Original Research

Jaceosidin, a natural flavone, promotes angiogenesis via activation of VEGFR2/FAK/PI3K/AKT/NF-κB signaling pathways in endothelial cells

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Abstract

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, plays an important role in physiological and pathological processes such as embryonic development wound healing and revascularization of tissues after exposure to ischemia. We investigated the effects of jaceosidin, a main constituent of medicinal herbs of the genus *Artemisia*, on angiogenesis and signaling pathways in endothelial cells. Jaceosidin stimulated proliferation, migration and tubulogenesis of ECs as well as *ex vivo* sprouting from aorta rings, which are phenomena typical of angiogenesis. Jaceosidin activated vascular endothelial growth factor receptor 2 (VEGFR2, FLk-1/KDR) and angiogenic signaling molecules such as focal adhesion kinase, phosphatidylinositol 3-kinase, and its downstream target, the serine-threonine kinase AKTWe also demonstrated that jaceosidin activated the NF-κB-driven expression of a luciferase reporter gene and NF-κB binding to DNA. Jaceosidin-induced proliferation and migration of human umbilical vascular endothelial cells were strongly inhibited by the phosphatidylinositol 3-kinase inhibitor LY294002 and NF-κB inhibitor BAY11-7082, indicating that the PI3K/AKT/NF-κB signaling pathway is involved in jaceosidin-induced angiogenesis. Our results suggest that jaceosidin stimulates angiogenesis by activating the VEGFR2/FAK/PI3K/AKT/NF-κB signaling pathway and that it may be useful in developing angiogenic agents to promote the growth of collateral blood vessels in ischemic tissues.

Keywords: Angiogenesis, endothelial cell, NF-κB, signaling pathway, jaceosidin, *Artemisia princeps* Pampanini

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, plays an important role in pathophysiological processes such as embryonic growth, the female reproductive cycle, wound healing, tumor growth, and revascularization of ischemic tissues.[1–4] The formation of functional microvasculature has been reported to enhance neurogenesis and functional recovery after stroke,[5,6] and neurogenesis and neuroplasticity are tightly co-regulated with angiogenesis.[7] In tissue repair and regeneration, angiogenesis supports increasing demands for metabolic supplies such as nutrients, various growth factors, and molecular oxygen.[8] Vascular endothelial growth factor (VEGF) is the most important factor in the process of angiogenesis.[9,10] Recent studies have suggested that angiogenic factors, such as VEGF and fibroblast growth factors (FGF), are effective therapeutic agents for the treatment of tissue repair and ischemic cardiovascular diseases.[2,11] However, the biological activity of growth factors may not last long *in vivo* due to poor protein stability.

VEGF family members bind to the three overlapping vascular endothelial growth factor receptors (VEGFRs), which are crucial receptors in angiogenesis and induce cellular processes including cell migration, survival, and proliferation.[12] VEGFR2 plays crucial roles in mediating VEGF signal transduction in endothelial cells (ECs) by activating several different signaling pathways, which leads to different physiological functions including proliferation, migration, tubulogenesis, and gene expression. Focal adhesion kinase (FAK) is a key mediator of VEGF-mediated cell migration.[13] Stimulation of VEGFR2 by its ligands leads to activate phosphatidylinositol 3-kinase (PI3K) and AKT, a main signaling pathway for neovascularization and EC proliferation.[14,15]

Artemisia princeps Pampanini (APP) cv. Sajabal (family Asteraceae) is a medicinal plant, of which the aerial portions have widely been used in traditional medicine in Korea as a hepatoprotective, antioxidant, anti-inflammatory, and antibacterial agent. Recently, *Artemisia* leaf extract was reported to induce apoptosis in human endometriotic cancer cells[16] and to promote glucose uptake in cultured L6 muscle

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cells.[17] A water soluble extract of AP stimulated the proliferation of cultured ECs and A10 smooth muscle cells and enhanced bFGF accumulation.[18,19] Jaceosidin is a pharmacologically active flavone and one of the main constituents of *Artemisia* medicinal plants. Jaceosidin has been reported to have various biological activities, such as antioxidant, anti-inflammatory, and immunosuppressive activities.[20,21] In addition, Jaceosidin has exhibited pharmacological activities such as inhibiting COX-2 and MMP-9 in human epithelial cells and anticancer activity in U87 glioblastoma cells.[22,23] However, the effect of jaceosidin on angiogenesis is not known.

In the present study, we analyzed the effects of *A. princeps* Pampanini extract and its active component, jaceosidin on angiogenic processes. We showed that extracts of *A. princeps* Pampanini stimulated EC proliferation and migration of human ECs, and we found that jaceosidin, among compounds isolated from APP extracts, was responsible for angiogenesis. In the present study, we demonstrated that jaceosidin promotes EC proliferation and migration and induces micro vessel sprouting in rat aortic tissues. Jaceosidin stimulated VEGFR2 and the angiogenic signal mediators such as FAK, PI3K, and AKT. Inhibitor studies showed that PI3K/AKT and NF-κB activation is involved in jaceosidin-induced proliferation and migration of ECs. Taken together, our results demonstrated that jaceosidin promoted angiogenesis via activation of NF-κB and its upstream signaling molecules.

Materials and methods

Cell culture and reagents

Human umbilical vascular ECs (HUVECs) were obtained from American Tissue Culture Collection and passages 5–8 were used. HUVECs were grown in M199 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL bFGF, and 5 units/mL heparin at 37°C in a humidified 95% and 5% (v/v) mixture of air and CO_2, respectively. Vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, MN) and used as the positive control in each experiment. Antibodies for phospho-VEGFR2, total VEGFR2, phospho-AKT, total AKT, phospho-IκB kinase (IKK)α/β, total IKKα/β, phospho-PI3K, total PI3K, and phospho-FAK, total FAK were obtained from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). Specific antibodies against phospho-IκB-α, IκB-α, p65, and proliferating cell nuclear antigen (PCNA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The signal inhibitor LY294002 (a PI3K/AKT inhibitor) was obtained from Cell Signaling Technology, and BAY11-7082, PD98059 were obtained from Calbiochem (La Jolla, CA). Other chemicals were obtained commercially from Sigma Aldrich (Sigma Aldrich, St. Louis, MO).

Isolation of compounds from the extract of *A princeps* Pampanini cv. Sajabal

MeOH extract of APP and isolation of flavonoid compounds were performed as described previously.[24,25] Briefly, the dried, aerial parts of *A. princeps Pampanini* (Sajabalssuk) were extracted at room temperature with 80% MeOH. The extract was partitioned with water, EtOAc, and normal butanol, successively. Four flavonoids, eupatilin, apigenin, eupafolin, and jaceosidin were isolated from the EtOAc extract through repeated silica gel and octadecyl silica gel column chromatography. The chemical structures of the flavonoids were confirmed by spectroscopic analyses, including nuclear magnetic resonance spectrometry, mass spectrometry, and infra red spectroscopy.

MTT assay for ECs growth

HUVECs were seeded in 96-well plates at a density of 5×10^3 cells/well and allowed to attach for 24 h. After discarding the growth medium, HUVECs were treated with *A. princeps Pampanini extract* or various concentrations of phytochemicals isolated from *A. princeps Pampanini extract* in serum-free medium for 48 h. Vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) was used as a positive control. After incubation, cells were treated with 100 μg/mL of 3-[4,5-dimetnythiazol-2-yl]-2,5-diphenyl-thetazolium bromide (MTT) for 1 h. Formazan precipitates were dissolved in 200 μL of dimethyl sulfoxide, and the absorbance at 560 nm was determined spectrophotometrically. Analyses were repeated three times and the results are expressed as the means of three independent experiments.

Cell migration assay

Migration of HUVECs was assessed in 48-well Boyden chemotaxis chambers (Neuro Probe Inc., Cabin John, MD), as described previously.[26] A polyvinylpyrrolidone-free polycarbonate membrane with 12 μm pores (Neuro Probe Inc.) was coated with 0.1% gelatin. The bottom chamber was loaded with 30,000 cells, and a polycarbonate membrane was laid over the cells. The chemotaxis chamber was then inverted and incubated at 37°C for 2 h. The upper wells were then loaded with RPMI 1640 containing 0.1% BSA and various concentrations of jaceosidin. VEGF was used as a positive control. The chamber was incubated at 37°C for 2 h, and the filters were fixed and stained using Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL).

BrdU incorporation assay and cell viability

A 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay was performed using a BrdU labeling and detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. In brief, HUVECs were seeded in each well of gelatin-coated 96-well plates for 24 h. After removing the medium, cells were starved for 6 h, and then serum-free medium containing various concentrations of jaceosidin or VEGF was added for 24 h. The cell monolayer was incubated for 24 h in culture medium containing 10 μM BrdU. The cells were washed and fixed, and the incorporated BrdU was detected using a Wallace Victor^2 1420 multilabel counter (Perkin–Elmer, Monza, Italy). Analyses were repeated three times and the results are the means of three independent experiments.

Scratch wound closure assay

Confluent HUVEC monolayers in six-well plates (SPL Inc. Seoul, Korea) were scratched and wounded using a universal sterile 200-μL pipette tip and rinsed with phosphate buffered saline. Each cell was treated with various concentrations of *princeps pampanini* extract and jaceosidin for 8 h. VEGF was added as a positive control. Cells were stained with Diff-Quick. The width of the wound was observed using an Olympus digital camera, and the wound area was analyzed using the Scion image program.

Tube formation assay on Matrigel

Unpolymerized growth factor reduced-Matrigel (BD Biosciences, San Jose, CA) was added to 24-well plates, with a total volume of 300 μL in each well, and allowed to polymerize for overnight at 37°C. Various concentrations of jaceosidin or VEGF were plated onto the layer of Matrigel at a density of 1×10^5 cells/well in control medium. After 12 h, cells were photographed using an Olympus digital camera, and the extent of tube formation was analyzed using the Scion image program.

Ex vivo rat aortic ring assay

Animal experiments were approved by the Institutional Review Board, Kyung Hee University. Rat aortic rings were prepared as previously described.[27] The thoracic and abdominal aorta was obtained from 200 to 250 g male Sprague-Dawley rats (Central Lab. Animal Inc. Korea). Excess perivascular tissues were removed, transverse sections were made and the resulting aortic rings were washed in full growth medium. The rings were then embedded in matrigel (BD Biosciences, San Jose, CA) in a 48-well plate. After the matrigel gelled, various concentrations of jaceosidin or VEGF were added to each well for 48 h. After the incubation, the rings were fixed, stained with Diff-Quick solution and photographed. Sprouting micro vessels were quantified using the Scion image program.

Chick chorioallantonic membrane assay

The chorioallantonic membrane (CAM) assay was carried out as described previously.[27] Briefly, 10 μL of jaceosidin (1, 10 μg) and VEGF (100 ng) in type I collagen were applied onto Thermonox disk (BD Biosciences, San Jose, CA) and polymerized at room temperature. The disks were loaded onto the CAM of 10-days-old embryos. After four days incubation, the area around the load disk was photographed and the number of newly formed micro blood vessels was counted by two observers in double-blind test

Western blot analysis

The cell extracts were prepared from HUVECs treated with stimuli and were fractionated by electrophoresis on 8–12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in Tris-buffered saline-Tween 20 buffer (0.5 mol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, and 1 g/L Tween 20) containing 3–5% non-fat dry milk. Peroxidase activity on the membrane was visualized on X-ray film (Fuji Photo Film Co. Ltd. Japan) with a standard enhanced chemiluminescence procedure.

Transfection and luciferase reporter assays

Transient transfections were carried out using lipofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After transfection, cells were incubated for 12 h in growth medium and treated with various concentrations of jaceosidin or VEGF for 8 h. Transfected cells were harvested and lysed in report lysis buffer (Promega, Madison, WI). Luciferase activity was measured using an enhanced Renilla luciferase assay kit (Promega).

Electrophoretic mobility shift assay

HUVEC cells were treated with various concentrations of jaceosidin or VEGF for 2 h. Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSAs) were performed as described previously.[26] A double-stranded NF-κB-specific DNA oligomer (5′-AGTTGAGGGGACTTT CCCAGGC-3′) was used as a probe and competitors. Oligomers were annealed and radiolabeled by phosphorylation with [γ-^{32}P] adenosine triphosphate using T4 polynucleotide kinase and purified on sodium Tris-EDTA (STE)-10 columns (BD Biosciences, San Jose, CA). The reaction products were separated on non-denaturing 6% polyacrylamide gels in 0.25X Tris-borated EDTA buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

Statistical analysis

Unless stated otherwise, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as mean ± SD, and statistical comparisons between groups were performed using one-way analysis of variance followed by Student's *t*-test.

Results

A. princeps Pampanini extract stimulates proliferation and migration of human ECs

Proliferation and migration of human ECs play an important role in angiogenesis. We investigated the effects of *A. princeps Pampanini* (APP) extract on the proliferation of human ECs at concentrations up to 10 μg/mL with an MTT assay. As shown in Figure 1(a), APP extract enhanced HUVEC proliferation by about 1.4-fold ($P < 0.05$) at 10 μg/mL compared to that of control untreated cells. Our results indicate that APP extract stimulates proliferation of human ECs in the range of concentrations tested in this study. We next investigated the effects of APP extract on HUVEC migration with wound scratch assays. When a confluent monolayer of ECs was scratched, cell migration into the wounded area after 8 h increased significantly in the presence of APP extract compared to migration in media alone (Figure 1(b)). APP contains flavonoids such as eupatilin, jaceosidin, apigenin, and eupafolin (Figure 2(a)). To identify the active compound responsible for angiogenic activity in

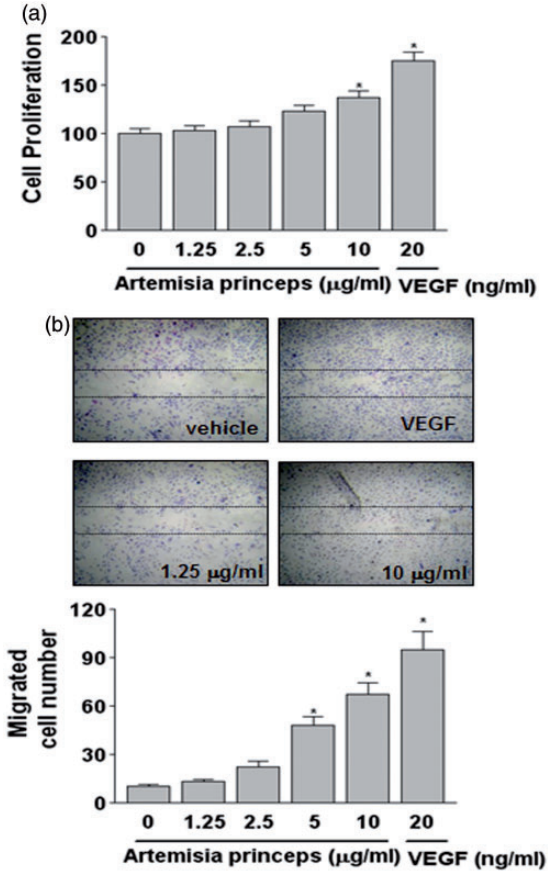

Figure 1 *A. princeps Pampanini* extracts promote endothelial cell proliferation and migration. (a) HUVEC proliferation was measured by the MTT assay. HUVECs were plated in 96-well plates, allowed to attach for 24 h, and then treated with the indicated concentrations of APP extracts for 48 h. Cell growth was measured using a spectrophotometer at 560 nm. (b) HUVECs were treated with various concentrations of APP extracts for 8 h, and cell migration was measured by using scratch wound closure assays. APP extracts significantly accelerated wound closure. The number of migrated cells was quantified using double-blind direct cell counting. One representative of three experiments is shown. $^*P < 0.05$ compared with control. (A color version of this figure is available in the online journal.)

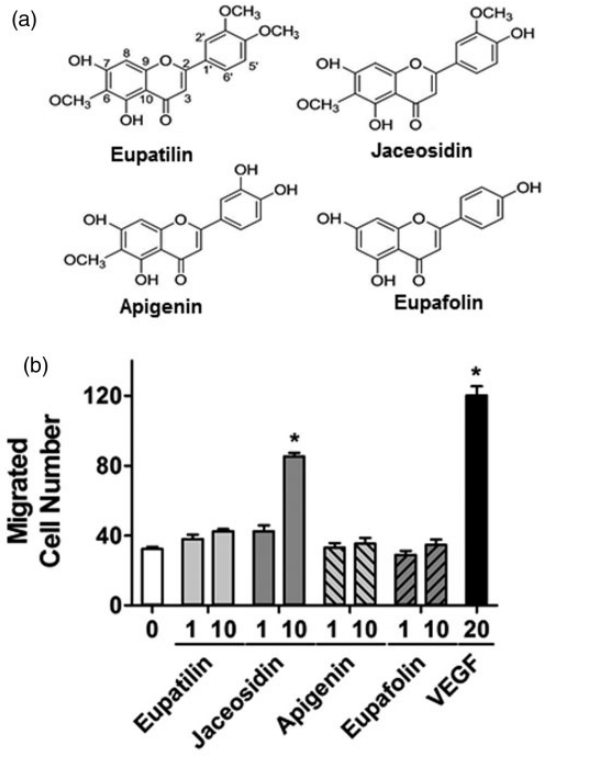

Figure 2 Effects of phytochemicals isolated from *A. princeps Pampanini* (APP) extracts on migration of human endothelial cells. (a) Chemical structures of various compounds isolated from APP extracts. (b) HUVECs were treated with each compound or VEGF at the indicated concentrations. Endothelial cell migration was quantified with the cell migration assays described in the Materials and Method section. The number of migrated cells in the presence of jaceosidin was compared with that in the presence of either medium alone or VEGF. $^*P < 0.05$ vs. medium alone. Data shown are the mean ± SD of more than three independent experiments conducted on triplicate samples

APP extract, we examined the effects of isolated compounds on HUVEC migration. We found that eupatilin, apigenin, and eupafolin did not promote migration of ECs at concentrations up to 10 μM, while jaceosidin at to 10 μM stimulated HUVEC migration by two-fold compared to that of untreated control cells (Figure 2(b)).

Jaceosidin induces EC proliferation *in vitro*

We next examined the effects of jaceosidin on EC growth. Incubation of HUVECs with various concentrations of jaceosidin significantly increased HUVEC proliferation in a dose-dependent manner at concentrations up to 10 μg/mL. Jaceosidin enhanced HUVEC proliferation by about 1.5-fold ($P < 0.05$) at concentrations of 5 to 10 μg/mL (Figure 3(a)). Jaceosidin enhanced DNA synthesis in ECs by more than two-fold at concentrations of 5 to 10 μg/mL, confirming that jaceosidin stimulates EC growth. EC proliferation and DNA synthesis stimulated by 10 μg/mL of jaceosidin were almost comparable to the positive control, VEGF, at 20 ng/mL (Figure 3).

Jaceosidin induces EC migration *in vitro*

We determined whether jaceosidin-induced migration of ECs by using Boyden chamber and wound scratch assays (Figure 4). Our results demonstrated that jaceosidin stimulates EC migration in a dose-dependent manner (Figure 4(a)). After 2 h, cell migration in response to 10 μg/mL jaceosidin was enhanced by two- to three-fold ($P < 0.05$) over migration in medium alone. The positive control, 20 ng/mL VEGF, increased EC migration by about 4.5-fold compared with the control. HUVEC migration was also demonstrated by wound scratch assays (Figure 4(b)), which appeared to be consistent with the results of the Boyden chamber assays.

Jaceosidin promotes EC differentiation

To determine whether jaceosidin stimulates differentiation of ECs, we next examined tube formation of capillary-like structures by ECs on basement membrane matrigel after stimulation with various concentrations of jaceosidin. This assay can evaluate EC differentiation, which is an important step in angiogenesis. Under free medium condition, the

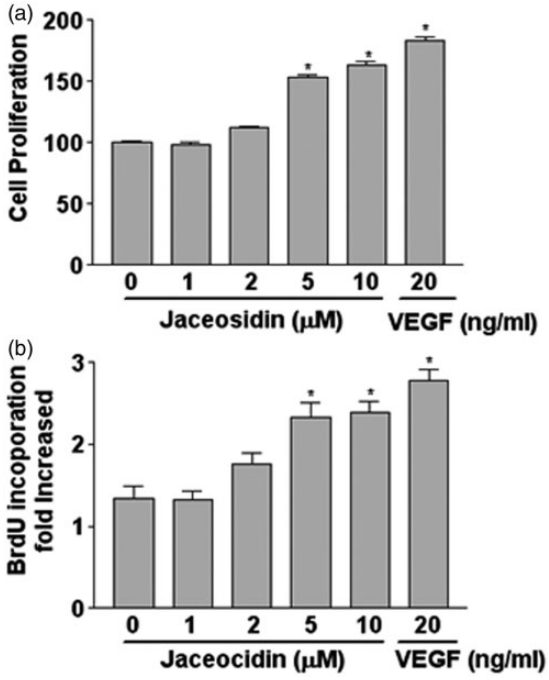

Figure 3 Jaceosidin promotes endothelial cell proliferation. (a) HUVEC proliferation was measured with the MTT assay. HUVECs were plated in 96-well plates, allowed to attach for 24 h, and treated with the indicated concentrations of jaceosidin for 48 h. Cell growth was measured using a spectrophotometer at 560 nm. (b) HUVECs were grown, starved for 6 h and incubated in serum-free medium in the presence or absence of jaceosidin. BrdU incorporation was measured as relative luminescence units. $^*P < 0.05$ vs. media alone. Data shown are the mean ± SD of more than three independent experiments conducted on triplicate samples

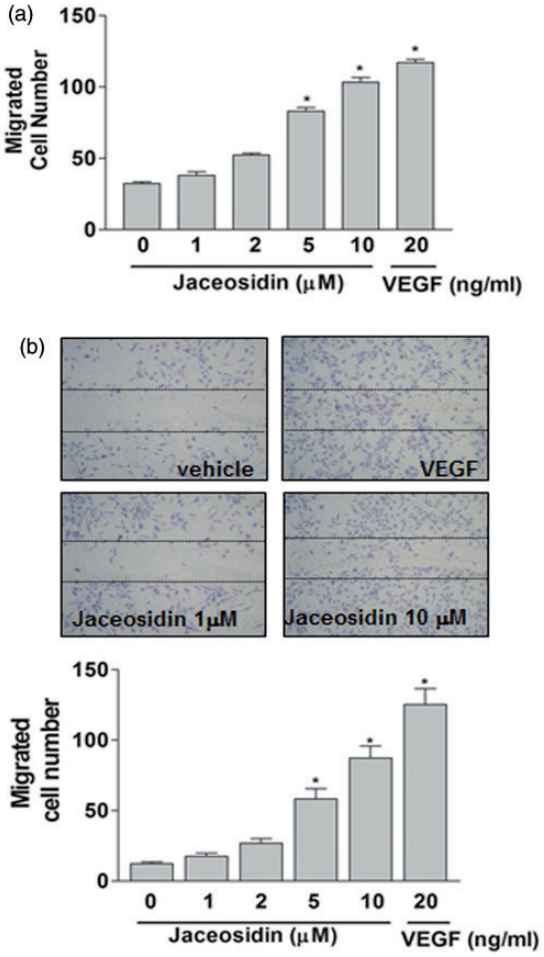

Figure 4 Jaceosidin stimulates endothelial cell migration. (a) The chemotactic response of HUVECs to jaceosidin was measured using Boyden chamber assays. Jaceosidin significantly induced HUVEC migration compared with migration in medium alone. $^*P < 0.05$ vs. media alone. (b) HUVECs were treated with various concentrations of jaceosidin for 8 h. Jaceosidin significantly accelerated wound closure. Representative photographs of the medium two different jaceosidin concentrations and VEGF as a positive control. The number of migrated cells was determined by direct, double-blind cell counting. $^*P < 0.05$ vs. media alone. Data shown are the mean ± SD of more than three independent experiments conducted on triplicate samples. (A color version of this figure is available in the online journal.)

mean intensity of HUVEC tube-like structures formed was 1284 ± 31.3, whereas jaceosidin significantly increased the formation of tube-like structures in a dose-dependent manner. With jaceosidin at 10 μM, the mean intensity of tube-like structures formed was 4257 ± 127.5 tubes/field, about 3.4-fold higher than for the control (Figure 5(a)). The activity observed with 10 μM jaceosidin was comparable with that of VEGF (4.7-fold increase). These results suggest that jaceosidin may promote angiogenesis.

Jaceosidin enhances angiogenesis in *ex vivo* and *in vivo*

To investigate the *ex vivo* angiogenic activity of jaceosidin, we performed rat aortic ring sprouting assays. Transverse sections of rat aorta tissue embedded in growth factor reduced matrigel were cultured with various concentrations of jaceosidin. The degree of sprouting vessels was examined. Jaceosidin induced numerous capillary sprouts in a dose-dependent manner (Figure 5(b)). The angiogenic activity of 10 μM jaceosidin was almost comparable to that of 20 ng/mL VEGF. Furthermore, we examined angiogenic activity of jaceosidin *in vivo* by using CAM assays. Jaceosidin significantly induced neovascularization from pre-existing blood vessels. The presence of 1 and 10 μg of jaceosidin per embryo chick egg caused an approximate two- and five-fold increase, respectively, in number of newly formed micro blood vessels compared with that of negative control (Figure 6).

Jaceosidin activates the VEGFR2, FAK, PI3K, and AKT signaling pathways

VEGFR2 is almost exclusively expressed in ECs and plays crucial roles in mediating angiogenic processes including proliferation, migration, and differentiation in ECs. We examined whether jaceosidin would activate VEGFR2 signaling pathways in ECs. Growth factor-starved HUVECs were exposed to jaceosidin, and VEGFR2 phosphorylation was investigated at different time intervals. VEGFR2 was rapidly phosphorylated after exposure to jaceosidin, peaking at about 15 min and decreasing after 30 min (Figure 7(a)). Jaceosidin increased VEGFR2 phosphorylation in HUVECs in a dose-dependent manner (Figure 7(b)). Meanwhile, total VEGFR levels appeared to be unchanged in jaceosidin-treated ECs. Treatment of HUVECs with jaceosidin strongly stimulated phosphorylation FAK, PI3K, and AKT in a time and dose-dependent manner. The

phosphorylation reached each signaling molecules a maximum level at 10 to 30 min after exposure, and then decreased. Additionally, the level of FAK, PI3K, and AKT phosphorylated by 10 μM of jaceosidin was similar to the level observed for the positive control, VEGF (20 ng/mL). Because VEGFR2 is upstream of the FAK and PI3K/AKT signaling pathways, we examined the effects of jaceosidin on the downstream signaling pathways. Jaceosidin activated phosphorylation of FAK, PI3K, and AKT in a similar pattern to that of VEGFR2 (Figure 7).

Jaceosidin induces NF-κB activation in ECs

Various external stimuli activate the IκB kinase complex, resulting in phosphorylation, ubiquitination, and degradation of IκB-α and permitting nuclear translocation and activation of NF-κB. As NF-κB can be activated through many different signaling pathways, including the FAK and AKT/PI3K pathways,[13,28] we determined whether jaceosidin could activate IKK α/β and IκB-α in ECs. Treatment of HUVECs with jaceosidin resulted in marked increase in phosphorylation of IKK α/β and IκB-α in a time- and dose-dependent manner (Figure 8(a) and 8(b)). Phosphorylation of IKK α/β and IκB-α was comparable to that of the positive control at 10 μM jaceosidin. Next, we examined the effects of jaceosidin on nuclear translocation of NF-κB in ECs. Jaceosidin treatment increased the level of phosphorylated IκB-α, a subunit of the inhibitor protein for NF-κB. Meanwhile, total IκB-α was degraded after jaceosidin treatment (Figure 8(a) and 8(b)). IκB-α phosphorylation is considered to induce its degradation, which then releases NF-κB and allows transcriptional activation. Thus, we performed immunoblot analysis to detect nuclear translocation of p65, a subunit of NF-κB, after treating ECs with jaceosidin. Basal levels of p65 were observed in nuclei of unstimulated cells, but treatment of cells with jaceosidin for 2 h dramatically induced nuclear translocation of p65 (Figure 8(c)).

Jaceosidin stimulates transcription of NF-κB reporter gene and DNA binding of NF-κB

We next investigated the effects of jaceosidin on transcriptional activation and site-specific DNA binding by NF-κB. HUVEC cells were transfected with an NF-κB-driven luciferase construct and treated with jaceosidin. As shown in Figure 9(a), jaceosidin dose-dependently increased the transcription of the NF-κB/luciferase reporter gene in transfected HUVECs, indicating that jaceosidin up-regulates the expression of NF-κB-regulated genes at transcriptional levels. We further examined whether treating HUVECs with jaceosidin stimulates NF-κB binding to its DNA binding site. DNA binding of proteins in nuclear extracts of HUVECs incubated in the presence or absence of jaceosidin

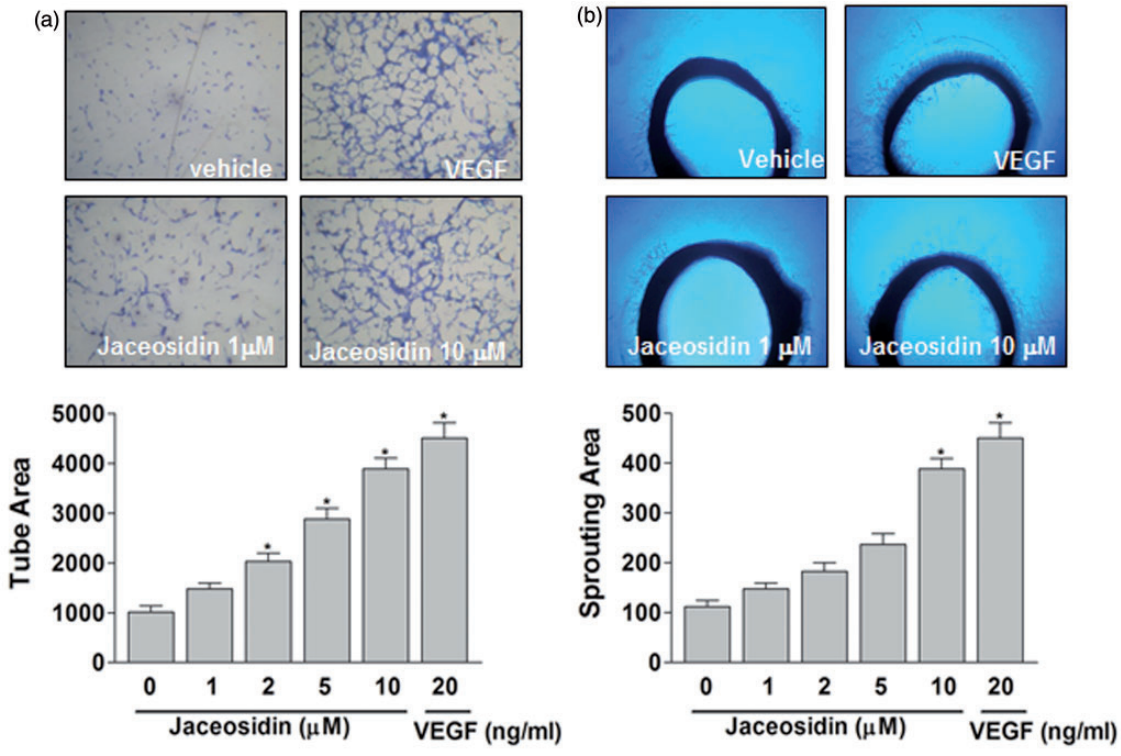

Figure 5 Jaceosidin induces endothelial cell differentiation and rat aortic microvessel sprouting. (a) HUVECs were treated with various concentrations of jaceosidin or VEGF on Matrigel. After incubation for 12 h and fixation, cells were observed under a microscope (magnification, 100×) and photographed. Digital images of HUVECs treated with free-medium (control), jaceosidin and VEGF at 20 ng/mL. Tubes per field were counted in at least four fields, and results are expressed as the number of tubes formed. (b) Rat aortic ring capillary sprouting in response to various jaceosidin concentrations from 0 to 10 μM. Capillary sprouting occurred from the edge of the rings. VEGF at 20 ng/mL was used as a positive control, 100× magnification. Sprouting microvessels were quantified using the Scion image program. One representative of three experiments is shown. *$P < 0.05$ compared with control. (A color version of this figure is available in the online journal.)

was analyzed using ^{32}P-labelled oligonucleotides corresponding to the NF-κB site. When HUVECs were treated with 10 μM jaceosidin, NF-κB-DNA complexes markedly increased compared with unstimulated cells. Cold oligomer at 50× molar excess almost completely inhibited the formation of NF-κB-DNA complexes. Antibodies against p65 also inhibited the formation of protein-DNA complexes (Figure 9(b)). Taken together, our data suggest that jaceosidin induces transcription of NF-κB-regulated genes involved in angiogenesis by activating NF-κB in ECs.

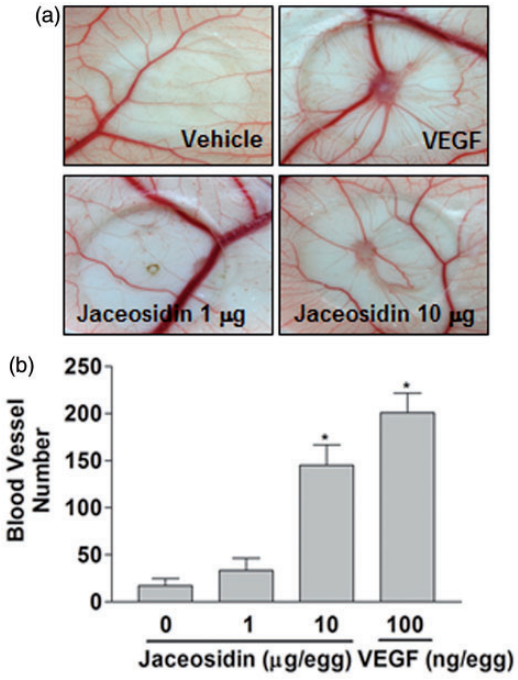

Figure 6 Jaceosidin promotes micro blood vessels formation *in vivo*. Jaceosidin or VEGF was loaded onto the CAMs of 10 days old chick embryos as indicated concentrations. After four-day incubation, a fat emulsion was injected under the CAMs for better visualization of the vessels. Disk and surrounding CAMs were photographed. Representative photographs of the vehicle, VEGF (100 ng/egg), and jaceosidin (1, 10 μg/egg). Quantification of newly formed micro blood vessels. *$P < 0.05$ compared with vehicle. (A color version of this figure is available in the online journal.)

Inhibition of PI3K/AKT and NF-κB reduces jaceosidin-induced proliferation and migration of ECs

To evaluate whether PI3K/AKT and NF-κB signaling affect jaceosidin-induced proliferation and migration of ECs, HUVECs were treated for 1 h with signal pathway inhibitors before stimulation with jaceosidin. The signaling inhibitors, LY294002 (PI3K inhibitor) and BAY11-7082 (NF-κB inhibitor), reduced jaceosidin-induced EC proliferation to the levels of untreated control cells (Figure 10(a)). Moreover, LY294002 and BAY11-7082 also abrogated EC migration by more than 90% (Figure 10(b)). Our results indicate that jaceosidin-induced proliferation and migration of ECs by activating the PI3K/AKT and NF-κB signaling pathways.

Discussion

In the present study, we identified an angiogenic role for jaceosidin isolated from *A. princeps* P*ampanini* cv. Sajabal, the leaves of which have been traditionally used for various diseases in Korea. Our data demonstrated that jaceosidin significantly enhanced EC proliferation and that the proliferative effect of jaceosidin was nearly comparable to that of a potent angiogenic growth factor, VEGF, as measured by MTT assays and DNA synthesis in ECs. Furthermore, jaceosidin-induced EC migration and stimulated tube formation of capillary-like structures by ECs *in vitro*. In aortic ring

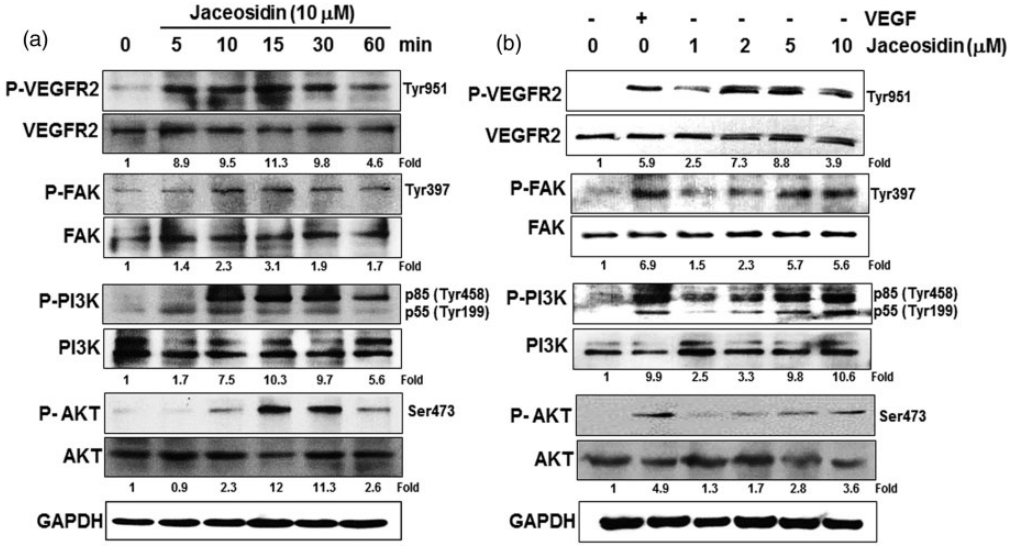

Figure 7 Jaceosidin stimulates phosphorylation of VEGFR2 (KDR/Flk-1) and other signaling molecules. HUVECs were incubated in the presence of 10 μM jaceosidin for various times (a) or in the presence of various jaceosidin concentrations for 15 min. (b) Cytosolic extracts were prepared from the cells, and the proteins in the extracts were fractionated by SDS-gel electrophoresis and subjected to Western blotting with phospho-specific antibodies against VEGFR2 and signaling molecules. The blots were then stripped and re-probed with antibodies against non-phosphorylated proteins

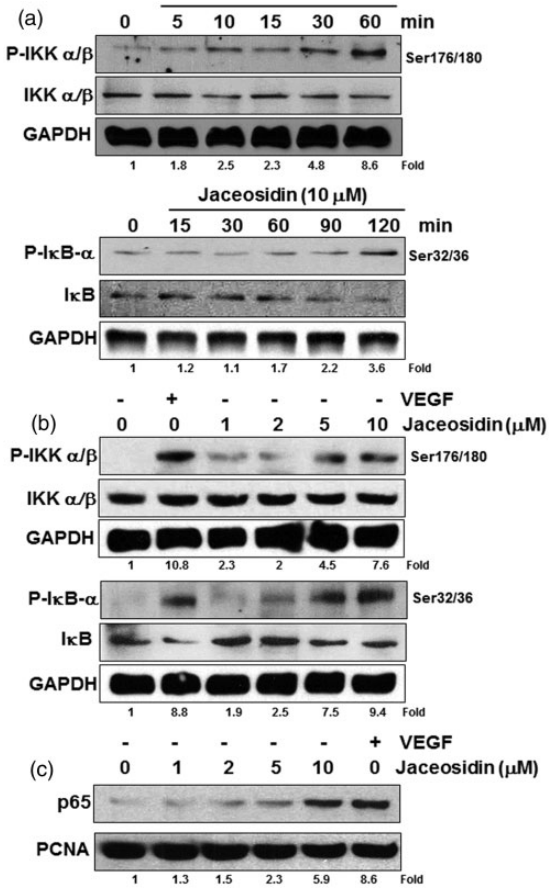

Figure 8 Jaceosidin induced NF-κB activation in endothelial cells. HUVECs were incubated in the presence of 10 μM jaceosidin for various times (a) or in the presence of various jaceosidin concentrations for 60 min. (b) Cytosolic extracts were prepared, and the proteins in the extracts were subjected to Western blotting with phospho-specific antibodies against IKK α/β and IκB-α. The blots were then stripped and re-probed with antibodies against IKK α/β, IκB-α. (c) HUVECs were incubated in the presence of various jaceosidin concentrations for 2 h. Nuclear extracts were prepared, and the proteins in the nuclear extracts were subjected to Western blotting with an antibody against NF-κB p65. The blot was stripped and re-probed with an antibody against PCNA. PCNA was used as a loading control for nuclear proteins

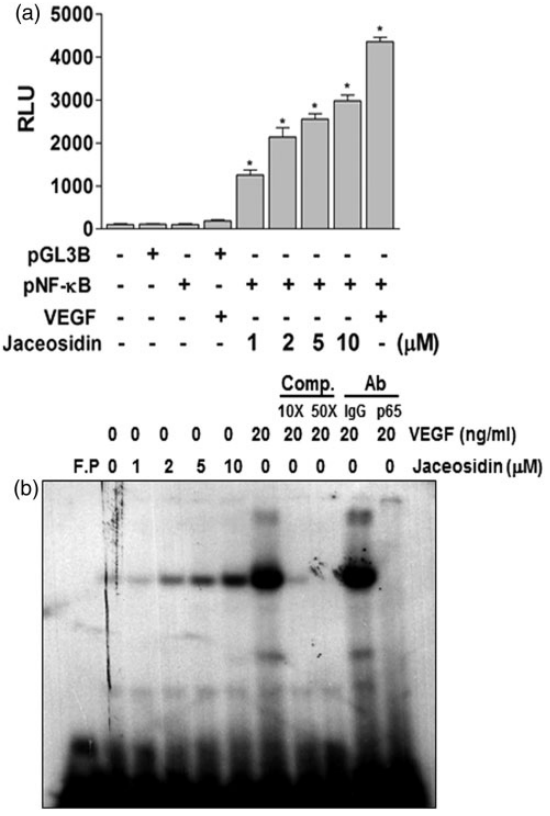

Figure 9 Jaceosidin activated NF-κB-driven transcription and NF-κB binding to its consensus sequences. (a) HUVECs were transiently transfected with an NF-κB-dependent reporter gene. After transfection, cells were incubated for 12 h in growth media and stimulated with jaceosidin or VEGF for 8 h. Luciferase activities were determined according to the manufacturer's instructions. Data shown are the mean ± SD ($n = 3$). *$P < 0.05$ compared with negative control values. (b) Nuclear extracts were analyzed for NF-κB binding to an oligonucleotide containing an NF-κB consensus sequence by electrophoretic mobility shift assays. For specific competition, a 10- and 50-fold excess unlabeled NF-κB oligonucleotides were added to the reaction mixture

assays and CAM assays, microvessels sprouted from pre-existing aorta tissue, indicating that jaceosidin has prominent *ex vivo* and *in vivo* proangiogenic activity. Thus, the angiogenic effect of jaceosidin was evident in a variety of assays, such as the *in vitro* migration assay, wound closure assay, matrigel tube formation assay, *ex vivo* rat aortic assay, and *in vivo* CAM assay.

Jaceosidin, a natural flavone isolated from APP, stimulated VEGFR2 phosphorylation. Activated VEGFR2 has been reported to recruit and activate signaling molecules such as FAK.[13] VEGFR2 activation by jaceosidin in ECs correlated with increased FAK phosphorylation. Phosphorylation of tyrosine 397 of FAK provides a binding site for the Src homology two domain of the p85 subunit of PI3K.[29] Our data showed that jaceosidin strongly activated phosphorylation of PI3K and its downstream effector, AKT, in ECs. Activated PI3K and AKT strongly induce neovascularization and EC proliferation. Taken together, our results indicate that jaceosidin may promote EC proliferation, migration, and microvessel sprouting by activating the VEGFR2/FAK/PI3K/AKT signaling cascade in ECs.

NF-κB can be activated through many different pathways including PI3K and its downstream kinase AKT.[30] Our results demonstrated that NF-κB activation was effectively increased by treating HUVECs with jaceosidin. A variety of external stimuli such as growth factors, cytokines, chemokines, and active compounds from plants stimulate angiogenesis via various signaling pathways that converge to activate NF-κB. NF-κB resides in cytoplasm, which is associated with IκB proteins, such as IκB-α and IκB-β, in unstimulated cells. External stimuli activate IKKs, which phosphorylate IκBs, leading to the ubiquitination and proteasomal degradation of IκBs. Activated IKK α/β induces IκB degradation,[28] which then stimulates nuclear translocation and NF-κB binding to its DNA elements. Our data showed that jaceosidin activated IκB-α phosphorylation, thereby releasing the IκB inhibitor and allowing NF-κB translocation in ECs. Luciferase reporter assays revealed

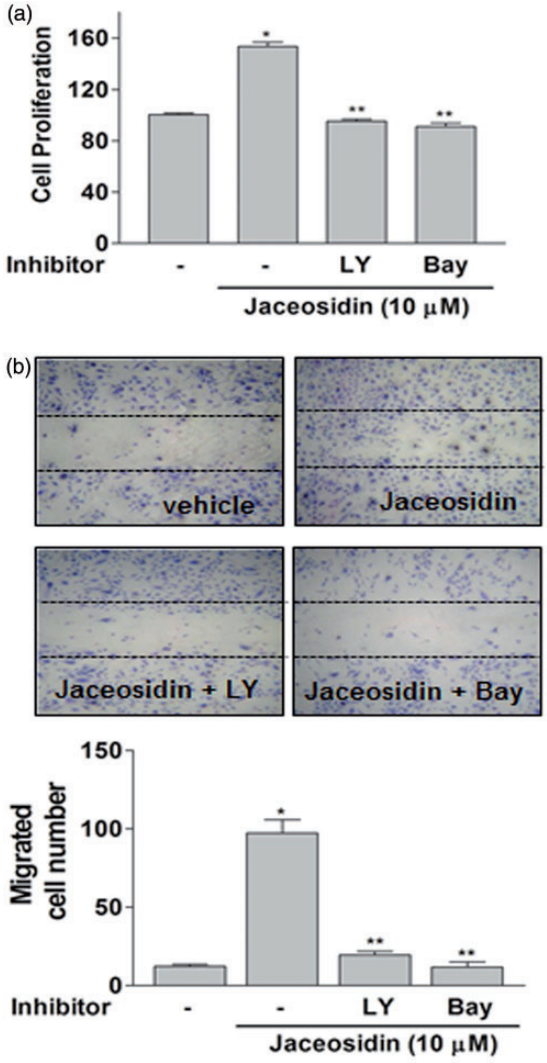

Figure 10 Inhibitors for PI3K and NF-κB decreased jaceosidin-induced endothelial cell proliferation and migration. (a) HUVEC cells were pretreated for 1 h with LY294002 (PI3K inhibitor) or BAY11-7082 (NF-κB inhibitor) and treated with 10 μM jaceosidin for 48 h. Cell growth was measured by MTT assay using a spectrophotometer at 560 nm. (b) HUVECs were pretreated for 1 h with LY294002 or BAY11-7082 and treated with 10 μM jaceosidin for 8 h. The number of migrated cells was determined by direct, double-blind cell counting. Data shown are the mean ± SD ($n = 3$). *$P < 0.05$ compared with negative control values. **$P < 0.05$ compared with jaceosidin-treated control values. (A color version of this figure is available in the online journal.)

that jaceosidin stimulated transcription of an NF-κB/Luc reporter gene in ECs. Gel mobility shift assays showed that jaceosidin increased complex formation between NF-κB and its consensus binding sites.

Angiogenic factors, including vascular endothelial growth factor, fibroblast growth factor, and placental growth factor, are potent angiogenic agents and have been used for angiogenic therapy to treat ischemic diseases and peripheral artery disease. However, the use of protein drugs is limited by their short half-life, high cost, and adverse effects such as increased vascular permeability and inflammation. Therefore, interest is increasing in developing natural agents from medicinal herbs with proangiogenic activity, high therapeutic efficacy, and few side effects. A variety of plant extracts have been reported to have proangiogenic activities,[31,32] including Panax ginseng,[33] Cinnanomum cassia,[34] and Stewartia koreana.[26] In fact, many efforts are being made to develop agents that can promote neovascularization in ischemic tissues.[35] Our results suggest that jaceosidin may be a proangiogenic agent useful for treatment of diseases that require angiogenesis including wound healing, and ischemic and cardiovascular diseases.

In conclusion, the present study has demonstrated that jaceosidin isolated from *A. princeps* Pampanini promotes proliferation, migration, and differentiation of human endothelia cells *in vitro* and microvessel sprouting *in vivo*, which may be primarily attributed to enhanced angiogenesis. Our results demonstrate for the first time that jaceosidin induces EC migration and differentiation and that this induction may be associated with activated VEGFR2/FAK/PI3K/AKT/NF-κB signaling. Properties of jaceosidin support further investigation of its effects on angiogenesis, which may lead to the development of new topical treatments for ischemic failure.

Author contributions: THL and JK designed the project; THL, HJ, and KHP performed the experiments; MHB and NIB isolated jaceosidin and other compounds from *A. princeps Pampanini* and determined the structures of the compounds. THL and JK analyzed the data and wrote the manuscript.

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