

In Vitro and *Ex Vivo* Antiangiogenic Activity of *Salvia officinalis*

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INTRODUCTION

Angiogenesis, the formation of new blood capillaries from pre-existing capillaries, links with physiological processes such as growth and repair and pathological conditions such as diabetic retinopathy, atherosclerosis, tumor growth and metastasis (Murray, 2001; Plank and Sleeman, 2004). Angiogenesis is tightly controlled through a balance of positive and negative regulatory factors and is triggered by pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which induce activation of their respective receptors on the surface of endothelial cells (Murray, 2001; Ferrara, 2004). Inhibition of angiogenesis is one of the promising approaches for the treatment of some chronic diseases, tumor growth and metastasis (Murray, 2001; Liekens *et al.*, 2001; Varet *et al.*, 2004). Since the identification of angiostatin and endostatin as endogenous inhibitors of angiogenesis (O'Reilly *et al.*, 1994, 1997), a variety of antiangiogenic compounds, such as soybean trypsin inhibitor (Shakiba *et al.*, 2007), withaferin A from *Withania somnifera* (Mohan *et al.*, 2004), a peptide from shark cartilage (Hassan *et al.*, 2005) and green tea catechin (Tang *et al.*, 2007) have been isolated from natural products. Antiangiogenic agents are known to inhibit proteases, to suppress receptor phosphorylation or disrupting endothelial cell tube formation (Murray, 2001; Ferrara, 2004).

Salvia officinalis (*Lamiaceae*) is reported to have a wide range of biological activities, including antibacterial, fungistatic, virustatic, astringent and antihydrotic effects (Eidi *et al.*, 2005; Horiuchi *et al.*, 2007). The antimicrobial properties of *S. officinalis* (active ingredient of dental-care herbal medicinal preparations) include the reduction in plaque growth, the inhibition of gingival inflammation and have positive effects on caries prophylaxis. Furthermore, due to the antiviral activity, *S. officinalis* extract is included for treatment of acute and chronic bronchitis (Schnitzler *et al.*, 2008; Horiuchi *et al.*, 2007; Eidi *et al.*, 2005; Bors *et al.*, 2004). Other experimental studies on *S. officinalis* extracts showed that some constituents of this plant such as triterpenes oleanolic and ursolic acids or diterpene carnosol, have antiinflammatory properties or antiprotease and anti-metastatic activity on lung colonization of B16 mouse melanoma cells (Baricevic *et al.*, 2001; Jedinák *et al.*, 2006). In the present study, it is demonstrated that ethanol extract and its hexane fraction of *S. officinalis* possess potent antiangiogenic activity on three dimensional cultures of rat aorta and HUVEC.

MATERIALS AND METHODS

Materials. Rat tail collagen (Sigma, St Louis, MO, USA) at 2 mg/mL in 0.5 M acetic acid, 10× minimum essential medium (10× MEM), 1.4% (w/v) sodium bicarbonate solution, sodium hydroxide solution (1 M), medium, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal bovine serum (FBS) (Gibco, New York, USA), dextran-coated cytodex 3 microcarriers (Amersham Pharmacia Biotech), human umbilical vein endo-

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thelial cells (HUVEC) obtained from the American Type Culture Collection, mouse embryo fibroblast cells (3T3) obtained from the Pasteur Institute (Tehran, Iran), trypan blue 0.4%, LDH cytotoxicity assay kit (Roch Chemical Co.). Rat aorta was isolated from 8–12 weeks-old male Wistar rats purchased from the Pasteur Institute (Tehran, Iran).

Plant material. Aerial parts of *S. officinalis* were collected from a field of pharmacological plants, Medical University, Kermanshah, Iran in summer and identified in the Agricultural College (voucher number 2402, deposited in the Herbarium of Razi University, director: Dr S. M. Maassoumi). The plant was cleaned, shed dried at 25 °C, and the dried material was ground with a blender. The powder was kept in nylon bags in a freezer until the time of experiments.

Preparation of the ethanol extract and fractions. The powder of dried aerial parts (500 g) was extracted with 70% (v/v) ethanol and concentrated to dryness under reduced pressure to give an ethanol extract (80 g). The extract was successively fractionated using *n*-hexane, ethyl acetate, *n*-butanol and aqueous solvents for the presence of different classes of phyto-constituents (Wagner *et al.*, 1984; Jones *et al.*, 1989; Ghayur and Gilani, 2007). Briefly, saponins were detected on observation of any froth formation following rigorous shaking of the extract dissolved in distilled water. Detection for steroids in the extract involved treating the extract with petroleum ether, then extracting with CHCl₃ and treating with acetic anhydride and HCl. The appearance of a green or pink color was indicative for the presence of steroids. Testing for flavonoids required mixing the extract with AlCl₃ and the appearance of yellow coloration indicated a positive result. The presence of phenolic and tannins was determined after the appearance of any green or dark green color following dissolution of the extract in aqueous FeCl₃. The presence of proteins and peptides was determined using the method of Bradford (1976). Absorbance at 595 nm was recorded against a ribulose 1,5-diphosphate carboxylase-oxygenase standard in NaOH.

Cytotoxicity. To determine cytotoxic concentrations of ethanol extract and its fractions, HUVECs were grown in culture medium supplemented with 10% FBS and containing different concentrations (0.0–400 µg/mL) of the ethanol extract and its fractions. After 72 h of incubation, cell viability was determined by trypan blue exclusion and lactate dehydrogenase (LDH) assays compared with controls. The absorbance of converted dye in LDH assay was measured at 490 nm with background subtraction at 630 nm (Decker and Lohmann-Matthes, 1988).

Antiproliferative assay. The antiproliferative assay was performed on HUVECs as a good source of endothelial cells. The cells were seeded on to 24-well culture plates at a density of 2×10^4 cells/well in MCDB131 supplemented with 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin. After 24 h incubation at 37 °C and 5% CO₂, ESO (0.0–400 µg/mL) and HSO (0.0–300 µg/mL) were added to the wells, and the cells were cultured for an additional 72 h, then trypsinized and counted with a cell counter (KX-21 Sysmex Co.) against control

wells. In addition, the antiproliferative effect of ESO (0.0–400 µg/mL) and HSO (0.0–300 µg/mL) was assayed in fibroblast cells (3T3) at the same condition in comparison with HUVECs.

Wound repair assay by endothelial cells. Endothelial cells were cultured in 24-well culture plates. When these cells were confluent, a wound was made. After being washed with PBS, the cells were incubated with MCDB131 supplemented with 2% FBS (concentration of FBS which allows cell survival but not cell proliferation) in the presence of ESO (0.0–400 µg/mL) or HSO (0.0–300 µg/mL). After 48 h incubation, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were then stained with Giemsa and photographed with a camera connected to an inverted microscope at appropriate magnifications (Varet *et al.*, 2004).

HUVEC capillary tube formation in three-dimensional collagen matrix. HUVECs were grown in MCDB131 supplemented with 10% FBS at 37 °C and 5% CO₂.

The cells were mixed with cytodex 3 microcarriers at a ratio of 30 cells per bead in 1 mL of MCDB131 medium (Auerbach *et al.*, 2003). Beads with cells were shaken gently every 20 min for 4 h at 37 °C and 5% CO₂. The mixture was transferred to a 24-well tissue culture plate and left for 12–16 h in 1 mL of MCDB131 at 37 °C and 5% CO₂. The following day, beads with cells were re-suspended in type 1 collagen gel as described above, and 50 µL of collagen/bead mixture was added to each well of a 96-well tissue culture plate and allowed to clot for 20 min at 37 °C, 5% CO₂. Then, 250 µL of MCDB131 medium was added to each well and after 8–12 h, different concentrations of the extracts were added. After 3–5 days of treatment, the antiangiogenic effects of the extracts were monitored microscopically.

Rat aorta model of angiogenesis. Angiogenesis also was studied by culturing rings of rat aorta in collagen matrix (Auerbach *et al.*, 2003; Zhe and Nicosia, 2002). The thoracic aorta was removed from rats killed by cervical dislocation and immediately transferred to a culture dish containing serum-free minimum essential medium (MEM). The peri-aortic fibroadipose tissue was removed carefully and 1–2 mm long aortic rings (approximately 24 rings per aorta) were sectioned and extensively rinsed in five consecutive washes of MEM. The ring explants were then embedded in each well of a 24-well culture plate (Berger *et al.*, 2004). 7.5 volumes of 2 mg/mL collagen solution, 1 volume of 10× MEM, 1.5 volume of 1.4% (w/v) NaHCO₃ were mixed in an ice bath and the pH adjusted to 7.4 with 0.1 volume of 1 M NaOH (Murray, 2001). Then, 400 µL of the mixture was added to each well. After clotting was completed, 1 mL of DMEM + RPMI (containing 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) was added. The culture plates were kept at 37 °C and 5% CO₂ in a humidified environment, and the medium was changed three times a week starting from day 3 (Nicosia and Ottinetti, 1990). After 3 days of culture, the extract and its fractions at different concentrations were added to the culture plates and after 4–7 more days, the results examined microscopically at appropriate magnification.

Statistical analysis. The results were expressed as mean \pm SE. Comparison between experimental groups was performed by ANOVA followed by the Tukey's multiple range tests. Values of $p < 0.05$ were considered to be significant.

RESULTS

An ethanol extract of *S. officinalis* was successively fractionated in to *n*-hexane (14.7%), ethyl acetate (3.1%), *n*-butanol (28.8%) and aqueous (53.4%) fractions. The fractions subjected to preliminary phytochemical screening using chemical methods showed the presence of steroids and saponins (*n*-hexane fraction), flavonoids and tannins (ethyl acetate fraction), flavonoids and phenolic compounds (*n*-butanol fraction) and poly peptides (aqueous fraction).

The ethanol extract of *S. officinalis* (ESO) in the range of 50–200 $\mu\text{g}/\text{mL}$ had no significant effect on the proliferation of HUVECs after 72 h, but at 300 $\mu\text{g}/\text{mL}$ and higher concentrations, a significant inhibition was observed (Fig. 1). On the other hand, HSO at concentrations higher than 50 $\mu\text{g}/\text{mL}$ inhibited endothelial cell proliferation in a dose dependent manner significantly. The antiproliferative effect of HSO did not significantly result from a cytotoxic effect, because nearly 90% of the endothelial cells were alive at 100 $\mu\text{g}/\text{mL}$ and more than 70% at 200 $\mu\text{g}/\text{mL}$ of this fraction after 72 h of treatment (Fig. 2). In comparison, ESO at 0.0–400 $\mu\text{g}/\text{mL}$ and HSO at 0.0–300 $\mu\text{g}/\text{mL}$ had no significant effects on the proliferation of fibroblast cells (data not shown).

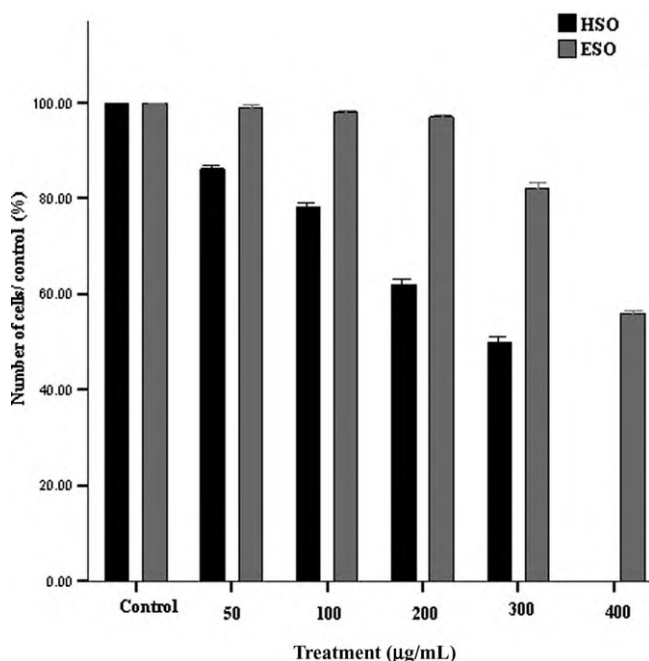


Figure 1. Dose response effects of ESO and its hexane fraction (HSO) on endothelial cell proliferation after 72 h. The HUVECs were seeded at 2×10^4 cells per well and incubated at the indicated concentrations of ESO and HSO. Data are expressed as the ratio of the number of treated cells to the number of vehicle-treated control cells (without ESO, HSO). Each column represents mean \pm SE of the three independent experiments.

Wound repair by endothelial cells was observed in untreated control wells. In contrast, inhibition of migration was clearly observed in wells with ESO (100–300 $\mu\text{g}/\text{mL}$) or HSO (50–200 $\mu\text{g}/\text{mL}$) (Fig. 3). Inhibition of wound repair was begun at 100 and completed at 200 $\mu\text{g}/\text{mL}$ of ESO, and in comparison, was begun at 50 and completed at 100 $\mu\text{g}/\text{mL}$ of HSO. These results revealed that ESO and HSO at 100–300 and 50–100 $\mu\text{g}/\text{mL}$, respectively, perfectly prevented the migration of HUVEC to fill the wound without considerable toxic effects.

Three-dimensional culture of HUVECs is an *in vitro* model to screen the inhibitory activity of ESO and its fractions on vascular development. After 3–5 days of treatment, untreated control wells showed a branching pattern of tube-like capillaries (Fig. 3A). In contrast, capillary tube formation was strongly suppressed in wells which were treated with ESO (100–300 $\mu\text{g}/\text{mL}$) (Fig. 3C–E). ESO was successively fractionated using hexane, ethyl acetate and *n*-butanol to determine the chemical characters of the active principle(s) present in ESO. Among the obtained fractions, the ethyl acetate and aqueous fractions showed partial antiangiogenic effects at concentrations lower than 300 $\mu\text{g}/\text{mL}$ and perfect antiangiogenic effects at doses higher than 300 $\mu\text{g}/\text{mL}$ (data not shown). On the other hand, the hexane fraction showed the highest inhibitory activity on three-dimensional culture of HUVEC, and this inhibition was dose-dependent in the range of 50–200 $\mu\text{g}/\text{mL}$ (Fig. 3F–H). The IC_{50} (the concentration of extract which showed half-maximal inhibition of the average number of capillary-like formations per micro carrier (cap/MC) of ten micro carriers from three independent

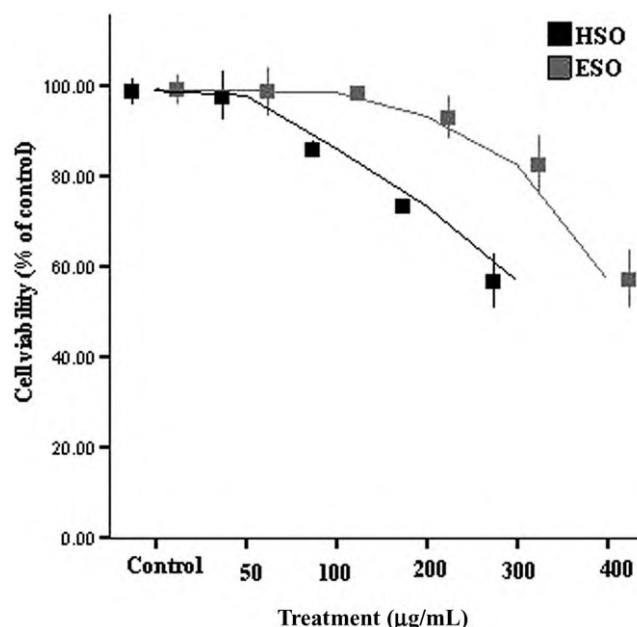


Figure 2. Dose response effects of ESO and its hexane fraction (HSO) on endothelial cell viability after 72 h. The HUVECs were seeded at 2×10^4 cells per well and incubated at the indicated concentrations of ESO and HSO. Cell viability was assessed by trypan blue exclusion assay compared with controls. Data are expressed as the ratio of the number of treated cells to the number of vehicle-treated control cells (without ESO, HSO). Each column represents mean \pm SE of the three independent experiments.

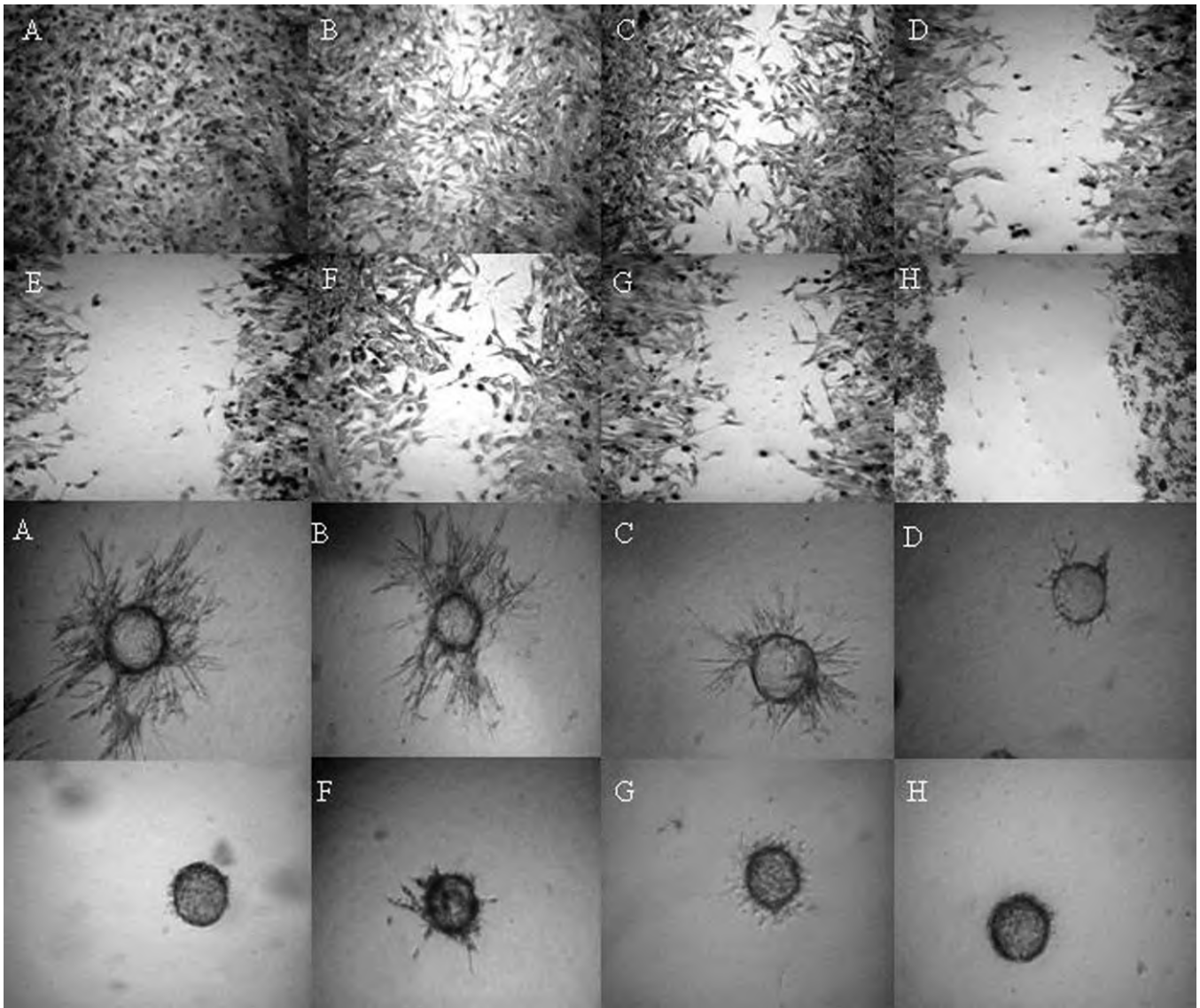


Figure 3. The effects of ESO and its hexane fraction (HSO) on the endothelial cell migration (upper half) and capillary tube formation (lower half). The effects of ESO and HSO on endothelial cell migration in the wound-healing model after 48 h (magnification $\times 25$). A, control; B, ESO 50 $\mu\text{g}/\text{mL}$; C, ESO 100 $\mu\text{g}/\text{mL}$; D, ESO 200 $\mu\text{g}/\text{mL}$; E, ESO 300 $\mu\text{g}/\text{mL}$; F, HSO 50 $\mu\text{g}/\text{mL}$; G, HSO 100 $\mu\text{g}/\text{mL}$; H, HSO 200 $\mu\text{g}/\text{mL}$. The effects of ESO and HSO on HUVEC capillary tube formation, in collagen gel. Capillary tube formation was analysed in vehicle-treated control well and wells treated with ESO and HSO, for 3 days, with an inverted microscope. A, control; B, ESO 50 $\mu\text{g}/\text{mL}$; C, ESO 100 $\mu\text{g}/\text{mL}$; D, ESO 200 $\mu\text{g}/\text{mL}$; E, ESO 300 $\mu\text{g}/\text{mL}$; F, HSO 50 $\mu\text{g}/\text{mL}$; G, HSO 100 $\mu\text{g}/\text{mL}$; H, HSO 200 $\mu\text{g}/\text{mL}$.

experiments) of the hexane fraction (HSO) was calculated to be 125 $\mu\text{g}/\text{mL}$ compared with 200 $\mu\text{g}/\text{mL}$ of ESO (data not shown).

Since the rat aorta model is an *ex vivo* model that mimics the *in vivo* situation, it was used to screen antiangiogenic activities of ESO and HSO on vascular development. After 7–10 days of culture of rat aorta, the branching pattern of capillary-like structures obviously was evident in control wells. In contrast, the formation of these structures was strongly inhibited in wells treated with ESO at 200–300 $\mu\text{g}/\text{mL}$ or HSO at 100–200 $\mu\text{g}/\text{mL}$ (Fig. 4). Taken together, ESO possesses potent antiangiogenic activity and its hexane fraction may contain major active antiangiogenic compound(s) responsible for the antiangiogenic properties of *S. officinalis*.

DISCUSSION

Over the recent years, more attention has been focused on the antiangiogenic and antineoplastic effects of non toxic compounds from natural products. Angiogenesis can be separated into several main steps including: digestion of basement membrane and extracellular matrix, migration, proliferation and rearrangement of endothelial cells to form new blood vessels (Plank and Sleeman, 2004). Inhibition of angiogenesis has been considered to be advantageous for the prevention of neoplastic growth and inflammatory reactions. Some antiangiogenic substances were identified to be effective in animal models of arthritis, and several antirheumatic drugs such as methotrexate, contain antiangiogenic

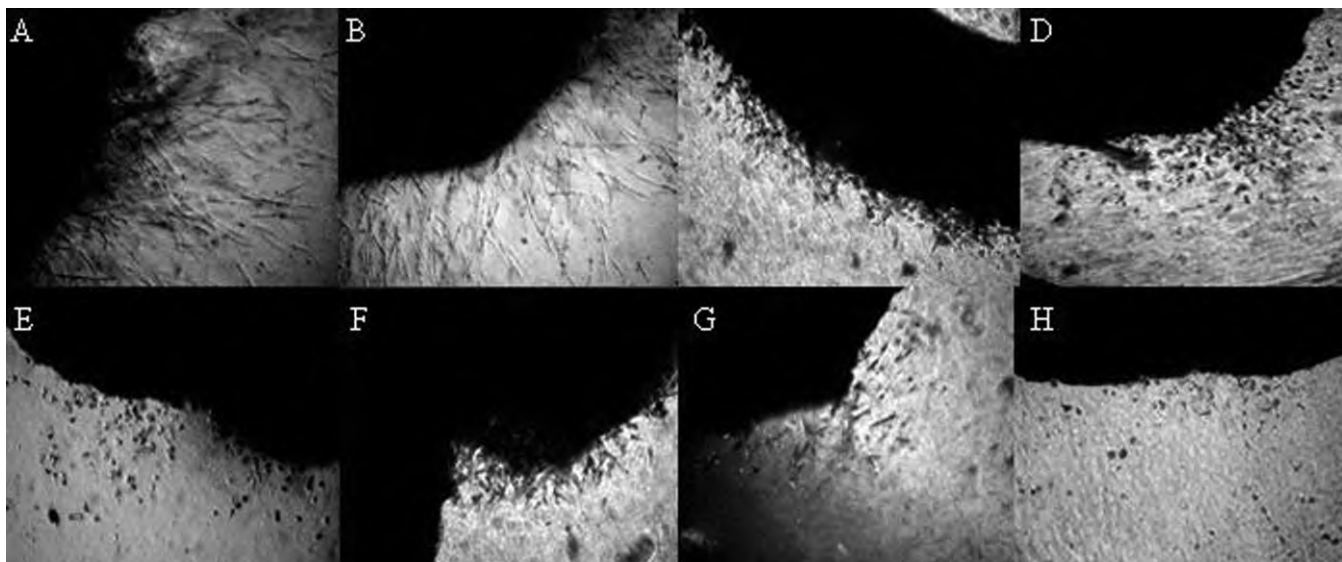


Figure 4. The effects of ESO and hexane fraction (HSO) on capillary tube formation of cultured rat aorta in collagen matrix. Capillary tube formation was analysed in vehicle-treated control well and wells treated with ESO and HSO, for 7 days, with an inverted microscope. A, control; B, ESO 50 µg/mL; C, ESO 100 µg/mL; D, ESO 200 µg/mL; E, ESO 300 µg/mL; F, HSO 50 µg/mL; G, HSO 100 µg/mL; H, HSO 200 µg/mL.

activity (Folkman, 2006). Currently antiangiogenic strategies are based on inhibition of endothelial cell adhesion and migration, and interference with metalloproteinases (Murray, 2001; Ferrara, 2004). Many researchers have been trying to screen novel antiangiogenic compounds from various natural products (Shakiba *et al.*, 2007; Mohan *et al.*, 2004; Park *et al.*, 2003; Hassan *et al.*, 2005; Tang *et al.*, 2007). *S. officinalis* is a member of the *Laminaceae* family with a wide range of biological activities including antibacterial, antiinflammatory, antiproteases and antimetastatic (Fiore *et al.*, 2006; Horiuchi *et al.*, 2007; Baricevic *et al.*, 2001). In this study, an ethanol extract was prepared from aerial parts of *S. officinalis* and the extract fractionated to its fractions. Thereafter, the antiangiogenic effects of ESO and its fractions were assessed using HUVEC capillary tube formation and rat aorta models of angiogenesis. The results clearly indicated that ESO contains antiangiogenic activity in a concentration dependent manner. To determine the chemical characters of active antiangiogenic principle(s) present in ESO, it was successively fractionated into *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions. Among the fractions, the hexane fraction showed the highest inhibitory activity on the two mentioned models that are reliable *in vitro* and *ex vivo* models to study angiogenesis, respectively. Based on the results of proliferative and wound healing assays (Figs 1 and 3), it may be concluded that antiangiogenic activity of ESO is due to an antimigratory more than an antiproliferative effect. This extract inhibited the migration of endothelial cells perfectly at 200–300 µg/mL without toxic effect at this range. The antiangiogenic activity of ESO theoretically can be attributed to anti-protease and/or interference of cytoskeleton organization, which are known to play important roles in cell locomotion and capillary tube formation (Plank and Sleeman, 2004; Dixelius *et al.*, 2002). On the other hand, the antiangiogenic activity of HSO was associated with a decrease in endothelial cell proliferation and migration in a dose dependent manner. This fraction, for

example, could prevent the migration of endothelial cells perfectly, and reduce the proliferation of these cells by more than 25% at 100 µg/mL. Because of a considerable toxic effect of HSO at doses higher than 200 µg/mL (Fig. 2), it was concluded that antiangiogenic effect of this fraction at the mentioned doses may be collectively due to the cytotoxic, antiproliferative and antimigratory activities. Even though the results of the wound healing assay and HUVEC capillary tube formation model of angiogenesis indicated that most of endothelial cells were alive at these doses, they were prevented to repair the wound or to make tube-like structures, respectively.

The concentrations of the hexane fraction required to inhibit angiogenesis in both models were relatively high in comparison with the ratio of this fraction in the ESO extract (14.7%). So far, it is possible that the phytoconstituents of HSO from *S. officinalis* may not act alone, but rather in concert with other agents. In addition, the ESO and its hexane fraction act more selectively on endothelial cells.

All together, the present study apparently is the first to demonstrate that *S. officinalis* extracts at pharmacological concentrations inhibit angiogenesis both *in vitro* and *ex vivo*. However, further investigations are required to elucidate the responsible component(s) and to ascertain the potential beneficial role of *S. officinalis* on the inhibition of angiogenesis *in vivo*.

CONCLUSION

In conclusion, the present study demonstrated that ESO at concentrations of 100–300 µg/mL, could inhibit the capillary tube formation both in HUVEC and rat aorta models of angiogenesis. This effect can be attributed mainly to inhibition of endothelial cell migration that is one of the most important steps of angiogenesis. The results also showed that the hexane fraction was the

most responsible fraction of the *S. officinalis* extract. The HSO at 50–200 µg/mL showed strong antiangiogenic activity both *in vitro* and *ex vivo*, and this effect can be attributed mainly to inhibition of endothelial cell migration and proliferation. These findings provide additional pharmacological information of the therapeutic efficacy of *S. officinalis*, and it would be considered as a novel starting point for the development of a new antiangiogenic drug.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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