Anti-angiogenic effects of total flavonoids from *Scutellaria barbata* D. Don

Zhi-Jun Dai\(^1\)*, Wang-Feng Lu\(^1\)†, Jie Gao\(^2\)*, Hua-Feng Kang\(^1\), Yu-Guang Ma\(^1\), Shu-Qun Zhang\(^1\), Yan Diao\(^1\), Shuai Lin\(^1\), Xi-Jing Wang\(^1\)*, and Wen-Ying Wu\(^3\)*

\(^1\) Department of Oncology, the Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710004, China; E-Mails: luwangfengsl@126.com (W.-F.L.); kanghf73@yahoo.com.cn (H.-F.K.); mygxmj@hotmail.com (Y.-G.M.); zhangshuqun1971@yahoo.com.cn (S.-Q.Z.); dy971203@163.com(Y.D.); linshuai420@stu.xjtu.edu.cn (S.L.)

\(^2\) Department of Nephrology, the Second Affiliated Hospital of Xi’an Jiaotong University, 710004 Xi’an, China; E-Mail: gxej_cn@sina.com (J.G.)

\(^3\) Department of Pharmacology, the Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710004, China

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: dzj0911@126.com (Z.-J. D.); wangxijing@21cn.com (X.-J. W.); wwy0413@126.com (W.-Y. W.);

Tel.: +86-29-8767-9226(Z.-J. D.); Fax: +86-29-8767-9282(Z.-J. D.).

**Abstract**

**Background:** Angiogenesis is closely related to the growth, invasion and metastasis of the tumor, also anticancer therapy key targets. *Scutellaria barbata* D. Don (*S. barbata*), a traditional Chinese medicine, is being used for treatment of many diseases including cancer. However, the antitumor molecular mechanism of
S. barbata was 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 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5- diphenyte- trazoliumromide (MTT) assay. The activity of TF-SB on migration of HUVECs by the scratch assay. The ability of HUVECs forming network structures *in vitro* was tested 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 could significantly suppress the process of *in vitro* angiogenesis of HUVECs on matrigel, and the angiogenesis of CAM *in vivo*. Furthermore, TF-SB treatment down-regulated the expression of VEGF in both HUVECs and MHCC97-H human hepatocarcinoma cells.

**Conclusion:** TF-SB could significantly suppress the process of *in vitro* angiogenesis of HUVECs on matrigel, and the angiogenesis of CAM *in vivo*. 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

**Background**

The incidence of hepatocellular carcinoma (HCC) in worldwide is the rise trend. According to the statistics in the world each year about seven million people died of liver cancer [1]. The treatment of HCC patients with tumor heterogeneity, biological behavior and liver function is closely related [2]. Chemotherapy is one of the main methods for the treatment of HCC. Although chemical anti-cancer drugs
have definite effect, they often cause severe side-effects. Moreover, many chemotherapy drugs bring about multiple drug resistance. Therefore, it is very important to develop new anti-cancer pharmaceuticals from Chinese herbal medicine [3, 4]. Many herbs have been discovered of anti-tumor activity and become the main source of anti-cancer drug research [5].

Angiogenesis, the formation of new blood vessels from an existing vasculature, has been associated with the growth and dissemination of solid tumors [6]. Tumor angiogenesis is a complex process and including a series of process of the injure locally of basement membrane, migrate and proliferate of endothelial cells, influence of angiogenic factors [7]. The expression of many cytokines have involved in this formation process, such as vascular endothelial growth factor (VEGF) and angiopoietin (Ang) [8-10]. The cytokines played an important role in the regulation of process of tumor angiogenesis [11]. Therefore, the search for effective inhibition of vascular endothelial cell proliferation and expression of angiogenesis related cytokines in natural herbs, will be better for the treatment of cancer [12].

**Scutellaria barbata** D. Don (S. barbata) is a medicine herb, which widely distributed in some areas of China and Korea. The S. barbata have anti-inflammatory and anti-tumor effects from Chinese Pharmacopoeia records. 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 [13-17]. Our previous results show that the extracts of S. barbata have antitumor activities in mouse hepatoma both *in vitro* and *in vivo* [18]. Currently, there are a large number of flavones, alkaloids, polysaccharides and steroids separated from the S. barbata [19-23]. In the present study, we investigated the anti-angiogenic effect of total flavonoids of S. barbata (TF-SB) using 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(-z-y1)-3,5- diphenye- trazoliumromide (MTT) was provided by Sigma-Aldrich (St. Louis, MO, USA); Matrigel (BD Biosciences, San Jose, CA, USA); human VEGF ELISA kit was 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. 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 material was coarsely ground before extraction. A total of 300 g of the material was extracted two times with 95% ethanol for 3 h in 50°C. The fluid was filtered through a 1-mm pore-size filter. Then the filtrate was evaporated. The crude extract were isolated by AB-8 macroporous adsorption resin column in which 70% aqueous ethanol was used to elute flavonoids.

Cell line and Cell culture

The human hepatocarcinoma cell line (MHCC97-H) and human umbilical vein endothelial cell line (HUVEC), 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, 5% CO₂ 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 a density of 1×10⁴ cells/well. After 12 h, the cells were treated
with different concentrations (0, 20, 40, 80 and 160 µg/mL) TF-SB for 48h or 72h. At the end of the treatment, 20 µL MTT (5 mg/mL in PBS) were added to each well the cells were incubated for another 4 hours. The supernatants were removed carefully and 150 µL of dimethyl sulfoxide (DMSO) were added to each well. The absorbance was measured at 490 nm using an Enzyme-labeling instrument (ELX800, Bio-Tek, Winooski, VT, USA).

**In vitro scratch assay**

To assess the activity of TF-SB on migration of HUVECs by the scratch assay [24]. 2×10^5 HUVECs were seeded on a 12-well plate in complete medium overnight to obtain a full confluent monolayer. After 24 h, cells were scraped away vertically in each well using a 100 µL 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 concentration 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 [25], 96-well plates were plated with 50µL of 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 ×. 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 [24]. Briefly, 60 fertilized chicken eggs were incubated in a constant-temperature incubator (Heraeus, Germany) maintained at 37 °C and 40–60% humidity for 4 d. The eggs were randomly divided into 4 groups. 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 by hematoxylin and eosin (HE) staining. The relationship between leukocyte infiltration and small angiogenesis were analyzed quantitatively. Different concentrations of TF-SB samples in 20µL 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 treatment cells in different concentrations of TF-SB. The VEGF level in MHCC97-H cells and HUVECs culture supernatants was measured by using ELISA kit (Boshide) according to the manufacturer's instructions. The each well were plated with 0.1mL diluted samples in samples buffer and incubated 90 minutes at 37°C. The wells were coated with 100 µL anti-human VEGF antibody working solution and incubated 60 minutes at 37°C. After three washes, ABC working solution were added and incubated 30 minutes at 37°C. After five washes with PBS, followed by the addition of 90 µL TMB color liquid in the dark for 30 minutes. Then, 100µL TMB Stop Solution were added and the absorbance was measured at 450 nm.

**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 SPSS 13.0. \( p < 0.05 \) were 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 inhibit the cell growth of lung cancer, leukemia, colon cancer, hepatoma, and skin cancer [14-17]. It was reported by Lee *et al.* that reduced the HCG-promoted proliferation of myometrial and leiomyomal cells [26].

In this study, cell viability was examined using 3-(4, 5) -dimethylthiazio(-z-y1)-3, 5- diphenyte- trazoliumromide (MTT) assay. HUVECs were treated with different concentrations of TF-SB for 0-72 h. As shown in Figure 1, after 48 h treatment, cell viabilities of the TF-SB treated (20, 40, 80 and 160 \( \mu g/mL \)) groups were suppressed by 24.3±0.1, 30.9±1.5, 55.4±0.9 and 73.2±0.6\% respectively, compared with the control group. After treatment for 72 h, the inhibition rate in the TF-SB treated groups were further reduced, the inhibitory rate of 160 \( \mu g/mL \) group was as high as (78.13±0.6) \%. 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. In this study, the effect of TF-SB on HUVECs migration was determined by the scratch assay. As shown in Figure 2, after 48 h treatment, the cell migration in TF-SB groups were inhibited with different levels. The inhibition rate in 120 \( \mu g/mL \) TF-SB group was the highest, compared with the control group (\( p<0.01 \)). The cell migration of HUVECs were dose-dependently inhibited after treatment with TF-SB for 48h.

*TF-SB inhibits HUVEC tube formation*
Tumor neovascularization is defined as the process of new blood vessel formation in solid neoplasms [27]. Activation of angiogenic pathways is required for tumor spreading, as well as for proliferation of metastatic cells in distant organs [28]. 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 [29]. In a colorectal cancer mouse xenograft model, the ethanol extract of *S. barbata* could inhibit tumor angiogenesis via suppression of the SHH pathway [30].

As an essential step for angiogenesis, the formation of tube-like structures involves matrix degradation, rearrangement and apoptosis of endothelial cells. Therefore, we used tube formation assay on matrigel to observe the process of *in vitro* angiogenesis of HUVECs. As shown in Figure 3, HUVECs placed on the basement membrane matrigel formed to the capillary tube structures in the control group. 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**

To confirm the effect of TF-SB on angiogenesis in vivo, we used a chick embryo chorioallantoic membrane (CAM) model. The CAM assay is widely used for both developmental and post-developmental studies of angiogenesis due to easy access to the vascularized CAM membrane [31, 32]. An additional advantage is that in many countries animal license is not needed for chicken embryo experimentation. The assay is rapid, inexpensive and suitable for large-scale screening of substances thought to regulate angiogenesis [33].

After 7 days fertilized, chick eggs were incubated and treated with various concentrations of TF-SB (40, 80 and 120 µg/mL). After treatment for 48h, the structure of blood vessels were observed with an anatomical microscope. Normal
vascular pattern with numerous branching was observed in the control group. As shown in Figure 4, TF-SB treatment significantly reduced the total number of blood vessels on the chick chorioallantoic membrane compared with untreated control. The results indicated that TF-SB is able to suppress angiogenesis \textit{in vivo}.

\textbf{The relationship between leukocyte infiltration count and formation of blood vessels}

Infiltration of macrophages, lymphocytes, mast cells are often present in tumors microenvironment that these cells may contribute to tumor progression, suggests that these inflammatory cells have promoted neoplastic progression by stimulating tumor revascularization and have closely related with tumor angiogenesis [34]. In the present study, the leukocyte infiltration count was observed surrounding sponge angiogenesis on the CAM by HE staining. As shown in Figure 5, the results showed that the leukocyte infiltration had no correlation with the formation of big blood vessels, but a positive correlation with the formation of small blood vessels.

\textit{2.6. TF-SB suppresses the expression of VEGF in both MHCC97-H cells and HUVECs}

VEGF is a potent mitogen responsible for the induction of angiogenesis [35]. It was investigated the humanized monoclonal antibody of VEGF, bevacizumab, as potential treatment for several cancers in clinical trials [36]. 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 24h or 48h. The supernatant was collected and detected the expression level of VEGF. As shown in Figure 6, The VEGF expression levels were obviously decreased after TF-SB treatment in both MHCC97-H cells and HUVECs. Furthermore, the results showed that the VEGF expression level had an apparent relationship 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 used in the treatment of various tumors. However, these therapies have limited efficiency and expensive for most patients. Traditional Chinese medicine have 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 [37]. The crude extract of S. barbata have anticancer and anti-angiogenic activity in vitro and in vivo [18, 38].

S. barbata is one of the conventional anticancer drugs in China, it have significant anti-tumor activity and inhibition of angiogenesis effect [39]. The chemical composition of S. barbata includes flavonoids, diterpenoids, polysaccharides, etc. The flavonoids is considered to be 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. MTT assay showed that TF-SB inhibited the proliferation of HUVECs in both dose- and time- dependent manner (p<0.05). Moreover, in modified scratch assay and tube formation assay, we demonstrated that TF-SB inhibited the migration and capillary tube formation of HUVECs in a dose- dependent manner.

Inflammation, cytokines and angiogenesis are common feature of tumor microenvironment in the progression of the malignancy [41]. Both biological processes of angiogenesis and inflammation often share common pathways are closely related to progression of cancer [41]. Inflammatory cells, macrophages/monocytes are induced under in a variety of cytokines, become
tumor-associated macrophages (TAM), and help creation of a microenvironment that favors angiogenesis [42]. 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. There was positively correlated between the leukocyte infiltration and small blood vessels.

VEGF is highly expressed in the process of tumor angiogenesis, which is secreted by endothelial cells and tumor cells and functions autocrine and via paracrine signaling pathways by VEGF primarily binds to its specific receptors located on endothelial cells, triggering the process of angiogenesis [43]. The VEGF can be combined with the specific receptors on the endothelial cells, and promote angiogenesis process. In the present study, the results showed that TF-SB down-regulated the expression of VEGF in both MHCC97-H human hepatocarcinoma 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 scutellarein, apigenin, scutellarin 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.

Acknowledgments

This study was supported by National Natural Science Foundation of China, No.81102711, No.81274136; the Fundamental Research Funds for the Central Universities, China, No.xjj2011039; Sci-tech Program of Administration of Traditional Chinese Medicine of Shaanxi Province, China, NO. 2009jc86.

References


Figure 1. Growth inhibiting effects of TF-SB on HUVECs. Cell viability was determined by MTT method and treated with different concentrations drug for 48h or 72h. This assay was performed in triplicate. ($p<0.05$, ANOVA analysis).
Figure 2. 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 ± SEM from three independent experiments. *p<0.05, **p<0.01 versus the control group.
Figure 3. 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: 40μg/mL TF-SB group; C: 80μg/mL TF-SB group; D: 120μg/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 ± SEM from three independent experiments. *p<0.05, **p<0.01 compared with the control group.
Figure 4. 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 ± SEM from fifteen eggs. *p<0.05, **p<0.01 compared with the control group.
Figure 5. 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 6. 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 48h. 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 control group.