Original Article

Anti Tumor Activity of *Salvia officinalis* is Due to Its Anti-Angiogenic, Anti-Migratory and Anti-Proliferative Effects

Maryam Keshavarz, M.Sc.[1, 2], Ali Bidmeshkipour. Ph.D.[2*], Ali Mostafaie, Ph.D.[1], Kamran Mansouri, M.Sc.[1], Hamid-Reza Mohammadi-Motlagh, M.Sc.[1]

1. Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
2. Department of Biology, Faculty of Sciences, Razi University, Kermanshah, Iran

**** Corresponding Address: P.O. Box: 6714967346, Department of Biology, Faculty of Sciences, Razi University, Kermanshah, Iran***
Email: abidmeshki@razi.ac.ir

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Abstract

Objective: The significant factor contributing to the distant invasion of cancer cells is the ability of tumors to produce large numbers of new blood vessels, known as angiogenesis. Many natural products inhibit angiogenesis. Herein, ethanol extract of *Salvia officinalis* (SO) has been analyzed for its anti-angiogenic, anti-proliferation and anti-migration activities.

Materials and Methods: The anti-angiogenic effect of the SO extract was evaluated on chicken chorioallantoic membrane (CAM) neovascularisation model, microscopically. The inhibitory effect of the extract on human umbilical vein endothelial cells (HUVECs) migration was tested on the wound-healing model with an inverted microscope. In addition, SO extract was screened for its possible anti-proliferative effects by separately counting HUVECs, Wehi and K562 cells with cell counter against their control wells.

Results: So extract exhibited a significant inhibitory activity in CAM assay in a dose dependent manner. CAM angiogenesis was gradually prevented to from at 100 µg/ml of SO extract, but completely inhibited to form at 200 µg/ml. After human umbilical vein endothelial cells (HUVECs) were suppressed by dose-dependent SO extract, their migrations were detected by wound healing model, yet they were unable to show a dose response effect on proliferation of the different cells (50-200 µg/ml). As observing in this study, SOextract could inhibit proliferation of the different cells at the concentrations above 200 µg/ml without toxic effect on the cells in doses ranged from 0-500 µg/ml.

Conclusion: These findings indicated that SO extract might be a promising candidate for anti-angiogenic treatment.

Keywords: *Salvia offificinalis*, Angiogenesis, Migration, Proliferation

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Introduction

Angiogenesis is a process of new blood vessel formation by endothelial cells that plays a critical role in normal physiology, such as development and pathological conditions including spreading of tumor, diabetic retinopathy, and rheumatoid arthritis (1, 2). Most primary solid tumors are dependent on angiogenesis for survival, growth, invasion, and metastasis. Therefore, targeting the angiogenesis process has become one of the important strategies in the treatment and prevention of cancer progression (3, 4). In the angiogenesis process, vascular endothelial cells migrate out from the parental vessels, invade through the matrix, proliferate, and form capillary tubes (5, 6). Currently, there are a variety of angiogenesis inhibitors being used in clinical trials such as soybean trypsin inhibitor (7), withaferin A derived from *Withania somniferous* (8), a HYPERLINK "http://linkinghub.elsevier.com/retrieve/pii/S1567576905000068" low molecular weight peptide extracted from shark cartilage (9), green tea catechin (10, 11) and aqueous extract of shallot (12) have been isolated from natural sources. Anti-angiogenic agents were known to inhibit proteases, suppress receptor phosphorylation or disrupt endothelial tube formation (13, 14). Drug development from natural products has become a rapidly emerging and highly promising strategy to identify novel anti-angiogenic and anti-tumor agents.

The genus salvia (Laminaceae family) comprises many species and shows diverse biological activities in the plant materials and/or extracts which are manifested by the different components that allow applying for the many medicinal and pharmaceutical applications. Indeed, *Salvia officinalis* has been

shown to have anti-bacterial, fungistatic, virustatic, astringent and anti-hydrotic effects (15-19). Other experimental studies on *S. officinalis* extracts show that some constituents of this plant such as triterpenes oleanolic, ursolic acids and diterpene carnosol for anti-inflammatory properties, or antiprotease and anti-metastatic activities on lung colonization of B16 mouse melanoma cells (20, 21). The present study was conducted to examine the effects of *S. officinalis* ethanolic extract on the proliferation, migration and anti- angiogenic activities.

Materials and Methods

The research was conducted in accordance with the accepted ethical principles of Kermanshah University of Medical Sciences.

Chemicals

MCD131 medium, Dulbecco's modified minimum essential medium (DMEM), RPMI 1640, fetal bovine serum (FBS) (Gibco, New York, USA), trypan blue 0.4% (Gibco, USA), Human umbilical vein endothelial cells (HUVECs), K562 (human chronic myeloid leukemia) and Wehi (Mouse fibrosarcoma) were obtained from the Pasture Institute (Tehran, Iran).

Plant material

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

Preparation of SO extract

Dried and ground aerial parts of the plant were extracted with ethanol 80% (v/v) for 24 hours and the dissolved fraction evaporated under reduced pressure by a rotavapor. The precipitant was redissolved in phosphate buffer saline and used in the different steps of the experiments.

Cytotoxicity

Cytotoxic concentrations of SO extract determined viability of cell lines reduction. The cells were grown in the medium containing the different concentrations (0-500 µg/ml) of the extract in triplicate samples for each dose. 48 hours after of the incubation, cell viability was determined by trypan blue exclusion assay compared to the control (22, 23).

Proliferation assays of different cell types

The following cell lines were used for this study: HUVECs, K562 and Wehi. The cells were plated and then treated with different concentrations of the SO extracts (0-500 µg/ml) in triplicate samples for each dose. Antiproliferative assay was performed on HUVEC because they were representative of microvascular endothelial cells; then after 4th passage, the cells were seeded into a 24-well culture plate at a density of 2×10^4 cells/well in M131 supplemented with 10% FBS, 100 IU/ml penicillin, and 100µg/ml streptomycin. 24hrs after the incubation at 37ºC in a 5% CO_2 incubator, SO was added to the wells (0-500 µg/ml) and the cells were further cultured for additional 3 days, then trypsinised and counted with cell counter (KX-21 SYSMEX Co.) against control wells.

K562 cells were grown as suspensions in RPMI+DMEM medium supplemented with 10% FCS in a 24-well plate (2×10^4 cells/well) and Wehi cells were cultured in RPMI supplemented with 10% FCS in a 24-well plate (2.5×10^4 cells/well) followed by incubating for 24 hours with adding different concentrations of SO extract (50-500µg/ml). 48 hours after the incubation, Wehi and K562 cells were harvested and counted with cell counter.

Chick embryo Chorioallantoic membrane (CAM) assay

In vivo anti-angiogenic activity of the different concentrations of SO extract utilizing chicken eggs was measured using CAM assay as previously described (24-26). The fertilized chicken eggs used in this work were kept in a humidified incubator at 37ºC. Three days after the incubation, about 2 ml of albumin was aspirated from the eggs through the small hole drilled at the narrow end of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane.

The shell covering the air sac was punched out and removed by a forceps, and the shell membrane on the floor of the air sac was peeled away. On the 4-day-old chick embryo, a methylcellulose cover slip with the various concentrations of SO extract was placed in the areas between vessels of the eggs. Another 48 hours after the incubation, the CAMs were carefully isolated and fixed in cold phosphate buffer saline, then neovascular zones of CAM located under the disks in each treatment group were photographed through a stereoscope equipped with a digital camera. Local vessel density was measured and the inhibitory effects on CAM angiogenesis were evaluated. Assays were repeated three times for each experimental group comprised 8 eggs.

Wound repair assay by endothelial cells

Endothelial cells were cultured in a 24-well culture plate. When HUVECs were confluent, they were wounded with a tip from the center of each well. After being washed with PBS, the cells were incubated with M131 supplemented with 2% concentration of FBS allows cell survival but not any cell proliferation in the absence or presence of different concentrations of SO (0-200 µg/ml) in triplicate samples for each dose. After 48 hours the incubation, cells were washed twice with PBS and fixed with 4 % paraformaldehyde in PBS for 10min at the room temperature. The cells were then stained with Giemsa and photographed with camera connected to an inverted microscope at the appropriate magnifications (27, 28). Cell migration ability was determined by the areas between the parallel lines represented the artificial wound.

Statistical analysis

The data were analyzed by one way ANOVA and differences were compared by Tukey multiple comparison post hoc test. All data were presented as mean ± SEM and differences were considered as significant at $p<0.001$.

Results

In this study, the SO extract up to 200 µg/ml did not show anti proliferative effect on HUVEC, Wehi and K562 cells; whereas higher concentrations of SO (300-500 µg/ml) inhibited proliferation of these cells (Fig 1).

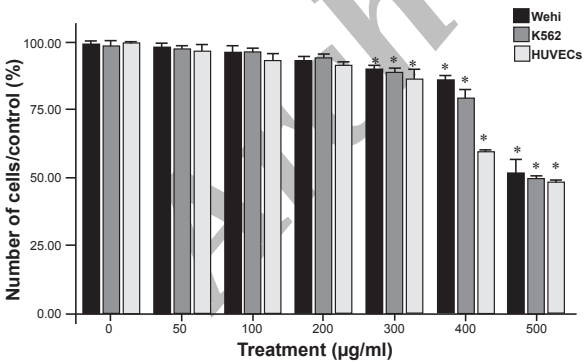

Fig 1: Effect of dose-dependent SO extract on the different cell types. These cells were incubated at the indicated concentrations of SO. Using a cell counter to express the data as a ration of the treated cells to the number of control cells (without SO).
Each column represents mean ± SEM of the three independent experiments. * p< 0.001, compared with control group. A value of p< 0.001 was regarded to be statistically significant.

Furthermore, this inhibitory effect of SO extract in the range concentration of 0-500 µg/ml did not result cytotoxic effect because the cell lines maintained viability above 75%, as being assessed by trypan blue exclusion assay and compared with the controls (Fig 2).

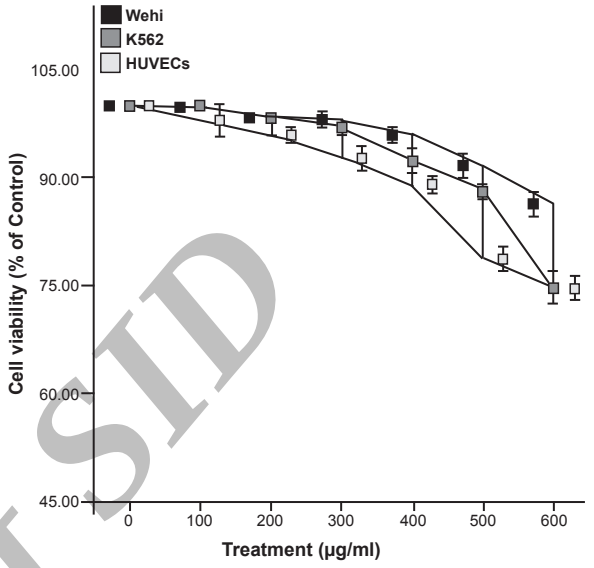

Fig 2: Cytotoxicity effects of SO extract on HUVECs, Wehi cells and K562 cells. Cells were treated with the various concentrations of SO extract for 48 hours, and then cell viability was detected by trypan blue exclusion assay.

To evaluate the effect of SO extract on the angiogenesis *in vivo*, we performed CAM assay. The SO extract significantly suppressed angiogenesis in a dose-dependent manner (Fig 3).

CAM assay is a widely-used model to determine angiogenesis *in vivo*. In this model, in the control group, endothelial sprouts progressively invaded and branched after 9 days treatment; however, in different concentration of SO extract the following observations were recorded: 50 µg/ml did not have any significant effect in inhibition of branching, 100 µg/ml had mild inhibitory effect ,and 200 µg/ml completely suppressed the growth of sprouts (Fig 4).

The average numbers of capillary-like formation of three independent experiments under the methyl cellulose membrane were counted (Fig 3). These result suggested that SO extract had an inhibitory effect on *in vivo* angiogenesis in CAM model.

Wound repair assay by endothelial cells was observed in the control wells without SO extract. In contrast, inhibition of migration was clearly observed in the wells with SO extract in a dose dependent fashion. These results revealed that this extract prevented migration of HUVEC to fill the wound.

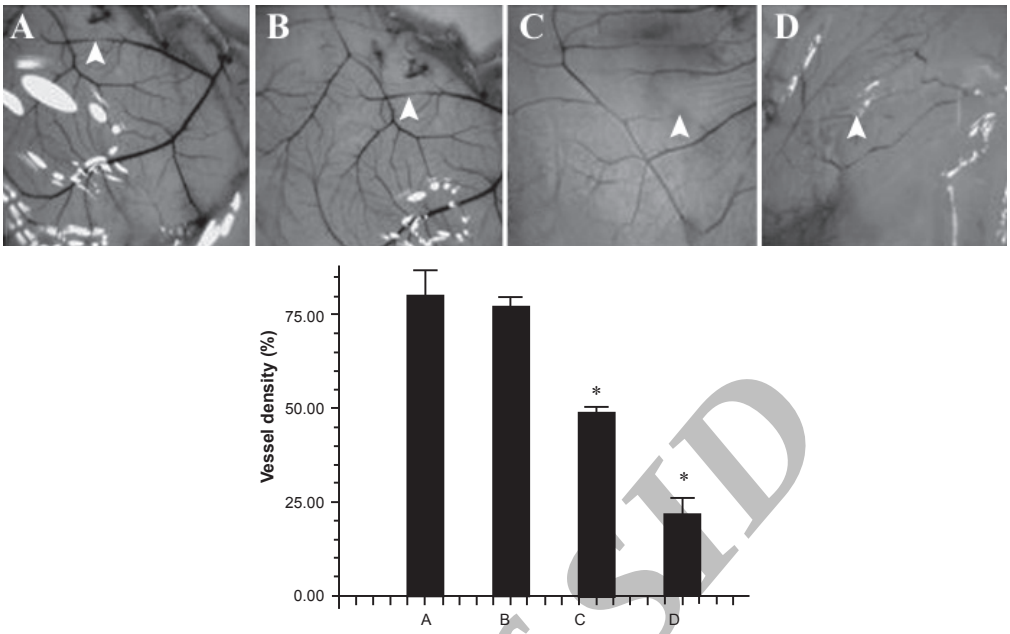

Fig 3: Inhibitory effects of SO extract on in vivo chick embryo chorioallantoic membrane (CAM) model. A. CAMs were treated with different concentrations of SO for 48 hours, which showed an inhibitory effect on angiogenesis. A. control (solvent of SO); B. 50 µg/ml of SO, C. 100 µg/ml of SO; D. 200 µg/ml of SO. The density of CAM microvessels was observed under a microscope and photographed with a digital camera. Antiangiogenic responces were scored as counting the average numbers of capillary-like formation of three independent experiments under the methyl cellulose membrane and each experiment group included 8 eggs. B. Vessel density in the scale of CAM was evaluated by determining the average vessel number (%). Each column represents mean ± SEM of the three independent experiments. A. control (solvent of SO); B. 50 µg/ml of SO, C. 100 µg/ml of SO; D, 200 µg/ml of SO. * P< 0.001, compared with the control group. A value of p<0.001 was regarded to be statistically significant.

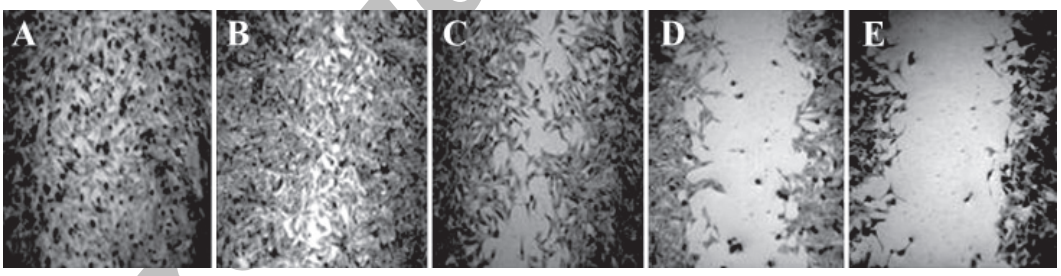

Fig 4: Effects of SO extract on cell migration in the wound-healing model. Inhibitory effect induced by SO extract on migration, is shown in a wound-healing model, 48 hours after incubation. A. Control (solvent of SO); B. 50 µg/ml of SO; C. 100 µg/ml of SO extract; D. 200 µg/ml of SO extract; E. Before addition SO extract. Cell migration ability is determined by the areas between the parallel lines representing the artificial wound.

Discussion

Over the recent years, more attention has been focused on the anti-angiogenic and anti-tumor effects of non toxic compounds from natural products. Angiogenesis mainly depends on proper activation, proliferation, adhesion, migration, and maturation of endothelial cells. Therefore, most approaches to modulate angiogenesis have focused on endothelial cells functions during blood vessel formation (3, 29). Inhibition of angiogenesis has been considered to be advantageous for prevention of tumor growth and metastatic activity. Some anti-angiogenic substances were identified to be effective in animal models of arthritis, and several antirheumatic drugs such as methotrexate, contain anti-angiogenic activity (6).

Some plant extracts contain many active ingredients. They are complex chemical cocktails with medicinal properties that affect tumor angiogenesis. A wide range of plants that contains compounds with angiogenesis-modulating properties were identified and their phytochemicals were isolated and characterized (30).

S. officinalis is a member of Salvia genus has a wide range of biological activities (17, 20, 21). Previous studies have shown that dihydrotanshi-

none I from Salvia miltiorrhiza Bunge could inhibit endothelial cell invasion and tube formation (31). In addition, *in vitro* antiproliferative activity of the methanol crude extracts of six salvia species in human cancer cell lines were tested and the data suggest that there are great differences among the various species, and the results strengthen the evidence that the genus Salvia could be considered a natural resource of antitumor agents (23). B-ursolic acid isolated from *S. officinalis*, significantly inhibited some proteases as included in tumor invasion and metastasis to combat with the pathologies including cancer (21). In this study, ethanolic extracts (SO) of the aerial parts of *S. officinalis* were prepared and the different concentrations of the extract tested on CAM model.

The chick chorioallantoic membrane assay (8, 24, 25) was used for examining the anti-angiogenic activity of SO. The results indicated that SO in a dose dependent manner inhibits angiogenesis in vivo. Anti-angiogenic activity of *S. officinalis* extract was associated to inhibit migration of endothelial cells, as assessed by wound healing method under conditions in which repair was mostly due to cell migration rather than cell proliferation because of presence of very low concentrations of fetal bovine serum in the cell culture medium.

The potential effect of SO extracts on the proliferation of following cell lines: HUVEC, K562 and Wehi were showed SO extracts in the range of 50-200 µg/ml had no significant effect on the proliferation of these cell lines, but at concentrations higher than 300 µg/ml, a significant inhibition was observed. It may be concluded that anti-angiogenic activity of SO extract is due to anti-migratory more than anti-proliferative effect, anti-migratory activity are probably attributed to anti-protease and/or interference of cytoskeleton organization, which are known to play important roles in cell locomotion and capillary tube formation (29, 32).

When Ethanolic extract of *S. officinalis* were subjected to preliminary phytochemical screening using chemical method, showed presence of steroids, saponins, flavonoids, tannins, phenolic compounds and poly peptides. In according to previous result (21), B-ursolic could be one of the potent candidates which affected endothelial cell migration. However, further investigations are required to elucidate the responsible component (s) and ascertain the potential beneficial role of *S. officinalis* on the inhibition of angiogenesis *in vivo*.

Conclusion

Indeed, this inhibitory effect of SO extract in the range of 0-500µg/ml did not result from cytotoxic effect as being assessed by trypan blue exclusion assay and compared with the control. All together, the result of this study apparently demonstrated that *S. officinalis* extract at pharmacological concentrations inhibits angiogenesis *in vivo*. 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 anti-angiogenic drug.

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