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ORIGINAL PAPER

The natural compound *n*-butylidenephthalide derived from the volatile oil of *Radix Angelica sinensis* inhibits angiogenesis in vitro and in vivo

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Abstract *Radix Angelica sinensis* is a Chinese medicinal herb that has been used extensively in the East for the treatment of cardiovascular diseases (CVDs). Angiogenesis plays an important role in the pathogenesis of CVDs. We hypothesized that *Radix A. sinensis* may contain angiogenesis modulators. In the current study, we investigated the effects of a volatile oil of *Radix A. sinensis* (VOAS) and *n*-butylidenephthalide (BP), one of the bioactive components in VOAS, on angiogenesis in vitro and in vivo. The results suggested that VOAS exerted anti-angiogenic effects by inhibiting human umbilical vein endothelial cell proliferation, migration and capillary-like tube formation on Matrigel. BP was also shown to be anti-angiogenic and its mechanisms were through inhibition of cell cycle progression and induction of apoptosis. Western blotting analysis indicated that the anti-angiogenic actions of BP were associated with the activation of p38 and ERK 1/2 but not SAPK/JNK and Akt signaling pathways. Further investigations showed that BP inhibited endothelial sprouting in an ex vivo mouse aortic ring model and was a potent inhibitor of the development of zebrafish subintestinal vessels in vivo. Our data using the volatile oil contrast with previous findings, which showed an aqueous extract of *Radix A. sinensis* was pro-angiogenic. This highlights the importance of identifying pro- and anti-angiogenic substances in *Radix A. sinensis*, not only for the development

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of novel angiogenesis modulators for the treatment of CVDs, but also to ensure the proper use of *Radix A. sinensis* as a nutraceutical.

Keywords *Radix Angelica sinensis* · *n*-butylideneph-thalide · Angiogenesis

Introduction

Angiogenesis is the process of forming new blood vessels from pre-existing vasculature. In recent years, it has become increasingly evident that excessive, insufficient or abnormal angiogenesis contributes to the pathophysiology of cardiovascular diseases (CVDs) [1, 2]. Modulating angiogenesis using angiogenesis inhibitors or stimulators therefore becomes an attractive strategy for the treatment of CVDs. For example, pro-angiogenic molecules vascular endothelial growth factor (VEGF-A) and fibroblast growth factor (FGF) have been used to promote therapeutic angiogenesis in ischemic cardiovascular disease in clinical trials and animal studies [3–6], whereas the anti-angiogenic approach has been used to promote atherosclerotic plaque stabilization and to delay plaque progression [7–9].

Many of today's medicines were originally derived from natural products. Traditional Chinese medicine, which involves using herbal remedies as prescription medicines, has been developed over 3,000 years. Recently, there has been a renewed interest in mechanistic studies of, and identification of active compounds from, herbal formulations [10, 11]. Such evidence-based approaches are not only important to the validation of traditional medicine, but also fundamental to future drug discovery.

Radix Angelica sinensis (also referred to as danggui or dongquai) is one of the most widely used traditional Chinese medicines and is often applied in composite formulae to treat CVDs and gynecological dysfunction in Asia. Earlier investigations suggested anti-atherosclerotic, cardio- and endothelial-protective actions of *Radix A. sinensis* [12–14].

The most studied bioactive constituents of *Radix A. sinensis* are alkylphthalides, ferulic acid and polysaccharides [15]. Previous reports showed that *n*-butylidenephthalide (BP) (Fig. 1), an alkylphthalide derived from the volatile oil of *Radix A. sinensis* (VOAS), exhibited anti-platelet, anti-anginal and anti-cancer activities [16–19]. In addition, BP acted synergistically with the nitric oxide donor sodium nitroprusside to cause vasorelaxation in rat aortic rings [20]. However, the role of *Radix A. sinensis* in angiogenesis modulation has not yet been sufficiently investigated. A study by Lam and colleagues indicated that an aqueous extract of *Radix A. sinensis* (AQAS), which contained 60% polysaccharide, was pro-angiogenic [21].

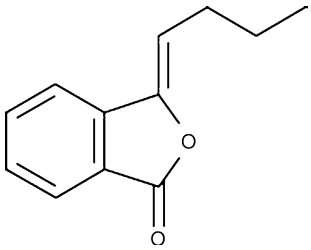


Fig. 1 Chemical structure of *n*-butylidenephthalide (BP)

However, here we report that BP and VOAS, which has a very different composition compared with AQAS, are anti-angiogenic. The mechanistic action of BP on human umbilical vein endothelial cells (HUVECs) was explored.

Materials and methods

Chemicals

BP was purchased from Lancaster Synthesis Ltd (Newgate, Morecambe, UK). VOAS was a gift from Professor Guanghua Lu, Chengdu University of Traditional Chinese Medicine, China. The raw *Radix A. sinensis* material was purchased from Good Agricultural Practice site in Gansu Province, China. Volatile oil extracted from raw plant material was through steam distillation, followed by chromatographic separation and purification. Chemical composition of VOAS was determined by gas chromatography-mass spectroscopy (GC–MS) analysis. Further information on GC–MS analysis is available in the Online Data Supplement. Both BP and VOAS were dissolved in dimethylsulfoxide (DMSO).

Cell culture

HUVECs were isolated from human umbilical veins by collagenase perfusion as described by Jaffe et al. [22]. Umbilical cords were collected with Ethics Committee approval and written informed patient consent. Harvested primary cells were grown in endothelial cell basal medium-2 supplemented with EGM-2 SingleQuots (Lonza Biologics, Slough, UK). Cells were maintained at 37°C in 5% CO_2 humidified tissue culture incubator. HUVECs from passages 4–5 were used in these studies.

Trypan blue dye exclusion assay

Cell proliferation was measured by trypan blue dye exclusion assay. HUVECs (8–10 × 10^4 cells/well) were plated into 12-well plates and incubated with VOAS (1–40 μg/ml) and BP (20–60 μg/ml) for different periods

of time. The attached cells were harvested with trypsin (Sigma–Aldrich, Dorset, UK) and stained with 0.4% trypan blue (Sigma–Aldrich). Trypan blue negative (viable) cells were counted using a hemocytometer under a phase-contrast microscope (Nikon; 100X).

In vitro wound healing assay

We employed an in vitro endothelial wound healing assay for the assessment of cell migration. Confluent HUVEC monolayers were grown on 1.5% gelatin-coated Thermanox TM coverslips (Nunc, Loughborough, UK) and wounded with a multi-channel wounder [23]. The coverslips were then transferred to new 24-well plates containing various concentrations of VOAS and BP. After 8 h, cells were fixed with methanol for 25 min. Images of wound closure were captured using a phase-contrast microscope (Nikon; 40X). The width of each wound from each picture was measured four times using ImageJ 1.36b software (National Institutes of Health, USA). The degree of wound regeneration was calculated as the percentage of the remaining cell-free area compared to the vehicle control.

Capillary-like tube formation on matrigel

Pre-chilled 96-well plates were coated with 50 μl/well growth factor-reduced Matrigel (Trevigen, MD, USA) which was allowed to polymerize at 37°C. HUVECs were subsequently plated on the Matrigel followed by addition of VOAS and BP and incubation for 22 h. Cells were photographed using a phase-contrast microscope (Nikon; 40X). To quantify the results, we counted the number of branch points, in which at least 3 tubes joined.

Determination of apoptosis

Apoptosis was monitored by active caspase-3 immunostaining and Hoechst nuclear staining. Briefly, confluent HUVECs were treated with BP for 2 h, fixed with 10% neutral buffered formalin and permeabilized with 0.2% Triton X-100 in PBS. Cells were then incubated with anti-active caspase-3 antibody (1:250 dilution; Promega, Southampton, UK) in blocking solution (5% goat serum in 0.1% Tween 20) at 4°C overnight. Antibody binding was visualized with a secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG; 1:100 dilation in PBS; Promega). Cell nuclei were counterstained with Hoechst 33342 (Molecular Probes, Invitrogen, Paisley, UK). The images were captured by a camera (Nikon, Coolpix 4500) attached to a fluorescence microscope (Leica; 100X).

Flow cytometry

We performed flow cytometry to investigate the effects of BP on cell cycle and apoptosis. The detailed protocols can be found in the Online Data Supplement.

Western blotting analysis

Analysis information is available in the Online Data Supplement.

MTS assay

The MTS assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega) is a colorimetric assay for measuring cell viability. The detailed protocols were described in the Online Data Supplement.

Capillary spouting from mouse aortic rings

Mouse aortae from C57/black mice were cut into 1–2 mm long rings and positioned in 48-well plates with the lumen oriented horizontally. Aortic rings were then embedded in a fibrin gel obtained by adding bovine fibrinogen solution (3 mg/ml) and thrombin (1.5 U/ml) to each well. The organ culture was maintained at 37°C in 5% CO_2 in DMEM medium containing 10% fetal calf serum. After 24 h, the aortic rings were treated with BP and carboxyamidotriazole (CAI as a positive control) in medium containing 2% serum. The medium with test substances was replaced after 2 days. After 3 days of treatment, the area covered by the pseudo-capillaries that had sprouted from aortic rings was measured using an ocular grid under an inverted microscope (100X). The result is expressed as the number of grid units (NGU) [24].

In vivo zebrafish assay

A zebrafish (*Danio rerio*) breeding colony (wild-type AB strain) was maintained at 28°C as described at the Zebrafish Facilities of the University of Brescia [25]. Maintenance of adult fish, collection of fertilised eggs, and compound exposure were all performed in water adjusted to a conductivity of approximately 1,500 AS/cm, pH 7.5–8.0, with Instant Ocean Salts (Aquarium System, Mentor, OH). Exposure, initiated at the 13 somite stage (ss), was carried out in 35 mm dishes with 30 embryo/well in 3.0 ml of 0.01 μg/ml of BP or vehicle (0.2% DMSO) at 28°C. At 72 h post-fertilization (hpf), zebrafish embryos were fixed in 4% paraformaldehyde for 2 h at room temperature and stained for endogenous alkaline phosphatase (AP) activity [26]. Embryos were subsequently mounted in agarose-coated

petri dishes and photographed under an inverted microscope equipped with DFC480 digital camera and ICM50 software (Leica) at 10× magnification. To evaluate the anti-angiogenic activities of BP, we analysed its ability to modify the development of subintestinal vessels (SIVs) as evidenced by AP staining. SIV plexus was scored as absent, reduced, disorganized, or normal.

Statistical analysis

Data were shown as mean ± standard deviation. Statistical significance was analyzed using unpaired Student's *t*-test, one-way analysis of variance (ANOVA) or linear regression model. Values of $P < 0.05$ were considered to be statistically significant.

Results

VOAS inhibits angiogenesis in cultured endothelial cells

To investigate the effect of VOAS on angiogenesis, we first performed a series of in vitro angiogenesis studies on endothelial cell proliferation, migration and tube formation on Matrigel using HUVECs. Treatment with VOAS markedly decreased cell proliferation in a concentration-dependent manner (Fig. 2a). Over 48 h of culture, VOAS 1 μg/ml had no effect on cell proliferation compared with vehicle control. However, a persistent inhibitory effect was observed by incubating HUVECs with VOAS 10–20 μg/ml. Treatment with VOAS 30–40 μg/ml resulted in loss of viable cells within 24 h. Three different batches of VOAS were studied and all produced similar inhibitory effect on cell proliferation (data not shown). When endothelial cell migration was assessed after 8 h of incubation, it was found that treatment with VOAS inhibited cell migration into denuded areas (representative images in Fig. 2b), and such inhibitory effect was concentration-dependent ($P < 0.001$, linear regression analysis) (Fig. 2c). VOAS was also shown to inhibit the ability of HUVECs to form capillary-like tubes on a Matrigel matrix after 22 h of treatment ($P < 0.001$, unpaired Student's *t*-test) (Fig. 2d). Linear regression analysis indicated that the inhibitory effect of VOAS on tube formation was concentration-dependent ($P < 0.001$) (Fig. 2e). These results suggested that VOAS is anti-angiogenic.

Chemical characteristics of VOAS

VOAS exhibited anti-angiogenic properties which contrast with Lam and colleagues' findings in 2008 [21]. They reported that AQAS contained 60% polysaccharide and was pro-angiogenic. Therefore, we determined the chemical composition of VOAS using GC–MS. Fifty-one compounds in VOAS were separated and identified according to retention indices and NIST Mass Spectral Library (Online Figures I, A- I, E and Online Table I). The relative contents were obtained on the basis of the peak area ratio. The volatile constituents present in the VOAS were mainly monoterpenes ($C_{10}H_{16}$) (*e.g.* α-pinene, 8.85% and 3-carene, 32.09%) and sesquiterpenes ($C_{15}H_{24}$) (*e.g.* bulnesene 3.16% and α-himachalene, 2.79%), which were chemically distinct from the compositions in AQAS.

BP derived from VOAS inhibits angiogenesis in vitro

It was previously shown that BP, a compound derived from *Radix A. sinensis*, exhibited anti-platelet and anti-anginal activities [16, 17]. We therefore, determined the BP content of VOAS and evaluated its effects on angiogenesis in vitro and in vivo. The GC–MS results indicated that VOAS contained ~2.5% of BP (Online Table I). Treatment with BP concentration-dependently inhibited endothelial cell proliferation (Fig. 3a). When incubating HUVECs with BP 30–40 μg/ml, the increase in cell number was initially inhibited but after 24 h in culture, there was some recovery. BP 50–60 μg/ml caused cytostasis during 48 h of treatment. In an endothelial wound healing assay, BP inhibited cell migration in a concentration-dependent manner (Fig. 3b, c) ($P < 0.001$, linear regression analysis). Moreover, BP interfered with endothelial tube formation on a Matrigel matrix and the effect was also shown to be concentration-dependent (Fig. 3d, e) ($P < 0.001$, linear regression analysis). These data suggested the anti-angiogenic activities of BP. We further studied the mechanistic actions of BP.

BP arrests endothelial cell cycle and causes apoptosis

A flow cytometry analysis was employed to investigate the effects of BP on cell cycle. We found that BP reduced cell proliferation through the inhibition of cell cycle progression and induction of apoptosis. Figure 4a illustrates an example of representative flow cytometric data of HUVECs treated with BP and vehicle control for 6, 18 and 36 h. Quantitative analysis of flow cytometric data showed that BP 40–50 μg/ml caused cell cycle arrest in G0/G1 phase at 18 h (Fig. 4b), where the proportion of cells in G0/G1 phase was increased with a corresponding decrease in the proportion of cells in S phase and G2/M phase. However, at 36 h, the cell cycle of BP-treated HUVECs was restored and the proportion of cells in each phase was similar to vehicle control. In addition, treatment with BP 40-50 μg/mL resulted in a small percentage of cells undergoing apoptosis. Apoptosis was also detected via

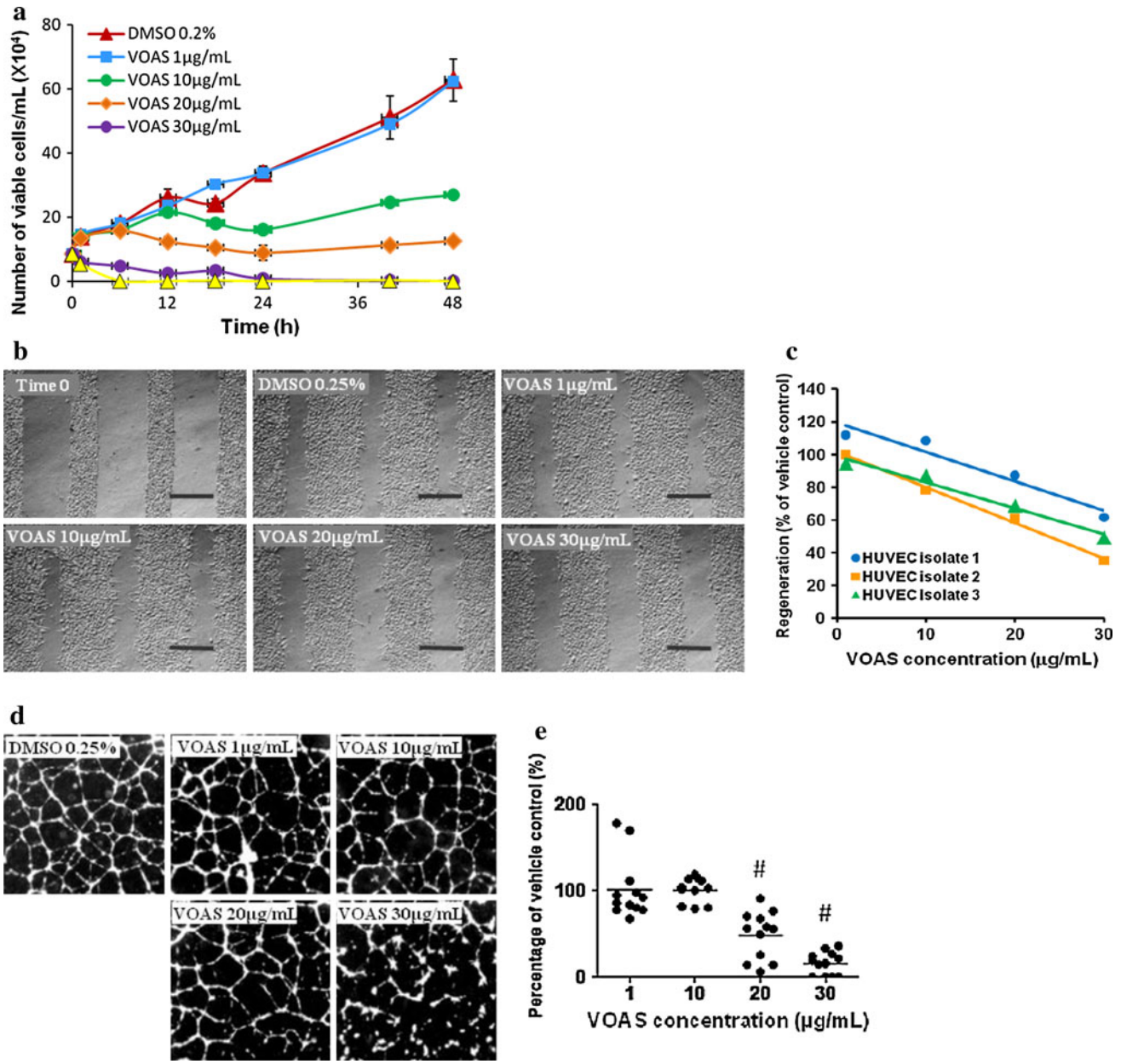


Fig. 2 VOAS inhibits angiogenesis in cultured endothelial cells. **a** HUVECs were plated in 12-well plates and incubated with VOAS and vehicle. Cells were counted at the indicated time points by Trypan blue exclusion. The results demonstrated that VOAS decreased HUVEC proliferation in a concentration-dependent manner. Data shown as the mean number of viable cells ±SD in triplicate. **b** Confluent monolayers of HUVEC on Thermanox coverslips were wounded using a multi-channel mechanical scraper and treated with VOAS and vehicle for 8 h. The images of wound closure were captured under a phase-contrast microscope (40X). *Scale bar* 400 µm. **c** The data from three different HUVEC isolates showed that VOAS concentration-dependently inhibited endothelial cell migration into denuded areas ($P < 0.001$, linear regression analysis). Total monolayer regeneration was expressed as a percentage of mean wound width of each treatment compared to vehicle control. The regression lines represent the relationship between wound regeneration and VOAS concentrations. **d** HUVECs were seeded on Matrigel-coated 96-well plates and incubated with VOAS and vehicle for 22 h. Branch points were counted to quantify endothelial tube formation (40X). This figure shows representative images of HUVEC tube formation treated with VOAS and vehicle. **e** VOAS ≥ 20 µg/ml reduced endothelial tube formation in a concentration-dependent manner ($P < 0.001$, linear regression analysis). *Dots* represent the number of branch points for treatment, as percentage compared to vehicle control ($n = 3$). # indicates significant inhibitory effect compared to vehicle control ($P < 0.001$, unpaired Student's *t*-test)

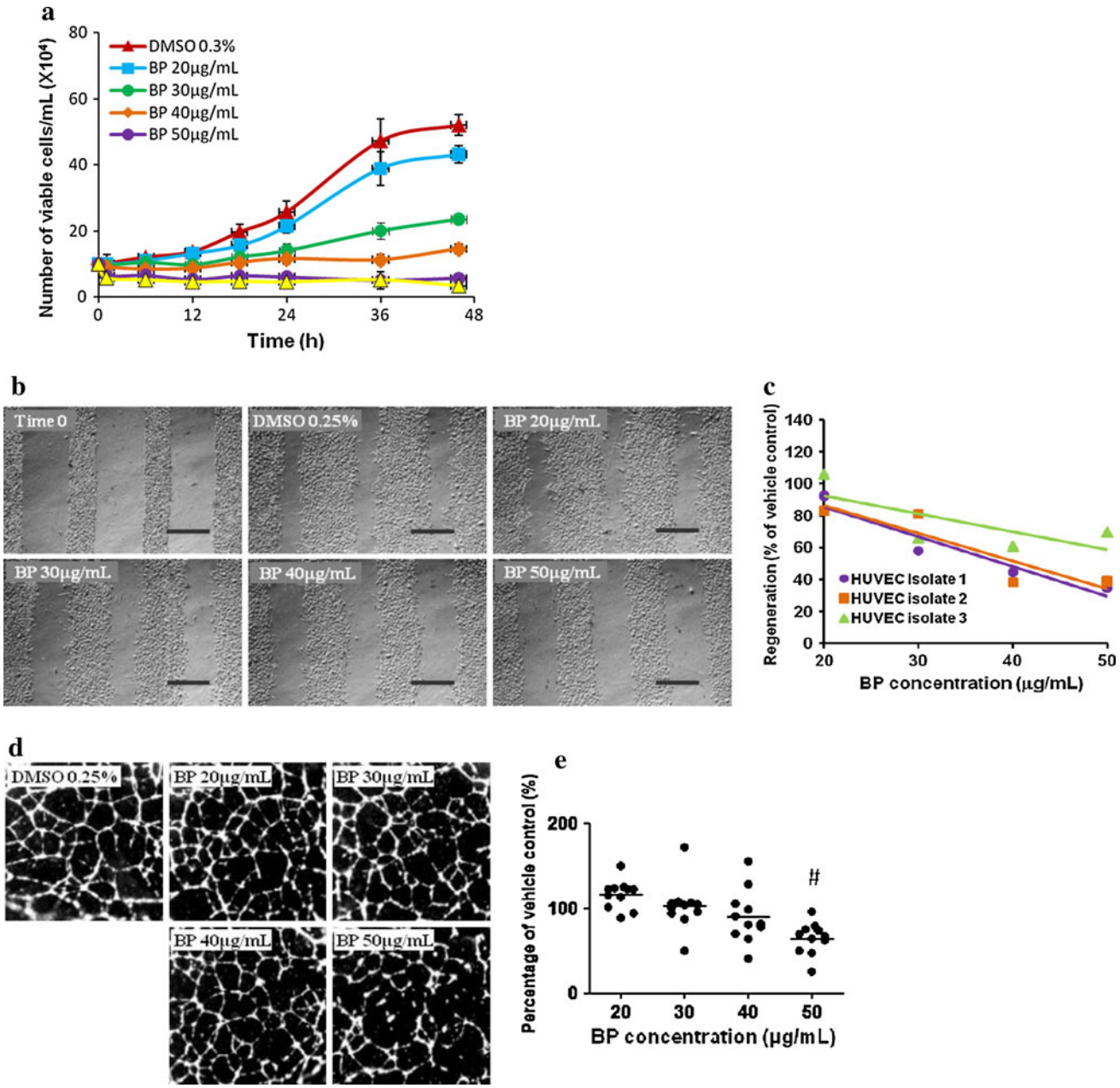


Fig. 3 BP inhibits angiogenesis in vitro. **a** HUVECs were plated in 12-well plates and incubated with BP and vehicle. Cells were counted at the indicated time points by Trypan blue exclusion. The results demonstrated that BP decreased HUVEC proliferation in a concentration-dependent manner. Data shown as the mean number of viable cells ±SD in triplicate. **b** Confluent monolayers of HUVEC on Thermanox coverslips were wounded using a multi-channel mechanical scraper and treated with BP and vehicle for 8 h. The images of wound closure were captured under a phase-contrast microscope (40X). *Scale bar* 400 µm. **c** The data was from three different HUVEC isolates showed that BP concentration-dependently inhibited endothelial cell migration into denuded areas ($P < 0.001$, linear regression analysis). Total monolayer regeneration was expressed as a percentage of mean wound width of each treatment compared to vehicle control. The regression lines represent the relationship between wound regeneration and BP concentrations. **d** HUVECs were seeded on Matrigel-coated 96-well plates and incubated with BP and vehicle for 22 h. Branch points were counted to quantify endothelial tube formation (40X). This figure shows representative images of HUVEC tube formation treated with BP and vehicle. **e** BP ≥ 50 µg/ml reduced endothelial tube formation in a concentration-dependent manner ($P < 0.001$, linear regression analysis). *Dots* represent the number of branch points for treatment, as percentage compared to vehicle control ($n = 3$). # indicates significant inhibitory effect compared to vehicle control ($P < 0.001$, unpaired Student's *t*-test)

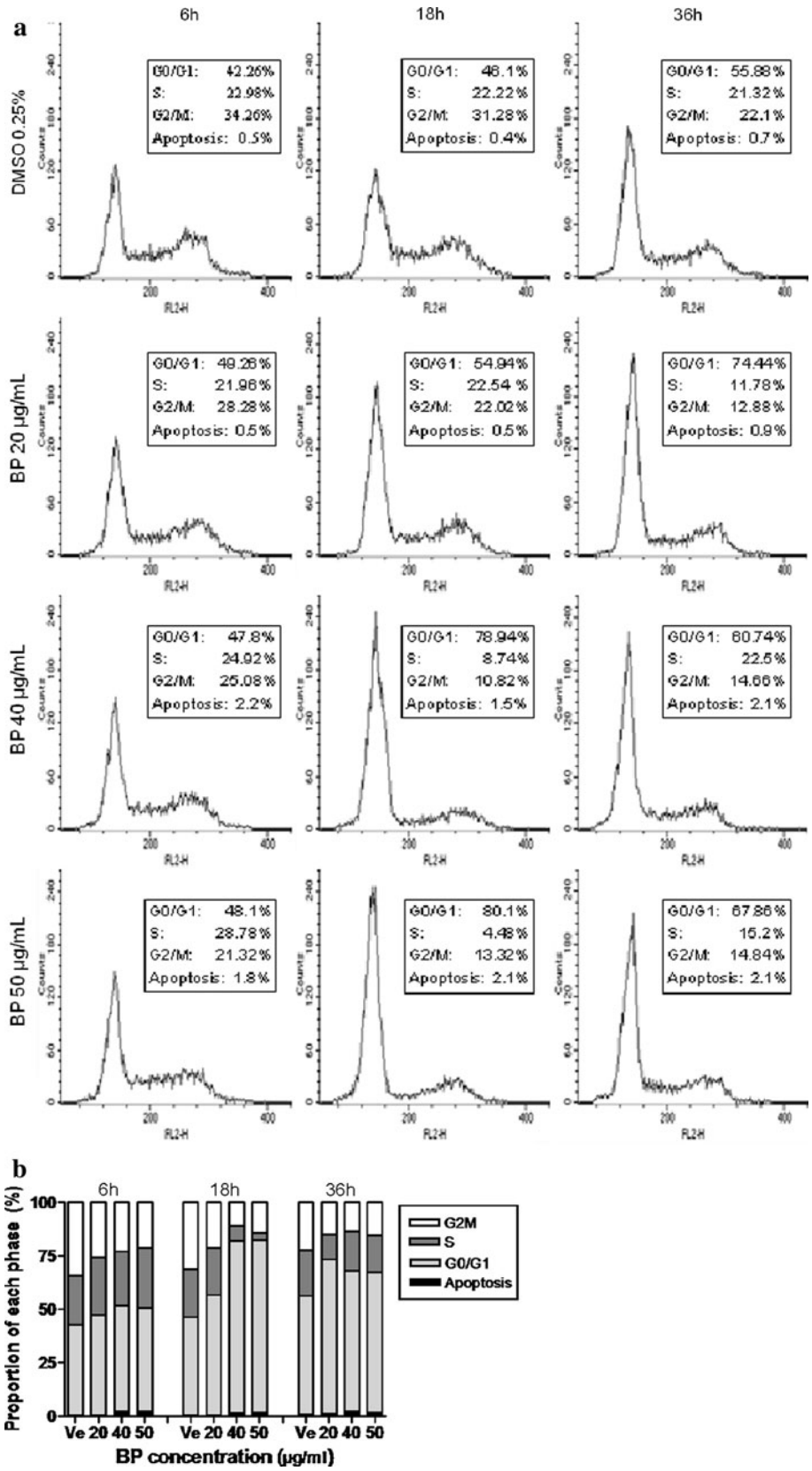


Fig. 4 BP arrests endothelial cell cycle and induces apoptosis. HUVECs were incubated with BP for 6, 18 and 36 h. Both floating and adherent cells were collected and analyzed by flow cytometry. **a** An example of flow cytometric data of HUVECs treated with BP and vehicle control. The inserts show the proportion of cell in each phase. **b** Quantitative analysis showed that BP 40–50 µg/ml caused cell cycle arrest in G0/G1 phase at 18 h. However, at 36 h, the cell cycle of BP-treated HUVECs was restored, as the proportion of cells in each phase was similar to vehicle control. Treatment with BP 40–50 µg/ml resulted in a small percentage of cells undergoing apoptosis. The data are expressed as the mean percentage of each phase from three individual experiments. *Ve* Vehicle control

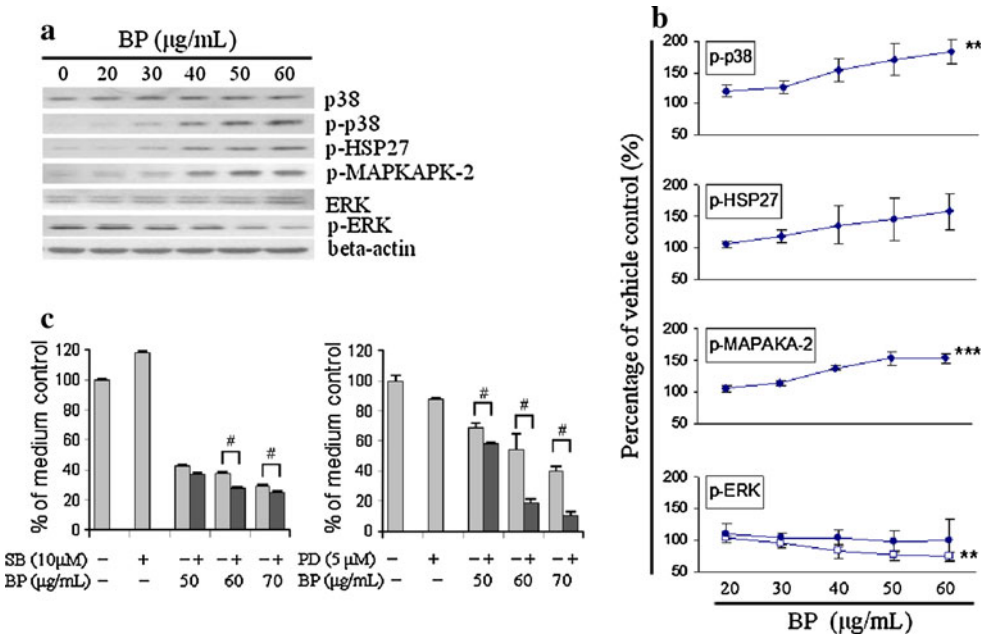


Fig. 5 Effects of BP on HUVEC signaling pathways. **a** HUVECs were incubated with BP (20–60 μg/ml) for 1 h. Whole cell lysates were analyzed by Western blotting using specific antibodies. **b** The results showed that BP activated the p38 pathway by up-regulating the phosphorylation of p38 and MAPKAPK-2 proteins. In addition, BP at 3 h decreased ERK1/2 phosphorylation in a concentration-dependent manner. Quantitative densitometry of protein phosphorylation shown as percentage of vehicle control (%). The protein levels were normalized to beta-actin or Ponceau S ($n = 3$). Treatment time = 1 h (*filled circles*). And for p-ERK also at 3 h (*open squares*). $^{**}P < 0.01$; $^{***}P < 0.001$; significant increase or decrease of protein phosphorylation (one-way ANOVA). **c** The MTS data demonstrated that the anti-proliferative effect caused by BP was p38-independent, for which the treatment with p38MAPK inhibitors SB 203580 and PD169316 did not attenuate the inhibitory effect of BP on HUVECs. Instead, two inhibitors enhanced the sensitivity of HUVECs to BP treatment. # indicates a significant decrease of cell viability compared to BP treatment only ($P < 0.001$, unpaired Student's *t*-test)

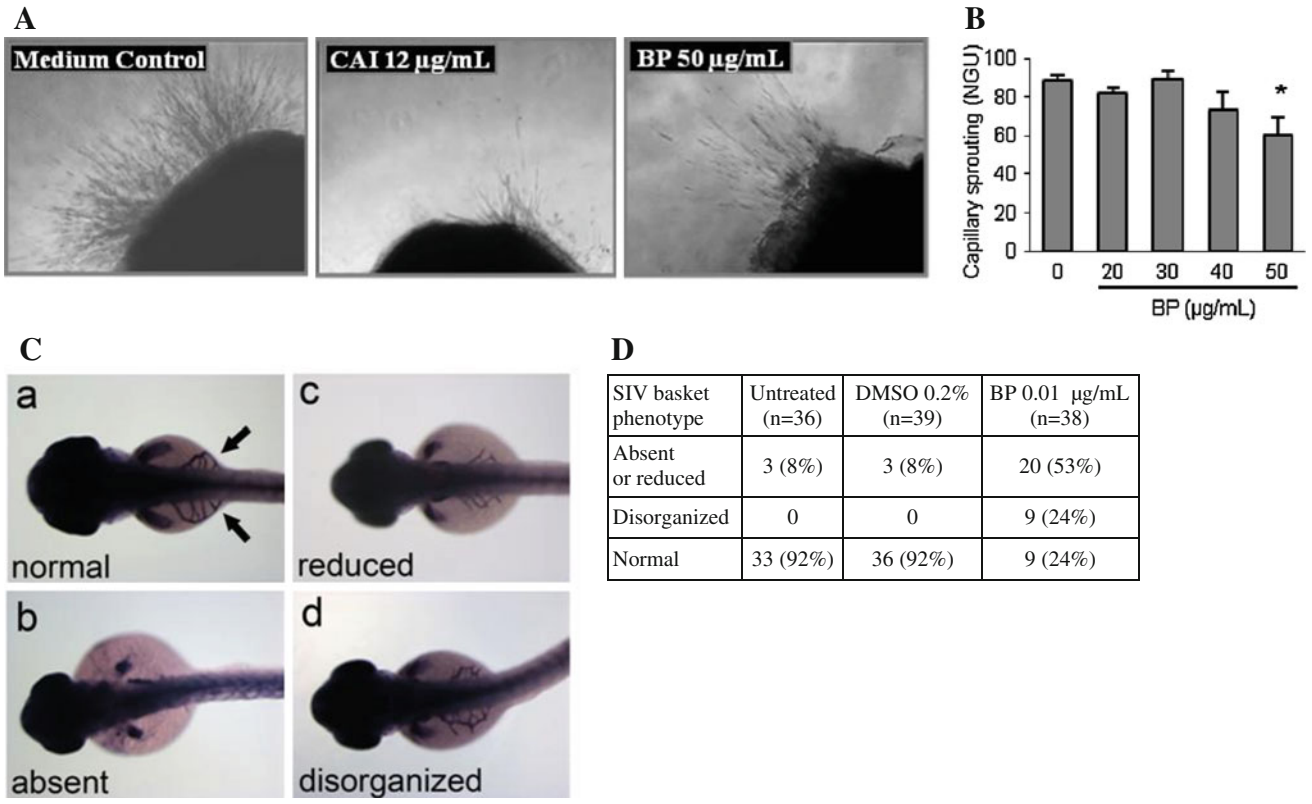
active caspase-3 immunostaining and Hoechst nuclear staining (Online Figures II, A and II, B).

Effects of BP on endothelial signaling pathways

We used Western blotting to investigate the effects of BP on a selection of proteins involved in signal transduction in HUVECs. Figure 5a, b show that BP activated the p38 pathway by up-regulating the phosphorylation of p38 and MAPKAPK-2 proteins after 1 h of incubation ($P < 0.05$, one-way ANOVA). BP at 3 h decreased ERK1/2 phosphorylation in a concentration-dependent manner ($P < 0.01$, one-way ANOVA). However, BP had no significant effect on the phosphorylation of SAPK/JNK or Akt at 1 h or 3 h (data not shown). We then used MTS assay to determine if the anti-proliferative effect caused by BP was p38-dependent. The results showed that treatment with the p38 MAPK inhibitors SB 203580 and PD169316 did not attenuate the inhibitory effect of BP on HUVEC viability (Fig. 5c). Instead, both p38 MAPK inhibitors increased the sensitivity of HUVECs to BP treatment, resulted in a lower MTS reading compared with the reading of BP treatment only ($P < 0.001$, unpaired Student's *t*-test).

BP inhibits capillary spouting from mouse aortic rings and impairs SIV vascularization in zebrafish

We performed an ex vivo angiogenesis study using mouse aortic ring model to assess the effects of BP on endothelial capillary outgrowth. Figure 6A shows examples of mouse aortic rings treated with BP 50 μg/ml and CAI 12 μg/ml, which both inhibited pseudo-capillary sprouting compared to medium control. BP concentration-dependently inhibited capillary sprouting (one-way ANOVA analysis) (Fig. 6B). For the zebrafish assay, zebrafish embryos were incubated with BP 0.01 μg/ml or vehicle at 13 ss. The development and organization of the SIV plexus was scored at 72 hpf after whole mount AP staining of the fixed embryos (Table in Fig. 6). BP 0.01 μg/ml profoundly affected the SIV plexus with 53% (20/38 embryos) of the treated embryos showing a dramatic impairment of SIV development, characterized by the absence or reduction of the vessels originating from the plexus (Fig. 6C, b and C, c). BP 0.01 μg/ml was the highest non-toxic concentration and did not cause any gross alteration in zebrafish development. A second phenotype observed in 24% (9/38 embryos) of the treated embryos was instead characterized by a profound



D

SIV basket phenotype	Untreated (n=36)	DMSO 0.2% (n=39)	BP 0.01 µg/mL (n=38)
Absent or reduced	3 (8%)	3 (8%)	20 (53%)
Disorganized	0	0	9 (24%)
Normal	33 (92%)	36 (92%)	9 (24%)

Fig. 6 BP produces anti-angiogenic actions ex vivo and in vivo. **A** Examples of mouse aortic rings treated with DMEM medium control, positive control (CAI 12 µg/ml) and BP 50 µg/ml (100X). Both CAI and BP exhibited inhibitory effects on pseudo-capillary sprouting. Representative pictures of vessel sprouting were taken at day 3 of treatment. **B** Capillary sprouting from mouse aortic rings was quantified as area covered by outgrowing capillaries and was expressed as the number of grid units (NGU). Quantitative analysis indicated that BP produced concentration-dependent inhibitory activities on capillary sprouting. Data are expressed as mean ± SD of NGU. $n = 4$ experiments run in quadruplicate. $^*P < 0.05$ compared to medium control (One-way ANOVA). **C** Zebrafish embryos were exposed to BP 0.01 µg/ml at 13 ss and stained for endogenous AP activity at 72 hpf. SIV plexus appearance was scored as normal **a**, absent **b**, reduced **c**, or disorganized **d**. Top view, head on the *left*. Arrows mark a normally developed SIV plexus. Original magnification (10X). **D** BP 0.01 µg/ml markedly affected the development of SIVs, characterized by the absence or reduction (53%, 20/38 embryos) and disorganized (24%, 9/38 embryos) of the vessels originating from the plexus. More than 90% of the vehicle-treated (0.2% DMSO) or untreated embryos, showed a normal developmental organization of the SIVs. These differences were highly significant ($P < 0.001$, *chi-squared*)

disorganization of the SIVs (Fig. 6C, d). More than 90% of the vehicle-treated (0.2% DMSO) or untreated embryos, showed a normal developmental organization of the SIVs (Fig. 6C, a). These differences were highly significant ($P < 0.001$, *chi-squared*).

Discussion

The present study demonstrated that VOAS (10–30 µg/ml) and BP (20–50 µg/ml) exhibited anti-angiogenic activities in vitro, as shown in the results of endothelial cell proliferation, migration and Matrigel tube formation assays (Figs. 2 and 3). Further studies using flow cytometric analysis and active caspase-3 immunostaining indicated that BP, at concentration range 40–50 µg/ml, inhibited cell proliferation via inhibition of cell cycle progression and induction of apoptosis (Fig. 4 and Online Figures II). Consistent with our in vitro data, BP 50 µg/ml inhibited capillary sprouting from mouse aortic rings (Fig. 6A, B). In addition, BP at 0.01 µg/mL impaired SIV vascularization in zebrafish embryos (Fig. 6C and Table in Fig. 6).

Our findings are in contrast to the previous study by Lam et al. [21] who reported that an aqueous extract of *Radix A. sinensis*, AQAS, was pro-angiogenic and enhanced HUVEC proliferation, migration, invasion and tube formation on Matrigel. In addition, their results showed that AQAS at 50 and 100 µg/ml promoted angiogenesis in vivo by stimulating angiogenesis in SIVs of zebrafish. To identify the chemical constituents of AQAS, Lam and colleagues utilized high-performance liquid chromatography analysis and found that AQAS contained mainly polysaccharides (60%), although ferulic acid, *Z*-ligustilide and BP were also detected [21]. However, we

showed in the current study that VOAS was anti-angiogenic and contained mainly monoterpenes and sesquiterpenes, neither ferulic acid nor polysaccharide was detected (Online Figure I and Online Table I). Traditional preparation of herbal formulae is a process called decoction, which is defined as the process of boiling herbs to concentrate and extract their active ingredients. Many volatile compounds of VOAS will not be present in the aqueous extract, although traces of some may remain. Hence Lam et al. might have found pro-angiogenic effects but not anti-angiogenic effects in AQAS. These findings suggest that distinct constituents present in the different extracts of *Radix A. sinensis* are likely be the main cause of the contrasting effects on angiogenesis.

We previously demonstrated that *Panax ginseng* contains ginsenosides which exert opposite effects on angiogenesis [27]. The ginsenoside Rg1 stimulated proliferation of HUVECs in vitro and promoted angiogenesis in vivo. The pro-angiogenic effect of Rg1 was mediated by nitric oxide synthase and the phosphatidylinositol-3 kinase-Akt pathway and also via the activation of the glucocorticoid receptor and the generation of VEGF-A [27, 28]. However, ginsenoside Rb1 and its metabolite Rg3 were anti-angiogenic [27, 29, 30]. The inhibitory mechanism of Rb1 is through estrogen receptor beta and by regulating pigment epithelium-derived factor [30].

In summary, the current data and those of Lam et al. [21] suggest that *Radix A. sinensis* contains both anti-angiogenic and pro-angiogenic components. This work emphasizes the importance of characterizing active substances present in *Radix A. sinensis*, not only for the development of novel angiogenesis modulators for the treatment of diseases associated with abnormal angiogenesis, but also to ensure the proper use of *Radix A. sinensis* as nutraceutical. For example, the aqueous extract of *Radix A. sinensis* might be contra-indicated for patients with atherosclerosis or cancer. On the other hand, the volatile oil of *Radix A. sinensis* might negate therapeutic angiogenesis of the heart or prevent the healing of chronic wounds. This work also highlights the need for regulation and standardization of herbal medicine, including their methods of preparation.

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