African Journal of Pharmacy and Pharmacology Vol. 5(24), pp. 2647-2654, 29 December, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.266
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Anti-angiogenic effect of *Spica prunellae* extract *in vivo* and *in vitro*

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Accepted 15 December, 2011

Angiogenesis is a fundamental component of cancer growth and metastasis; therefore, inhibition of angiogenesis has become a promising strategy for the discovery and development of cancer chemotherapeutics. Recently, natural products have attracted significant interest as therapeutic agents for cancer since they have relatively fewer side effects as compared to modern chemotherapeutics and have long been used clinically to treat a variety of diseases, including cancer. *Spica prunellae* is an important component in several traditional Chinese medicine (TCM) formulations for cancer treatment. Recently, we reported that *S. prunellae* inhibits cancer cell growth through the induction of mitochondrion-dependent apoptosis. However, the precise mechanisms of its overall anti-cancer activity remain largely unknown. In the present study, we investigated the anti-angiogenic effects of the ethanol extract of *S. prunellae* (EESP) *in vitro* on human umbilical vein endothelial cells (HUVECs) and *in vivo* using the chick embryo chorioallantoic membrane (CAM) assay. We found that EESP inhibited the proliferation of HUVECs via blockade of cell cycle G1 to S progression. In addition, EESP inhibited the migration and tube formation of HUVECs. Moreover, EESP treatment decreased VEGF-A expression in HT-29 human colon carcinoma cells as well as the expression of VEGF-A and VEGFR-2 in HUVECs. Furthermore, EESP exposure reduced the formation of blood vessels in chick embryos. These results suggest that the inhibition of angiogenesis is one of the mechanisms by which *S. prunellae* can mediate an anti-cancer effect.

Key words: *Spica prunellae*, angiogenesis, cancer treatment, herbal medicine.

INTRODUCTION

Both drug resistance and toxicity on normal cells limit the efficacy of current cancer chemotherapies (Gorlick and Bertino, 1999; Boose and Stopper, 2000; Longley et al., 2006), thus increasing the necessity for the development of new therapeutic approaches. Natural products, including traditional Chinese medicines (TCMs), have relatively few side effects and have been used clinically for thousands of years as important alternative remedies for a variety of diseases including cancer (Tang and Eisenbrand, 1992; Huang, 1999; Gordaliza, 2007; Ji et al., 2009; Zhao et al., 2010). Therefore, interest in the use

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natural products as therapeutic agents for cancer has recently increased. *Spica prunellae*, the fruit-spikes of the perennial plant *Prunella vulgaris* L., is a well-known Chinese folk medicinal herb with traditional properties of heat-clearing and detoxification (Pharmacopoeia of the People's Republic of China, 2010). According to TCM theory, accumulation of heat and toxic dampness is a major causative factor in the pathogenesis of cancer. Therefore, *S. prunellae* has been used as a major component in TCM formulations designed for the treatment of several types of cancer (Liu et al., 2003; Sun et al., 2003; Wang and Zhang, 2010). Previous studies by our group and others demonstrated that extracts of *S. prunellae* inhibit the growth and induce apoptosis of several cancer cell types (Wang et al., 2000; Zhang et al., 2005, 2006; Ma et al., 2006; Du et al., 2009; Zheng et al., 2011), however, the precise mechanism of its anti-cancer activity remains to be further elucidated.

Angiogenesis involves the growth of new blood vessels from the pre-existing vasculature and plays an important role in a wide range of physiological processes, such as wound healing, reproduction and embryonic development (Folkman and Shing, 1992). However, the angiogenic process is also essential for continued growth of the tumor and provides an avenue for hematogenous metastasis (Folkman, 1971, 1986, 1995, 2006; Jain, 1987; Cook and Figg, 2010). The induction of angiogenesis is mediated by a variety of molecules secreted from the cells within the tumor (Weidner et al., 1991; Breier and Risau, 1996; Stromblad and Cheresh, 1996). Vascular endothelial growth factor A (VEGF-A) is one of the most effective biologic inducers of angiogenesis (Risau, 1997; Jain, 2002; Ferrara, 2002). VEGF-A is highly expressed in and secreted from a wide variety of human cancers and is associated with cancer progression, invasion and metastasis, and poor patient prognosis (Maeda et al., 1996; Kaya et al., 2000; Ferrara et al., 2003).

In order to extend the clinical observations of the potential anti-cancer effect of *S. prunellae* and help to establish a scientific foundation for further research, in this study we evaluated the effect of ethanol extract of *S. prunellae* (EESP) on angiogenesis *in vitro* and *in vivo*, and investigated the underlying molecular mechanisms.

MATERIALS AND METHODS

Reagents

Roswell Park Memorial Institute Medium 1640 (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and TriZol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Cell cycle assay kit was purchased from BD Biosciences (San Jose, 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). Human VEGF-A and VEGFR2 (KDR) ELISAs were obtained from R&D Systems (Minneapolis, MN, USA). All other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of ethanol extract from *S. prunellae* (EESP)

The fruiting spikes of *P. vulgaris (S. prunellae)* were collected in Hubei province, China, in July, 2008. EESP was prepared as described previously (Zheng et al., 2011). Stock solutions of EESP were prepared by dissolving the EESP powder in DMSO to a concentration of 200 mg/ml and were stored at -20°C. The working concentrations of EESP were made by diluting the stock solution in the cell culture medium. The final concentration of DMSO in the medium for all experiments was < 0.5%.

Cell culture

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

Evaluation of cell viability

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 1×10^4 cells/well in 0.1 ml medium. The cells were treated with various concentrations of EESP for 24 h. Treatment with 0.5% DMSO was included as the vehicle control. 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. The absorbance was measured at 570 nm using a microplate reader (BioTek, Model EXL800, USA).

Evaluation of cell confluency

HUVECs were seeded into 6-well plates at a density of 2×10^5 cells/well in 2 ml medium. The cells were treated with various concentrations of EESP for 24 h. Cell confluency was evaluated using a phase-contrast microscope (Olympus, Japan). Photographs were taken at a magnification of 200×.

Cell cycle analysis

After incubation with various concentrations of EESP for 24 h, HUVECs were harvested and adjusted to a concentration of 1×10^6 cells/ml. Cell cycle progression of HUVECs was determined by flow cytometry analysis with a propidium iodide (PI) staining cell cycle assay kit (BD Biosciences). Staining was performed according to the manufacturer's instructions. 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).

Wound-healing assay

HUVECs were seeded into 12-well plates at a density of 2×10^5 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

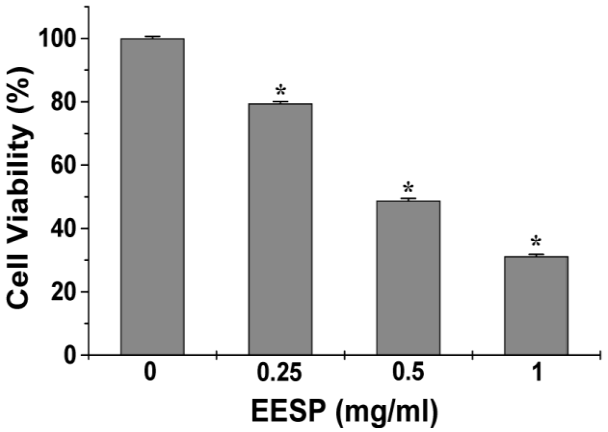

Figure 1. Effect of EESP on the HUVEC viability. Cells were treated with the indicated concentrations of EESP for 24 h. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%, treated with 0.5% DMSO vehicle). Data are means with S.D. (error bars) from at least three independent experiments. *P < 0.01, versus control cells.

randomly selected views along the scraped line were photographed on each well using phase-contrast inverted microscope at a magnification of 100×. Cells were then treated with various concentrations of EESP for 24 h, and another set of images were taken by the same method. A reduction in the scraped area is indicative of wound-healing and cell migration.

Capillary-like tube formation assay

The effect of EESP on tube formation by HUVECs was evaluated using a commercially available angiogenesis assay (*In Vitro* Angiogenesis Assay Kit, Millipore) following the manufacturer's instructions. Briefly, confluent HUVECs were harvested and diluted (1×10^4 cells) in 50 µl of medium, containing the indicated concentrations of EESP. The treated cells were seeded onto a solid gel of basement proteins (ECMatrix gel) within 96-well plates and incubated for 9 h at 37°C. Cellular morphorphology and the development of capillary tube networks were evaluated using phase-contrast inverted microscope. Photographs were taken at a magnification of 100×.

VEGF-A and VEGFR-2 RT-PCR analysis

2×10^5 HUVECs or HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESP for 24 h. Total RNA was isolated with TriZol Reagent (Invitrogen). Oligo(dT)-primed RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The obtained cDNA was used to determine the amount of VEGF-A or VEGFR-2 mRNA by PCR with Taq DNA polymerase (Fermentas) using the following primers: VEGF-A forward 5'-GCCTTGCCTTGCTGCTCTA-3' and reverse 5'-GATGTCCACCAGGGTCTCG-3'; VEGFR-2 forward 5'-ACGCCGATTATGTGAGA-3' and reverse 5'-AGGCAGGAGTTGAGTATGT-3'. GAPDH was used as an internal control (forward 5'-GTCATCCATGACAACTTTGG-3' and reverse 5'-GAGCTTGACAAAGTGGTCGT-3').

VEGF-A and VEGFR-2 ELISA

2×10^5 cells were seeded into 6-well plates in 2 ml medium and were treated with various concentrations of EESP for 24 h. Cell culture media were collected to measure the secretion level of VEGF-A in both HUVECs and HT-29 cells, whereas cell lysates were used to determine the protein expression of VEGFR-2 in HUVECs. Measurement was performed using Quantikine ELISA kits (R&D, USA) according to the manufacturer's instructions.

Chick chorioallantoic membrane (CAM) assay

A CAM assay was performed to determine the *in vivo* anti-angiogenic activity of EESP. Briefly, 10 µl of EESP (10 or 100 µg/µl) was loaded onto a 0.5 cm diameter Whatman filter paper. The filter was then applied to the CAM of a seven-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 in a circular perimeter surrounding the implants, at a distance of 0.25 cm from the edge of the filter was counted manually.

Statistical analysis

All data are the means of three determinations except for the CAM assays in which 10 determinations were made for each data point. The data were analyzed using the SPSS package for Windows (Version 11.5). Statistical analysis of the data was performed with the Student's t-test and analysis of variance (ANOVA). Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

EESP inhibits the proliferation of HUVECs

Endothelial cells have a robust capacity to proliferate and this characteristic is an essential component of angiogenesis. Therefore, we first evaluated the effect of EESP on the growth of HUVECs. HUVEC viability was determined after treatment with various concentrations of EESP for 24 h. As shown in Figure 1, treatment with 0.25 to 1 mg/ml of EESP for 24 h dose-dependently reduced cell viability by 20 to 70% as compared to untreated control cells ($P < 0.01$). To further verify these results, we evaluated the effect of EESP on HUVEC confluency via phase-contrast microscopy. As shown in Figure 2, EESP treatment led to a gradual decrease in confluency of the monolayer with an increase in drug concentration. Taken together, these data demonstrate that EESP inhibits the proliferation of HUVECs.

EESP blocks cell cycle progression of HUVECs

G1/S transition is one of the two main checkpoints used by cell to regulate the cell cycle progress and thus, the cell proliferation. Therefore, we investigated the effect of EESP on the G1 to S progression in HUVECs by FACS analysis. As shown in Figure 3, the percentage proportion of S phase cells following treatment with 0, 0.25, 0.5 and 1 mg/ml of EESP was 35.39 ± 0.2, 23.87 ± 0.7, $18.87 \pm$

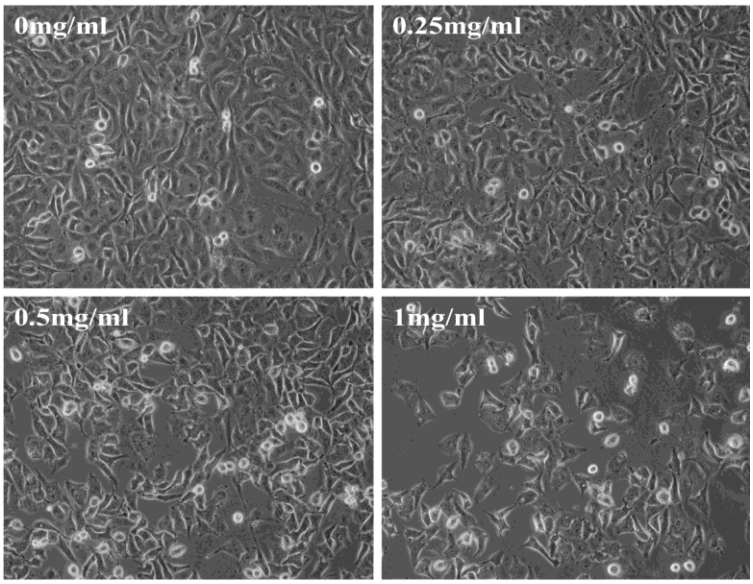

Figure 2. Effect of EESP on HUVEC confluency. Cells were treated with the indicated concentrations of EESP for 24 h and confluent changes were observed using phase-contrast microscopy. The photographs were taken at a magnification of 200×. Images are representative of three independent experiments.

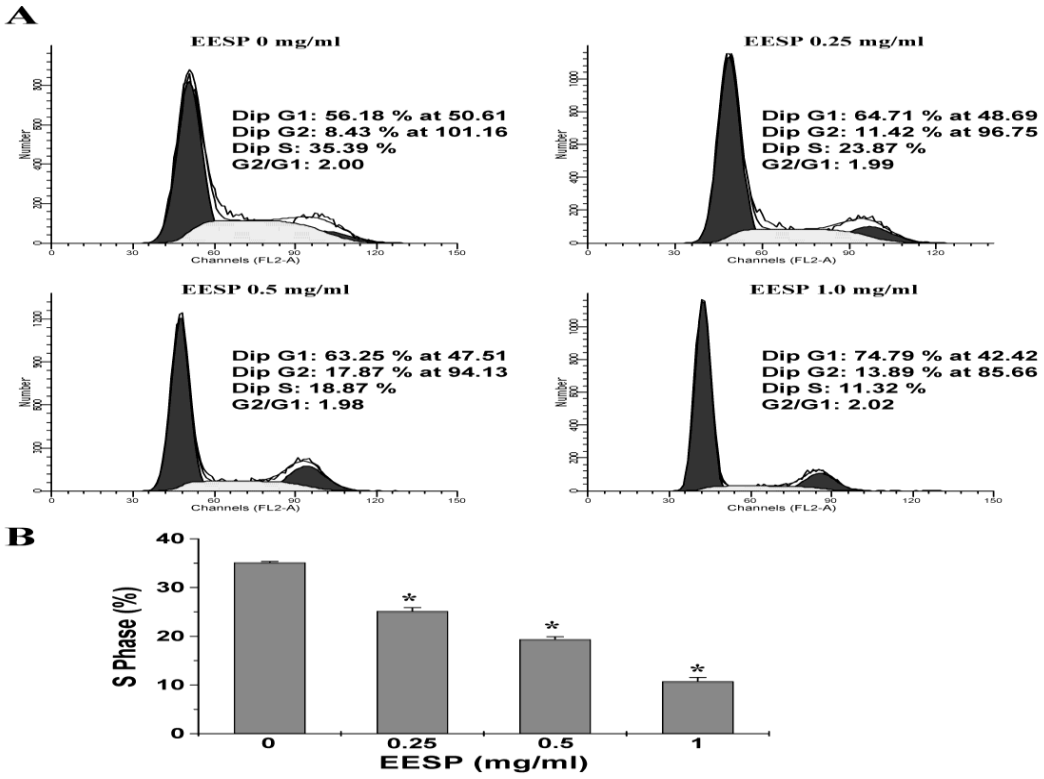

Figure 3. Effect of EESP on the HUVEC cell cycle progression. (A) Cells were treated with the indicated concentrations of EESP for 24 h, stained with PI, and analyzed by FACS. The proportion of cells in each phase of the cell cycle was calculated using ModfitLT Version 3.0 Software. Representative assays are shown for each concentration of EESP. (B) The percent of cells in S phase for each concentration of EESP were compared. Data shown are means with S.D. (error bars) from three independent experiments. *$P < 0.01$, versus control cells.

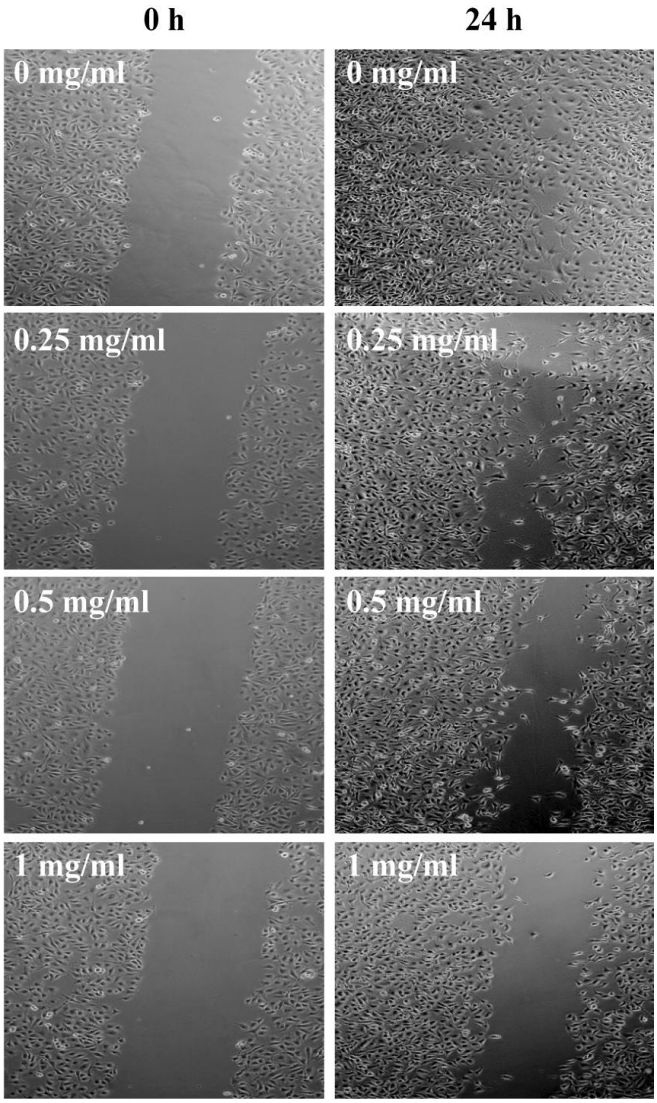

Figure 4. Effect of EESP on HUVEC migration. After treatment with the indicated concentrations of EESP for 24 h, the migration pattern of the HUVECs was observed using phase-contrast microscopy. The photographs were taken at a magnification of 100×. Images are representative of three independent experiments.

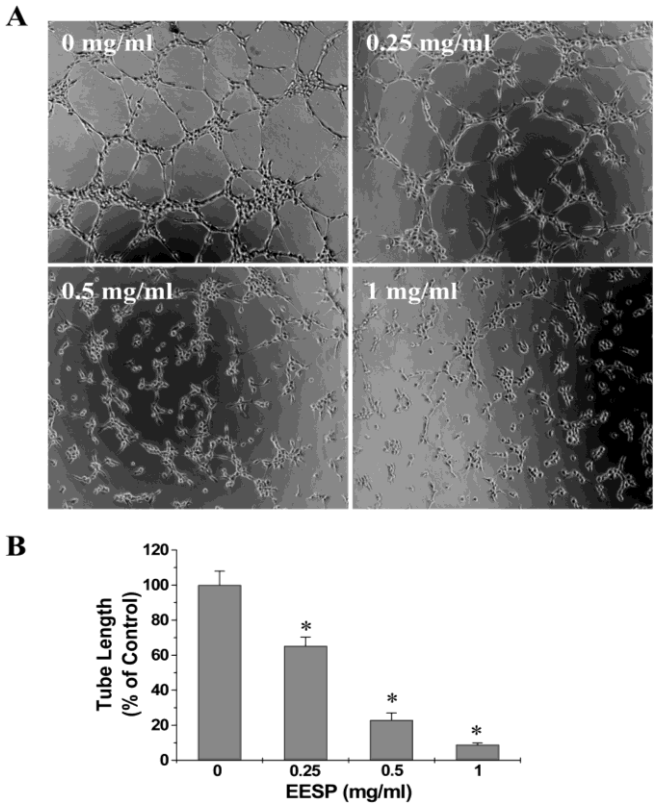

Figure 5. The effect of EESP on HUVEC tube formation. (A) HUVECs were harvested and suspended in medium containing various concentrations of EESP. The harvested cells were then seeded into basement protein (ECMatrix, Millipore) coated plates and incubated for 9 h at 37°C. The development network-like, tube structures were examined by phase-contrast microscopy. The photographs were taken at a magnification of 100×. Images are representative of three independent experiments. (B) The total length of the capillary-like tubes was measured and normalized to the untreated control. Data shown are means with S.D. (error bars) from three independent experiments. *P < 0.01, versus control cells.

0.5, and 11.32 ± 0.8%, respectively ($P < 0.01$), suggesting that EESP inhibits HUVEC proliferation by blocking the cell cycle G1 to S progression.

EESP inhibits HUVEC migration and tube formation

Angiogenesis consists of several features, including endothelial cell migration and alignment of these cells into capillary tubular structures. Therefore, we evaluated the effect of EESP on each of these processes. Endothelial cell migration was evaluated in a wound-healing assay. As demonstrated in Figure 4, 24 h post-wounding, untreated HUVECs migrated into the wounded (clear) area of the cell monolayer, whereas EESP treatment inhibited HUVEC migration dose-dependently. To test the effect of EESP on endothelial capillary tube formation, HUVECs were grown on a solid gel containing mouse basement proteins (ECMatrix, Millipore). This gel induces cultured endothelial cells to rapidly align and form hollow tube-like structures. As shown in Figure 5A and B, untreated HUVECs formed elongated tube-like structures. In contrast, EESP treatment resulted in a significant decrease in capillary tube formation in a dose-dependent fashion.

EESP suppresses the expression of VEGF-A and VEGFR-2

The process of cancer-associated angiogenesis is a highly

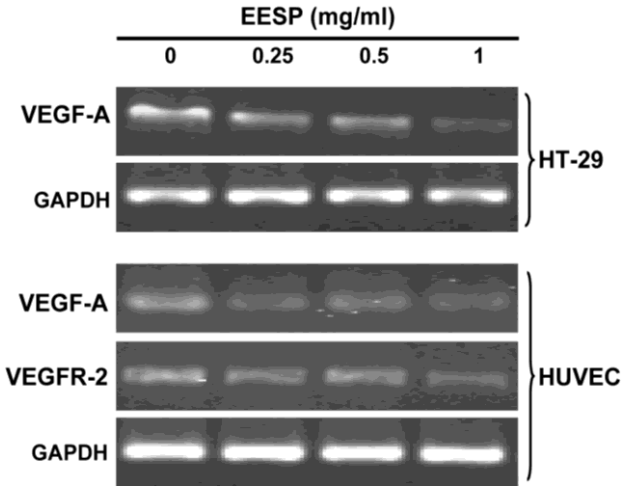

Figure 6. Effect of EESP on VEGF-A and VEGFR-2 mRNA expression. Cells were treated with indicated concentrations of EESP for 24 h. The mRNA levels of VEGF-A and VEGFR-2 were determined by RT-PCR in HUVECs and HT29 cells, as indicated. GAPDH was used as an internal control. Data are representative of three independent experiments.

regulated process involving several growth factors. VEGF-A, one of these growth factors, is secreted by cancer cells and endothelial cells. VEGF-A exerts its biologic effect primarily through the interaction with specific receptors present on the surface of vascular endothelial cells, such as VEGFR-2 (Ishigami et al., 1998; Ferrara, 2002; Ferrara et al., 2003). Binding of VEGF-A to VEGFR-2 triggers a tyrosine kinase signaling cascade, inducing endothelial cell proliferation, migration, survival, sprouting and eventually tube formation (Gille et al., 2001; Ferrara, 2002). To further investigate the underlying mechanism of EESP's anti-angiogenic activity, we examined the effect of EESP on VEGF-A expression and secretion in HUVECs and HT-29 human colon carcinoma cells and on the expression of VEGFR-2 in HUVECs. The results of the RT-PCR assay showed that EESP treatment dose-dependently reduced VEGF-A mRNA expression in both HT-29 cells and HUVECs, as well as suppressed VEGFR-2 mRNA expression in HUVECs (Figure 6). Moreover, the protein expression pattern of VEGF-A and VEGFR-2 followed the changes in the respective mRNA levels (Figure 7).

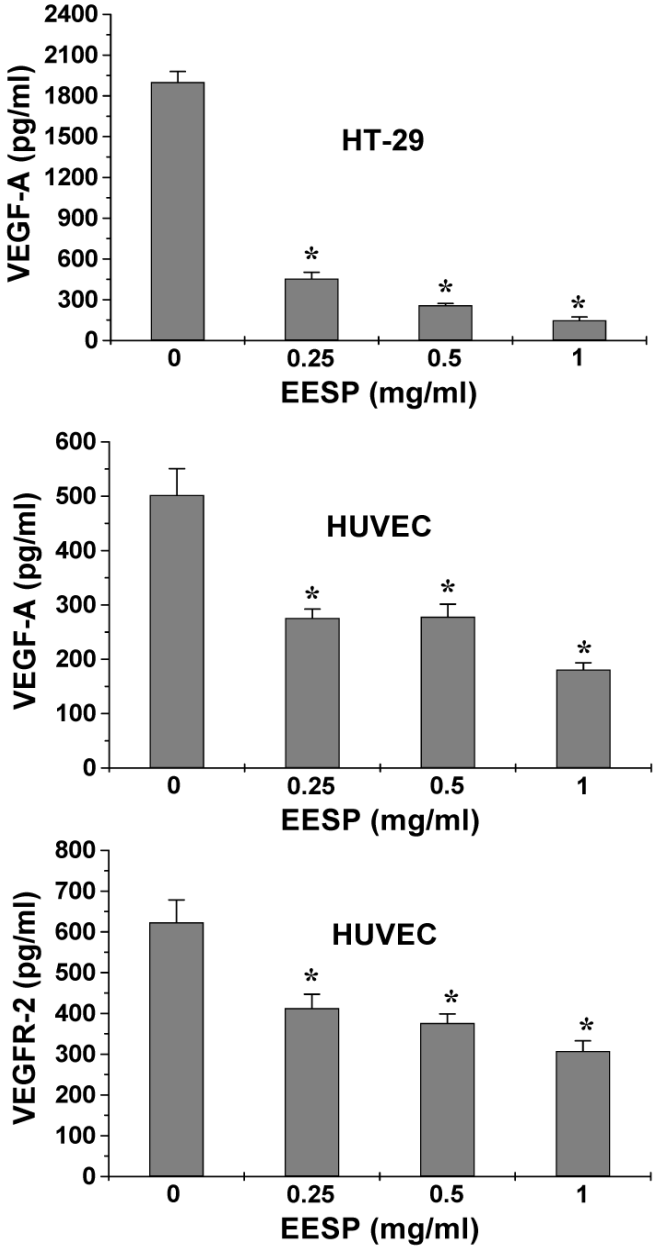

Figure 7. Effect of EESP on VEGF-A and VEGFR-2 protein expression. HUVECs and HT29 cells were treated with indicated concentrations of EESP for 24 h. The protein levels of VEGF-A (cell culture media, that is secreted) and VEGFR-2 (cell lysates) were determined by ELISA. Data are means with S.D. (error bars) from at least three independent experiments. *$P < 0.01$, versus control cells.

EESP inhibits angiogenesis *in vivo*

To further verify the aforementioned *in vitro* observations, we tested the anti-angiogenic effect of EESP *in vivo* using a classic chick chorioallantoic membrane (CAM) model. We observed that EESP treatment significantly reduced the total number of blood vessels in the chicken embryos in a dose-dependent fashion as compared to untreated control (Figure 8), indicating that EESP is able to suppress angiogenesis *in vivo*.

In conclusion, we report for the first time that the ethanol extract of EESP can inhibit angiogenesis, both *in vitro* and *in vivo.* Based on this present and our previous studies, it is clear that *S. prunellae* inhibits cancer growth via at least two distinct mechanisms: (1) the induction of mitochondrion-dependent apoptosis and (2) the inhibition of angiogenesis.

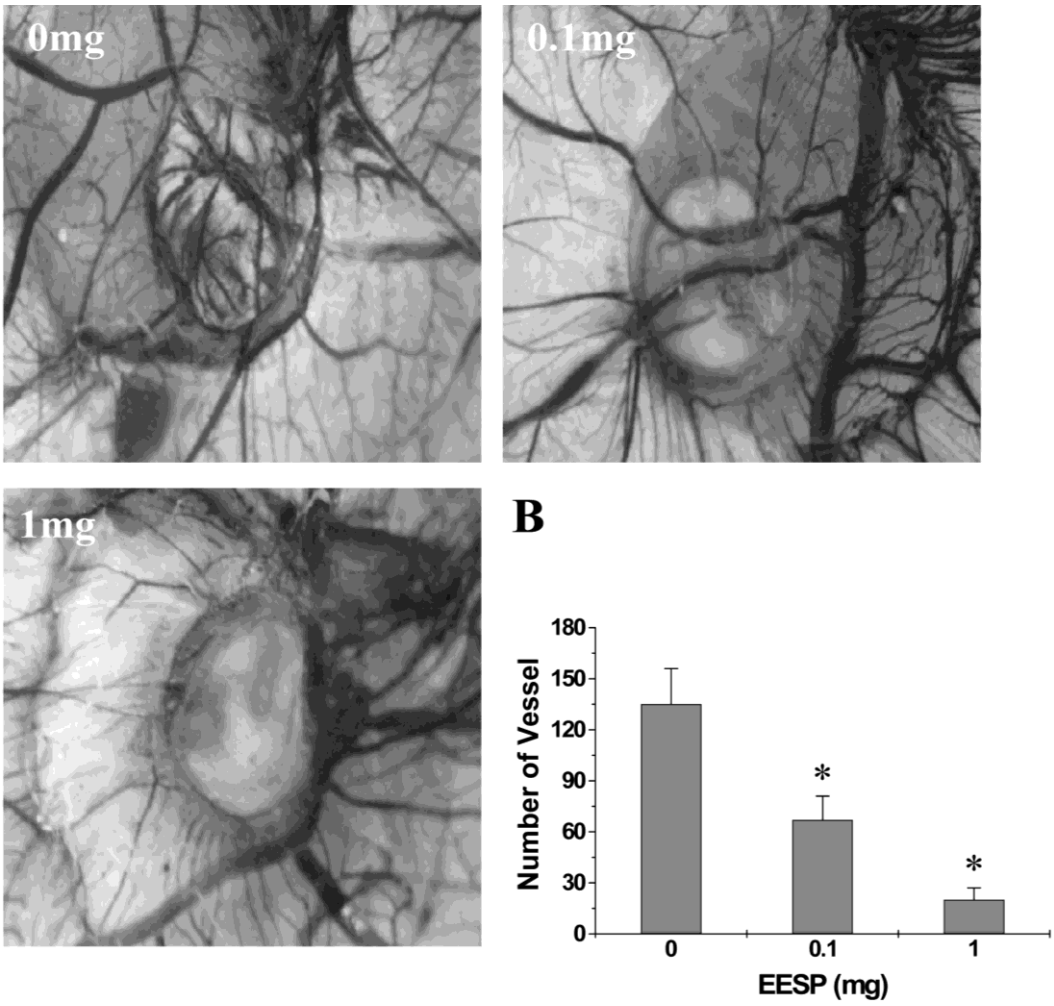

Figure 8. Effect of EESP on the angiogenesis *in vivo*. (A) A 0.5 cm-diameter filter paper loaded with 0, 0.1 and 1 mg of EESP was applied to the chick chorioallantoic membrane and incubated at 37°C for 72 h. Blood vessels surrounding the filter were photographed. Images are representative photographs of the results obtained at each dose of EESP. (B) The number of blood vessels was quantified manually in a circular perimeter 0.25 cm from the edge of the filter paper inserts. Assays were performed twice with a total of 10 eggs for each tested concentration of EESP. Data are means with S.D. (error bars). *P < 0.01, versus control.

ACKNOWLEDGEMENTS

This work was sponsored by the Natural Science Foundation of Fujian province of China (2009J01173), the Research Foundation of Education Bureau of Fujian province of China (JA10168), the National Natural Science Foundation of China (81073097) and the Developmental Fund of Chen Keji Integrative Medicine (CKJ 2010019).

Abbreviations: EESP, ethanol extract of *Spica prunellae*; **TCM,** traditional Chinese medicine; **CAM,** chorioallantoic membrane; **HUVEC,** human umbilical vein endothelial cell; **MTT,** 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMSO,** dimethyl sulfoxide.

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