Scutellaria barbata inhibits angiogenesis through down-regulation of HIF-1 α in lung tumor

Ai-Li Shiau[1], Yu-Ting Shen[1], Jeng-Long Hsieh[2], Chao-Liang Wu[3] and Che-Hsin Lee[4]*

[1]Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan

[2]Department of Nursing, Chung Hwa University of Medical Technology, Tainan, Taiwan

[3]Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan

[4]Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan.

*Correspondence: Dr. C.H. Lee, Department of Microbiology, School of Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan. E-mail: chlee@mail.cmu.edu.tw Fax: +886-4-22053764; Tel: +886-4-22053366 ext. 2173

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ABSTRACT

Hypoxia, a hallmark of many solid tumors, is associated with angiogenesis and tumor progression. Hypoxia-inducible factor-1 (HIF-1) plays a significant role in tumor angiogenesis. In this study, we constructed a selective platform to screen the traditional Chinese medicine as anti-angiogenic agent. We examined the molecular mechanism by which *Scutellaria barbata* (*S. barbata*) regulates HIF-1-dependent expression of vascular endothelial growth factor (VEGF), which is an important angiogenic factor. Hypoxia promotes angiogenesis by increasing VEGF expression and secretion. Herein, the expression of VEGF was decreased by treatment with *S. barbata* in tumor cells. Meanwhile, *S. barbata* reduced the migration and proliferation of endothelial cells under hypoxic condition. *S. barbata* inhibited the expression of HIF-1α, as well as phosphorylated their upstream signal mediators AKT. *S. barbata* significantly inhibited the tumor growth *in vivo* and immunohistochemical studies in the tumors revealed decreased intratumoral microvessel density. These results suggest that the traditional Chinese medicine therapy using *S. barbata*, which exerts anti-angiogenic activities, represents a promising strategy for the treatment of tumors.

Keywords: Traditional Chinese Medicine; *Scutellaria Barbata*; HIF-1, Angiogenesis; Tumor

INTRODUCTION

Hypoxic microenvironment is a potential risk factor for tumor progression. Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that mediates responses to hypoxia by binding to a hypoxia-response element (HRE) present within target genes. The HIF-1 transcription factors are composed of oxygen-sensitive HIF-1α subunit and a constitutively expressed HIF-1β subunit. Several studies have demonstrated overexpression of HIF-1α in a variety of tumor types compared to the respective normal tissues, including brain, colon, breast, gastric, lung, skin, ovarian, prostate, and renal carcinomas (Zagzag et al., 2000; Lee et al., 2007). Several HIF-1 regulated genes in tumor cells are essential for tumor growth and metastasis. Therefore, it is important to develop novel therapeutic strategies that can inhibit HIF-1 activity in tumors.

Scutellaria barbata (*S. barbata*) is widely used as anti-inflammatory and anti-tumor agent. Extracts from *Scutellaria barbata* inhibit the tumor growth including leukemia, colon cancer and hepatoma (Yu et al., 2007). In this study, we suggested that the extracts of *S. barbata* decreased the transcriptional activity of HIF under hypoxic condition. Meanwhile, the extracts of *S. barbata* can inhibit the migration and proliferation of endothelial cell. Our results suggest that the anti-angiogenic activity of *S. barbata* may have potential therapeutic value for cancer therapy.

MATERIAL AND METHODS

Herbal extract, Plasmid construction and lentivirus

Dimethyl sulfoxide (DMSO) and luteolin were purchased from Sigma (Sigma, St. Louis, MO). Crude *Lonicera japonica (L. japonica)*, *Glycyrrhiza glabra (G. glabra)*, and *S. barbata* powders obtained from Chuang Song Zong Pharmaceutical Co. (Kaohsiung, Taiwan) was dissolved in 70% pyrogen-free ethanol at a concentration of 320 mg/mL. The mixture was then vortexed rigorously for 2 min followed by vigorous shaking for 1 h at 37 °C. After being centrifuged at 3000*g* for 10 min, the extract was collected and stored at −20 °C. The extract (1 mL) was lyophilized to dryness, weighed, and redissolved to 1 mL of 70% ethanol. All concentrations used in this study were based on the dry weight of extract. The luteolin component was dissolved in DMSO as the stock (10 mM) and stored at −20 °C until use. The 24-bp HRE (5'-CAC ACG TGG GTT CCC GCA CGT CCG-3') of the human lactic dehydrogenase A gene was obtained by polymerase chain reaction, and 6 copies of this fragment were individually tandemly ligated to the 5'region of the CMV minimal (CMVmini) promoter derived from pTRE vector (Clontech, Palo Alto, CA) at the *Stu*I/*Eco*RI sites (Lee et al., 2007). To construct luciferase reporter plasmids, six copies of HRE ligated to the CMV minimal promoter (6×HRE/CMVmini) were excised from the pTRE-based plasmids by digestion with *Kpn*I and *Hin*dIII and

subcloned into pGL3 (Promega, Madison, WI) at the *Kpn*I/*Hin*dIII sites. To construct lentiviral vector, the fragment of 6×HRE/CMVmini-Luc was excised from pGL3by digestion *Kpn*I/*Bgl* II sites and subcloned into pMECA (Thomson et al., 1998). The fragment of 6×HRE/CMVmini-Luc was released from pMECA-based plasmid by digestion with *Cla* I/ *Swa* I sites and cloned into pWPXL (Shiau et al., 2010). To produce recombinant lentiviruses encoding luciferase, 293T cells were transfected with pWPXL-6×HRE/CMVmini-Luc, together with psPAX2 packaging vector and pMD2.G envelope vector by calcium phosphate precipitation, and the conditioned medium containing viral particles was harvested 48 h after transfection (Shiau et al., 2010). For transduction of cells (CL1-5, H1299 and LL2) with the luciferase gene, cells were infected with recombinant lentiviruses carrying *luciferase* gene under the control of 6×HRE responsive elements. As the lentiviral vectors did not contain selectable markers, luciferase-expressing stable cell clones were identified by monitoring luciferase expression in each isolated clone. Stable clones with high level luciferase expression were used for further studies. Constitutively active AKT plasmid was kindly provided by Dr. Chiau-Yuang Tsai (Department of molecular immunology, Osaka University).

Cell lines and mice

Human CL1-5 lung adenocarcinoma, human HEL299 embryonic lung, human 293T

and murine LL2 Lewis lung carcinoma (ATCC, Manassas, VA) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum at 37℃ in 5% CO_2. Human HMEC-1 microvascular endothelial cells were cultured in EGM endothelial growth medium containing 30 mg/ml bovine brain extract, 10 ng/ml human EGF, 1 μg/ml hydrocortisone, 2% FBS, 50 μg/ml gentamicin and amphotericin B (Cambrex, East Rutherford, NJ). Hypoxic conditions were generated with a GasPak chamber and anaerobic system envelope with palladium catalyst (BBL Gas Pak Plus, Becton Dickinson, Cockeysville, MD) (Lee et al., 2007). Cells were cultured under hypoxic conditions for 16 h. Male C57BL/6 mice at the age of 6~8 weeks were obtained from the Laboratory Animal Center of the National Cheng Kung University. The animals were maintained in specific pathogen-free animal care facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan, and was approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University.

Analysis of hypoxia-inducible transcriptional activities

Cell lysates were harvested and assessed for their luciferase activities determined by a luciferase reporter gene assay system (Applied Biosystem, Foster City, CA) using a luminometer (Minilumate LB9506, Bad Wildbad, Germany). Relative luciferase

activity was measured per microgram protein. The protein content in each sample was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). An index of hypoxic responsiveness, the hypoxia/normoxia ratio was determined by a comparison of the luciferase activity for the cells treated under hypoxic conditions with that for the aerobic control. Stable clones with high level luciferase expression were transfecting control or constitutively active AKT plasmids. The luciferase activity was assessed by luminometer as previously described.

Assay of endothelial migration and proliferation

HMEC-1 cells (2×10^3/well) were cultured in 96-well plates overnight. The culture medium was then removed and replaced with *S. barbata*-contained medium. After 48 h, cell proliferation was assessed by the colorimetric WST-1 assay (Dojindo Labs, Tokyo, Japan) according to the manufacturer's instructions. Cell migration was assessed using a modified Boyden Chamber (Corning Costar, Cambridge, MA). The membrane was coated with gelatin (100 ng/ml) for migration assay. HMEC-1 (1×10^4 cells/well) treated with *S. barbata* were added to the upper compartment and incubated for 4 h. The filter was then fixed with 100% methanol for 8 minutes and stained by Giemsa stain solution for 1 h. Cells were counted randomly three images per well under a microscope (Shiau et al., 2010).

Assessment of vascular endothelial growth factor (VEGF) expression

The levels of mouse and human VEGF in supernatant of cells after *S. barbata* administration were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). The levels of VEGF mRNA in liver and tumor were determined by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated and reversed transcribed into cDNA using standard methods. The specific primer pairs used for detecting human VEGF, and β-actin were 5'-CTG CTG TCT TGG GTG CAT TG, 5'-CAC GCC TCG GCT TGT CAC AT, and 5'-GCA CTG CCG CAT CCT CT as well as 5'-GTA CAG GTC TTT GCG GAT GTC C, respectively.

Western blot analysis

For detection of HIF-1α induction in cancer cells in response to hypoxia, cells treated with *S. barbata* were harvested. Proteins from total cell extracts were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont), and probed with anti-mouse HIF-1α antibody (H1alpha67, Novus Biologicals, Littleton, Co), rabbit anti-Akt (9272, Cell signaling Technology, Danvers, MA), rabbit-anti-phospho-Akt (9721s, Cell Signaling Technology) or mouse anti-β-actin monoclonal antibody (NB100-449, Sigma). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Jackson, West Grove, PA) were used as secondary antibodies, and

protein-antibody complexes were visualized by enhanced chemiluminescence system (Amersham).

Experimental lung metastatic models

In the experimental metastatic model, groups of 4 C57BL/6 mice receiving 5×10^4 of LL2 cells via tail vein injection at day 0 were intraperitoneally injected with 500 mg/kg of the extracts of *S. barbata*, solvent or with PBS daily for 22 consecutive days. Moreover, lungs from the tumor-bearing mice treated with *S. barbata*, solvent or PBS were collected and weighted at day 23. To analyze microvessel density in the tumor sites, tumor-bearing mice were injected intraperitoneally with *S. barbata*, solvent, or PBS at day 1. The whole tumors were excised and snap frozen at day 23. Tumor angiogenesis was assessed by immunostaining as previously described (Shiau et al., 2010).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± SD of triplicate determinations. The unpaired, two-tailed Student's *t* test was used to determine differences between groups for the comparisons of cell survival, luciferase activity, and lung weight. Any *P* value less than 0.05 is regarded as statistically significant.

RESULTS

S. barbata decreased the transcriptional activity of HIF-1α

L. japonica has been known as an anti-inflammatory tradition al Chinese herb and is used constantly for upper respiratory tract infections. *L. japonica* has a spectrum of biological activities especially with anti-oxidative and anti-inflammatory properties. *L. japonica* was taken to treat the wind-heat, epidemic febrile diseases, sores, carbuncles and some infectious diseases. *G. glabra* is a common Chinese medicinal herb with antitumor activity. *G. glabra* has shown various pharmacological activities, including anti-tumor, anti-parasitic, and anti-oxidative effects. Some components in *G. glabra* have been shown to induce cell cycle arrest or apoptosis in cancer cells. *S. barbata* is also widely used as anti-inflammatory and anti-tumor agent. Herein, we used the three tradition Chinese to examine their anti-angiogenic activity. First, we evaluated the potential cytotoxic effects of various traditional Chinese medicines (*L. japonica*, *G. glabra* and *S. barbata*) in the range of 0.1 mg/ml~3 mg/ml by using proliferation assay. The half maximal inhibitory concentrations (IC50) of *L. japonica*, *G. glabra* and *S. barbata* were 1.2759 mg/ml, 1.2730 mg/ml and 1.9457 mg/ml, respectively. At concentration up to 0.8 mg/ml, these traditional Chinese medicines showed no cytotoxic effects on CL1-5 treated for 24 h. Therefore, we performed the following experiments using concentration less than or equal to 0.8 mg/ml. Next, we constructed

lentiviral vectors carrying luciferase reporter gene under the control of CMVmini promoter combined with 6 copies of the HRE derived from the human lactic dehydrogenase A gene. By using lentiviral transduction, we established the stable cells (CL1-5, LL2 and HEL299) carrying luciferase reporter gene under the control of CMVmini promoter combined with 6 copies of the HRE as a screening platform. We examined the effect of the traditional Chinese medicines on the hypoxia-inducible gene expression by the luciferase reporter assay (Fig 1). An index of hypoxic responsiveness, the hypoxia/normoxia ratio was determined by a comparison of the luciferase activity for the cells treated with traditional Chinese medicines under hypoxic conditions with that for the aerobic control. The extent of hypoxic responsiveness of various traditional Chinese medicines in lung cancer cells varied, ranging from 2.5 to 5 folds. The ratio of luciferase activity in cells was higher in *L. japonica* and *G. glabra* treatment than that in *S. barbata* treatment (Fig.1). This result points out that *L. japonica* and *G. glabra* had less activities to influence the transcriptional activity of HIF-1 under hypoxic condition .As shown in Figure 1, *S. barbata* significantly inhibited the transcriptional activity of HIF-1α under hypoxic condition.

Responsiveness of HRE to hypoxia in cells treated with *S. barbata*

Because *S. barbata* significantly inhibited the HIF-1 transcriptional activity under

hypoxic condition, we used *S. barbata* to treat various cells whether it had the same activity to decrease the transcriptional activity of HIF under hypoxic condition. The extent of hypoxic responsiveness of *S. barbata* treatment in different cells varied, ranging from 1 to 1.8 folds (Fig. 2). *S. barbata* significantly reduced the transcriptional activity of HIF-1 in human and mouse lung tumor cells (Fig. 2A and B). The phenomenon was not observed in control cells (HEL299) (Fig. 2C). Taken together, these results suggest that *S. barbata* inhibited the transcriptional activity of HIF-1 in tumor cells but not in normal cells.

S. barbata reduces hypoxia-induced VEGF expression

Since hypoxia and HIF-1α transactivate VEGF, a growth factor which can induce angiogenesis in tumor growth (Koshikawa et al., 2000; Lee et al., 2008) VEGF expression in the tumor cells treated with *S. barbata* was measured. As shown in Figure 3A, the mRNA of VEGF was induced by hypoxic condition. The mRNA levels of VEGF were dramatically decreased in CL1-5 cells after *S. barbata* treatment. Figure 3A shows that VEGF mRNA was overexpressed in control cells, but almost undetectable in *S. barbata*-treated cells. In the protein levels, VEGF increased 1.5-fold under hypoxic condition. The levels of VEGF were significantly reduced in the tumor cells after *S. barbata* treatment regardless of oxygen condition (Fig 3 B and

C). Collectively, treatment of *S. barbata* decreases transcriptional activity of HIF-1α and inhibits the expression of VEGF gene in response to hypoxia in tumor cells.

S. barbata inhibited the migration and proliferation of endothelial cells

The HMEC-1 cells treated with *S. barbata* were tested for their ability to inhibit the proliferation and migration of endothelial cells. Figure 4A demonstrates that migration of HMEC-1 cells was dramatically decreased upon addition of *S. barbata* compared with that from control groups. Meanwhile, *S. barbata* dramatically reduced hypoxia-induced HMEC-1 proliferation (Fig. 4B). Taken together, these observations suggested that *S. barbata* inhibited the proliferation and migration of endothelial cells.

S. barbata reduced hypoxia-induced HIF-1α expression by inactivation of AKT

HIF-1 α protein level were suppressed by inhibitors of phosphatidylinositol 3-kinase (PI3K), suggesting that the PI3K/AKT pathways are involved in a translational increase in HIF-1α (Ban et al., 2010). The loss of tumor suppressors, such as von Hippel-Lindau (pVHL), PTEN or p53, and constitutive activation of AKT have been implicated in promoting HIF-1α-mediated gene expression (Krieg et al., 2000; Semenza et al., 1999; Yoo et al., 2003). As shown in Figure 5 *S. barbata* reduced AKT phosphorylation in CL1-5 cells induced by hypoxic condition *in vitro*. The luteolin may be the bioactive compound in the extract of *S. barbata* (Yu et al., 2007).

We found that luteolin inhibits HIF-1α expression by reducing AKT phosphorylation (Fig. 5B). The transcriptional activity of HIF-1 was induced by transfecting constitutively active AKT plasmid. Suppressive effect of Luteolin and *S. barbata* on the transcriptional activity of HIF-1 was relieved by transfecting constitutively active AKT (Fig. 5C). Our results suggest that AKT is required for tumor growth and angiogenesis through HIF-1α and VEGF expression.

Inhibition of tumor growth by *S. barbata*

Because inhibition of metastatic tumor growth is still a major challenge for tumor treatment, we next investigated whether *S. barbata* inhibited established pulmonary tumor nodules. Mice were injected intravenously with LL2 cells at day 0 followed by intraperitoneal treatment with *S. barbata* (500 mg/kg), solvent, or PBS for 22 consecutive days and killed at day 23 for determining wet lung weight. While solvent treatment did not exert any antitumor effect on mice bearing tumor, the wet lung weight of tumor-bearing mice treated with *S. barbata* was decreased by 56.4% compared with that treated with solvent (Fig. 6A). Twenty-three days after systemic treatment of mice bearing metastatic tumor with *S. barbata*, solvent or PBS, microvessel density within the tumors was analyzed by immunohistochemistry. Fewer microvessels were observed in the tumors from *S. barbata*-treated mice than those from solvent- or PBS-treated mice, whereas no difference was found between solvent-

and PBS-treated groups (Fig. 6B). As shown in Figure 6C, the number of factor VIII-positive intratumoral microvessels in *S. barbata*-treated mice was reduced by more than 29% compared with that in mice treated with solvent or PBS. Altogether, these results indicate that systemic delivery of *S. barbata* can delay tumor burden in the lungs and reduced the capillary density.

DISCUSSION

Hypoxia is an abnormal physiological condition that arises from the rapid growth of tumor relative to its vascular supply (Brown and Giaccia 1998). Tumor hypoxia is associated with resistance to radiation and chemotherapy, which may affect treatment outcome (Frederiksen et al., 2003). Low oxygen conditions initiate a cascade of physiological responses resulting from the induction of genes involved in glycolysis, erythropoiesis, and angiogenesis (Semenza et al., 1996). Hypoxia in the tumor microenvironment can accelerate malignant progression and metastasis (Graebe et al., 1996) Therefore, it is important to develop novel therapeutic strategies that can specifically target hypoxic areas of tumors. Chinese herbal medicine has been used for the treatments of various diseases for years. However, it is often difficult to analyze their biological activities and molecule mechanisms because of their complex nature (Chen et al., 2010). In this study, we applied the HIF-1 transcriptional activity of

platform to analyze the antitumor activity induced by herbal formulae. *S. barbata* has been explored the possibility of treatment of inflammation, tumor and gynecological diseases in China (Dai et al., 2008). Previously, the Chinese traditional medicines has been described specifically induce apoptosis in various cancer cells and immuno-modulating activity. In this study, we further evaluated the anti-metastatic, anti-angiogenic and anti-tumor activities of *S. barbata* with a series of *in vitro* and *in vivo* experiments. Our observations point out that *S. barbata* not only could inhibit the migration and proliferation of endothelial cells but also could inhibit tumor cells to secrete VEGF under hypoxia by decreasing HIF-1α expression.

This study indicates that several activities of *S. barbata* may account for its inhibitory effect on tumor metastasis. *S. barbata* could inhibit the expression of HIF-1α, a transcription factor that promotes metastasis by inducing the metastasis-related gene in tumor cells under hypoxic condition. Furthermore, *S. barbata* inhibit the proliferation and migration of endothelial cells. With regard to the effect of *S. barbata* on tumor cells, *S. barbata* decrease the expression of HIF-1 α and the secretion of VEGF in tumor cells. The blocking of HIF-1 α and VEGF expression in tumor cells can thus lead to inhibit the angiogenesis in tumor microenvironment. These results suggest that *S. barbata* has multiple anti-tumor activities. This finding is similar to that reported earlier for the antitumor activities of Chinese herbal cocktail

Tien-Hsien liquid (Chia et al., 2010).

The expression of VEGF mRNA and protein were not significant compared with the transcriptional activity of HIF-1 α under hypoxic condition. Previously, we constructed retroviral vectors carrying luciferase reporter gene under the control of CMVmini promoter alone or combined with 3, 6, or 10 copies of the HRE. Meanwhile, we examined the effect of increasing HRE copy number on the hypoxia-inducible gene expression by the luciferase reporter assay. However, reporter constructs carrying 6 copies of HRE conferred higher hypoxic responsiveness than those carrying 3 copies of HRE. However, a saturation effect was observed for vectors with 6 copies of HRE. Thus, 6 copies of HRE upstream of the CMV basal promoter resulted in maximal hypoxia-inducible gene expression (Lee et al., 2007). The promoter activity of six copies of HRE ligated to the CMVmini promoter was 36.35% compared with the activity of the intact CMV promoter. Although hypoxic condition can induce the expression of VEGF, its promoter only has a single HRE that located at nucleotide positions -947 to -939 relative to the transcription start site (Olenyuk et al., 2004). The copy number of HRE in promoter may result the different gene expression under the same condition. Herein, we used the selective platform to screen tradition Chinese medicines as anti-angiogenic agent.

In conclusions, it will be of interest to identify the active chemical compounds in the

extract of *S. barbata* that target the HIF -1 α pathway. Our results also indicate that luteolin which was the major compound in *S. barbata* could inhibit the protein expression of HIF-1α (Fig. 5). By taking advantages of the anti-angiogenic effects of *S. barbata*, the traditional Chinese medicine appears to hold promise for the treatment of tumors. Our findings suggest that *S. barbata* is a novel HIF-1α inhibitor with antitumor potential.

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FIGURE LEGENDS

Fig. 1. The traditional Chinese medicines induced hypoxia-inducible transcriptional activities in lung cancer cell lines.CL1-5 cells expressed luciferase under the HRE control were treated with *L. japonica*(0.8 mg/ml), *G. glabra* (0.8 mg/ml), *S. barbata* (0.8 mg/ml), solvent, or PBS for 16 h under hypoxic condition. Ratio of luciferase activity was defined as the relative luciferase activity in hypoxia compared with that in normoxia. Data shown were the mean ± SD (n=4). *, $P<0.05$; ***, $P<0.001$.

Fig. 2. *S. barbata* induced hypoxia-inducible transcriptional activities in various cell lines. (A) CL1-5 and (B) LL2, as well as (C) HEL299 cells were treated with *S. barbata* (0.8 mg /ml), solvent, or PBS under hypoxic condition for 16 h. Ratio of luciferase activity was defined as the relative luciferase activity in hypoxia compared with that in normoxia. Data shown were the mean ± SD (n=4). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig. 3. *S. barbata* reduced the expression of VEGF in various cell lines. (A) VEGF mRNA expressions in CL1-5 cells were determined by RT-PCR. VEGF levels in (B) CL1-5 and (C) LL2 cells treated with S. barbata (0.8 mg/ml) were measured by ELISA. Data shown were the mean ± SD (n=4). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. N: normoxia; H: hypoxia.

Fig. 4. *S. barbata* reduced the migration and proliferation of endothelial cells. The HEMC-1 cells treated with *S. barbata* (0.8 mg/ml) were examined the migration and proliferation activity. (A) Migration of HMEC-1 cells was examined by using a 48-well Boyden chemotaxis chamber with membranes coated with gelatin. (B) Cell viability was assessed by the WST-1 assay. Data shown were the mean ± SD (n=6-8). *, $P<0.05$; ***, $P<0.001$.

Fig. 5. Effect of *S. barbata* on AKT phosphorylation and HIF-1 α expression.CL1-5 cells treated with (A) *S. barbata* (0.8 mg/ml) or (B) luteolin (100μM) for 16h. Total cellular protein extracts were prepared and Western blot analyses were performed using antibodies specific for total AKT, pAKT, and HIF-1α. (C) The stable clone CL1-5 cells transfected control or constitutively active AKT plasmids were treated with *S. barbata* (0.8 mg/ml), luteolin (100 μM) solvent, or PBS for 16 h under hypoxic condition. Ratio of luciferase activity was defined as the relative luciferase activity in hypoxia compared with that in normoxia. Data shown were the mean ± SD (n=4). N: normoxia; H: hypoxia.

Fig. 6. *S. barbata* retarded tumor growth and reduced the vessel density.

Groups of mice were inoculated with LL2 cells via tail vein at day 0. *S. barbata* (500

mg/kg), solvent or PBS were treated with tumor-bearing mice daily for 22 consecutive days. Mice were sacrificed at day 23, and their lungs were weighed (A). (B) Tumors were excised at day 23, snap frozen and immunostaining with rabbit antibody against factor VIII-related antigen (×200). (C) Intratumoral microvessel density was determined by averaging the number of vessels in three areas of highest vessel density at × 400 magnification in each section. Data shown were the mean ± SD (n=4). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

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