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# Extract of *Artemisia lavandulaefolia* Inhibits In Vitro Angiogenesis in Human Umbilical Vein Endothelial Cells

SHORT COMMUNICATION

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Angiogenesis is important processes for tumor growth and metastasis. Anti-angiogenesis target therapy has recently been known to be new anti-cancer therapeutic strategies. Natural products such as traditional medicine comprise a major source of angiogenesis inhibitors. *Artemisia lavandulaefolia* has been known to use in the traditional medical practices. However, its molecular mechanism on the tumor protection and therapy was not clearly elucidated. In this study, we investigated the possibility that extract of *A. lavandulaefolia* inhibits in vitro angiogenesis. Therefore, we examined the effect of extract of *A. lavandulaefolia* on the vascular network formation of human umbilical vein endothelial cells (HUVECs). We found that the treatment of *A. lavandulaefolia* extract suppressed the tube formation of HUVECs without any influence on the viability of HUVECs. In addition, extract of *A. lavandulaefolia* inhibited the migration and invasion of HUVECs. These results suggest that extract of *A. lavandulaefolia* could be act for an angiogenic inhibitor.
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**Key Words:** Angiogenesis, Natural products, *Artemisia lavandulaefolia*, Tumor

## INTRODUCTION

Angiogenesis is a process of remodeling such as sprouting and growth of blood vessels.[1] It is regulated under the control of balance between stimulators and inhibitors.[2] In addition, angiogenesis involve complex and diverse cellular actions such as degradation of extracellular matrix, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes.[3,4]

Many traditional oriental medicines consist of medicinal products from plants and animals that are used for treatments.[5] It has been practiced in China, Korea, Japan, and other Asian countries for many centuries.[6] In many cases, traditional medicinal products showed anti-tumor activities and they have been considered as candidates for novel cancer therapeutics.[7,8]

*Artemisia lavandulaefolia* is the Chrysanthemum family plant and is used in traditional medicine as a perennial plant that is widely distributed in Korea.[9] As well as being used as food material, *A. lavandulaefolia* has been used for the treatment of various diseases in traditional medicine in Korea.[10] *A. lavandulaefolia* has been used as a digestive, anthelmintic, and effective odor remover. In addition, *A. lavandulaefolia* has known effects about gastrointestinal diseases, constipation, pain, belly pain, asthma, and gynecological problems.[10] Recently, it has been reported that *A. lavandulaefolia* has anti-bacterial and anti-fungal activity against many kinds of the pathogenic bacterium and fungi in the Chinese medicine.[11-14]

Although many studies on the effects of *A. lavandulaefolia* have been conducted, no exists the information concerning relationship with angiogenesis and its molecular mechanisms. Therefore, we examined the anti-angiogenic effects by *A. lavandulaefolia* in human umbilical vein endothelial cells (HUVECs).

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## MATERIALS AND METHODS

### 1. Materials and reagents

HUVECs were purchased from InnoPharmaScreen Inc. (Asan, Korea). Basic fibroblast growth factor (bFGF) and heparin were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). M199, fetal bovine serum (FBS), penicillin and streptomycin were purchased from WELGEN Inc. (Daegu, Korea). Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA, USA) and used for the tube formation assay. Trans-well filter chambers (8-μm pores) were purchased from Corning-Costar (Cambridge, MA, USA).

### 2. Preparation of *Artemisia lavandulaefolia* extract

*A. lavandulaefolia* was purchased from Kyeongdong Medicinal Herb Market (Seoul, Korea). The biomass was dried root and leaf of *A. lavandulaefolia*. It was converted to a powdered form by cold-extraction using grain alcohol, and was sonicated at room temperature and ambient pressure. The *A. lavandulaefolia* extract was mixed with 70% grain alcoholic solution (30% pure water). The concentration of *A. lavandulaefolia* was 100 mg/mL, and the extract was diluted in distilled water. Finally, we used 50, 100, 500, and 1,000 μg/mL *A. lavandulaefolia* for cytotoxic tests and used 100 μg/mL for in vitro angiogenesis assays.

### 3. Cell culture

HUVECs were grown in M199, supplemented with heat-inactivated 20% FBS (WELGEN Inc.), 20 ng/mL of bFGF, 100 units/mL of penicillin and 100 μg/mL of streptomycin in a 37°C incubator with a humidified atmosphere containing 5% $CO_2$.

### 4. MTT assay for cell viability

The effect of extract of *A. lavandulaefolia* on the viability of HUVECs was determined using the MTT assay, which is based on the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,-5-diphenyltetrazoliumbromide) to insoluble MTT-formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes in living cells. Briefly, HUVECs were grown in M199 with 20% FBS at a density of $2 \times 10^4$ cells on 24-well culture plates. After one night, the media was re-placed with M199 containing 1% FBS, and crude extract of *A. lavandulaefolia* and the cells were then incubated for 24 hours at 37°C under a humidified atmosphere that was comprised of 5% $CO_2$. Cells were treated with various concentrations of extract of *A. lavandulaefolia* (50, 100, 500 mg, and 1 mg). Next, MTT solution (5 mg/mL in $H_2O$) was added to the well, followed by the addition of 0.3 mL of dimethyl sulfoxide to dissolve the MTT-formazan. The amount of MTT-formazan was then determined by measuring the absorbance at 540 nm. Each sample was assayed in triplicate, and the experiment was repeated three times.

### 5. In vitro tube formation assay

Before performing the test, 0.3 mL matrigel was transferred to 24-well plate and incubated for 30 minutes. HUVECs ($2 \times 10^4$ cells) were plated on a layer of polymerized matrigel and treated with or without extract of *A. lavandulaefolia* at 37°C for 24 hours. Cell morphological changes were captured through a phase contrast microscope and photographed at 40× magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

### 6. In vitro wounding migration assay

HUVECs were seeded onto 24-well culture plate until confluence and left overnight. Media was aspirated the next day, and cells were scratched with a 200 μL pipette tip along the diameter of the well. Cells were washed twice with PBS and incubated at 37°C and 5% $CO_2$. After wounding, the cells were incubated in M199 with 1% serum, 1 mM thymidine, and/or extract of *A. lavandulaefolia*. These culture conditions minimized proliferation of HUVECs. Wound diameters were photographed at 24 hours. Wound closure was determined with optical microscopy at 40× magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

### 7. In vitro invasion assay

Invasion assay was performed using a trans-well chambers system (Corning Inc., Cambridge, MA, USA) with 8.0-μm pore polycarbonate filter inserts. The upper side of trans-well was coated with 10 μL of matrigel (0.5 mg/mL) at room temperature for 1 hour. Complete media was plated in the lower parts of the trans-well chamber filters, and HUVECs ($2 \times 10^4$ cells) and extract of *A. lavandulaefolia* in serum-free media were placed in the upper part. Cells were incubated at 37°C for 24 hours, fixed with methanol, and then stained with hematoxyline/eosin. Cells on the upper surface of the filter membrane were removed by wiping with a cotton swab. Invaded cells were determined with optical microscopy at 40× magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

### 8. Data analysis and statistics

Data are presented as means ± standard deviation. $P < 0.05$ was considered significant.

## RESULTS

### 1. Extract of *Artemisia lavandulaefolia* do not show any cytotoxic effect on the viability of human umbilical vein endothelial cells

The cytotoxic effect of the *A. lavandulaefolia* extract on the HUVECs was examined by treatment with various concentration of extract of *A. lavandulaefolia* (from 50 to 1,000 mg/mL) and determined by MTT assay. Treatment with the *A. lavandulaefolia* extract for 24 hours has no effect on the viability of HUVECs (Fig. 1). This result indicates that the *A. lavandulaefolia* has no cytotoxic effect in HUVECs.

### 2. Extract of *Artemisia lavandulaefolia* suppress tube formation of human umbilical vein endothelial cells

To examine the effect of *A. lavandulaefolia* on the differentiation of endothelial cells, we conducted the tube formation assay. The capillary tube formation was conducted on polymerized matrigel beds and tube formation with elongated tube networks was observed in 6 to 24 hours in HUVECs (Fig. 2). Importantly, when HUVECs were treated with *A. lavandulaefolia* extract, tube network was completely abrogated (Fig. 2). This finding demonstrates that extract of *A. lavandulaefolia* suppressed the tube formation of HUVECs.

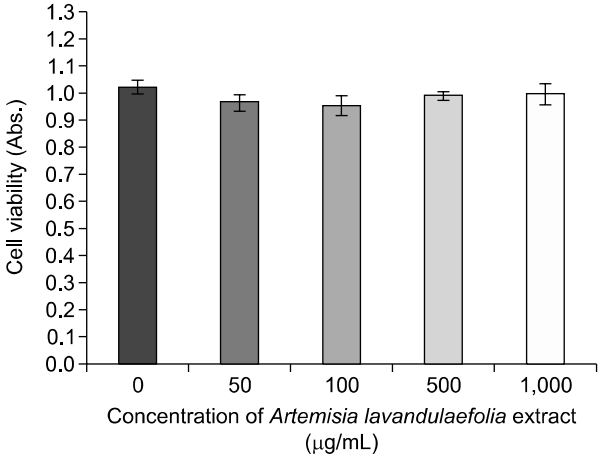


**Figure 1.** Extract of *Artemisia lavandulaefolia* has no effect on the cell viability. The cytotoxic effect of the *A. lavandulaefolia* extract on the human umbilical vein endothelial cells (HUVECs) was determined by the MTT assay.

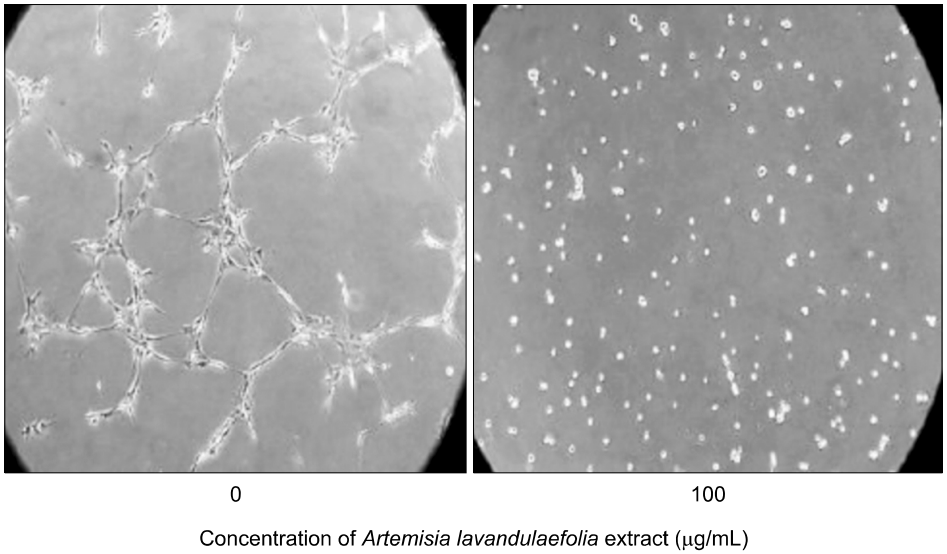


**Figure 2.** Extract of *Artemisia lavandulaefolia* suppresses the vascular network formation. The effect of extract of *A. lavandulaefolia* on tube formation of human umbilical vein endothelial cells was examined by in vitro tube formation assay. Extract of *A. lavandulaefolia* were added and incubated for 24 hours. The changes of cell morphology were captured through a phase contrast microscope (×40) and photographed. Representative photographs reveal the inhibitory effect of the *A. lavandulaefolia* extract on the formation of capillary-like structure. This independent experiment was repeated three times.

### 3. Extract of *Artemisia lavandulaefolia* inhibit migration and invasion of human umbilical vein endothelial cells

Endothelial cell migration and invasion are one of the critical steps in the formation of new blood vessels.[15] Therefore, we investigated the effect of extract of *A. lavandulaefolia* on the movement of HUVECs from a wounded edge to an open area by wound healing assay. Treatment with *A. lavandulaefolia* extract for 24 hours markedly decreased the migration of HUVECs compared with that of control (Fig. 3A). Migration of endothelial cells was decreased by about 63% by *A. lavandulaefolia* extract treatment compared with that of control (Fig. 3B).

To examine the effect of extract of *A. lavandulaefolia* on the invasiveness of HUVECs, we performed invasion assay with a trans-well system. Trans-wells were prepared such that the upper sides of the filter were coated with matrigel, and used for invasion assay. Extract of *A. lavandulaefolia* inhibited the

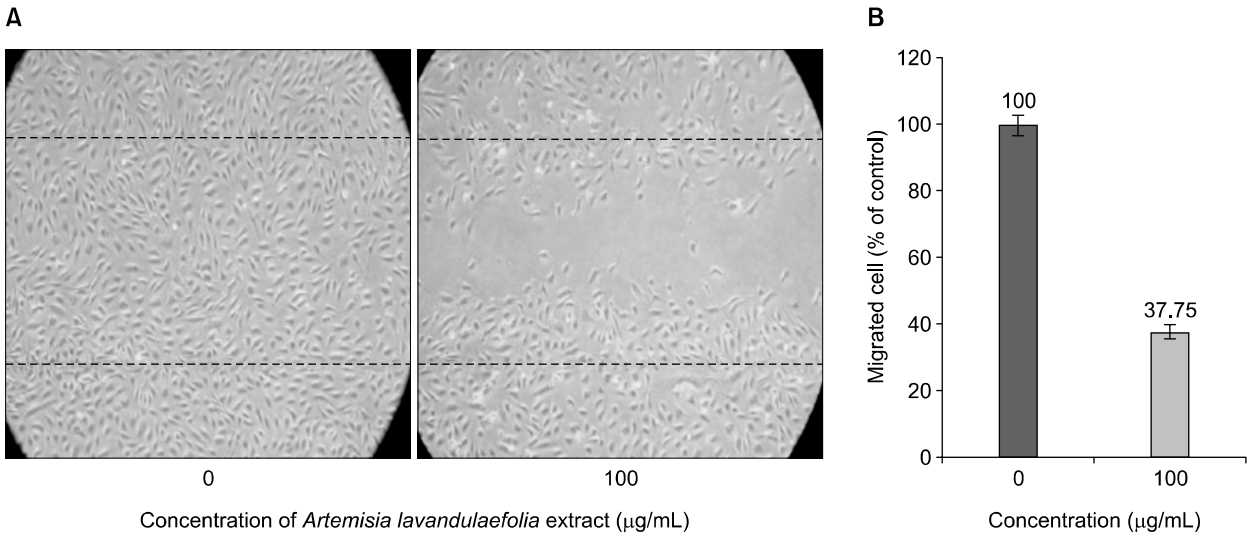


**Figure 3.** Extract of *Artemisia lavandulaefolia* inhibits migration of human umbilical vein endothelial cells. (A) Migration ability of HUVECs was measured by wound healing assay (×40). (B) Migrated cells were quantified under a phase-contrast microscope and photographed. This independent experiment was repeated three times.

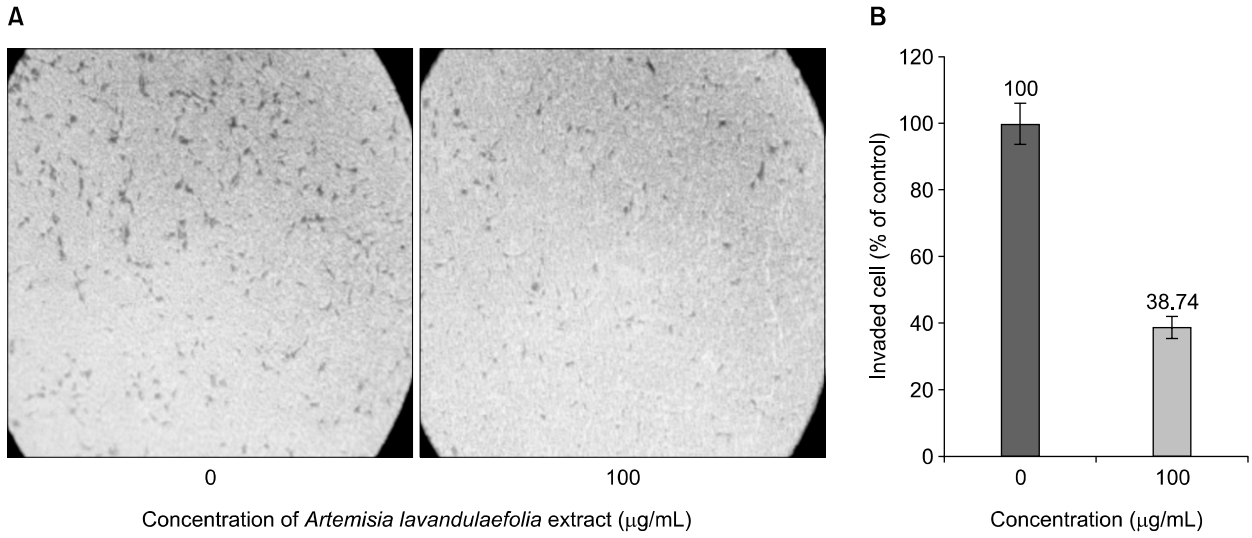


**Figure 4.** Extract of *Artemisia lavandulaefolia* inhibits invasion of human umbilical vein endothelial cells. (A) Invasion capacity was examined using a trans-wells system coated with matrigel (×40). (B) Invaded cells were quantified under a phase-contrast microscope and photographed. This independent experiment was repeated three times.

invasiveness of HUVECs compared with that of control after 24 hours of incubation (Fig. 4A). The invasiveness of endothelial cells was inhibited by about 62% by *A. lavandulaefolia* extract treatment compared with that of control (Fig. 4B). Taken together, extract of *A. lavandulaefolia* strongly suppressed the migration and invasion of HUVECs.

## DISCUSSION

Angiogenesis is the developmental process of new capillaries by sprouting from pre-existing vasculature,[16,17] and is required as a significant component of a wide variety of physiological process and pathological conditions.[18] In 1971, Folkman[15] was firstly built up that angiogenesis is essential process in tumor growth. Since then, angiogenesis have been recognized as important strategies for cancer therapy.[19,20] Angiogenesis are initiated by the secreted growth factors, chemokines/cytokines and other mediators.[3] Tumor initiation and progression are also closely linked to angiogenesis. Thus, angiogenic responses in tumor could be targets for development of anti-cancer therapeutic drugs.[5]

The studies for novel anti-cancer drugs from natural products have been continued through the research of scientists worldwide in looking for new bio-active compounds.[20] *A. lavandulaefolia* is one of the traditional medicinal products and is usually used for food materials.[11] Effects of *A. lavandulaefolia* have been known to use for treatment in the traditional medicinal field. However, scientific studies on the effects of *A. lavandulaefolia* were not clearly elucidated. Recently, *A. lavandulaefolia* has been known to show anti-bacterial and anti-fungal effects. *A. lavandulaefolia* has been also reported that it has induced apoptosis and necrosis of hela cells.[21]

In this study, we investigated whether extract of *A. lavandulaefolia* has anti-angiogenic activities in HUVECs. The MTT assay was first carried out. Because the MTT assay is one of the most widely used in cell viability assay, which measures the cytotoxicity of molecules. The result is that extract of *A. lavandulaefolia* did not affect the viability of HUVECs (Fig. 1). This result was expected because extract of *A. lavandulaefolia* widely using as food materials. To determine the effect on the anti-angiogenesis of extract of *A. lavandulaefolia*, we examined in vitro tube formation assay. It is important step of angiogenesis which promote morphological differentiation into capillary-like structure. In the absence of extract of *A. lavandulaefolia*, HUVECs formed capillary-like networks. However, tube-like structure was suppressed in the presence of extract of *A. lavandulaefolia* in HUVECs (Fig. 2). Endothelial cell migration and invasion are fundamental step during angiogenesis.[4] Therefore, we determined the effect of extract of *A. lavandulaefolia* on the migration and invasion of HUVECs. As shown in Figures 3 and 4, migration and invasion were remarkably reduced by treatment extract of *A. lavandulaefolia* in HUVECs.

Research of the functional ingredients from the traditional medicinal products is very important for therapy development. Chemical composition of *A. lavandulaefolia* essentioal oil has been previously reported in several studies.[22,23] The main components of *A. lavandulaefolia* essentioal oil was reported caryophyllene, b-thujone, eucalytol and b-farnesene.[24] However, the numerous activities of these components for medical therapy were not clearly elucidated. Thus, a functional study of the chemical composition of traditional medicine material will be added for effective disease treatment.

In summary, the major findings reported here are that extract of *A. lavandulaefolia* inhibited angiogenesis in HUVECs. Therefore, extract of *A. lavandulaefolia* may have potential to be a useful angiogenesis inhibitor. Further study is required to elucidate the mechanism of action on angiogenesis by extract of *A. lavandulaefolia*.

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## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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