# Hizikia Fusiformis Hexane Extract Decreases Angiogenesis *in Vitro* and *in Vivo*

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Angiogenesis, the formation of blood vessels from pre-existing vessels, is a multistep process regulated by modulators of angiogenesis. It is essential for various physiological processes, such as embryonic development, chronic inflammation, and wound repair. Dysregulation of angiogenesis causes many diseases, such as cancer, autoimmune diseases, rheumatoid arthritis, cardiovascular disease, and delayed wound healing. However, the number of effective anti-angiogenic drugs is limited. Recent research has focused on identifying potential drug candidates from natural sources. For example, marine natural products have been shown to have anti-cancer, anti-oxidant, anti-inflammatory, antiviral, and wound-healing effects. Thus, this study aimed to describe the angiogenesis inhibitory effect of *Hizikia fusiforms* (brown algae) extract. The hexane extract of *H. fusiformis* has shown inhibitory effects on *in vitro* angiogenesis assays, such as cell migration, invasion, and tube formation in human umbilical vein endothelial cells (HUVECs). The hexane extract of *H. fusiformis* (HFH) inhibited *in vivo* angiogenesis in a mouse Matrigel gel plug assay. In addition, the protein expression of vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK)/extracellular signal kinase, and AKT serine/threonine kinase 1 decreased following treatment with *H. fusiformis* extracts. Our results demonstrated that the hexane extract of *H. fusiformis* (HFH) inhibits angiogenesis *in vitro* and *in vivo*.

**Key words :** Anti-angiogenesis, brown algae, Hizikia fusiformis, natural extracts, vascular endothelial growth factor (VEGF)

## Introduction

Angiogenesis plays essential roles in several physiological processes, including embryonic development, chronic inflammation, and wound repair [8], and is the process of forming new microvessels from preexisting parent vessels, thereby expanding the microvasculature [10, 18, 41]. Angiogenesis is vital for tissue healing and homeostasis [10, 41] and is regulated by the balance between pro-angiogenic and anti-angiogenic factors [18, 41]. Upregulated angiogenic proteins, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) are critical in pathological conditions, including cancer, atherosclerosis, and diabetic retinopathy

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[25]. Therefore, anti-angiogenic agents are being intensively studied for the prevention and treatment of angiogenesis-related diseases [14]. There is currently great interest in natural extracts and synthetic drugs for use as ingredients in foods and drinks [27].

Natural marine products have various chemical diversities and have been studied as candidates for pharmacological research for a long time because of their unique biofunctional properties [12]. The secondary products of seaweeds are rich in a variety of bioactive compounds, such as polyphenols, pigments, and polysaccharides [17, 34, 40], and have great potential in the nutraceutical, pharmaceutical, and cosmetic industries [17, 40]. In particular, one edible brown algae, *Hizikia fusiformis(H. fusiformis)*, is rich in bioactive polysaccharides with antioxidant, anticancer, anti-hypertensive, antibacterial, and anti-inflammatory properties [17, 40]. Recent studies have also been reported on *H. fusiformis* extract and their anticancer effects, using cancer cell lines [17]. In this study, we examined the effects of *H. fusiformis* extract on angiogenesis *in vitro* and *in vivo*.

## Materials and Methods

### Materials

Endothelial basal medium (EGM-2; Cat. No: CC-3156), EGM-2 Endothelial SingleQuots™ Kit (Cat.No: CC-4176), and ReagentPack™ Subculture Reagents (Cat. No. CC-5034) were purchased from Lonza (Basel, Switzerland). The Endothelial cell growth medium (EGM) (Cat. No: C-22010) was purchased from PromoCell (Heidelberg, Germany). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent was purchased from Sigma-Aldrich (MO, USA). The Matrigel Matrix (Cat. No: 354234) and standard Transwell ® permeable supports (Cat no.: 3422) were purchased from Corning (NY, USA). The hemoglobin assay kit was purchased from Sigma-Aldrich (MO, USA). Pro-PREP lysis buffer was purchased from iNtRON (Daejeon, Korea). Antibodies against horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Bio-technology (Santa Cruz, CA, USA). ERK, p-ERK, Akt, p-Akt, and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The VEGF anti-body was purchased from Invitrogen (Waltham, MA, USA).

### Primary cell culture

Human umbilical vein endothelial cells(HUVECs, Lonza, Heidelberg, Germany) were cultured in endothelial basal me-dium (EGM-2, Lonza) supplemented with the EGM-2 Endo-thelial SingleQuots™ Kit (Lonza) containing 2% Fetal bovine serum (FBS), hydrocortisone (0.20 ml), Human fibroblastic growth factor-B (hFGF-B; 2.00 ml), Vascular endothelial growth factor(VEGF; 0.50 ml), Insulin-like growth factor-1 (R3-IGF-1; 0.50 ml), ascorbic acid (0.50 ml), Human epi-dermal growth factor (hEGF; 0.50 ml), Gentamicin sulfate-Amphotericin-1000 (GA-1000; 0.50 ml), and heparin (0.50 ml). In a few experiments, cells were cultured in Endothelial cell growth basal medium (EGM) supplemented with a mix comprising 2% FBS, 0.004 ng/ml endothelial cell growth sup-plement, 0.1 ng/ml EGF (recombinant human), 1 ng/ml basic FGF (recombinant human), 90 μg/ml heparin, and 1 μg/ml hydrocortisone. Cells between passages 2 and 9 were used in all experiments. The cells were then incubated for 24 hr at 37℃ in 5% $CO_2$.

### Preparation of extracts from *Hizikia fusiformis*

The test materials were extracted from *H. fusiformis*, as previously reported [7] . In brief, powder was extracted three times with ethanol at room temperature using a stirring apparatus. After concentrating the ethanol extract, the remain-ing material was fractionated into n-hexane, chloroform ($CHCl_3$), and ethyl acetate fractions based on polarity. The n-hexane fraction was used in this study. The n-hexane ex-tract was mixed with DMSO solution.

### Cell cytotoxicity assay

HUVECs were seeded at 10,000 cells in 96-well plates (SPL Life Science, Gyeonggi-do, Korea) with EGM-2, bFGF (25 ng/ml), and HFH (0.1–10 μg/ml) and incubated for 12 hr in an atmosphere of 5% $CO_2$. The MTT reagent (Sigma-Aldrich) was added directly to each well to a final concen-tration of 0.5 mg/ml. After 4 hr, the medium was removed, the formazan crystals formed in the cells were dissolved in dimethyl sulfoxide, and the absorbance of the formazan sol-ution was measured using a Synergy HTX Multi-Mode Reader (BIO-TEK, Vermont, USA) with a 540 nm filter. Each sample was assayed in triplicate.

### Cell proliferation assay

HUVECs were seeded at $2 \times 10^4$ cells in a 24-well plate and incubated overnight in an atmosphere of 5% $CO_2$. bFGF (25ng/ml) and HFH (0.1~10 μg/ml) treated to the cell for 48 hr. MTT reagent (Sigma-Aldrich) was added directly to each well to a final concentration of 0.5 mg/ml. After 4 hr, the medium was removed, the formazan crystals formed in the cells were dissolved in dimethyl sulfoxide, and the ab-sorbance of the formazan solution was measured using a Synergy HTX Multi-Mode Reader (BIO-TEK) with a 540 nm filter. Each sample was assayed in triplicate.

### Wound healing assay

HUVECs were seeded at $2 \times 10^5$ in 24-well plates (SPL) and incubated overnight. Upon reaching confluency, the cells were scratched using a P1250 pipette tip for wounding, and the cultures were further incubated in EGM supplemented with bFGF (25 ng/ml) and HFH (0.25 μg/ml) for 18 hr. The migration patterns were observed using a phase-contrast mi-croscope and photographed. The wound diameters were pho-tographed at 18 hr. Wound closure was determined using op-tical microscopy at 40× magnification. Migration was quanti-fied by counting the number of cells that moved beyond the reference line.

### Transwell assay

The invasion capacity of the cells was determined using a 24-well Transwell system (Corning). The upper side of the

Transswell membrane was coated with 10 µl/well of 1 mg/ml Matrigel (Corning). The cells were seeded at a density of $2\times10^4$ cells in 100 µl of serum-free media containing with bFGF (25ng/ml) and HFH (0.25 µg/ml) in the upper compartment of the Transwell and the full medium in the lower compartment. Then, the cells were incubated for 12, 18, and 24 hr at 37℃ in 5% $CO_2$. The cells were incubated at 37℃ for 18 hr, fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surface of the membrane were removed by wiping with a cotton swab. Cell invasion was determined by counting the whole cell number in a single filter using optical microscopy at 40× magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

### *In vivo* mouse Matrigel plug assay

To evaluate the effect of *H. fusiformis* extract on angiogenesis *in vivo*, we performed an *in vivo* mouse Matrigel plug assay, as previously reported [43]. Briefly, nine-week-old C57BL/6 mice were injected subcutaneously with 500 µl of Matrigel containing bFGF (50 ng) and heparin (10 U) with or without HFH (1.25 µg). After 7 d, the mouse skin was pulled back to expose the Matrigel plug, which remained intact. The Matrigel plugs were photographed, and the hemoglobin content was measured using a hemoglobin assay kit (Sigma-Aldrich), following the instructor's manual for quantification of blood vessel formation.

### *In vitro* tube formation assay

HUVECs ($2\times10^4$ cells) were seeded on a layer of previously polymerized Matrigel (Corning) and treated with bFGF (25ng/ml) and HFH (0.25 µg/ml and 5 µg/ml). The Matrigel culture was incubated at 37℃. After 4 hr, changes in cell morphology were captured using a phase-contrast microscope and photographed at 40× magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

### Western blot analysis

HUVECs were treated with bFGF (25 ng/ml) with or without HFH (0.25 µg/ml and 5 µg/ml) for 24 hr in the medium. Total cell lysates were prepared by adding Pro-PREP Protein Extraction Solution (iNtRON) containing 1 mM sodium orthovanadate. Equal amounts (30 µg) of the samples were separated on a 10% SDS polyacrylamide gel, transferred to a membrane, and sequentially probed with an antibody. The following primary antibodies were used at the indicated dilu-

tions: VEGF (1:500), total ERK, phospho-ERK, total Akt, phospho-Akt, and GAPDH (1:1,000) in 1× blocking solution.

### Ethics approval and consent to participate

The Institutional Animal Care and Use Committee (IACUC) of the Center of Animal Care and Use at the Lee Gil Ya Cancer and Diabetes Institute, Gachon University (Incheon, Korea) approved the animal protocol used in this study (approval number: LCDI-2021–0038).

### Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical comparisons between groups (cytotoxicity, invasion, tube formation, and Matrigel plug assay) were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and Two-way ANOVA for proliferation and migration. *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$; ****$p<0.0001$ were considered statistically significant, and statistical analyses were performed using Prism 9.1.0 (GraphPad Software, San Diego, CA, USA).

## Results

### *H. fusiformis* extract did not alter the viability of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were treated with various concentrations of *H. fusiformis* extract, namely hexane (HFH) (0.1~10 µg/ml), and their cytotoxic effects and proliferation rates were analyzed using the MTT assay. Our results showed that treatment with HFH for 24 hr did not affect the viability of HUVECs (Fig. 1a). Similarly, treatment with HFH for 48 hr did not affect the proliferation rate of HUVECs (Fig. 1b). These results indicate that HFH did not alter the viability of HUVECs.

### HFH inhibits migration and invasion of HUVECs

Endothelial cell migration and invasion are critical steps involved in sprouting angiogenesis [9, 29]. Therefore, we examined the effects of HFH on HUVECs migration and invasiveness using a wound healing assay and a Transwell invasion assay, respectively. Treatment with HFH for 18 hr markedly decreased the migration of HUVECs compared to that in the bFGF group (Fig. 2a). When treated with bFGF, the area where the cells migrated was greater than 70%. However, the migrated area was reduced to 13~30% when treated with HFH, similar to that of the control (Fig. 2b).

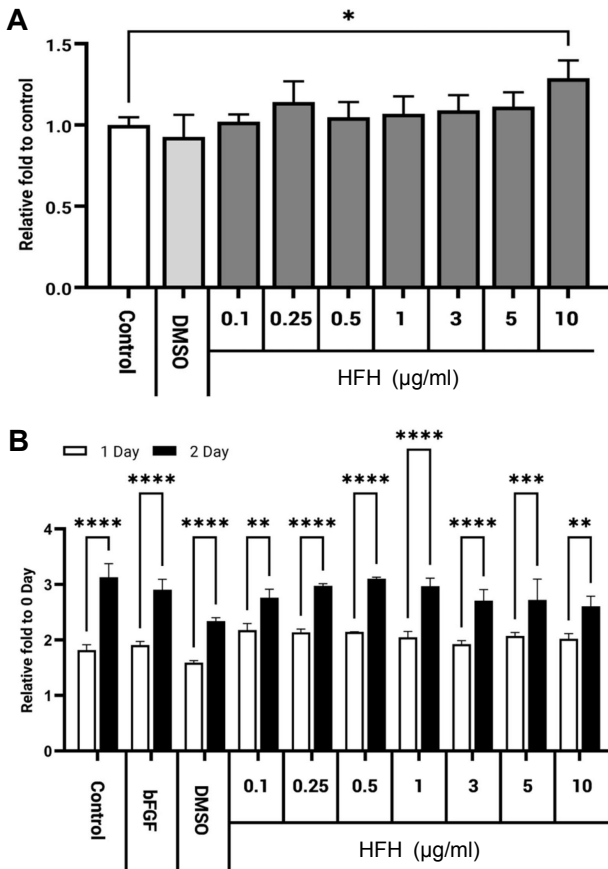HFH inhibited the invasiveness of HUVECs compared to

**A**



**B**



Fig. 1. HFH did not affect cell viability and proliferation. (a) The cytotoxic effect of the HFH on the human umbilical vein endothelial cells (HUVECs) was determined by the MTT assay; (*$P$; Control vs HFH 10 μg/ml); (b) The effect of HFH treatment on proliferation rate was confirmed using MTT assay (****$P$; 1 day vs 2 day).

that of the control after 12 to 24 hr of incubation (Fig. 3a). When treated with HFH for 12 hr, the number of invaded cells was fewer and similar to the control or bFGF treatment (Fig. 3B, left). However, prolonged treatment with HFH (18 hr) significantly inhibited the invasiveness of endothelial cells to approximately 60% compared with bFGF treatment (Fig. 3b, central panel). Similarly, when treated for 24 hr, HFH suppressed endothelial cell invasiveness to approximately 60 and 80%, respectively, compared with bFGF treatment (Fig. 3b, right panel). Our results suggest that the extract of *H. fusiformis* (HFH) strongly suppress the migration and invasion of HUVECs.

### HFH inhibits vascular network formation in HUVECs

During angiogenesis, the differentiation of endothelial cells into a capillary-like network marks a critical step [29]. Therefore, we analyzed the effect of HFH on capillary-like network formation *in vitro*. The HUVECs were placed on Matrigel-coated plates and incubated to allow the formation of weak capillaries on the Matrigel beds that, over time, developed into strong elongated networks. HUVECs cultured on Matrigel formed a blood vessel-like network in the absence of HFH; however, treatment with HFH for 4 hr resulted in broken, shortened, and narrow tube networks (Fig. 4a). The number of master junctions, nodes, meshes, and the total mesh area significantly decreased following treatment with HFH (Fig. 4b and c). These results showed that HFH inhibit tube formation in HUVECs.
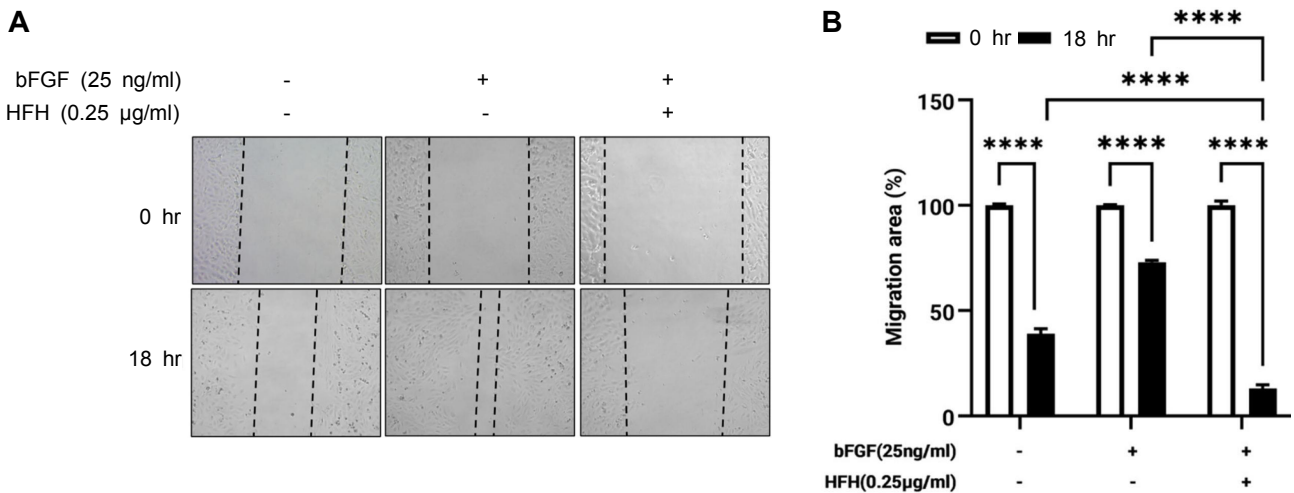
**A**



**B**



Fig. 2. HFH inhibits migration of HUVECs. (a) Migration ability of HUVECs was measured by wound healing assay (×40); (b) Migration area was quantified under a phase-contrast microscope and photographed. This independent experiment was repeated three times (****$P$; all group 0 h vs 18 hr and in 18 hr group control vs HFH, bFGF vs HFH).
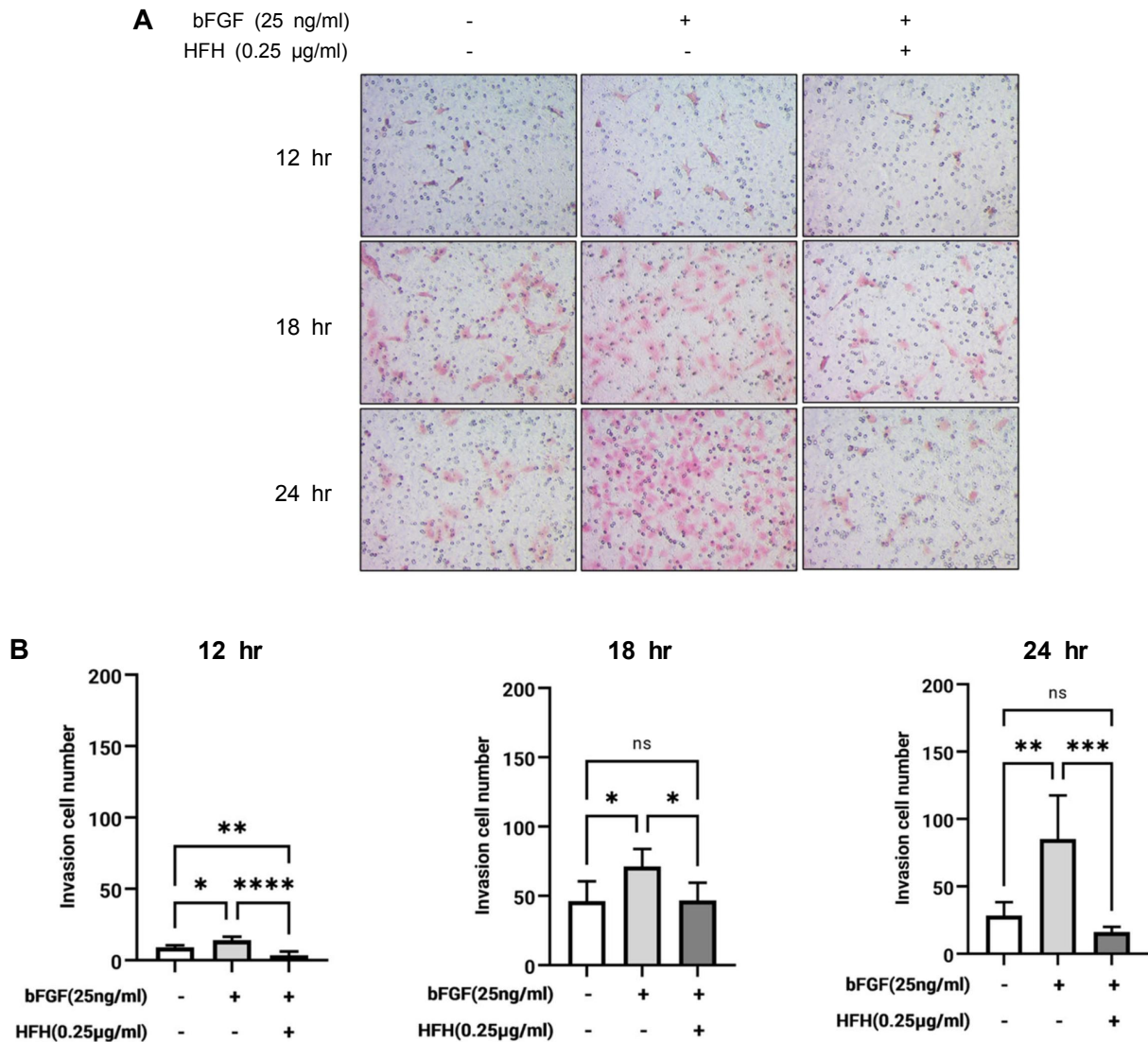
**A**

| bFGF (25 ng/ml) | - | + | + |
| HFH (0.25 μg/ml) | - | - | + |



**B**



Fig. 3. HFH inhibits invasion of HUVECs. (a) Invasion ability of HUVECs was measured by Transwell system (×40); (b) Invasion cells were quantified under a phase-contrast micro-scope and photographed. This independent experiment was repeated three times (*$P$; control vs bFGF; control vs bFGF and HFH; bFGF vs HFH).

### HFH suppresses ERK and Akt activation in HUVECs

VEGF and its receptors act specifically on endothelial cells and regulate their proliferation and migration, marking the primary steps involved in angiogenesis [1, 24]. We analyzed the effects of HFH on the expression of VEGF in HUVECs. Our results showed that HFH decreased the expressions of VEGF in a dose-dependent manner (Fig. 4d). As VEGF activation is mediated by ERK and Akt signaling pathways [42], we next examined ERK and Akt activation. Our results showed that HFH inhibited the phosphorylation of ERK and Akt in a dose-dependent manner (Fig. 4d). These results indicate that HFH interfere with ERK and Akt phosphorylation and further activated VEGF in HUVECs.

### HFH inhibits *in vivo* angiogenesis

To examine the effects of HFH on angiogenesis *in vivo*, we performed a mouse Matrigel plug assay, a well-established model of *in vivo* angiogenesis. Matrigel containing bFGF was subcutaneously implanted into mice with or without the extract of *H. fusiformis* at the indicated doses (Fig. 5). As shown in Fig. 5a, Matrigel plugs containing bFGF were abundantly filled with intact red blood cells compared to Matrigel plugs alone (blank), indicating the formation of functional vasculature inside the Matrigel. However, HFH dramatically suppressed the bFGF-induced angiogenic activity. In addition, the hemoglobin content in the Matrigel plugs was significantly reduced upon treatment with HFH.
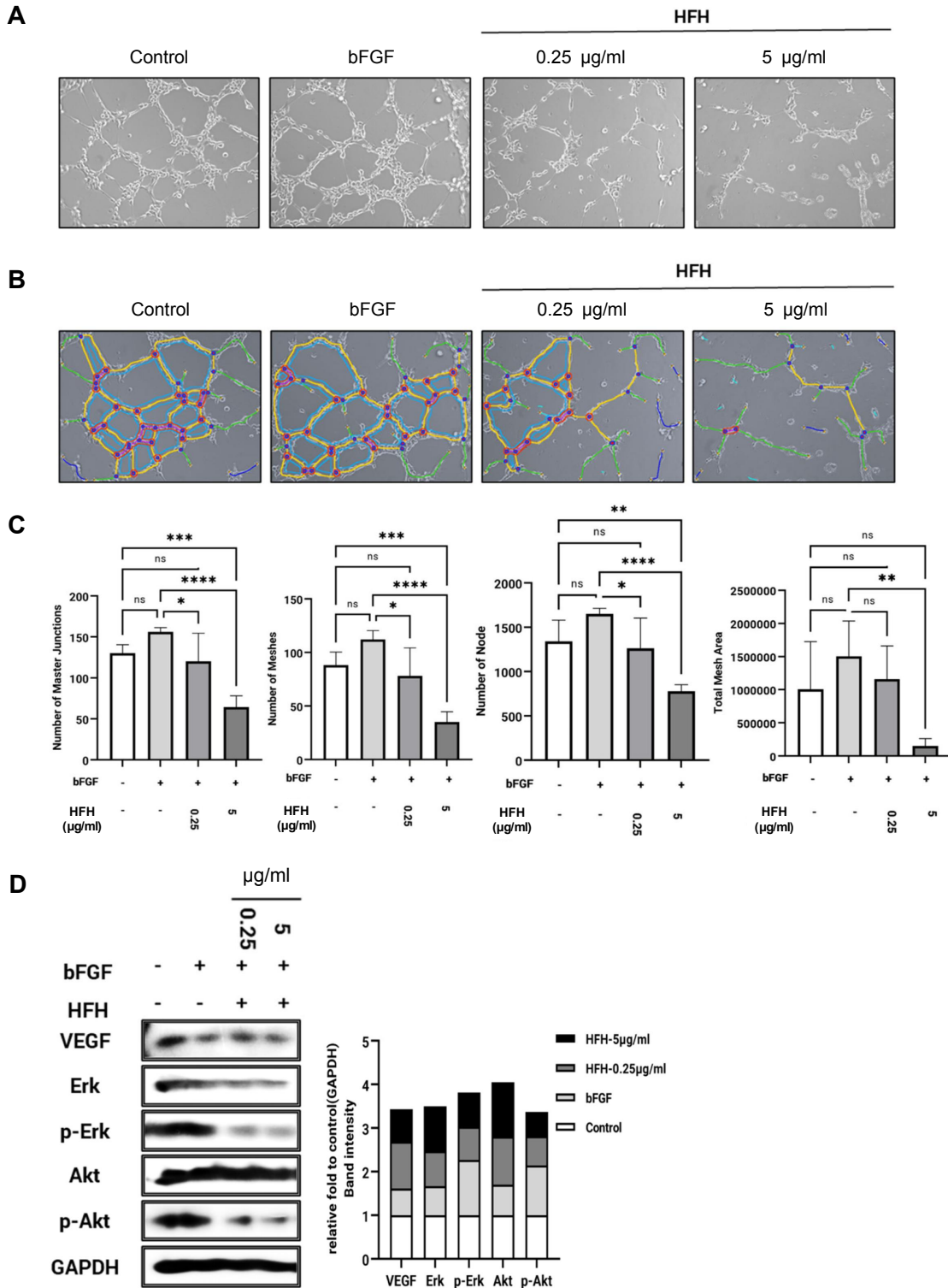
**A**



**B**



**C**



**D**



Fig. 4. HFH inhibits tube formation of HUVECs. (a) HFH inhibits tube formation of HU-VECs. The effect of HFH on tube formation of HUVECs was examined using an *in vitro* tube formation assay. HFH (0.25, 5 μg/ml) were added, and the cells were incubated for 4 hr. Changes in cell morphology were observed using a phase-contrast microscope (×40) and photographed; (b) Result of using Angiogenesis analyzer for Image J (Mesh: sky blue, Master junction: red circle, Branch: green); (c) The number of master junctions, nodes, mesh, and total mesh area were calculated using Image J; (*P*; control vs bFGF, HFH; bFGF vs HFH). (d) Related molecules of angiogenesis were confirmed by western blot analysis (left panel) and western blot intensity was quantified using Image J (right panel).
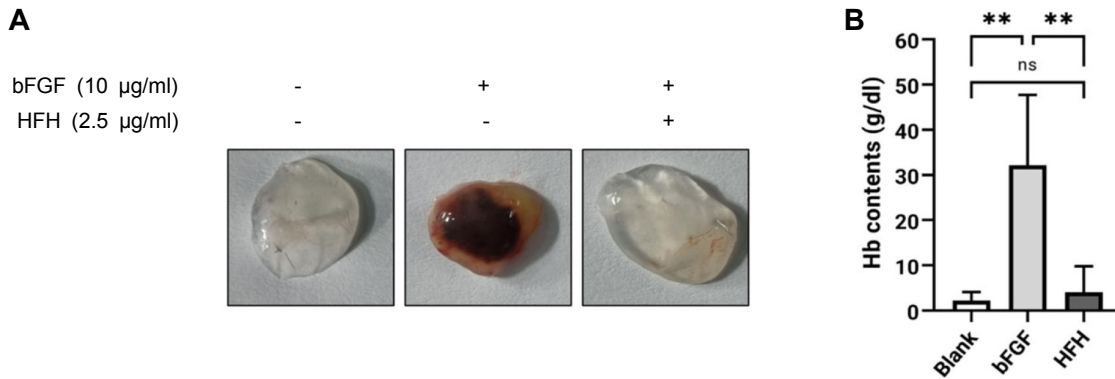
**A**

| | | | |
|---|---|---|---|
| bFGF (10 µg/ml) | - | + | + |
| HFH (2.5 µg/ml) | - | - | + |

**B**



Fig. 5. HFH suppresses *in vivo* angiogenesis in a mouse Matrigel assay. (a) Matrigel plugs were photographed (×40); (b) Quantification of hemoglobin content. Each value represents the mean ± standard deviation (SD) value. (**$p<0.005$, bFGF vs blank, HFH).

These results indicated that HFH have strong anti-angiogenic activities *in vivo*.

## Discussion

This study examined the effects of *H. fusiformis* extract on angiogenesis *in vitro* and *in vivo*. The results indicated that (1) the extract suppressed the migration, invasion, and tube formation of HUVECs; (2) *H. fusiformis* extract inhibited *in vivo* angiogenesis in a mouse Matrigel assay; (3) the extract downregulated the expression of VEGF in HUVECs; and (4) the activities of ERK and Akt were decreased by the treatment of *H. fusiformis*.

Several anti-angiogenic compounds/drugs have been identified and developed for treating diseases caused by the overexpression of angiogenesis-related proteins [1, 13, 20, 24, 42]. However, owing to severe side effects, only a limited number of drugs have been approved by the food and drug administration (FDA), including bevacizumab, sunitinib, sorafenib, and pazopanib [22, 31]. Recent research has identified natural products with potent angiogenic properties, and due to their advantages over synthetic drugs [23, 33], they have been developed into drugs against diseases, including cancer, cardiovascular disease, and multiple sclerosis, with therapeutic effectiveness. The availability and unique structural properties pose an excellent advantage for natural products over synthetic ones, aiding drug discovery [2]. Interestingly, marine compounds have structural characteristics different from those of land due to their various evolutionary histories or environmental characteristics unique to the ocean [39] and are reported to have anti-inflammatory, anticancer [16, 28, 34], and anti-infective properties [34, 39]. Brown seaweeds contain several bioactive secondary metabolites such as

phlorotannin, fucoxanthin, fucoidan, and sterols, and therefore possess antioxidant, anticancer, anti-hypertension, antibacterial, and anti-inflammatory activities [17, 40]. Depending on the type of seaweed, the phytosterol content might vary, altering biological properties [32, 36]. The antioxidant and anticancer properties of *H. fusiformis*, an edible brown alga, have been reported previously [17, 40], and it has shown to suppress cancer growth and metastasis [17]. This study aimed to identify the anti-angiogenic effects of *H. fusiformis* extract (HFH) using *in vitro* and *in vivo* angiogenesis assays. This study demonstrated that HFH extract of *H. fusiformis* inhibits *in vitro* and *in vivo* angiogenesis. HFH strongly inhibited the migration, invasion, and tube formation of HUVECs. Moreover, HFH prominently inhibited the formation of neo-micro vessels in the Matrigel assay and reduced the hemoglobin content in the Matrigel plug.

Tumors produce several pro-angiogenic molecules during angiogenesis [11, 21]. VEGF, are widely known as potential stimuli for angiogenesis [3, 15, 18, 19, 25, 29, 30]. And fucoidan isolated from *H. fusiformis* has been shown to inhibit VEGF/VEGFR [4] and has been reported to have anti-cancer effects [5, 38]. Therefore, we analyzed the effect of HFH on the expression of angiogenic factors. HFH downregulated the expression of key angiogenic molecules (VEGF) in a dose-dependent manner.

ERK and Akt are multiunit transcription factors that play central roles in regulating angiogenic modulators [37]. We studied the effect of HFH on ERK and Akt activation by western blot analysis. Protein expression of Akt did not change following treatment with HFH; however, the expression of ERK was downregulated by treatment with HFH. In addition, the phosphorylation of ERK and Akt was suppressed in a dose-dependent manner.

Even though *H. fusiformis* extract (HFH) was reported to have no antioxidant effects [7], we demonstrated the strong anti-angiogenic activity of HFH in this study. In general, substances with antioxidant effects are known to exert anti-angiogenic effects [6, 26, 35]. Therefore, HFH may have anti-angiogenic mechanisms that are different from those of existing antioxidant substances.

In conclusion, we describe that the anti-angiogenic properties of HFH could be associated with the activation of VEGF and by inhibiting the ERK and Akt signaling pathways. HFH are promising candidates for treating angiogenesis-related diseases. Further studies focusing on isolating a single compound with anti-angiogenic activity against HFH are warranted. Moreover, understanding the anti-angiogenic mechanism of the isolated compound is also essential.

## Acknowledgment

## The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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**초록 : Hizikia fusiformis 추출물의 *in vitro* 및 *in vivo*에서 혈관신생 감소 연구**

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　　기존 혈관에서 새로운 혈관을 형성하는 혈관 신생은 혈관 신생 조절인자에 의해 조절되는 다단계 과정이며 배아 발달, 만성 염증 및 상처 복구를 포함한 다양한 생리학적 과정에 필수적이다. 혈관 신생의 조절 장애는 암, 자가 면역 질환, 류마티스 관절염, 심혈관 질환 및 상처 치유 지연과 같은 많은 질병을 유발한다. 그러나 효과적인 혈관신생 억제 약물은 제한되어 있으며, 최근 연구에서는 천연 자원에서 잠재적인 약물 후보를 식별하는 데 중점을 두고 있다. 예를 들어, 해양 천연물은 항암, 항산화, 항염증, 항바이러스 및 상처 치유 효과를 입증했다. 따라서 본 연구에서는 톳(갈조류) 추출물의 혈관 신생 억제 효과를 확인했습니다. *H. fusiformis* 추출물은 인간 제대 정맥 내피 세포(HUVECs)에서 세포 이동, 침윤 및 관 형성을 억제하며, 동시에 Matrigel 겔 플러그 분석을 통해 생체 내 혈관 신생을 억제를 확인했다. 또한, 톳 추출물 처리 후 VEGF, Erk, Akt의 활성이 감소하는 것을 확인했다. 이 결과를 토대로 *H. fusiformis* 추출물이 *in vitro* 및 *in vivo* 혈관 신생을 억제함을 시사한다.