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RESEARCH ARTICLE

# Inhibition of vascular endothelial growth factor expression by Chinese medicine of *Hedyotis diffusa Willd* herbal compounds

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**Abstract** The *Hedyotis diffusa Will*d herbal compounds (HDWHCs) are commonly used as Chinese medicine to treat cancer patients with established clinical therapeutic efficacy in China. However, the underlying mechanisms remain to be elucidated. In this study, we used freeze-dried powder of the water extracts of HDWHCs to investigate the potential mechanisms of HDWHCs in cancer treatment. HDWHCs treatment significantly inhibited vascular endothelial growth factor (VEGF) mRNA levels and VEGF transcriptional activation in cancer cells. HDWHCs also had a remarkable inhibitory effect on the expression of hypoxia-inducible factor 1alpha (HIF-1alpha). Forced expression of HIF-1α restored VEGF transcriptional activation inhibited by HDWHCs, indicating that HDWHCs suppressed VEGF expression through decreasing HIF-1alpha expression. Moreover, HDWHCs inhibited cyclooxygenase-2 (COX-2) expression, and overexpression of HIF-1alpha restored HDWHCs' inhibitory effect on COX-2 at transcriptional level. These findings may provide better understanding of HDWHCs' anti-cancer mechanism in cancer treatment.

**Keywords** *Hedyotis diffusa Willd*, Chinese medicine, vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1alpha (HIF-1alpha), cyclooxygenase-2 (COX-2)

## 1 Introduction

Traditional Chinese medicine is a valuable resource in treating various diseases, including human cancer. During the last decade, extensive studies have been focused on the clinical effects of pure compound extracted from Chinese medicine in cancer treatment, but most of these trials failed since the single component of Chinese medicine hardly worked effectively in the clinic. The *Hedyotis diffusa Willd* herbal compounds (HDWHCs), being well-known traditional Chinese medicine, are commonly used in cancer therapy in China, including liver, lung, colon, brain, pancreas, and other cancers (Liao et al., 1979; Shi et al., 2008). Although it has been indicated that HDWHCs may decrease tumor growth, the molecular mechanisms by which it contributes to cancer inhibition remain to be elucidated.

Vascular endothelial growth factor (VEGF) is one of the strongest angiogenesis factors and plays a pivotal role in regulating tumor growth, angiogenesis, metastasis, and progression (Plate et al., 1992; Anan et al., 1996; Ellis and Hicklin, 2008). VEGF can be induced by many stimuli, including hypoxia, growth factors, and oxidative stress (Mazure et al., 1996; Richard et al., 2000). VEGF expression is mainly regulated at transcriptional level by hypoxia-inducible factor 1 (HIF-1) through binding to the HRE region in the VEGF promoter in response to hypoxia (Forsythe et al., 1996). HIF-1 is composed of HIF-1α and HIF-1β subunits, and the expression of HIF-1α has been shown to be associated with tumorigenesis and angiogenesis (Maxwell et al., 1997; Carmeliet et al., 1998; Zhong et al., 1999). Cyclooxygenase-2 (COX-2), an enzyme

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involved in prostaglandin biosynthesis, is one of the angiogenesis inducers and prognostic markers in human cancers. It regulates the production of various angiogenesis factors, including VEGF (Tsujii et al., 1998; Matsubayashi et al., 2007). HIF-1α directly promotes COX-2 expression and induces the survival of tumor cells (Kaidi et al., 2006). Previous studies have demonstrated that COX-2 and VEGF are often co-expressed in human cancers, and both of them are important angiogenesis factors closely related with cancer prognosis (von Rahden et al., 2005; Noriyuki et al., 2007). Furthermore, COX-2 induced the expression of VEGF, and inhibition of COX-2 suppressed tumor angiogenesis and growth through decreasing VEGF levels (Sawaoka et al., 1999; Murono et al., 2001; Cheng et al., 2004).

It is reported that HDWHCs contain flavonol glycosides and iridoid glycosides that may have antioxidant effects (Lu et al., 2000). Our previous studies have shown that flavonoids derived from some natural plants such as apigenin and crysin inhibit tumor growth and angiogenesis through the blockade of VEGF expression (Liu et al., 2005; Fang et al., 2007; Fu et al., 2007). It is possible that HDWHCs may have an inhibitory effect on VEGF expression. To elucidate the molecular mechanisms of HDWHCs in cancer therapy, we used the freeze-dried powder of the water extracts of HDWHCs in this study to investigate: (1) whether HDWHCs inhibit VEGF expression in human prostate cancer cell line DU145 and gastric carcinoma cell line SGC-7901; (2) whether HDWHCs inhibit VEGF expression through regulating HIF-1α expression; and (3) whether HDWHCs inhibit COX-2 expression through regulating HIF-1α expression. The results may provide helpful information of the potential molecular mechanisms of HDWHCs in cancer therapy.

## 2 Materials and methods

### 2.1 Cell culture and reagents

Human prostate cancer cells DU145 and human gastric carcinoma cells SGC-7901 were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C and 5% $CO_2$ in a humid environment. JB6 cells stably transfected with COX-2 were maintained in a DMEM medium with 10% FBS and antibiotics.

Antibodies against β-actin were purchased from Sigma (Saint Louis, MO, USA). Antibodies against HIF-1α were from BD Biosciences (Franklin Lakes, NJ, USA). Nitrocellulose membranes were from Schleicher & Schuell Biosciences (Keene, NH, USA). The chemiluminescence detection reagent was from Pierce Biotechnology (Rockford, IL, USA). Trizol reagent and lipofectamine were from Invitrogen (Carlsbad, CA, USA). The reverse transcriptase was from TAKARA (Dalian, LN, China). The reporter lysis buffer and Luciferase Assay System were from Promega (Madison, WI, USA).

### 2.2 Preparation of HDWHCs

The raw *Hedyotis diffusa Willd* herbal compounds were purchased from Nanjing University of Traditional Chinese Medicine, China, containing the following ingredients: 13.3% *Hedyotis diffusa Willd*, 13.3% *Solanum lyratum Thunb*, 6.65% *Taxus chinensis Rehd*, 6.65% *Curcuma aromatica Salisb*, 13.3% *Taraxacum officinale Weber*, 6.65% *Radix Salviae Miltiorrhizae*, 6.65% *Paeonia suffruticosa Andrews*, 6.65% *Plantago depressa Willd*, 6.65% *Gleditsia japonica Miq*, 6.65% *Frifllaria thuubergii Miq*, and 6.65% *Poncirus trifoliate Raf.*

The raw *Hedyotis diffusa Willd* herbal compounds (600 g) were dipped in 4800 mL of water for 15 min, boiled for 15 min, and centrifuged at 4000 r/min for 10 min to obtain the supernatant water extracts. The water extracts were freeze-dried and stored at −80°C. For each experimental treatment, the freeze-dried powders of HDWHCs were dissolved in RPMI 1640 medium and added to the cells.

### 2.3 Western blotting

The cells were washed with ice-cold PBS buffer and harvested to obtain cell pellets by centrifugation. Total protein lysates were prepared using the RIPA buffer supplemented with protease inhibitors (100 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate acid, 0.1% SDS, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 2 mmol/L DTT, 20 μg/mL leupeptin, and 20 μg/mL pepstatin). Aliquots of protein lysates (50 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed with antibodies against HIF-1α and β-actin. Immunoreactivity signals were visualized with chemiluminescence detection reagent and quantified.

### 2.4 Semi-quantitative RT-PCR and real-time RT-PCR

Total cellular RNAs were extracted using Trizol reagent according to the manufacturer's instructions. Aliquots of total RNAs (1 μg) were used as templates to synthesize the first-strand cDNAs using reverse transcriptase. The following primers were used for PCR amplification: GAPDH forward, 5′-CCACCCATGGCAAATTCCATGGC-3′, reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′; VEGF forward, 5′-TCGGGCCTCCGAAACCATGA-3′, reverse, 5′-CCTGGTGAGAGATCTGGTTC-3′; COX-2 forward, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, reverse, 5′-AGATCATCTCTGCCTGAGTATCTT-3′.

PCR was performed for 30 cycles, with each cycle at

95°C for 1 min, 56°C for 1 min, and 72°C for 1 min. PCR products were separated on a 1.5% agarose gel, visualized with ethidium bromide, and photographed using the EagleEye II system (Stratagene, La Jolla, CA). Real-time PCR was performed using the same primers and SYBR Green Mastermix (TAKARA, Dalian, China). Reactions were analyzed on an ABI 7900 real-time PCR machine using the following cycle conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were determined by the ratio of VEGF or COX-2 to GAPDH and normalized to the control group.

### 2.5 Construction of plasmids

VEGF promoter reporter pMAP11wt containing 47 bp of human VEGF 5′-flanking sequence (from −985 to −939) was inserted into the pGL2 basic luciferase vector as described previously (Forsythe et al., 1996). The plasmid encoding human HIF-1α was inserted into the pCEP4 vector as described previously (Forsythe et al., 1996; Jiang et al., 1996). The COX-2 promoter reporter construct containing the 5′-flanking region of the human COX-2 gene (phPES2-1432/+59) was used to study the transcriptional activation of the COX-2 promoter (Cao et al., 2007).

### 2.6 Transient transfection and luciferase assay

The cells were seeded in 24-well plates and cultured to 90% confluence. To determine the effect of HDWHCs on VEGF transcriptional activation, the cells were transiently transfected with VEGF reporter and pCMV-β-galactosidase (β-gal) plasmid using lipofectamine. The transfected cells were cultured overnight, followed by treatment with HDWHCs for indicated time. Cells were then washed with PBS buffer once and lysed with reporter lysis buffer from Promega (Madison, WI, USA). The luciferase (Luc) and β-gal activities were determined. Relative Luc activity was calculated as the ratio of Luc/β-

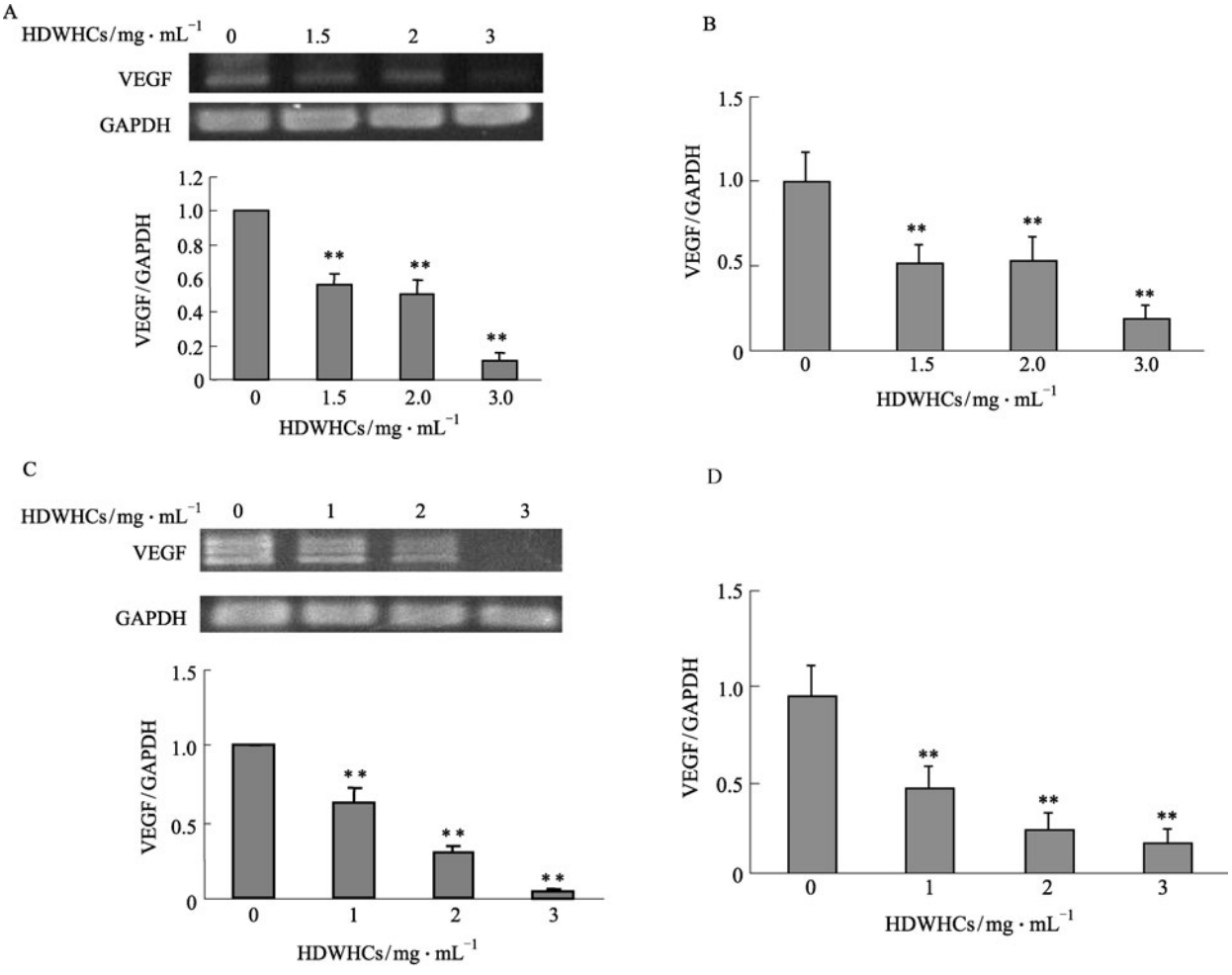


**Fig. 1** HDWHCs inhibited VEGF mRNA expression in DU145 and SGC-7901 cells. DU145 cells were treated with HDWHCs (0, 1.5, 2, and 3 mg/mL) for 12 h. Total RNAs were analyzed for VEGF and GAPDH mRNA levels using semi-quantitative RT-PCR (A) and real-time RT-PCR (B). SGC-7901 cells were treated with HDWHCs (0, 1, 2, and 3 mg/mL) for 12 h. Total RNAs were analyzed using semi-quantitative RT-PCR (C) and real-time RT-PCR (D). Data were presented by mean±SE from three duplicate experiments. *: $P < 0.05$; **: $P < 0.01$.

gal activity and normalized to that of the control.

2.7 Statistical analysis

Two-tailed Student's *t* test was used for the data analysis. A *P* value of less than 0.05 was considered significant.

## 3 Results

3.1 HDWHCs inhibited VEGF mRNA expression in a dose-dependent manner

VEGF is a key growth factor in regulating tumor growth and angiogenesis. To determine the effect of HDWHCs on VEGF expression, DU145 and SGC-7901 cancer cells were treated without or with various concentrations of HDWHCs for 12 h. The levels of VEGF and GAPDH mRNA were determined by both semi-quantitative RT-PCR and real-time RT-PCR. In DU145 cells, HDWHCs at 1.5 and 2 mg/mL suppressed VEGF expression by 50%, and HDWHCs at 3 mg/mL inhibited VEGF mRNA level by 80% (Fig. 1A and B). Similarly in SGC-7901 cells, HDWHCs at 1, 2 and 3 mg/mL inhibited VEGF expression in a dose-dependent manner by 40%–50%, 70% and 80%, respectively (Fig. 1C and D). These results indicate that HDWHCs treatment inhibited VEGF mRNA expression in both human prostate cancer and gastric carcinoma cells.

3.2 HDWHCs inhibited VEGF transcriptional activation

To study whether HDWHCs inhibit VEGF transcriptional activation, DU145 and SGC-7901 cells were co-transfected with VEGF promoter reporter plasmid pMAP11wt and pCMV-β-gal plasmid. After being cultured overnight, the cells were treated with different concentrations of HDWHCs for 24 h. The Luc and β-gal activities in the cells were analyzed. Relative Luc activity was determined by the ratio of Luc to β-gal activity and normalized to that of the control. Consistent with previous results, HDWHCs at 1, 2, and 3 mg/mL inhibited VEGF transcriptional activation by 24%, 80%, and 95%, respectively (Fig. 2A). In SGC-7901 cells, HDWHCs treatment at 1, 2, and 3 mg/mL decreased VEGF transcriptional activation by 43%, 57%, and 82%, respectively (Fig. 2B). These data suggest that HDWHCs inhibited VEGF transcriptional activation in a dose-dependent manner.

3.3 HDWHCs inhibited HIF-1α expression in DU145 and SGC-7901 cells

It is known that the human VEGF promoter contains HIF-1 DNA binding site (Forsythe et al., 1996). We hypothesized that HDWHCs can suppress VEGF expression through inhibiting HIF-1α expression. To test this hypothesis,

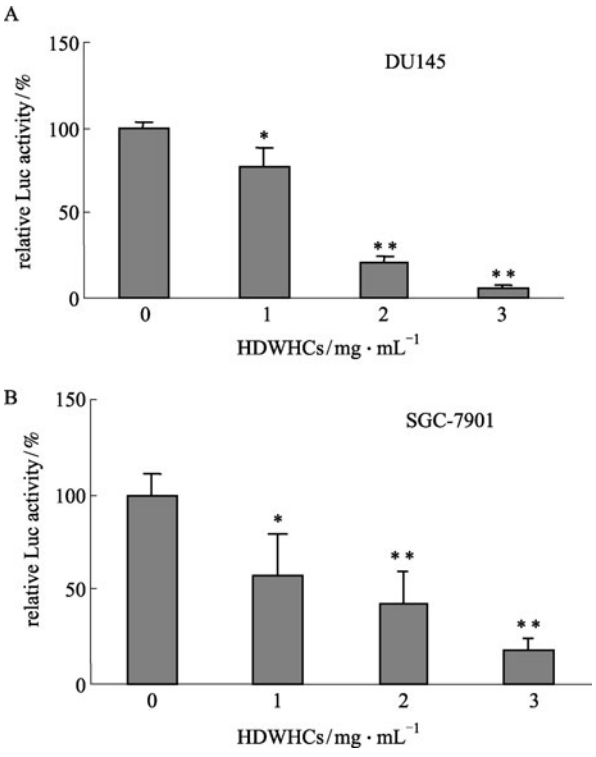


**Fig. 2** HDWHCs inhibited VEGF transcriptional activation. DU145 cells (A) and SGC-7901 cells (B) were co-transfected with a human VEGF reporter (0.5 μg) and β-gal plasmid (0.15 μg). After transfection, the cells were treated with HDWHCs (0, 1, 2 and 3 mg/mL) for 24 h. Relative Luc activity was determined by the ratio of Luc to β-gal activity and normalized to that of the control. Data were mean±SE. *: $P < 0.05$; **: $P < 0.01$.

DU145 and SGC-7901 cells were cultured in the absence or presence of different concentrations of HDWHCs for 12 h, and HIF-1α protein levels were analyzed by Western blotting. As shown in Fig. 3A and B, HDWHCs inhibited HIF-1α expression in a dose-dependent manner in both DU145 and SGC-7901 cells.

3.4 HDWHCs inhibited COX-2 expression at transcriptional level in a dose-dependent manner

It has been known that COX-2, one of the enzymes involved in prostaglandin biosynthesis, can modulate tumor growth and angiogenesis (Tsujii et al., 1998). To test whether COX-2 may be inhibited by HDWHCs, SGC-7901 cells were treated with various concentrations of HDWHCs. Total RNAs were extracted and used to determine COX-2 and GAPDH mRNA levels by semi-quantitative RT-PCR and real-time RT-PCR. As shown in Fig. 4A and B, HDWHCs treatment inhibited COX-2 mRNA expression in a dose-dependent manner. To study whether HDWHCs regulate COX-2 transcriptional activation, JB6 cells stably transfected with the COX-2 reporter were treated with different doses of HDWHCs for 24 h, and COX-2 reporter activities were analyzed. The results

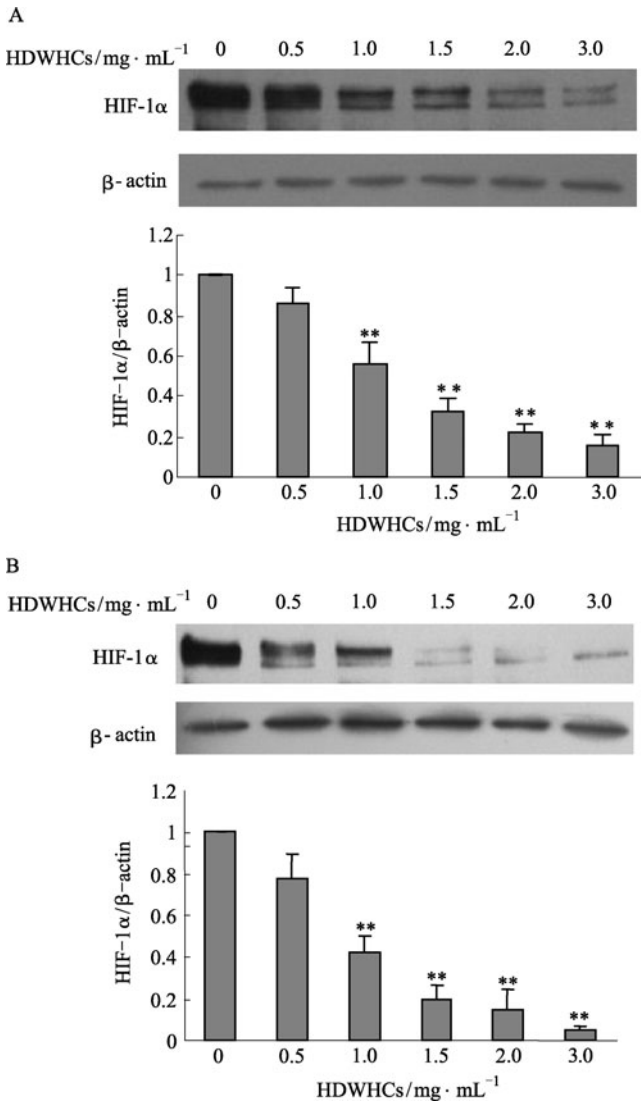


**Fig. 3** HDWHCs inhibited HIF-1α expression in DU145 and SGC-7901 cells. DU145 cells (A) and SGC-7901 cells (B) were treated with HDWHCs (0, 0.5, 1, 1.5, 2, and 3 mg/mL) for 12 h. HIF-1α and β-actin protein levels were analyzed by Western blotting (upper panel). The relative HIF-1α protein levels were determined by the ratio of HIF-1α to β-actin and normalized to that of the control. Data were mean±SE from three duplicate experiments (bottom panel). **: $P < 0.01$.

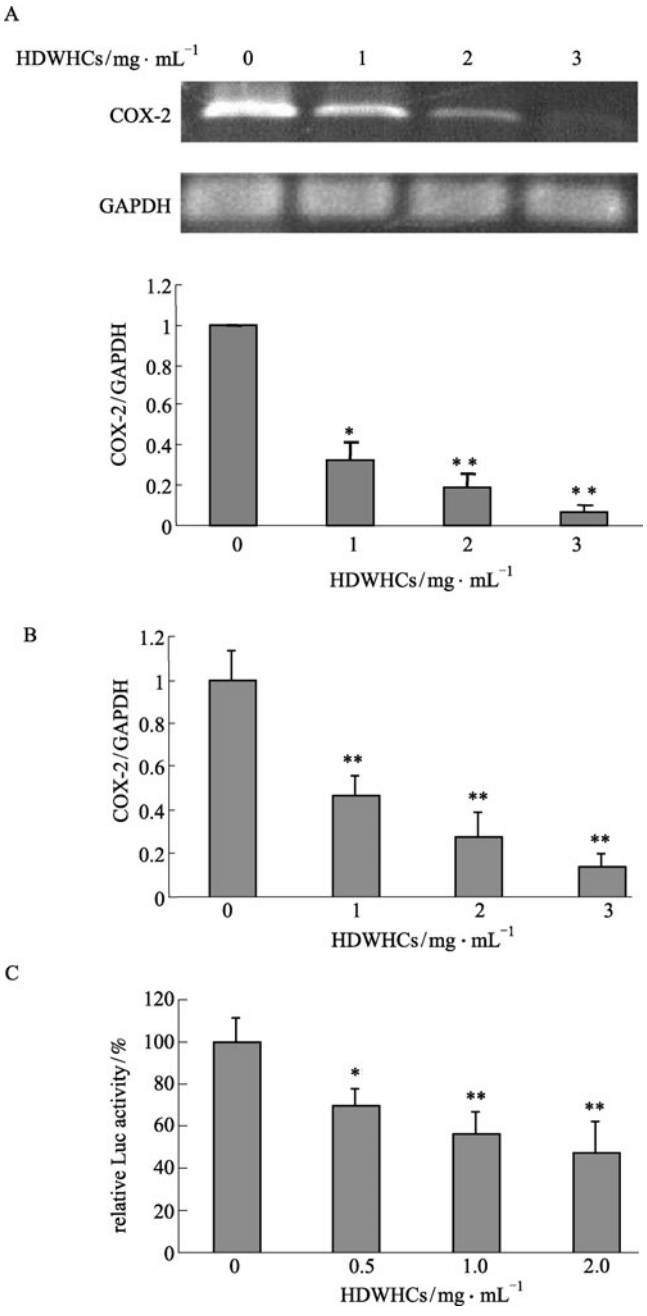


**Fig. 4** HDWHCs inhibited COX-2 expression at transcriptional level. (A) SGC-7901 cells were treated with HDWHCs (0, 1, 2, and 3 mg/mL) for 12 h. Total RNAs were analyzed using semi-quantitative RT-PCR (A) and real-time RT-PCR (B). The relative COX-2 mRNA levels were determined by the ratio of COX-2 to GAPDH mRNA. (C) JB6 cells stably transfected with COX-2 reporter were treated with HDWHCs (0, 0.5, 1, and 2 mg/mL) for 24 h. Relative Luc activity was determined. *: $P < 0.05$; **: $P < 0.01$.

show that HDWHCs decreased COX-2 transcriptional activation in a dose-dependent manner (Fig. 4C). These results indicate that in addition to VEGF, HDWHCs also inhibited COX-2 expression at transcriptional level.

### 3.5 Forced expression of HIF-1α restored HDWHCs' inhibitory activity on VEGF and COX-2 at transcriptional level

To determine whether overexpression of HIF-1α was sufficient to restore HDWHCs' inhibiting effect on VEGF at transcriptional level, DU145 cells were co-transfected with pMAP11 wt, pCMV-β-gal, and HIF-1α (0.5 μg) or empty vector plasmids. After transfection, the cells were cultured overnight, followed by the treatment without or with HDWHCs for 24 h. Cells transfected with the plasmids above and treated with HDWHCs for 12 h were

collected and subjected to Western blotting assay. As shown in Fig. 5A, HDWHCs treatment significantly suppressed HIF-1 protein level to 20% in DU145 cells, while exogenous expression of HIF-1α by transfection increased HIF-1α level, indicating that transient transfection of HIF-1α did partially restore HDWHCs' suppressing effect on HIF-1α protein level. Luc results show that 2 mg/mL of HDWHCs treatment significantly inhibited VEGF reporter activity to 16% of the control level, and HIF-1α overexpression completely restored VEGF transcriptional activation (Fig. 5B). It has been reported that HIF-1α promotes COX-2 expression (Kaidi et al., 2006). To further determine whether overexpression of HIF-1α can recover COX-2 activation suppressed by HDWHCs, similar experiments were performed in SGC-7901 cells showing that exogenous HIF-1α restored COX-2 activation under the treatment of HDWHCs (Fig. 5C). These results indicate that forced expression of HIF-1α was sufficient to restore VEGF and COX-2 transcriptional activation suppressed by HDWHCs and demonstrated that HDWHCs inhibited VEGF and COX-2 expression through suppressing HIF-1α expression.

## 4 Discussion

The HDWHCs are effective Chinese medicine used for the treatment of cancer and inflammatory diseases for a long time (Liao et al., 1979; Lu et al., 2000; Li et al., 2008). The HDWHCs can be fractioned into three major classes: iridoid glucosides, flavonoids, and anthraquinones (Lu et al., 2000; Kim et al., 2001; Li et al., 2008).

VEGF and its receptors are commonly expressed in various kinds of human cancers and play an important role in angiogenesis, tumor growth, and metastasis (Cebe-Suarez et al., 2006; Olsson et al., 2006; Shibuya, 2008). Angiogenesis is the process by which new blood capillaries are generated from the pre-existing vasculature. Angiogenesis is necessary for tumor cells to get oxygen and nutrients for growth (Folkman, 1995; Carmeliet and Jain, 2000). In this study, we found that HDWHCs, being well-established Chinese medicine for cancer therapy, inhibited VEGF mRNA expression in a dose-dependent manner. Further study showed that HDWHCs suppressed VEGF transcriptional activation, suggesting that the decrease of VEGF mRNA expression is due to the inhibition of its transcriptional activation.

HIF-1α is overexpressed in many human cancers and is associated with tumor growth, angiogenesis, and metastasis (Jiang et al., 1996; Maxwell et al., 1997; Zhong et al., 1999). In most cases, HIF-1α protein is constitutively expressed but rapidly degraded by the ubiquitin-proteasome pathway under normoxia (Salceda and Caro, 1997; Maxwell et al., 1999). HIF-1α is one of the key upstream regulators of VEGF (Forsythe et al., 1996;

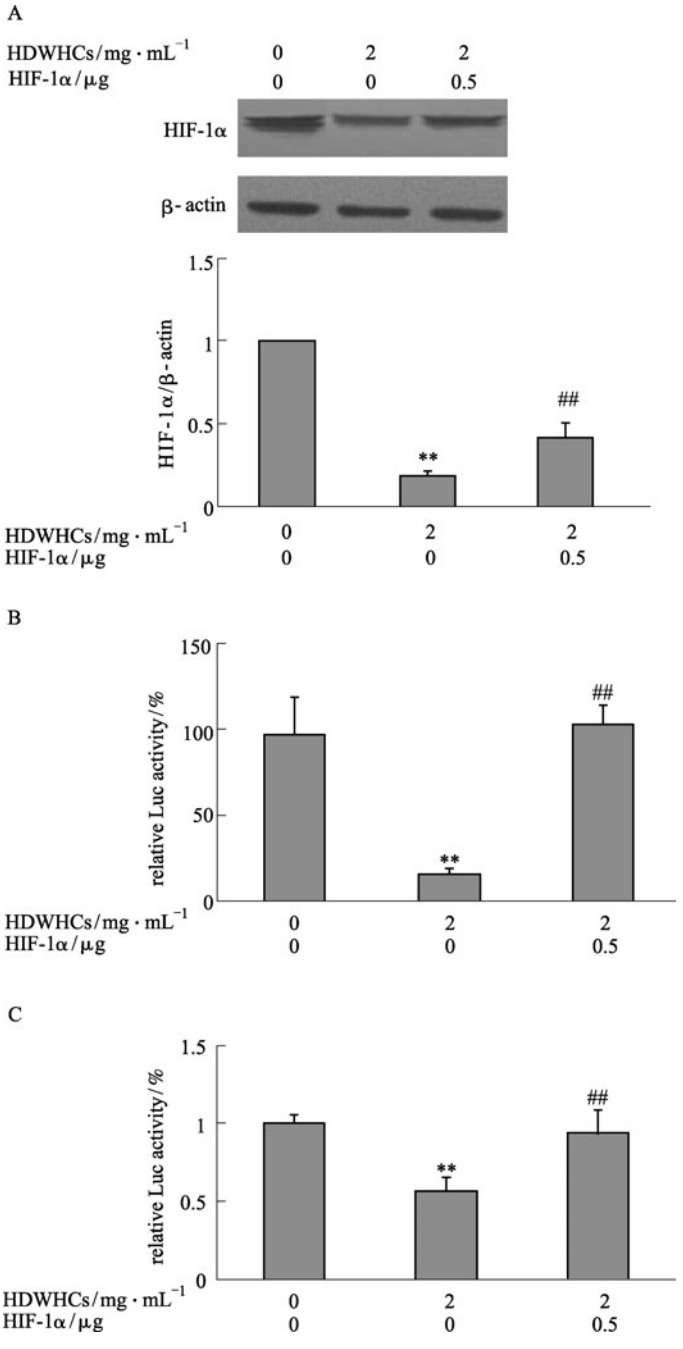


**Fig. 5** Overexpression of HIF-1α restored VEGF and COX-2 expression at transcriptional level suppressed by HDWHCs. A: DU145 cells were co-transfected with 0.5 μg of pMAP11wt reporter plasmid, 0.15 μg of β-gal plasmid, and 0.5 μg of HIF-1α plasmid. The cells were treated without or with HDWHCs at 2 mg/mL for 12 h. HIF-1α and β-actin protein levels were analyzed by Western blotting (upper panel). The relative HIF-1α protein levels were determined by the ratio of HIF-1α to β-actin. B: DU145 cells were transiently transfected and analyzed as above. C: SGC-7901 cells were transfected with COX-2 reporter plasmid and analyzed as above. **: $P < 0.01$ *vs* the control group; ##: $P < 0.01$ *vs* the HDWHCS treatment group.

Salceda and Caro, 1997). Cancer cells can increase HIF-1α expression under normoxia condition, leading to elevated expression of VEGF (Levy et al., 1997; Maxwell et al., 1999). Here we found that HDWHCs inhibited HIF-1α protein level in a dose-dependent manner. Our previous studies have demonstrated that flavonoids, the components of HDWHCs, can suppress HIF-1α expression through inhibiting AKT and ROS signaling pathways (Fang et al., 2007; Fu et al., 2007). Given the fact that flavonoids suppress ERK activation, and ERK signaling pathway is also involved in regulating HIF-1α expression (Spencer et al., 2009), it may be possible that HDWHCs may inhibit AKT, ROS, and ERK signaling pathways. However, further studies are needed to understand the molecular mechanism of HDWHCs in inhibiting HIF-1α expression. Moreover, forced expression of HIF-1α reversed VEGF transcriptional activation that had been reduced by HDWHCs, demonstrating that HDWHCs inhibited VEGF expression through downregulating HIF-1α expression.

Previous studies have shown that COX-2 is also closely associated with angiogenesis (Majima et al., 1997; Tsujii et al., 1998; Liu et al., 1999; Majima et al., 2000). HDWHCs treatment also inhibited COX-2 mRNA expression through suppressing its transcriptional activation. In addition, overexpression of HIF-1α restored COX-2 transcriptional activation reduced by HDWHCs, indicating that HDWHCs also inhibited another angiogenesis inducer COX-2 expression through suppressing HIF-1α expression.

In summary, HDWHCs suppress VEGF and COX-2 expression at transcriptional level through inhibiting HIF-1α expression. These results provide potential molecular mechanisms of HDWHCs in cancer therapy and indicate that HDWHCs may exert its antitumor effects through inhibiting VEGF and COX-2 expression.

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