Anti-angiogenic effects of total flavonoids from
Scutellaria barbata D. Don
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Abstract
Background: Angiogenesis is closely related to the growth, invasion and metastasis of the tumor, also considered as an anticancer therapy key target. Scutellaria barbata D. Don (S. barbata), a traditional Chinese medicine, is being used for treating many diseases including cancer. However, the antitumor molecular mechanism of S. barbata was still unclear. This study is aimed to...
investigate the inhibitory effect of total flavones from *S. barbata* (TF-SB) on angiogenesis.

**Methods:** Human umbilical vein endothelial cells (HUVECs) were treated with various concentrations of TF-SB. Cell viability was examined by MTT assay. The scratch assay was used to detect the migration of HUVECs after being processed by TF-SB. The ability of HUVECs forming network structures *in vitro* was demonstrated by tube formation assay. Chick embryo chorioallantoic membrane (CAM) assay was performed to detect the *in vivo* anti-angiogenic effect. The expression of VEGF was measured by ELISA assay.

**Results:** Our results showed that TF-SB inhibited the proliferation and migration of HUVECs in a dose dependent manner. At the same time, TF-SB significantly suppressed HUVECs angiogenesis *in vitro* and *in vivo*. Furthermore, VEGF were down regulated in both HUVECs and MHCC97-H cells after TF-SB treatment.

**Conclusion:** TF-SB could suppress the process of angiogenesis *in vitro* and *in vivo*. We presumed that TF-SB suppress angiogenesis in HUVECs by regulate VEGF. These data suggest that TF-SB may serve as a potent anti-angiogenic agent.

**Keywords:** *Scutellaria barbata*; Angiogenesis; Hepatocellular carcinoma; Human umbilical vein endothelial cells

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**Background**

The incidence of hepatocellular carcinoma (HCC) in worldwide is the rise trend. According to the global statistics each year, about seven million people died of liver cancer [1]. The HCC treatment selection depends on tumor heterogeneity, biological behhavior and liver function [2]. Chemotherapy is one of the main methods for the treatment of HCC. As the main treatment for HCC, chemotherapy eliminate cancer cells, however, they cause severe side-effects simultaneously. Moreover, many chemotherapy drugs often produce multiple drug resistance [3].
Therefore, it is very important to develop new anti-cancer pharmaceuticals from Chinese herbal medicine [4, 5]. Many herbs have been discovered of anti-tumor activity and become the main source of anti-cancer drug research [6].

Angiogenesis, the formation of new blood vessels from an existing vasculature, has been associated with the growth and dissemination of solid tumors [7]. Tumor angiogenesis is a complex process and including basal membrane injury, endothelial cells proliferate, migrate, and angiogenic factors activated, etc [8]. The expression of many cytokines have involved in this formation process, such as vascular endothelial growth factor (VEGF) and angiopoietin (Ang) [9-11]. The cytokines are important regulators in angiogenesis [12]. Therefore, the research on natural herbs is considered as new hope to inhibit vascular [13].

Scutellaria barbata D. Don (S. barbata) is a traditional Chinese medicine herb, which widely distributes in some areas of China and Korea. S. barbata orgined from China and Korea which was recorded for anti-inflammatory and anti-tumor effects. This herb has been used in clinics in treating inflammatory diseases and cancer. In recent years, the crude extracts of S. barbata have in vitro growth inhibitory effects on numerous human cancers including hepatoma, colon cancer, lung cancer, and breast cancer [14-18]. Also our previous study support that the extract of S. barbata is an potent inhibitor in hepatoma both in vitro and in vivo [19]. Currently, a large number of flavones, alkaloids, polysaccharides and steroids were separated from the S. barbata [20-24]. In the present study, we investigated the anti-angiogenic effect of total flavonoids of S. barbata (TF-SB) in HUVECs (human umbilical vein endothelial cells) and human hepatocellular carcinoma MHCC97-H cell line.

**Methods**

**Reagents**

Fetal bovine serum (Gibco BRL, Rockville, MD, USA); DMEM medium (Gibco, USA); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was
purchased by Sigma-Aldrich (St. Louis, MO, USA); Matrigel (BD Biosciences, San Jose, CA, USA); human VEGF ELISA kit was purchased from WuHan Boshide Biotechnology Co, Ltd. (WuHan, China).

**Preparation of TF-SB from Scutellaria barbata D. Don**

Dried plant materials of S. barbata were purchased from Yi Shan Tang Chinese Herbal medicine store (Xi’an, China) and authenticated according to the descriptions found in the Chinese Pharmacopoeia. The original herb was identified as Scutellaria barbata D. Don (SB) by Run-Xia Liu at Medical School of Xi’an Jiaotong University (Xi’an, China). The voucher samples, ZLK-ZY-05 (S. barbata) was deposited at the department of oncology, the Second Affiliated Hospital of Xi’an Jiaotong University.

The material was coarsely ground before extraction. A total of 300 g of the material was extracted twice with 95% ethanol for 3 h in 50 °C. The infusion was filtered through a 1-mm pore-size filter. The leftover on the filter was collected after evaporated. The crude extract was isolated by AB-8 macroporous adsorption resin column in which 70% aqueous ethanol was used to elute flavonoids. After treatment with AB-8 resin, the flavonoids purity increased with a recovery of 69%. The total flavonoids were stored at 4 °C for use.

The component of TF-SB was identified with high performance liquid chromatography (HPLC). As shown in Figure 1, there was a main peak in HPLC, which was identified as scutellarin (A). There were also some other flavonoids in TF-SB which were identified as apigenin (B), baicalein (C), luteolin (D). The contents of these four flavonoids were 67.2% (A), 8.7% (B), 4.6% (C) and 4.3% (D).

**Cell line and Cell culture**

MHCC97-H cells and HUVECs were purchased from the Liver Cancer Institute of Fudan University (Shanghai, China). The cells were grown in DMEM maximal
medium containing 10 % inactivated fetal bovine serum. Both cell lines were cultured at 37 °C in 5 % CO$_2$ under humidified environment.

**MTT assay for the cell viability of HUVEC cells**

Viability of HUVECs was assessed by the MTT assay. Cells were seeded into 96-well plates at the density of $1 \times 10^4$ cells /well. After 12 h, the cells were treated with TF-SB in different concentrations (0, 20, 40, 80 and 160 µg/mL) for 48 h or 72 h, respectively. MTT were applied to each well after treatment. The supernatant were removed after 4h incubation. Subsequently, DMSO were added to each. The supernatants were removed carefully and 150 µL of dimethyl sulfoxide (DMSO) were added to each well. The absorbance was measured at 490 nm through an Enzyme-labeling instrument (ELX800, Bio-Tek, Winooski, VT, USA). This assay was performed in triplicate. The results represented the average value of absorbance from three independent experiments done over multiple days.

**In vitro scratch assay**

We used the in vitro scratch assay to assess the activity of TF-SB on migration of HUVECs [25]. HUVECs were seeded in 12-well plates ($2 \times 10^5$ /well) with complete medium overnight to obtain a full confluent monolayer. After 24 h, the cells were scraped away vertically 24h later by pipette tip. Each well was washed twice with PBS to remove debris, and then further incubated for 24 h in serum-free DMEM medium with different concentrations of TF-SB (0, 40, 80 and 120 µg/mL). The distances between the 2 edges of the scratch were photographed on each well using inverted microscope at a magnification of 100× and analyzed quantitatively.

**Tube formation assay**

The ability of HUVECs forming network structures was tested by tube formation assay. As previously described [26], 96-well plates were plated with 50 µL matrigel and allowed to polymerize at 37 °C for 30 min. HUVECs were subsequently
seeded on the matrigel followed by addition of different concentrations of TF-SB (0, 40, 80 and 120 µg/mL) and incubation for 22 h at 37 °C. The tube-like structures were photographed on each well using a phase-contrast microscope (Olympus, Tokyo, Japan) at a magnification of 100 x. To quantify the results, we counted the number of branch points, in which at least 3 tubes joined.

*Chick chorioallantoic membrane (CAM) assay*

The CAM assay was performed as previously described [25]. 60 fertilized chicken eggs (14 ± 2 d) were purchased from Huxian chicken farm (Xi’an, China). The animals were housed and handled in strict accordance with the guidelines of the institutional and national Committees of Animal Use and Protection. The protocol was approved by the Committee on the Ethics of Animal Experiments of Xi’an Jiaotong University College of Medicine (Certificate No. 22-9601018). All efforts were made to minimize animals’ suffering and to keep the numbers of animals used to a minimum.

Briefly, the eggs were incubated at 37 °C in 40–60 % humidity for 96 h. And then, the eggs were randomly divided into four groups that were treated with different concentrations of TF-SB. After 7 days, a window (1×1.5 cm²) was opened in the shell to expose a part of the CAM. Different concentrations of TF-SB samples in 20 µL PBS was loaded onto sterilized gelatin sponges (2 mm²) that was then applied to the CAM. After 48 h of incubation, the neovascular numbers in the CAM around the sponges were photographed with an anatomical microscope (YZ20T4 type). The CAM of the sponge around were observed after hematoxylin and eosin (HE) staining [26]. The relationship between leukocyte infiltration and angiogenesis were analyzed quantitatively. Different concentrations of TF-SB samples in 20 µL PBS was loaded onto sterilized gelatin sponges (2 mm²) that was then applied to the CAM.

*Measurement of VEGF levels by ELISA*
We used ELISA assay to measure the variation of VEGF levels in MHCC97H cells and HUVECs. The supernatant was collected from different treatment groups. The VEGF level in MHCC97-H cells and HUVECs were measured by ELISA kit (Boshide) according to the manufacturer’s instructions. The each well was plated with 0.1 mL diluted samples in samples buffer and incubated 90 min at 37 °C. Next, 100 µL anti-human VEGF antibody was added and incubated for another 60 min. After washing with PBS for three times, 90 µL TMB color liquid was added in the dark for 30 min. And then, the absorbance was measured at 450 nm after TMB Stop Solution was applied. All measurements were performed for three times. The data represented average of absorbance value from three independent experiments.

Statistical analysis

Data were presented as Mean ± standard deviation (SD). Statistical analysis of the data were performed with Student's t-test, one-way analysis of variance (ANOVA) test and linear regression analysis using the Statistical Package for Social Sciences version 13.0 (SPSS Inc, Chicago, IL). p value < 0.05 was considered statistically significant.

Results

Effects of TF-SB on cell viability of HUVECs

S. barbata has been shown to be effective against a wide range of tumors. The extracts of S. barbata greatly could greatly inhibit the cell growth in lung cancer, leukemia, colon cancer, hepatoma, and skin cancer [15-18]. It was reported by Lee et al. that S. barbata decreased myometrial and leiomyomal cells proliferation which promoted by HCG [27].

In this study, MTT assay showed that cell viabilities of the TF-SB treated (20, 40, 80 and 160 µg /mL) groups were suppressed by 24.3±0.1, 30.9±1.5, 55.4±0.9 and 73.2±0.6% respectively after 48 h treatment (Figure 2). After treatment for 72 h, the
inhibition rate in the TF-SB treated groups were further reduced, the inhibitory rate of 160 \( \mu \text{g/mL} \) group was as high as \( (78.1\pm0.6)\% \). There were significantly difference between 48 h and 72 h in 20, 40, and 80 \( \mu \text{g/mL} \) groups. Together all, anti-proliferative effect of TF-SB on HUVECs was in a time- and dose-dependent manner.

**Effect of TF-SB on migration of HUVECs**

Endothelial cells migration is a necessary step in the process of angiogenesis [9]. In the present study, the effect of TF-SB on HUVECs migration was determined by the scratch assay. As shown in Figure 3, the cell migration in TF-SB groups were inhibited in various degree at 48h. The maximum inhibition was obtained in 120 group which was higher than control group \( (p<0.05) \) and 40 group \( (p<0.05) \). The cell migration of HUVECs were dose-dependently inhibited after treatment with TF-SB for 48 h.

**TF-SB inhibits HUVEC tube formation**

Tumor neovascularization is defined as the process of new blood vessel formation in solid neoplasms [28]. Activation of angiogenic pathways is required for tumor spreading, as well as for proliferation of metastatic cells in distant organs [29]. In a phase IB, multicenter clinical trial in America, Bezielle, an aqueous extract of *S. barbata*, was safe and showed promising clinical evidence of anticancer activity in this heavily pretreated population of women with metastatic breast cancer [30]. In a colorectal cancer mouse xenograft model, the ethanol extract of *S. barbata* could inhibit tumor angiogenesis via suppression of the SHH pathway [31].

As an essential step for angiogenesis, the formation of tube-like structures involves matrix degradation, rearrangement and apoptosis of endothelial cells. Therefore, the HUVECs angiogenesis was observed through tube formation assay. As shown in Figure 4, in the control group, the capillary tube structures were
observed in basal membrane after HUVECs was placed in the well. However, TF-SB treatment significantly reduced in tube-like structures formation, with a dose-dependent manner. There were few tube-like structures formed in 120 µg/mL TF-SB treated group.

Effect of TF-SB on angiogenesis in vivo

The CAM assay is widely used for both developmental and post-developmental studies of angiogenesis due to easy access to the vascularized CAM membrane [32, 33]. An additional advantage is that in many countries animal license is not compulsory for chicken embryo experimentation. The assay is rapid, inexpensive and suitable for large-scale screening potential angiogenesis regulators [34].

In this study, we used a CAM model to confirm the effect of TF-SB on angiogenesis in vivo. After treatment for 48h, the structure of blood vessels were observed with an anatomical microscope. As shown in Figure 5, normal vascular pattern with numerous branching was observed in the control group. The total number of blood vessels in TF-SB treatment significantly decreased compared with the control group ($p<0.05$). The results indicated that TF-SB could suppress angiogenesis in vivo.

The relationship between leukocyte infiltration count and formation of blood vessels

Infiltration of macrophages, lymphocytes, mast cells are often presented in tumors microenvironment that these cells may contribute to tumor progression. It is suggested that these inflammatory cells promoted neoplastic progression by stimulating tumor revascularization and related to tumor angiogenesis [35]. In the present study, the leukocyte infiltration count was observed surrounding sponge angiogenesis on the CAM after HE staining. As shown in Figure 6, the results showed that the leukocyte infiltration had no significant correlation with the number
of big blood vessels. However, there was a positive correlation between the leukocyte infiltration and the small blood vessels.

**TF-SB suppresses the expression of VEGF in MHCC97-H cells and HUVECs**

VEGF is a potent mitogen responsible for the induction of angiogenesis [36]. It was well known that the humanized monoclonal antibody of VEGF, bevacizumab, had already been used to treating several cancers [37]. In the present study, the variation of VEGF level was measured by ELISA assay. The cells were treated with different concentrations of TF-SB for 24 h or 48 h. The VEGF was detected in medium supernatant. As shown in Figure 7, The VEGF expression levels were obviously decreased after TF-SB treatment in MHCC97-H cells and HUVECs. Furthermore, the results showed that the VEGF expression level correlated with concentrations of TF-SB ($p<0.05$).

**Discussion**

In recent years, some new treatments, such as targeted therapy and gene therapy have become an important means of cancer comprehensive treatment. It have been used in the treatment of various tumors. However, these therapies have not been used widely for limited efficiceny and costliness. Traditional Chinese medicine have been used in anti-tumor treatment for thousands of years in China. It has been widely applied to enhance immune function, reduce side-effects, and prevent recurrence and metastasis for cancer patients. With the deepening of the study of Chinese herbs anticancer mechanism, the anticancer activity of many extracts from herbs was found in vitro studies. For example, BZL101, an aqueous extract of *S. barbata*, has shown anticancer properties in many human cancers [38]. The crude extract of *S. barbata* have anticancer and anti-angiogenic activity in vitro and in vivo [19, 39].

*S. barbata* is one of the conventional anticancer drugs in China, it has significant anti-tumor activity and inhibition of angiogenesis effect [40]. The chemical composition of *S. barbata* includes flavonoids, diterpenoids, polysaccharides, etc.
The flavonoids is considered as a main component of *S. barbata* in anti-tumor effects. Herein our study found that total flavones of Scutellaria barbatae (TF-SB) could inhibit angiogenesis by some experimental studies of proliferation, migration and tube formation of endothelial cells. Our results showed that TF-SB inhibited the proliferation and migration of HUVECs in a dose dependent manner. TF-SB could significantly suppress the process of angiogenesis of HUVECs.

Inflammation, cytokines activation and angiogenesis are common feature of tumor microenvironment in the progression of malignancy [41]. Both biological processes of angiogenesis and inflammation often share mutual pathways which related to cancer progression [42]. Inflammatory cells, macrophages/monocytes that are induced by various cytokines, become tumor-associated macrophages (TAM) in tumorgenesis. They are considered contributing to creation of microenvironment for angiogenesis [43]. In this study, we found that TF-SB inhibited the angiogenesis of CAM *in vivo*. Simultaneously, also found that the sponge around have a large number of inflammatory cell infiltration by HE staining. Simultaneously, a positive correlation was found between leukocyte infiltration and small blood vessels.

The VEGF can be combined with the specific receptors on the endothelial cells, and promote angiogenesis process VEGF is highly expressed in angiogenesis, which is secreted by endothelial cells and tumor cells. It’s secreted by autocrine and paracrine pathways that is initiated by combination of VEGF and its receptors on endothelial cell, and finally triggering angiogenesis [44]. In the present study, the results showed that TF-SB down-regulated the expression of VEGF in MHCC97-H cells and HUVECs.

**Conclusion**

In conclusion, TF-SB could significantly suppress the process of *in vitro* angiogenesis of HUVECs on matrigel, and the angiogenesis of CAM *in vivo*. However, TF-SB is composed of many chemical compounds including scutellarin...
apigenin, baicalein, luteolin, etc. The anticancer effects of these single component or many ingredients are still unknown. Further experimental studies will be require to clarify the anticancer molecular mechanisms.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

DZJ, WXJ and WWY designed the research. DZJ, LWF, GJ, KHF and MYG performed the experiments throughout this research. ZSQ, DY and LS contributed to the reagents, and participated in its design and coordination. DZJ and GJ analyzed the data; DZJ and LWF contributed to the writing of the manuscript. Co-first authors: DZJ, LWF and GJ. All authors have read and approved the final manuscript.

**Abbreviations**

HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; HUVECs, Human umbilical vein endothelial cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CAM, Chick embryo chorioallantoic membrane; TF-SB, total flavones of Scutellaria barbatae; HPLC, high performance liquid chromatography.

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**References**


Figure legends

Figure 1. HPLC analysis of TF-SB. There was a main peak in HPLC, which was identified as scutellarin (A). There were also some other flavonoids in TF-SB which were identified as apigenin(B), baicalein(C), luteolin (D). (Wavelength=280 nm)

Figure 2. Growth inhibiting effects of TF-SB on HUVECs. Cell viability was determined by MTT method and treated with different concentrations drug for 48 h or 72 h. This assay was performed in triplicate ($p<0.05$, ANOVA analysis). *$p<0.05$, **$p<0.01$ versus the control group.

Figure 3. Effects of TF-SB on the cell migration of HUVECs. Cell migration was analyzed by the scratch assay. HUVECs were treated with various concentrations of TF-SB (40, 80 and 120 µg /mL) for 48 h. A: blank control group; B: 40 µg /mL TF-SB group; C: 80 µg /mL TF-SB group; D: 120 µg /mL TF-SB group. The images were captured under a phase-contrast microscope at a magnification of 100×. Values represent mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$ versus the control group.

Figure 4. The effect of TF-SB on HUVEC tube formation. HUVECs were seeded on Matrigel-coated 96-well plates and incubated in the diluted medium containing different concentrations of TF-SB for 9 h at 37 °C. A: blank control group; B: 40 µg /mL TF-SB group; C: 80 µg /mL TF-SB group; D: 120 µg /mL TF-SB group. The images were captured under a phase-contrast microscope at a magnification of 100 × and observed the network-like structures. Values represent mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$ compared with the control group.
Figure 5. The effects of TF-SB on the angiogenesis of CAM. The chick chorioallantoic membrane of 7-day-old chick embryos were treated with various concentrations of TF-SB and incubated for 48 h. A: blank control group; B: 40 µg/mL TF-SB group; C: 80 µg/mL TF-SB group; D: 120 µg/mL TF-SB group. The angiogenesis around the gelatin sponges was photographed with an anatomical microscope. Values represent mean ± SD from fifteen eggs. *p<0.05, **p<0.01 compared with the control group.

Figure 6. The relationship between leukocyte infiltration count and formation of blood vessels. The leukocyte infiltration and blood vessels on the CAM were observed under the microscope by HE staining. and no correlation between the formations of big blood vessels. A: The leukocyte infiltration was positively correlated with small blood vessels (r=0.883, p<0.05); B: The leukocyte infiltration had no correlation with formation of big blood vessels (r=0.067, p>0.05).

Figure 7. Effect of TF-SB on the expression of VEGF in both MHCC97-H cells and HUVECs. Cells were treated with different concentrations of TF-SB for 24 h or 48 h. The protein secretion levels of VEGF were examined by ELISA in MHCC97-H cells (A) and HUVECs (B). The VEGF expression in the two cell lines were significantly reduced. This assay was performed in triplicate. *p<0.05 versus the control group.
Figure 1
Figure 7