation ECs. Using the MTT assay we found that EEHDW inhibited the proliferation of ECs in a dose- and time-dependent manner. The anti-proliferative effect of EEHDW on ECs probably results from cell cycle arrest since EEHDW treatment dose-dependently blocked the G1 to S progression of ECs. Moreover, using the wounding-healing method and ECMatrix system we demonstrated that EEHDW inhibited the migration and capillary tube formation of ECs in a dose-dependent manner. The process of tumor angiogenesis is highly regulated by growth factors such as VEGF-A which is secreted by tumor cells and ECs and functions via paracrine and autocrine signaling pathways (13-15). Following secretion VEGF-A primarily binds to its specific receptor VEGFR-2 located on ECs (15,16,19,20), triggering the process of angiogenesis. In the present study we found that EEHDW down-regulated the expression of VEGF-A in HT-29 human colon carcinoma cells and ECs. Thus, based on the present study as well as our previous studies, it is evident that EEHDW inhibits cancer growth via at least two mechanisms, inducing mitochondrial-dependent apoptosis of cancer cells and inhibiting tumor angiogenesis.

Apoptosis and angiogenesis are regulated by various pathways, such as the STAT3, Ras/ERK, PI3K/mTOR and Wnt signaling pathways. In addition, EEHDW comprises numerous chemical compounds including ferulic acid, 2-methyl-3-hydroxyanthraquinone, 2-methyl-3-methoxyanthraquinone, scopolin, oleanolic acid, ursolic acid, quercetin, kaempferol, daucosterol, stigmasterol, β-sitosterol. However, the number of compounds involved in anticancer activity, and whether the anticancer compounds of EEHDW target different sites individually, or act on a single site additively or synergistically remains to be elucidated. Additionally, the signaling pathway(s) these compounds are involved in to exert their bioactivity remain to be determined. These issues should be addressed in future experiments to elucidate the molecular mechanism by which EEHDW is involved in cancer treatment to develop better multi-target drugs for cancer therapy.

Acknowledgements

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