School of Life Sciences[1]; State Key Laboratory of Applied Organic Chemistry[2], Lanzhou University; Medical College of Northwest University for Nationalities[3], Lanzhou, China

A novel flavonoid isolated from *Sophora flavescens* exhibited anti-angiogenesis activity, decreased VEGF expression and caused G0/G1 cell cycle arrest *in vitro*

Xiu-Li Zhang[1,*], Mei-Ai Cao[2,*], Li-Ping Pu[1], Shuang-Sheng Huang[3], Qing-Xiang Gao[1], Cheng-Shan Yuan[2], Chun-Ming Wang[1,2]

Received October 24, 2012, accepted November 23, 2012

Dr Chun-Ming Wang, School of Life Sciences, Lanzhou University, Lanzhou, 730000, China
cmwang@lzu.edu.cn
Dr Cheng-Shan Yuan, State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, 730000, China
yuancs@lzu.edu.cn
* *These authors contributed equally to this work*

Pharmazie 68: 369–375 (2013) *doi: 10.1691/ph.2013.2845*

Kushen, the dried root of *Sophora flavescens* Ait, is a traditional Chinese herbal medicine. Kushen alkaloids have been developed in China as anticancer drugs, and more potent antitumor activities have been identified in kushen flavonoids than in kushen alkaloids. In this study, the anti-angiogenic properties of (2S)-7,2',4'-triihydroxy-5-methoxy-8-dimethylallyl flavanone (Compound **1**, a novel flavonoid isolated from *Kushen*), were examined using the human umbilical vein endothelial cell line (ECV304) *in vitro*. The results indicated that compound **1** shows anti-angiogenesis activity *via* inhibitory effects on cell proliferation, cell migration, cell adhesion, and tube formation. Further studies indicated that compound **1** blocks cell cycles in the G0/G1 phase without inducing apoptosis, and down regulates vascular endothelial growth factor (VEGF) expression. The free radical scavenging activity of compound **1** was found through 2′,7′-dichlorofluorescin diacetate (DCFH-DA) incubation assay in cells. The anti-angiogenic properties of compound **1** and its anti-proliferative effect on endothelial cells without causing apoptosis make it a good candidate for development as a agent against development of tumors.

1. Introduction

Flavonoids comprise the most common group of polyphenols in human diet and are found ubiquitously in plants (Tsao 2010). Many medicinal properties of flavonoids have been identified, such as antioxidant activity, anti-inflammatory activity, vascular activity, oestrogenic activity and cytotoxic antitumor activities (Harborne and Williams 2000). In recent years, the health beneficial effects are expanded to cognition, dementia, potential therapeutic utility for Alzheimer disease (Williams and Spencer 2012), and even the cancer prevention activities (Birt et al. 2001; Yao et al. 2011). The function of cancer prevention is to cause delay in onset of cancer, progression from precancerous lesions or recurrence after treatment, as an alternative to treatment of cancer cases after clinical symptoms have appeared. The mechanisms are related to many activities, such as antioxidant, anti-inflammatory, induction of apoptosis, inhibition of cell proliferation, induction of cell differentiation, and anti-angiogenesis (Tsuda et al. 2004); all of these activities have already been found in flavonoids.

Angiogenesis is a key process in the promotion of cancer, the natural health products that inhibit angiogenesis are considered a potential source for investigational new agents to treat cancer (Sagar et al. 2006). Many flavonoids, such as xanthohumol, kaempferol and quercetin, have been reported exhibiting cancer prevention effect through anti-angiogenesis mechanism recently (Albini et al. 2006; Luo et al. 2009; Priyadarsini et al. 2011). As part of our continuous efforts to explore Chinese traditional medicinal herbs (Huang et al. 2011; Li et al. 2007; Wu et al. 2004), and to search novel natural products for cancer treatment (Wang et al. 2007, 2004), we explored the anti-angiogenesis effects of a new flavonoid from the traditional Chinese medicinal herb *Kushen* in this study.

The dry root of *Sophora flavescens* Ait, a traditional Chinese herbal medicine named *Kushen*, has long been used for the treatment of jaundice, leukorrhea, carbuncles, pyogenic infections of the skin, scabies, enteritis as well as dysentery (Chinese-Pharmacopoeia 2010). Quinolizidine alkaloids and flavonoids are the two main active components of this herb. The contents of matrine and oxymatrine are used as the standards to evaluate the quality of *Kushen* as a Chinese traditional medicinal herb (Chinese-Pharmacopoeia 2010). The bioactivities of matrine and oxymatrine have been widely studied, and the anticancer and anti-inflammation activities are still being reported (Ho et al. 2009; Liang et al. 2012; Zhang et al. 2011). Flavonoids isolated from *Kushen* are getting more and more attention in recent years; their antioxidant, anti-proliferative and anti-inflammatory activities are also being reported frequently (Jin et al. 2010; Zhou et al.

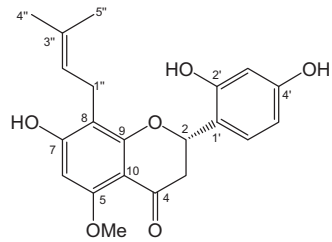

Fig. 1: Chemical structure of compound **1**

2009). We have reported 17 alkaloids isolated from *Kushen* in 2010 (Liu et al. 2010), and here we want to further report a new flavonoid isolated from this plant, (2*S*)-7,2',4'-triihydroxy-5-methoxy-8-dimethylallyl flavanone (**1**). Its anti-angiogenesis activities were studied through the effect on cell proliferation, cell migration, cell adhesion and tube formation; the related mechanisms were evaluated on antioxidative activity, human vascular endothelial cell growth factor (VEGF) expression and cell cycle interferes.

2. Investigations and results

2.1. Bidirectional regulation activity of compound 1 on cell proliferation

Bidirectional regulation activity of compound **1** on cell proliferation was detected in 96-well plate under different concentrations (10, 20, 30, 40 and 50 μg/ml) treated for 12 h. The results showed that compound **1** exhibited no inhibitory effect on cell proliferation under lower concentrations (10 and 20 μg/ml), and even a promotion effect under 20 μg/ml treatment, although the ratio was only 6%. While the higher concentrations (30, 40 and 50 μg/ml) treatments showed an inhibitory effect to a certain degree. The inhibition rates were 15.9, 31.9 and 33.3% respectively (Fig. 2).

2.2. Compound 1 inhibited cell migration under higher concentrations

It is an early event of angiogenesis that endothelial cells pass through a distance and migrate to a new site. No effects were found on migration under the treatment of 10, 20 and 30 μg/ml of compound **1**; while potent inhibition were detected under treatment of 40 and 50 μg/ml concentrations; the inhibition rate were 66.1 and 69.1% respectively (Fig. 3).

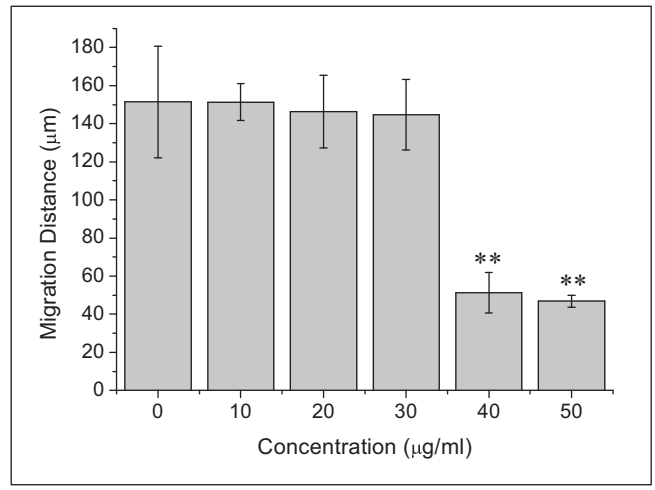

Fig. 3: Effect of compound **1** on cell migration of ECV304 cells. Monolayers of confluent cells in 6-well plates were scraped away to a portion and treated with different concentrations of compound **1** for 12 h at 37 °C. After fixation, cells were stained with Giemsa dye and cell migrations were quantified by measuring distance between wound edges before and after incubation. Values were expressed as mean ± SD of triplicate-independent experiments. $^{**}P < 0.01$ vs. control

2.3. Compound 1 inhibited cell adhesion activity

Cell adhesion activities were inhibited by compound **1** under all the concentrations treated. The inhibition rates were 27.8, 31.9, 34.8, 36.0, and 39.1% corresponding to 10, 20, 30, 40 and 50 μg/ml treatments (Fig. 4).

2.4. Compound 1 inhibited tube formation

The typical tube-like structures were formed after cells being seeded on fibrin gel for 12 h (Fig. 5A); and it was disturbed when compound **1** was added under different concentrations. The tube formation inhibition rates were 12.5, 13.3, 58.6, 63.8 and 70.3%,

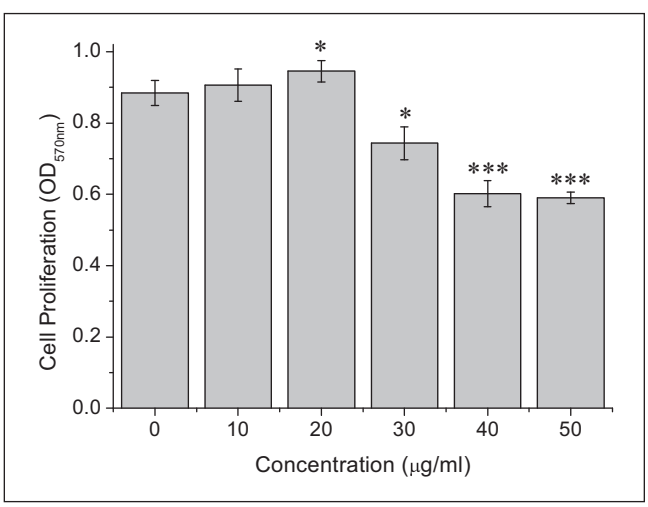

Fig. 2: Bidirectional effect of compound **1** on cell proliferation of ECV304 cells. Cells were seeded in 96-well plates (4×10^3 cells/well) and incubated for 24 h, then different concentrations of compound **1** were added and incubated for another 12 h. after fixation, cells were stained with SRB and measured at 570 nm on a microplate reader. Values were expressed as mean ± SD of triplicate-independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control

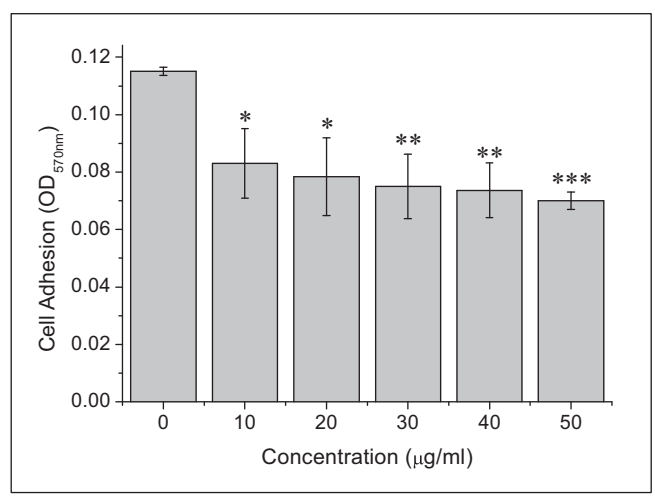

Fig. 4: Effect of compound **1** on cell adhesion of ECV304 cells. Cells (2.5×10^4 cells/well with different concentrations of compound **1**) were seeded in wells of 96-well plate coated with extracellular matrix protein type I collagen. After incubation for 1 h at 37 °C, cells were washed, fixed, stained with crystal violet and measured at 570 nm. Values were expressed as mean ± SD of triplicate-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control

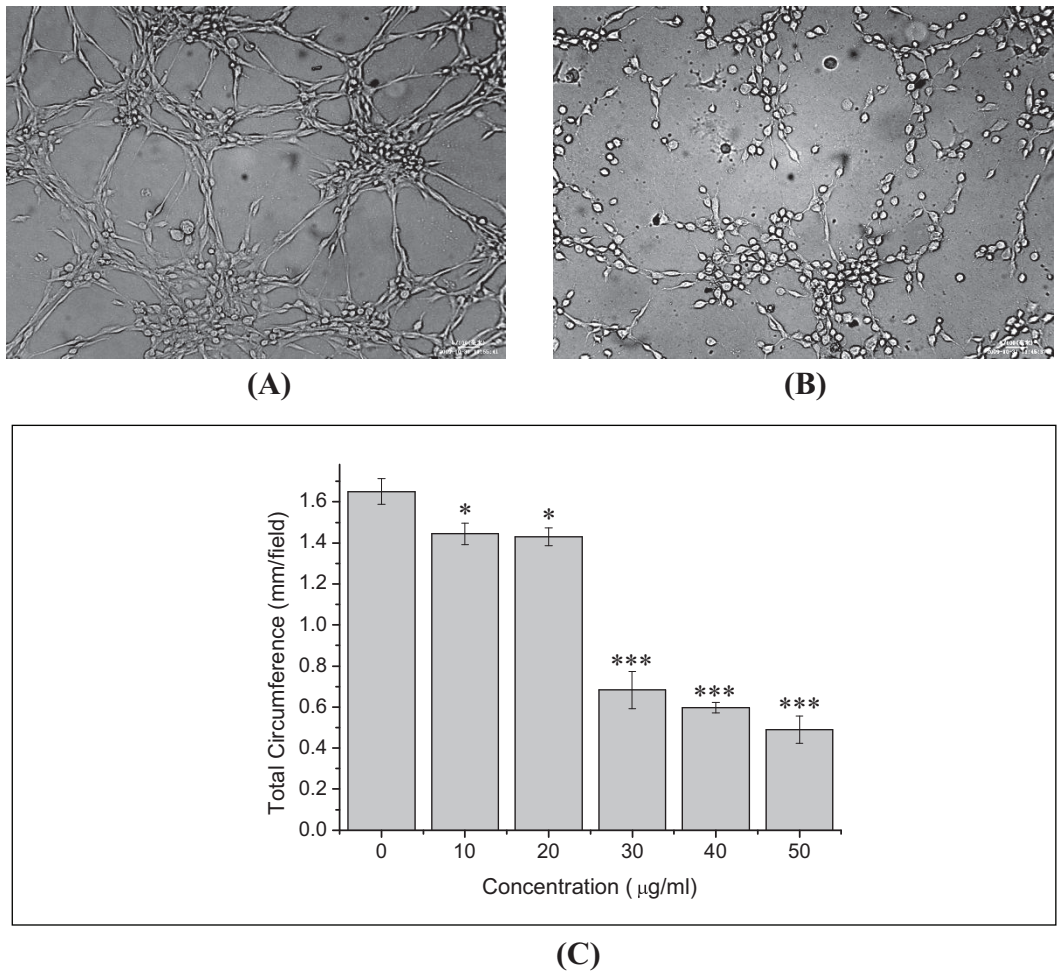

Fig. 5: Effect of compound **1** on tube formation of ECV304 cells. Cells (1.5×10^5 cells/well) were seeded in 24-well plate pre-coated with fibrin gel. After incubation for 12 h, wells were washed with PBS and fixed with 2.5% glutaraldehyde. Randomly selected fields under phase contrast microscopy were photographed. Tube formation was quantified by measuring the total circumference of tubular structures in three randomly selected fields using a computer-assisted image analyzer (CMIS 8.0, Beijing University of Aeronautics and Astronautics, Beijing, China). Tube formation images (400 ×) of control (A) and 50 μg/ml compound **1** treated (B) ECV304 cells were shown. Statistic graph showed the inhibitory effect of compound **1** on tube formation (C). Values were expressed as mean ± SD of triplicate-independent experiments. $^*P < 0.05$, $^{***}P < 0.001$ vs. control

under the treatments of 10, 20, 30, 40 and 50 μg/ml of compound **1** (Fig. 5C). Figure 5B showed the tube-like structures formed at 50 μg/ml compound **1** treatment, the tube-like structures were apparently destroyed compared to the control group.

All of these results shown above clearly figured out the anti-angiogenesis effect of compound **1** *in vitro*, which included many key steps of angiogenesis: proliferation, migration, adhesion, and tube formation.

2.5. Compound 1 down-regulated ROS levels in ECV304 cells

In view of the fact that antioxidant activity is a common bioactivity of flavonoids (Harborne and Williams 2000), the effect of compound **1** on ROS levels in ECV304 cells were evaluated in this study. The results showed that ROS levels in ECV304 cells were down-regulated by compound **1** in a dose-dependent manner (Fig. 6). The inhibitory rates were 15.3, 19.2, 31.6, 35.3 and 47.2% of control cells after treated with 10, 20, 30, 40 and 50 μg/ml compound **1** for 12 h.

2.6. Compound 1 down-regulated VEGF content

As exogenous ROS stimulate induction of VEGF in endothelial cells (Chua et al. 1998), and oxidative stress can increase VEGF expression in tumor cells (Ushio-Fukai and Nakamura 2008), we want to check if down-regulation of ROS levels in ECV304 cells by compound **1** has any effect on VEGF content. With VEGF ELISA Kit, we detected the effect of compound **1** on VEGF levels in ECV304 cell cultures. The results showed that VEGF contents were down-regulated by compound **1** in all of the treatments (Fig. 7). The inhibitory rates were 15.8, 47.5, 40.0, 33.7 and 39.3% of control when treated with 10, 20, 30, 40 and 50 μg/ml of compound **1** respectively.

2.7. Compound 1 induced cell cycle arrest in G0/G1 phase without apoptosis

In view of the bidirectional regulation activity of compound **1** on cell proliferation, we checked the cell cycles under different concentrations with PI staining flow cytometry method. Results indicated almost the same trends as the anti-proliferation assay: no effect on cell cycle progress was found under the lower concentrations (10 and 20 μg/ml); while an increase of G0/G1 phase accumulation was exhibited in a dose-dependent manner in the higher concentration treatments (30, 40, and 50 μg/ml) (Fig. 8). The percentages of G0/G1 cells were 65.8, 65.2 and 66.9% of control, 10 and 20 μg/ml treatment groups respectively. While the G0/G1 percentages of 30, 40 and 50 μg/ml treatments were increased to 76.9, 85.9 and 91.2%; and accompanied by the decrease of the percentages of S phase cells from 29.7% of control group to 19.6, 11.5 and 5.6% of 30, 40 and 50 μg/ml compound **1** treatments; suggesting that the cell cycles were arrested

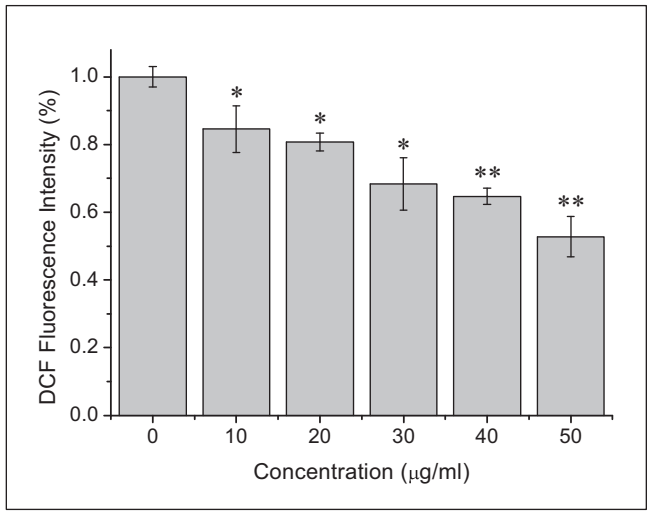

Fig. 6: Effect of compound **1** on ROS generation in ECV304 cells. ROS levels were assayed by measuring the oxidative conversion of cell permeable DCFH-DA to fluorescent DCF. Cells were treated with different concentrations of compound **1** for 12 h, and then incubated with 10 μM DCFH-DA for 30 min. Following washing with PBS, cells were collected in PBS, counted, sonicated and centrifuged for 15 min at 10,000×g at 4 °C. Fluorescent intensity of the supernatant was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. Data were adjusted for cell number. Values were expressed as mean ± SD of triplicate-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control

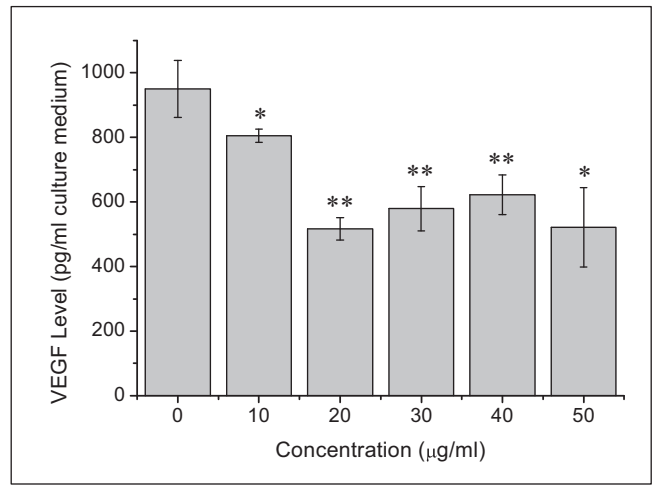

Fig. 7: Effect of compound **1** on VEGF expression in ECV304 cells. Cells were treated with different concentrations of compound **1** for 12 h. Cell culture supernatants were collected and centrifuged to remove any particulates. Samples and standards were then detected with a VEGF ELISA kit. The concentrations of VEGF in samples were determined by comparing the OD of the samples to the standard curve. Values were expressed as mean ± SD of triplicate-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control

in the G0/G1 phase. No significant changes of G2/M percentages were found between control and all of the other treatments. Although G0/G1 phase accumulations were found in a dose-dependent manner when treated with 30, 40 and 50 μg/ml of compound **1**, apoptosis was not found under the same conditions by analyzing the proportion of sub-G1 populations.

3. Discussion

Studies on *Kushen*, the dry root of *Sophora flavescens* Ait, are kept on booming in recent years. Kushen alkaloids containing oxymatrine, matrine, and total alkaloids have been developed in China as anticancer drugs, and more potent antitumor activities have been identified in kushen flavonoids than in kushen alkaloids (Sun et al. 2012). We have ever reported 17 alkaloids isolated from *Kushen* in 2010 (Liu et al. 2010). In this study, we illustrated the anti-angiogenesis activity of compound **1**, and its related mechanisms, that were down-regulated of ROS levels and VEGF expression, and induction of cell cycle arrested in G0/G1 phase. All these aspects indicated that compound **1** is a good candidate for anti-cancer drug development.

3.1. Compound 1 inhibited several key steps of angiogenesis including cell proliferation, migration, adhesion, and tube formation

Angiogenesis is a key process in the promotion of cancer to provide the source of nutrition and oxygen, and to remove waste products to support the growth of solid tumor. It can be divided into four main steps: (1) degradation of the basement membrane of exiting blood vessels by matrix metalloproteinase secreted by activated endothelial cells and making endothelial cells detached; (2) migration of these detached endothelial cells toward the angiogenic stimulus; (3) proliferation of the endothelial cells leading to the formation of solid endothelial cells sprouts in the stromal space; and (4) organization of endothelial cells into capillary tubes and vascular loops with the formation of tight junctions and the deposition of new basement membrane. In correspondence to these steps, the *in vitro* models of angiogenesis have focused on migration, adhesion, proliferation and tubule formation abilities of endothelial cells in response to exogenous stimulatory or inhibitory agents. To evaluate the effect of compound **1** on angiogenesis, we performed an *in vitro* anti-angiogenesis study with the human umbilical vein endothelial cell line ECV304. The results indicated that compound **1** could inhibit all of the four important steps of the angiogenesis process, proliferation, migration, adhesion, and tube formation. Bidirectional regulation activity of compound **1** on cell proliferation was detected in our study. The inhibitory effects were found in the treatments under the higher concentrations (30, 40 and 50 μg/ml) of compound **1** (Fig. 2). The inhibitory effect on cell proliferation is reasonable for the explanation on anti-angiogenesis activities. While no inhibitory effect on cell proliferation under lower concentrations (10 and 20 μg/ml), and even a promotion effect under 20 μg/ml treatment looked to be unusual. In fact, many flavonoids have already been reported to promote cell proliferation; such as genistein and calycosin (Chen et al. 2011; Yang et al. 2010), they promoted proliferation of estrogen receptor-positive cells via estrogen receptors. Although we did not check the estrogen receptor expression levels in our study, many papers have reported that ECV304 cells constitutively expressed estrogen receptor alpha (Wang and Passaniti 1999). This indicated that compound **1** may also promote cell proliferation in lower concentrations *via* the estrogen receptor.
It is an early event of angiogenesis that endothelial cells pass through a distance and migrate to a new site. The inhibitory effects of compound **1** on cell migration were found under treatment at 40 and 50 μg/ml concentrations (Fig. 3); and both cell adhesion and tube formation were inhibited by compound **1** under all the concentrations used (Fig. 4 and Fig. 5).
Based on this *in vitro* anti-angiogenesis model, our results demonstrated that compound **1** acquired its anti-angiogenesis activity through interference with the four key steps of angiogenesis: cell proliferation, migration, adhesion, and tube formation.

3.2. Down regulated ROS levels and VEGF content may be related to the mechanisms of anti-angiogenesis activity of compound 1

High levels of ROS have been found in various cancer cells, and have also been suggested to contribute to angiogenesis in tumors

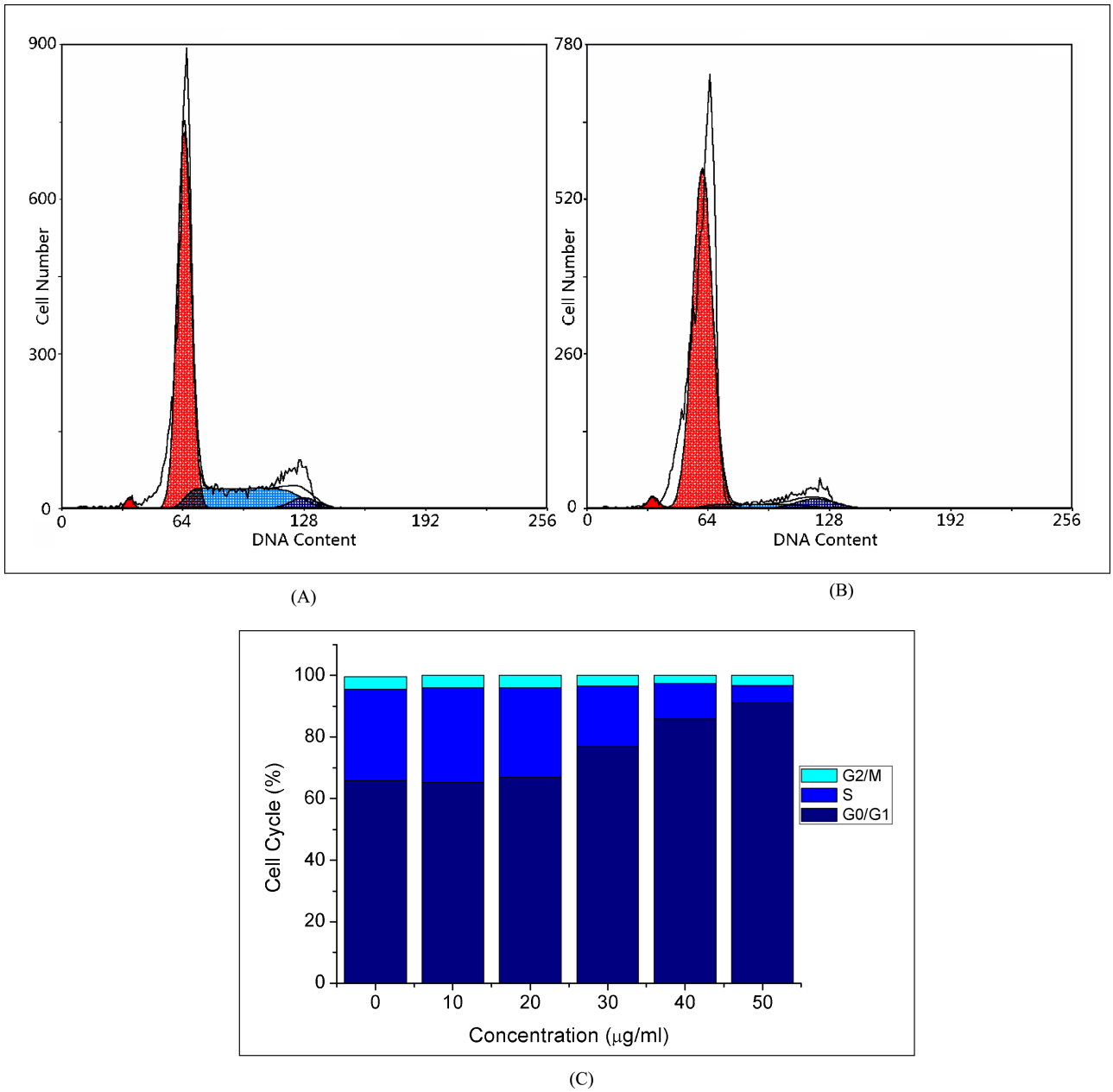

Fig. 8: Effect of compound **1** on cell cycle and apoptosis. Cells were treated with different concentrations of compound **1** for 12 h, then harvested and stained with propidium iodide. Cell cycle distribution was analyzed using a BD FACSCanto II flow cytometer. Apoptosis was identified by the formation of sub-G1 peak. (A) Representative cell cycle distribution of control (without treatment). (B) Representative cell cycle distribution after treatment with compound **1** 50 μg/ml. (C) G0/G1, S and G2/M populations' distributions after treatment with different concentrations of compound **1** for 12 h

(Ushio-Fukai and Nakamura 2008). We have illustrated that the inhibitory effect of rosmarinic acid on angiogenesis is at least partially mediated through its antioxidant activity (Huang and Zheng 2006). And in view of the fact that antioxidant activity is a common bioactivity of flavonoids (Harborne and Williams 2000), the effect of compound **1** on ROS levels in ECV304 cells were evaluated in this study. Our results showed that ROS levels in ECV304 cells were down-regulated by compound **1** in a dose-dependent manner (Fig. 6). These results represented an antioxidative activity of compound **1**; and it may also relate to the expression of VEGF, as it was reported that exogenous ROS stimulate induction of VEGF in endothelial cells (Chua et al. 1998), and oxidative stress can increase VEGF expression in tumor cells (Ushio-Fukai and Nakamura 2008).

VEGF is one of the major angiogenesis factors, which plays a major role in regulating angiogenesis through stimulates proliferation, migration and tube formation of endothelial cells (Sagar et al. 2006; Shojaei 2012). As we have found the anti-angiogenesis effect of compound **1** in all the aspects of cell proliferation, migration and tube formation in this study, it is logical to check if the VEGF content would be influenced at the same time. Our results demonstrated that VEGF levels were down-regulated in all of the groups treated with different concentrations of compound **1**. This indicated that down-regulation of VEGF was one of the mechanisms of the anti-angiogenesis activity of compound **1**.

3.3. G0/G1 arrest without apoptosis, accompanied with anti-proliferation activity indicate a cytostatic effect of compound 1

Compound **1** induced cell cycles arrested in G0/G1 phase while without apoptosis induction. If considered with the anti-proliferation effect together, these results may indicate a cytostatic effect of compound **1** on endothelial cells, which is

similar to the reports about flavonoids tangeretin and nobiletin (Lam et al. 2011; Morley et al. 2007). The authors reported that both tangeretin and nobiletin exhibited a significant growth inhibition of human breast cancer cell lines MDA-MB-435 and MCF-7, human colon cancer cell line HT-29, and human umbilical vein endothelial cells HUVECs with accumulation of cells in G0/G1 phase without any effect on apoptosis. They proposed that these flavonoids were clearly cytostatic and not cytotoxic in their anti-proliferation activity. The same way, compound **1** inhibited proliferation, arrested cell cycles in G0/G1 phase while without induction of apoptosis, and exhibited anti-angiogenesis ability. Our results indicated that compound **1** may be desirable in a cancer chemopreventative regimen for it does not cause cytotoxicity to normal cells while offering anti-proliferative effects on developing tumor cells as well as discouraging any new blood vessel formation through angiogenesis to feed these tumors.

Some flavonoids have been isolated from *Sophora flavescens* in recent years. Four flavonoids showed anti-proliferation activities against human myeloid leukemia HL-60 and human hepatocarcinoma HepG2 cells and induced apoptosis in both cell lines (Ko et al. 2000). Trifolirhizin, a pterocarpan flavonoid, was reported to show the anti-inflammation activity and inhibition effects on growth of human A2780 ovarian and H23 lung cancer cells (Zhou et al. 2009). Different from these flavonoids reported before, compound **1** exhibited anti-proliferation effects but without apoptosis inducing activity, and showed anti-angiogenesis activities in migration, adhesion and tube formation aspects. As the ideal botanical derivatives would specifically antagonize new vessel formation in tumors without significant toxicity to normal tissues and without major adverse reactions, our findings provide a rationale for future development of compound **1** for treatment of tumors.

In conclusion, we reported a new flavonoid isolated from *Sophora flavescens* Ait; its anti-angiogenesis activity, accompanied by its anti-proliferative effect on endothelial cells without causing apoptosis makes it a good candidate for development as a chemopreventive agent against development of tumors.

4. Experimental

4.1. Chemicals

Fibrinogen, thrombin from human plasma, 6-aminohexanoic acid, sulforhodamine B, type I collagen, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Human Vascular Endothelial cell Growth Factor (VEGF) ELISA Kit (Catalog No: E0143 h) was purchased from Wuhan EIAab Science Co., Ltd. (Wuhan, China). Cell culture medium RPMI 1640 was purchased from Gibco (CA, USA). Super neonatal bovine serum was the product of Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China).

4.2. Cell culture

Human umbilical vein endothelial cell line ECV304 cells (obtained from China Center for Type Culture Collection, Wuhan, China) were maintained in RPMI 1640 containing 10% inactivated super neonatal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.0 mg/ml $NaHCO_3$ at 37 °C with 5% CO_2 in a humidified atmosphere.

4.3. Cell proliferation assay

Cell proliferation was determined by the sulforhodamine B (SRB) method (Skehan et al. 1990). Briefly, exponentially growing cells were harvested and seeded in 96-well plates with the final volume of 100 μl containing 4×10^3 cells/well. After 24 h incubation, cells were treated with various concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) for 12 h. Each concentration was tested in three parallel wells. Cells without treatment were used as control. The cultures were then fixed at 4 °C for 1 h by addition of ice-cold 50% trichloroacetic acid (TCA) to give a final concentration of 10%. Fixed cells were rinsed five times with deionized water and stained for 10 min with 0.4% SRB dissolved in 0.1% acetic acid. The wells were washed five times with 0.1% acetic acid and left to dry. The absorbed SRB was dissolved in 150 μl unbuffered 1% Tris base (pH10.5). The absorbance of extracted SRB at 570 nm was measured on a microplate reader.

4.4. Cell migration assay

Cell migration activity was performed by the scratch wound assay as described previously (Huang and Zheng 2006). Monolayers of confluent cells in 6-well plates were scraped away to form a 2-mm sterile area with a rubber scraper. The cultures were washed twice with PBS and incubated with the medium containing 2% calf serum in the presence of different concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) for 12 h at 37 °C. Wells without compound **1** were used as control. After incubation, the cells were washed with PBS, fixed with 75% ethanol and stained with Giemsa dye. Cell migration from edge of the injured monolayer was quantified by measuring distance between wound edges before and after incubation over five points per cultured well.

4.5. Cell adhesion assay

The cell adhesion assay was performed as described previously (Huang and Zheng 2006). Part wells of 96-well plates were coated at room temperature overnight with 2 μg extracellular matrix protein type I collagen in PBS in a final volume of 50 μl. The wells were then washed three times with PBS and blocked for 2 h with 1% bovine serum albumin (BSA) in 100 μl PBS at 37 °C. The additional uncoated wells were incubated with BSA to serve as a negative control. The wells then washed three times with 100 μl PBS. Endothelial cells (2.5×10^4 cells/100 μl) suspended in culture medium with different concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) were added to each well. Cells without compound **1** were used as control. The plates were incubated for 1 h at 37 °C and then washed twice with PBS. The attached cells were fixed 1 h with 10% TCA and stained with 2% crystal violet for 10 min and washed with PBS. For quantification, cells were solubilized in 100 μl 2% sodium dodecyl sulfate (SDS) and measured at 570 nm.

4.6. In vitro angiogenesis assay

Tube formation of ECV304 cells on fibrin gel was performed following the method described previously (Huang and Zheng 2006). Briefly, fibrinogen in PBS was dialyzed against PBS overnight and sterilized with 0.2-μm syringe filter. To make a fibrin gel, 250 μl of 3 mg/ml fibrinogen solution was placed into each well of 24-well plates, with 6-aminohexanoic acid 50 μg/ml was added to modulate the degradation of gel by plasmin, and then the human thrombin was added to a final concentration of 0.625 U/ml. The gel was allowed to polymerize overnight at 37 °C. Endothelial cells (1.5×10^5 cells/well) in 1 ml of culture medium with 2% calf serum were seeded onto the surface of fibrin gel. The cells were incubated with different concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) for 12 h, and then washed three times with PBS and fixed with 2.5% glutaraldehyde in PBS. Cells without compound **1** treatment were used as control. Subsequently, randomly selected fields under phase contrast microscopy were photographed. Tube formation was quantified by measuring the total circumference of tubular structures in three randomly selected fields using a computer-assisted image analyzer (CMIS 8.0, Beijing University of Aeronautics and Astronautics, Beijing, China).

4.7. Intracellular reactive oxygen species assay

Intracellular reactive oxygen species (ROS) levels were assayed by measuring the oxidative conversion of cell permeable DCFH-DA to fluorescent dichlorofluorescein (DCF) as described previously (Huang and Zheng 2006). Nearly confluent endothelial cells were treated with different concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) for 12 h; cells without compound **1** treatment were used as control, and then after washing with culture medium, cells were incubated with 10 μM DCFH-DA for 30 min. Following washing with PBS, cells were collected in PBS, counted, sonicated and then centrifuged for 15 min at 10,000×g under 4 °C. Fluorescent intensity of the supernatant was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. Data were adjusted with cell number.

4.8. Determination of VEGF expression

VEGF protein released from the cultured cells was detected with ELISA kit. Cells (2×10^6) were plated in 12.5 cm^2 flasks and allowed to grow to 80% confluence in 24 h. After treated with different concentrations of compound **1** (10, 20, 30, 40 and 50 μg/ml) for 12 h, cell supernatants were centrifuged for 20 min at 1000 g 4 °C to remove any particulates. Cells without compound **1** treatment were used as control. VEGF assay was performed according to the manufacturer's instruction. Briefly, samples or standards (100 μl) were added to microtiter plate wells pre-coated with an antibody specific to VEGF.

After incubation for 2 h at 37 °C, liquid was removed from each well, and 100 µl Avidin conjugated Horseradish Peroxidase (HRP) was added and incubated for 1 h at 37 °C. After washing, a TMB substrate solution was added to each well. Only those wells that contain VEGF, biotin-conjugated antibody and enzyme-conjugated Avidin would exhibit a change in color. After incubation for 30 min at 37 °C, the enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of VEGF in samples were then determined by comparing the OD of the samples to the standard curve. VEGF protein levels were expressed as picogram of VEGF per ml culture medium.

4.9. Cell cycle analysis

Cell cycle and apoptosis were measured by flow cytometry; cells were stained with propidium iodide (PI). ECV304 cells pretreated with different concentrations of compound **1** (10, 20, 30, 40 and 50 µg/ml) for 12 h were collected and fixed with 75% ethanol at 4 °C overnight. Cells without compound **1** treatment were used as control. Fixed cells were washed with PBS and stained with PI in the presence of RNase A at 4 °C for 30 min, then washed with PBS and analyzed with flow cytometry immediately.

4.10. Statistics

Results were analyzed using the Student′s t-test, and expressed as means ± SD. Values of $P < 0.05$ were considered statistically significant.

Acknowledgment: This work was supported by the Fundamental Research Funds for the Central Universities (lzujbky-2011-37).

References

Albini A, Dell'Eva R, Vene R, Ferrari N, Buhler DR, Noonan DM, Fassina G (2006) Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-kappaB and Akt as targets. FASEB J 20: 527–529.

Birt DF, Hendrich S, Wang W (2001) Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 90: 157–177.

Chen J, Liu L, Hou R, Shao Z, Wu Y, Chen X, Zhou L (2011) Calycosin promotes proliferation of estrogen receptor-positive cells via estrogen receptors and ERK1/2 activation *in vitro* and *in vivo*. Cancer Lett 308: 144–151.

Chinese-Pharmacopoeia-Commission (2010) Kushen (Sophorae Flavescentis Radix). In: LI, Y., Zhao, Y.Y., Yu, H.P., Song, H.L. (Eds.), The People's Republic of China Pharmacopoeia. China Medical Science Press, Beijing, pp. 188–189.

Chua CC, Hamdy RC, Chua BH (1998) Upregulation of vascular endothelial growth factor by H_2O_2 in rat heart endothelial cells. Free Rad Biol Med 25: 891–897.

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55: 481–504.

Ho JW, Ngan Hon PL, Chim WO (2009) Effects of oxymatrine from Ku Shen on cancer cells. Anticancer Agents Med Chem 9: 823–826.

Huang HL, Wang CM, Wang ZH, Yao MJ, Han GT, Yuan JC, Gao K, Yuan CS (2011) Tirucallane-type triterpenoids from Dysoxylum lenticellatum. J Nat Prod 74: 2235–2242.

Huang SS, Zheng RL (2006) Rosmarinic acid inhibits angiogenesis and its mechanism of action *in vitro*. Cancer Lett 239: 271–280.

Jin JH, Kim JS, Kang SS, Son KH, Chang HW, Kim HP (2010) Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of Sophora flavescens. J Ethnopharmacol 127: 589–595.

Ko WG, Kang TH, Kim NY, Lee SJ, Kim YC, Ko GI, Ryu SY, Lee BH (2000) Lavandulylflavonoids: a new class of *in vitro* apoptogenic agents from Sophora flavescens. Toxicol *In Vitro* 14: 429–433.

Lam KH, Alex D, Lam IK, Tsui SK, Yang ZF, Lee SM (2011) Nobiletin, a polymethoxylated flavonoid from citrus, shows anti-angiogenic activity in a zebrafish *in vivo* model and HUVEC *in vitro* model. J Cell Biochem 112: 3313–3321.

Li PL, Wang CM, Zhanga ZX, Jia ZJ (2007) Five new eremophilane derivatives from Ligularia sagitta. Tetrahedron 63: 12665–12670.

Liang CZ, Zhang JK, Shi Z, Liu B, Shen CQ, Tao HM (2012) Matrine induces caspase-dependent apoptosis in human osteosarcoma cells *in vitro* and *in vivo* through the upregulation of Bax and Fas/FasL and downregulation of Bcl-2. Cancer Chemother Pharmacol 69: 317–331.

Liu XJ, Cao MA, Li WH, Shen CS, Yan SQ, Yuan CS (2010) Alkaloids from Sophora flavescens Aition. Fitoterapia 81: 524–527.

Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC (2009) Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer 61: 554–563.

Morley KL, Ferguson PJ, Koropatnick J (2007) Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. Cancer Lett 251: 168–178.

Priyadarsini RV, Vinothini G, Murugan RS, Manikandan P, Nagini S (2011) The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors. Nutr Cancer 63: 218–226.

Sagar SM, Yance D, Wong RK (2006) Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 1. Curr Oncol 13: 14–26.

Shojaei F (2012) Anti-angiogenesis therapy in cancer: current challenges and future perspectives. Cancer Lett 320: 130–137.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.

Sun M, Cao H, Sun L, Dong S, Bian Y, Han J, Zhang L, Ren S, Hu Y, Liu C, Xu L, Liu P (2012) Antitumor activities of kushen: literature review. Evid Based Complement Alternat Med 373219.

Tsao R (2010) Chemistry and biochemistry of dietary polyphenols. Nutrients 2: 1231–1246.

Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, Iigo M, Takasuka N, Moore MA (2004) Cancer prevention by natural compounds. Drug Metab Pharmacokinet 19: 245–263.

Ushio-Fukai M, Nakamura Y (2008) Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. Cancer Lett 266: 37–52.

Wang CM, Jia ZJ, Zheng RL (2007) The effect of 17 sesquiterpenes on cell viability and telomerase activity in the human ovarian cancer cell line HO-8910. Planta Med 73: 180–184.

Wang CM, Yang H, Wei YM, Jia ZJ, Zheng RL (2004) *In vitro* effects on proliferation, telomerase activity and apoptosis of an eremophilanoid sesquiterpene from Senecio oldhamianus maxim in cultured human tumor cell lines. Pharmazie 59: 802–806.

Wang W, Passaniti A (1999) Extracellular matrix inhibits apoptosis and enhances endothelial cell differentiation by a NfkappaB-dependent mechanism. J Cell Biochem 73: 321–331.

Williams RJ, Spencer JP (2012) Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic Biol Med 52: 35–45.

Wu QH, Wang CM, Cheng SG, Gao K (2004) Bieremoligularolide and eremoligularin, two novel sesquiterpenoids from Ligularia muliensis. Tetrahedron Letters 45: 8855–8858.

Yang X, Yang S, McKimmey C, Liu B, Edgerton SM, Bales W, Archer LT, Thor AD (2010) Genistein induces enhanced growth promotion in ER-positive/erbB-2-overexpressing breast cancers by ER-erbB-2 cross talk and p27/kip1 downregulation. Carcinogenesis 31: 695–702.

Yao H, Xu W, Shi X, Zhang Z (2011) Dietary flavonoids as cancer prevention agents. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 29: 1–31.

Zhang B, Liu ZY, Li YY, Luo Y, Liu ML, Dong HY, Wang YX, Liu Y, Zhao PT, Jin FG, Li ZC (2011) Antiinflammatory effects of matrine in LPS-induced acute lung injury in mice. Eur J Pharm Sci 44: 573–579.

Zhou H, Lutterodt H, Cheng Z, Yu LL (2009) Anti-Inflammatory and antiproliferative activities of trifolirhizin, a flavonoid from Sophora flavescens roots. J Agric Food Chem 57: 4580–4585.