Food Production, Processing and Nutrition

RESEARCH

Lactic acid fermented *Elaeagnus multiflora* Thunb. fruit: suppressive effect of its extracts on angiogenesis

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Abstract

Background Angiogenesis has been considered as one of the hallmarks of tumor progression and cancer malignancy. Meanwhile, the fruit of *Elaeagnus multiflora* Thunb. or cherry silverberry (CS), was found to have strong biological activities including anti-cancer and anti-angiogenesis. In this study, the influence of the extracts of *E. multiflora* Thunb. fruits fermented by mono- and co-culture *of L. plantarum* and *L. casei* on the new blood vessel formation in human umbilical vascular endothelial cell (HUVEC) stimulated by vascular endothelial growth factor (VEGF) as well as the underlying mechanism was elucidated.

Results Sulforhodamine B, scratch wound-healing, Transwell migration, capillary-like tube formation, rat aortic ring assays, and morphological analysis were performed to determine the effect of the fermented fruit extracts on the VEGF-induced angiogenic events (motility, migration, invasion, formation of capillary-like tube, blood vessel sprouting ex vivo). The findings showed that at concentration of 25–50 μg/ml, the extracts of CS fermented by mixed cultures is the most effective in inhibiting angiogenesis in HUVECs. Moreover, analysis of the biomarker proteins related to angiogenesis through Western blot technique showed that the fermented extracts exert their anti-angiogenic activity by regulating the VEGFR2 signaling pathway and its possible downstream targets namely Erk ½ and FAK.

Conclusion Taken together, the results suggest that the extracts of CS fruits fermented by co-culture of *L. plantarum* and *L. casei* has the potential to be utilized in the development of functional food and ingredient with anti-angiogenic properties.

Keywords *Elaeagnus multiflora* Thunb., Lactic acid fermentation, Functional food, Angiogenesis
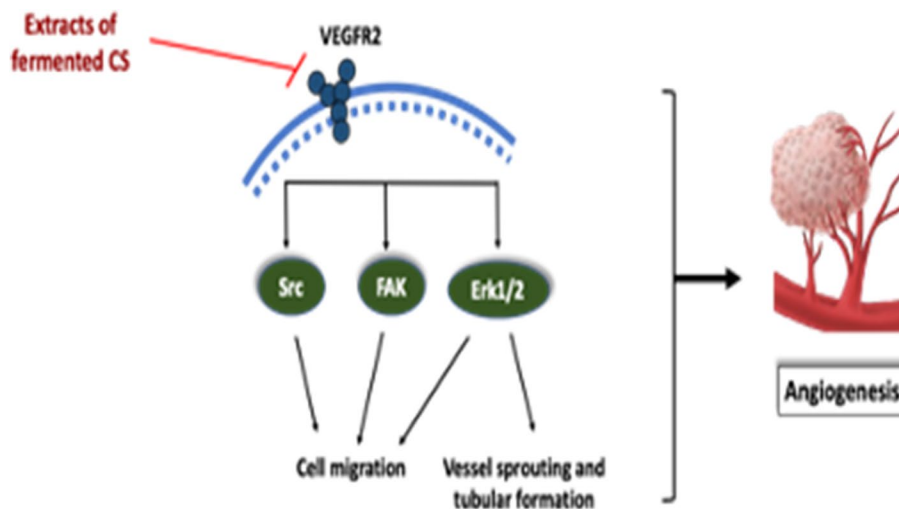
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Graphical Abstract

Introduction

Angiogenesis, the process involving the formation of blood vessels, is a vital physiological activity for the growth of organs, transport of oxygen and nutrients, as well as repair of body tissues. However, abnormalities in the growth and proliferation of blood vessels, as a result of imbalances in the activators and inhibitors, lead to the development and promotion of specific diseases (Carmeliet 2005a, b; Rajasekar et al. 2019). In cancer, formation of vascular vessels is an important factor involved in the progression of tumor growth and metastasis as this process is crucial to sustain the delivery of nutrients to the tumor cells (Carmeliet 2005a, b; Prager & Zielinski 2013). Angiogenesis and other related events that occur in tumor cells to support their growth and proliferation can be considered as hallmarks of cancer progression and malignancy (Ramjiawan, Griffioen, & Duda 2017). Consequently, regulation of angiogenesis has become an important target in developing treatment for cancer. Previous studies have shown that chemotherapeutic agents and radiation therapy in combination with angiogenesis inhibition could have promising impact in the strategies for cancer treatment and improvement in prognosis of cancer patients (Ferrara & Kerbel 2005; Prager & Zielinski 2013; Teleanu et al. 2019). In most cancers, the major regulators associated with angiogenesis such as the vascular endothelial growth factor (VEGF) family and associated receptors and signaling proteins were predominantly expressed. Particularly, VEGF-A is a primary inducer of angiogenic events and was also found to be upregulated in tumorigenic cells and various types of cancer. Moreover, among the receptors, vascular endothelial growth factor receptor-2 (VEGFR2), significantly influences the progression of cancer; hence, the VEGFR2 pathway has been considered as a viable target of several therapeutic agents (Carmeliet 2005a, b; Prager & Zielinski 2013).

Meanwhile, there has been an increasing interest in the development of therapeutic agents derived from phytochemicals as they are from natural sources and have limited side effects unlike the synthetically produced anti-cancer and anti-angiogenesis agents and drugs (Morbidelli 2016). Several studies have reported the chemotherapeutic properties of edible berries and their capacity to suppress or treat angiogenesis. Berries are rich source of phytochemicals such as polyphenols which have been proven to exhibit anti-cancer and angiogenesis-regulating properties (Folmer et al. 2014; Roy et al. 2002; Sarkar & Thirumurugan 2019; Tsakiroglou, Vandenakker, Del Bo', Riso, & Klimis-Zacas 2019). Prevailing studies have shown that among several berries, blueberry, cranberry, and raspberry exhibited the strongest anti-angiogenesis activities and that the extracts of these berries primarily target VEGF to inhibit angiogenic activities in vitro and in vivo (Folmer et al. 2014; Sarkar & Thirumurugan 2019). Moreover, polyphenols can modulate the processes involved in angiogenesis from the initial stage down to the signaling cascade that regulates several angiogenic events e.g. cell motility and formation of capillary tube-like structures (Diniz, Suliburska, & Ferreira 2017).

Elaeagnus multiflora Thunb., popularly known as cherry silverberry or 'gumi' is a native species in East Asia

where it has been utilized as food and medicinal plant (Lachowicz, Bieniek, Gil, Bielska, & Markuszewski 2019; Patel 2015). The fruit has been proven to exhibit strong antioxidant and cytotoxic properties (Oh & Lee 2008) as well as anti-inflammatory and anti-cancer activities (M. S. Lee, Lee, & Park 2010). Most of these biological activities could possibly be linked to the different phytochemicals, primarily polyphenolic compounds, found in the fruit (Lachowicz et al. 2019; Lee et al. 2011). Our previous research has also shown the potential *of E. multiflora* fruit extracts, particularly the lactic acid fermented fruit extract containing enhanced flavonoid content levels, to inhibit colorectal cancer proliferation and metastasis (Lizardo, Cho, Lee, Won, & Seo 2020). In this present study, we aimed to investigate the effect of the aqueous extracts of *E. multiflora* fruit fermented by lactic acid bacteria on the angiogenesis induced by VEGF in human umbilical vascular endothelial cells (HUVEC) as well as the underlying mechanisms. The findings of this study could provide additional information for the development of functional food and ingredients, with potential multi-faceted chemopreventive property involving angiogenesis, by utilizing bioconverted *E. multiflora* fruit.

Materials and methods
Chemicals and reagents
Vascular cell basal medium (VCBM) and endothelial cell growth kit – VEGF were obtained from the American Type Culture Collection (ATCC, Rockville, ND, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human VEGF was purchased from Peprotech (Rocky Hill, NJ, USA). Matrigel with reduced growth factor was purchased from BD Bioscience (NJ, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce™ Thermo Scientific (Rockford, IL, USA). Anti-Erk 1/2, anti-p-erk 1/2, anti-FAK, anti-p-FAK, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-VEGFR2, anti-pTyr951-VEGFR2, and anti-pTyr1175-VEGFR2 were obtained from Cell Signaling Technology (Danvers, MA, USA). Enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK).

Preparation of the extracts of fermented *E. multiflora* fruits
Fresh cherry silverberry (CS) fruits, were obtained from Gyeongnam province, Republic of Korea, at the ripened stage, from July to August. Only the fully mature fruits (completely red in color with average total soluble solids of 8.60 °Brix) were randomly collected from the fruiting trees. The harvested fruits were sorted based on uniformity of size and color, and absence of visible defects, crushed and homogenized using a commercial food

mixer. Fermentation and extraction were carried out using the procedure stated in our previous study (Lizardo, Cho, Won, & Seo 2020). Briefly, homogenized fruit mixture containing 80 g fruit puree added with 20 g distilled water was pasteurized at 85 °C for 30 min, cooled rapidly down to room temperature and inoculated with 5 log CFU/mL of single and co-culture of *L. plantarum* KCTC 33,131 and *L. casei* KCTC 13,086 (Korean Collection for Type Cultures, Seoul, Republic of Korea) at 1% of the total mixture volume. Fermentation was carried out at 35 °C and 150 rpm for 48 h. The unfermented and fermented cherry silverberry samples were freeze dried and subjected to extraction with triple distilled water using a Soxhlet apparatus for 30 min twice. The extracts were centrifuged at 3900 x *g* for 10 min and filtered using Whatman no. 2 filter paper. The supernatant was freeze dried to obtain the powdered product and was stored at -20 °C.

In our previous study, the flavonoid content of the extracts of unfermented and fermented fruits were analyzed using HPLC (Lizardo, Cho, Won et al. 2020). The main flavonoids found in the extracts of the fermented fruits, with the highest concentrations found in the extracts of fruits fermented by co-culture of *L. plantarum* and *L. casei*, are epigallocatechin (37.03 ± 0.05 μg/ml), catechin (10.41 ± 0.11 μg/ml), epicatechin (7.47 ± 0.10 μg/ml), epigallocatechin gallate (17.18 ± 0.05 μg/ml), rutin (11.87 ± 0.05 μg/ml), naringin (91.73 ± 0.03 μg/ml), and quercetin (13.10 ± 0.09 μg/ml).

Cell culture
Primary human umbilical vascular endothelial (HUVEC) cell line was purchased from American Type Culture Collection (ATCC, Rockville, ND, USA) and was cultured in vascular cell growth medium (VCBM) supplemented with endothelial cell growth factors and 2% FBS. The cells were incubated and maintained under a 5% CO_2 humidified atmosphere at 37 °C.

Sulforhodamine B (SRB) assay
To determine if the extracts have an effect on the viability of HUVECs, the cells were seeded at a density of 3×10^4 cells/well in 48-well culture plates and treated with 0-100 μg/mL of extracts of unfermented (UnF) cherry silverberry and those fermented by single cultures of *L. plantarum* (LP) and *L. casei* (LC) and mixed cultures of *L. plantarum* and *L. casei* (LPLC) for 24 h at 37 °C. After incubation, the medium was removed, and the cells were fixed using 12% trichloroacetic acid (Sigma-Aldrich). The plates were kept at 4 °C for 1 h then, each well was washed four times with distilled water and air dried. The cells were stained with 0.4% (w/v) SRB (Sigma-Aldrich, USA), incubated at ambient temperature for 1 h and then

washed four times with 1% acetic acid solution. Lastly, bound SRB was solubilized with 10 mM Tris and the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Inc. USA).

Wound healing migration assay

HUVECs were seeded in a 24-well plate at a density of 1×10^5 cells/well and cultured until confluence. An artificial wound was created on the cell monolayer using a sterile 1-mL pipette tip. The wells were washed with PBS to remove non-adherent cells, added with fresh VCBM supplemented with 0.5% FBS and 20 ng/ml VEGF, and treated with the CS extracts (25 and 50 µg/mL). After 16 h incubation at 37 ℃, cell images were taken using an inverted microscope (magnification, 100x).

Trans-well invasion assay

The cells were seeded into 24-well trans-well insert culture units at a density of 4×10^4 cells/well with VCBM containing 25 and 50 µg/mL of the CS extracts. The bottom wells were filled with 1 mL VCBM containing 20 ng/mL of VEGF for stimulation. After 12–16 h incubation, the cells which invaded the lower surface of the trans-well inserts were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (magnification, 100x).

Capillary-like tube formation assay

Firstly, the collagen thin layer was prepared by diluting Matrigel in VCBM containing 0.5% FBS, placing 75 µL of the 5 mg/mL Matrigel solution into the 48-well culture plates, and incubating at 37 ℃ for 1 h to allow the gel to solidify. In a 24-well cell culture plate, HUVECs were seeded at a density of 1×10^5 cells/well and cultured for 24 h in VCBM supplemented with 2% FBS. Then, the cells were starved for 4–6 h by replacing the medium with VCBM containing 0.5% FBS and subjected to treatment with corresponding concentrations of the CS extracts for 2 h. HUVECs were then harvested and seeded onto the Matrigel-coated wells, followed by the addition of 20 ng/mL VEGF. After 6–8 h of incubation at 37 ℃, the cells were photographed using an inverted microscope (magnification, 200x).

Rat aortic ring assay

The rat aortic ring assay was performed following the procedure as cited by Cho, Moon, Park, Lee, and Seo (2018). The procedures carried out in mice were approved by the Dong-A University Committee for the Care and Use of Laboratory Animals (DIACUC-16-12). Before the start of the assay, 96-well plates were initially coated with 30 µL of Matrigel solution per well. The gel was allowed to solidify at 37 ℃ for 1 h. Thoracic aortas isolated from 4-week old Sprague-Dawley rats were mechanically cleaned from periadventitial fats and connective tissues in cold sterile PBS. Using a surgical blade, the aortas were uniformly sliced into 1-1.5 mm rings. The aortic rings were placed into the Matrigel-coated wells and layered with 30 µL of Matrigel. After allowing the Matrigel overlay to solidify, 150 µL of VCBM (0.5% FBS) containing 20 ng/ml VEGF and with or without CS extracts was added to the wells. The medium was replaced with fresh VCBM every 2 days. After 6 days, microvessel sprouting was fixed and photographed using an inverted microscope (x100). The samples were scored from 0 (with least sprouting) to 5 (with most sprouting) in a blind manner. Each sample was scored six times.

Western blot analysis

HUVECs were seeded at a density of 5×10^6 cells in a 100-mm cell culture dish and incubated at 37 ℃ for 24 h. After culturing, the cells were first starved in serum-free VCBM for 4 h, treated with the CS extracts for 2 h, followed by stimulation with 20 ng/mL VEGF for 2 h. After incubation, the cells were collected and lysed by cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na4P2O7, 1 mM PMSF and 2 µg/mL aprotinin) at 4 ℃ for 30 min. The protein content of the supernatant was measured using BCA protein assay kit (Pierce, Rockford, IL, USA). The protein samples were loaded at 10 µg of protein per lane and resolved through 10% SDS-PAGE at a constant 100 V for 1.5 h. Following electrophoresis, the proteins were transferred onto nitrocellulose membrane. Blocking was done with 2.5% bovine serum albumin for 1 h at room temperature. Then, the membranes were incubated with primary antibodies at 4 ℃ overnight. After washing with TBS-Tween, the membranes were treated with horseradish peroxidase-coupled secondary antibody for 1 h. After incubation and washing, protein detection was performed using an ECL kit and densitometric quantification of the images was performed using Image StudioTM Lite software (LI-COR Co., NE, USA).

Statistical analysis

In each experiment, three independent measurements were carried out and data were expressed as mean ± standard deviation. One-way ANOVA technique was performed to determine statistical significance, followed by the Tukey–Kramer multiple comparisons test using R package for statistical analysis. Significant values were defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

Effect of CS extracts on the proliferation of HUVECs

The cytotoxic effect of the CS extracts against HUVEC cell line was determined using the SRB assay. As illustrated in Fig. 1A, both the unfermented and the fermented extracts did not significantly reduce the viability of HUVECs. The morphology of the cells as observed using an inverted microscope also showed no significant
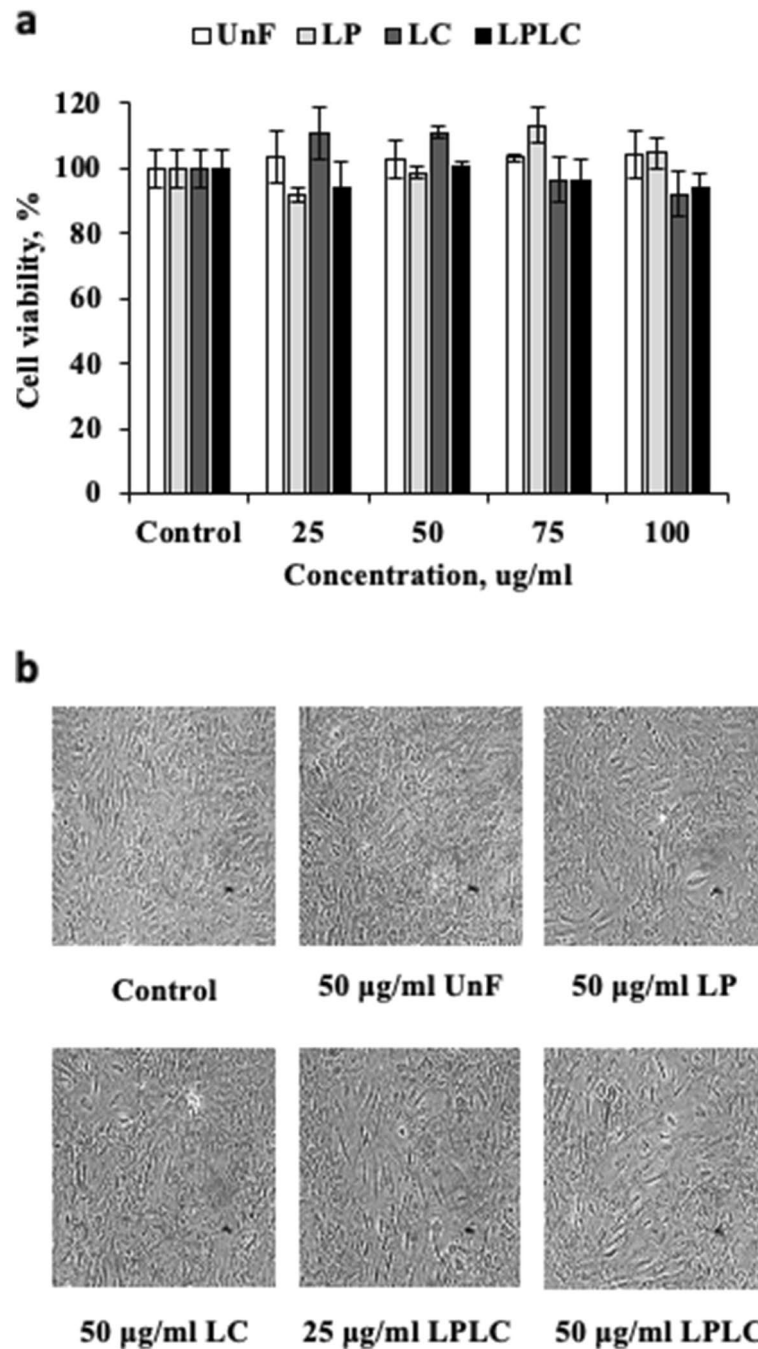

Fig. 1 Effect of the extracts of unfermented and fermented cherry silverberry fruit on the proliferation of human umbilical vein endothelial cells (HUVECs). Cells were treated with various concentrations of extracts of unfermented (UnF) cherry silverberry fruits and samples fermented by single and co-cultures (LPLC) of *Lactobacillus plantarum* (LP) and *L. casei* (LC). **a** After 24 h incubation, SRB assay was performed and **b** morphology was observed under an inverted microscope (magnification, 200x). Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

change after treatment with the CS extracts (Fig. 1B). These data indicate that the possible inhibitory effect of the CS extracts is not through the reduction in cell proliferation of HUVECs.

Inhibition of VEGF-induced endothelial cell migration and invasion by lactic acid fermented CS fruit extracts

The wound-healing assay is a simple method for the determination of the migration ability of a whole cell mass (Justus, Leffler, Ruiz-Echevarria, & Yang 2014). As shown in Fig. 2A, the extracts of the fermented CS fruits significantly decreased the chemotactic motility of HUVECs stimulated by 20 ng/ml VEGF. Moreover, LPLC most effectively reduced cell motility to 50–52% in comparison with the control without VEGF treatment.

The transwell migration and invasion assay evaluates the directional response of cells to a specific chemoattractant (Justus et al. 2014). VEGF stimulated the transwell migration and invasion of HUVEC (Fig. 2B). Significant reduction in the rate of migration and invasion of the cells was brought about by the treatment with fermented CS extracts. LPLC, at a concentration of 50 μg/mL, significantly reduced the relative invasiveness, with the relative invasiveness (93.75%) being comparable to the negative control (100%). These results indicate that the fermented extracts can cause significant decrease in the motility, migration and invasiveness of HUVECs; and with LPLC being the most effective one.

Lactic acid fermented extracts of CS fruits reduce the VEGF-stimulated capillary-like tube formation in HUVECs in vitro

In order to determine the effect of the CS extracts on the tube formation in HUVECs, the cells were seeded in Matrigel-coated wells, stimulated with 20 ng/ml VEGF, and treated with the unfermented and fermented CS extracts. The fermented extracts significantly reduced the tube formation in HUVECs upon being induced by VEGF to 5.33–8.67 average branching points compared to those of the UnF and VEGF control groups with 16.33 and 23.67 average branching points, respectively (Fig. 3). The tube formation in cells treated with the fermented extracts, except for the ones treated with LC, were also not significantly different with the control group not treated with VEGF (4.33 branching points). These results show that the fermented CS extracts can significantly inhibit the formation of capillary-like tubes in HUVECs.

Lactic acid fermented CS fruit extracts inhibit angiogenic sprouting in rat aorta ex vivo

Rat aortic ring assay is a valuable method to assess angiogenesis and factors that induce this process as the new vessels which sprout from the aortic rings are considered to be structurally and functionally similar to new blood vessels that form in vivo (Bellacen & Lewis 2009). In this study, thoracic aorta isolated from Sprague-Dawley rat was cut uniformly into 1-1.5 mm rings and fixed in Matrigel coated 96-well plates. The effect of the aqueous extract of fermented CS fruit on VEGF-induced sprouting of new blood vessels in rat aorta ex vivo was evaluated. Neovessel formation was scored using a scale of 0 (least sprouting) to 5 (most sprouting) in a double-blind manner. As illustrated in Fig. 4, treatment with the extracts of fermented CS significantly reduced neovessel sprouting from aortic rings, having average sprouting scores of 0.75–2.92, compared to the unfermented group (4.58 sprouting score). Moreover, the extracts of CS fruit fermented with co-culture of *L. plantarum* and *L. casei* markedly inhibited the sprouting (0.83 − 0.75 sprouting score) at concentrations of 25–50 μg/mL at $P < 0.001$.

Extracts of lactic acid fermented CS fruits inhibit VEGFR2 activation

VEGFR2 has become a promising target for anti-angiogenesis in cancer therapy as it regulates a number of angiogenesis-related events by mediating a series of downstream signaling pathway (Cho et al. 2018, 2019; Wang et al. 2012). In order to gain insight on the influence of extracts of lactic acid fermented CS fruits on the activation of VEGFR2, the relative protein levels expressed in HUVECs were evaluated. The fermented extracts markedly suppressed the phosphorylation of VEGFR2 (Tyr951 and Tyr1175) as illustrated in Fig. 5. These findings confirm that the extracts of fermented CS fruits effectively inhibited VEGF-induced angiogenesis in HUVEC via regulation of VEGFR2 activation.

Extracts of lactic acid fermented CS fruits regulate expression of pro-angiogenic kinases

VEGF-mediated angiogenesis is important particularly in cancer growth as it modulates a cascade of signaling pathways which prompts several angiogenic events (Carmeliet 2005a, b). As shown in Fig. 6, VEGF significantly induced the upregulation of phosphorylated Erk 1/2 and FAK. Treatment with fermented CS extracts effectively downregulated the expression of these pro-angiogenic kinases in HUVECs. These results indicate that the fermented CS extracts inhibit VEGF-induced angiogenesis by modulating the expression of Erk 1/2 and FAK.

Discussion

The physiological process of angiogenesis involves a series of steps and events that may have an impact in certain diseases such as in several types of cancer and in tumor progression. Tumor angiogenesis is a crucial step in the survival, malignancy and progression of
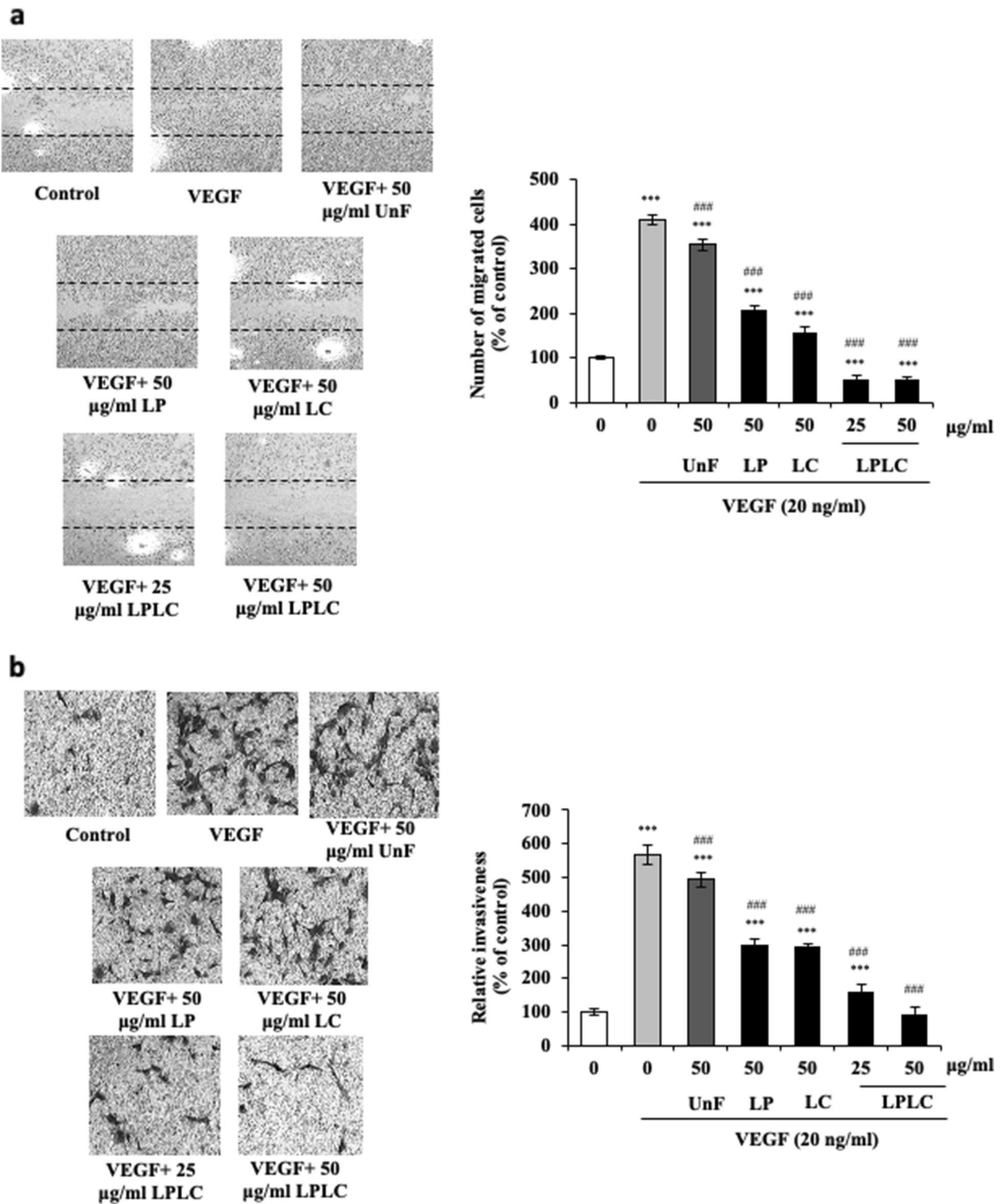
Fig. 2 Effect of extracts of unfermented and fermented cherry silverberry on VEGF-induced chemotactic motility and invasion of HUVEC. **a** Cell motility was analyzed using scratch wound healing assay and observed under an inverted microscope (magnification, 100x). **b** Invasive cells at the bottom of transwells were fixed and observed using an inverted microscope (magnification, 200x). Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and with the VEGF group at #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. VEGF, vascular endothelial growth factor; UnF, unfermented cherry silverberry; LP, *Lactobacillus plantarum* fermented extract; LC, *L. casei* fermented extract; LPLC, extract of fruits co-fermented w/ *L. plantarum* and *L. casei*
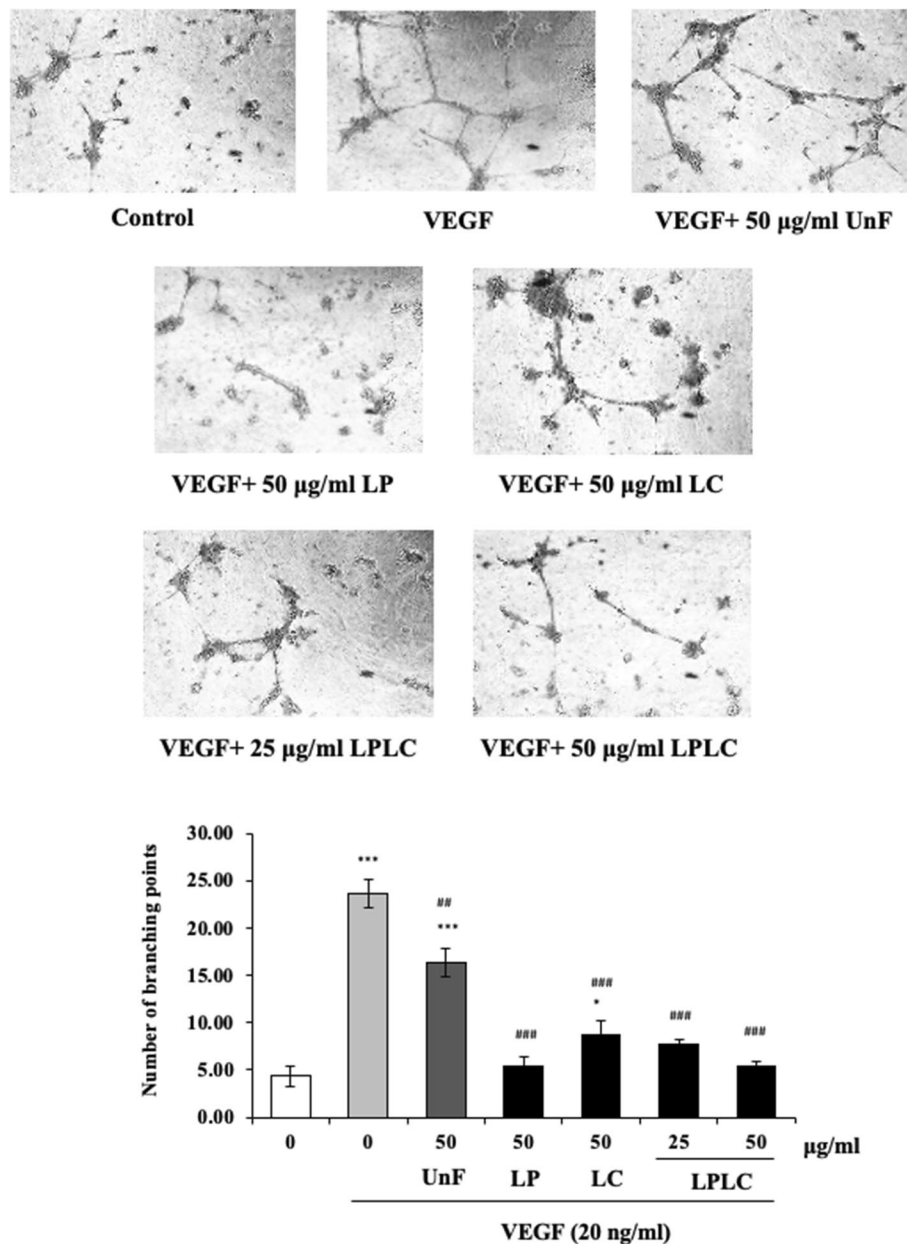
Fig. 3 Effect of cherry silverberry extracts on VEGF-induced capillary tube-like formation in HUVEC. Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and with the VEGF group at #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. VEGF, vascular endothelial growth factor; UnF, unfermented cherry silverberry; LP, *Lactobacillus plantarum* fermented extract; LC, *L. casei* fermented extract; LPLC, extract of fruits co-fermented w/ *L. plantarum* and *L. casei*

tumor cells. As the tumor grows, it requires more oxygen and nutrients hence, it secretes growth factors that binds to designated receptors specifically in endothelial cells to prompt signaling pathways related to formation of blood vessels necessary to support its growth (Marmé 2018; Prager & Zielinski 2013). In this present study, the effect of the extracts of cherry silverberry (CS) fruits fermented by single and co-culture of *L. plantarum* and *L.*

casei on angiogenesis, using the human umbilical vascular endothelial cell (HUVEC) as model induced by vascular endothelial growth factor (VEGF), was evaluated. The anti-angiogenic activity of the extracts of fermented CS fruits through the regulation of the VEGFR2 signaling pathway and the expression of downstream components extracellular-signal-regulated kinase 1 and 2 (Erk 1/2) and focal adhesion kinase (FAK) were investigated.
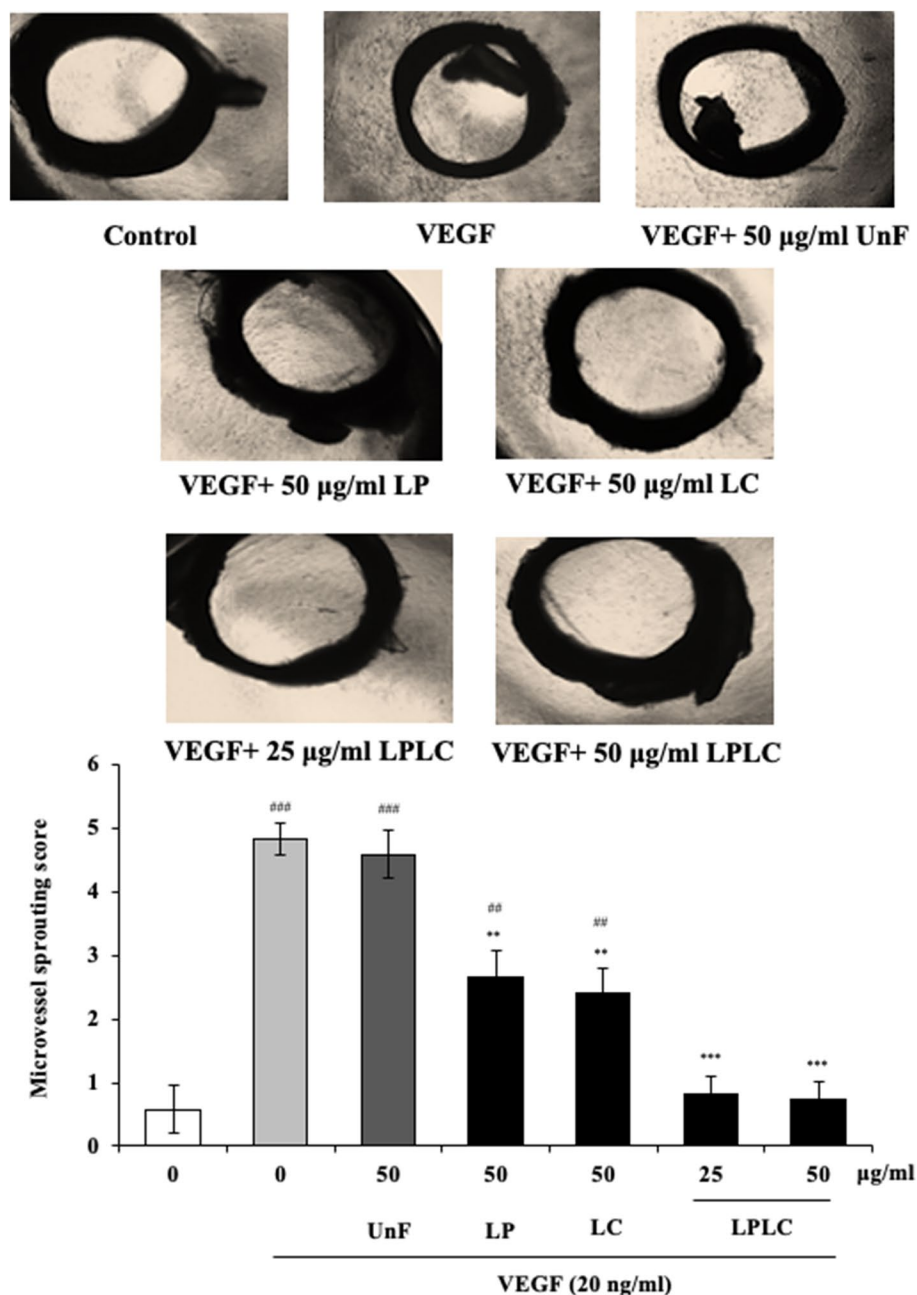
Fig. 4 Effect of extracts of unfermented and fermented cherry silverberry on VEGF-induced microvessel sprouting in rat aortic ring ex vivo. Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and with the VEGF group at #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. VEGF, vascular endothelial growth factor; UnF, unfermented cherry silverberry; LP, *Lactobacillus plantarum* fermented extract; LC, *L. casei* fermented extract; LPLC, extract of fruits co-fermented w/ *L. plantarum* and *L. casei*

Initially, the fermented extracts of CS fruits did not show cytotoxicity against HUVECs as these treatments did not significantly affect the cell viability. Nevertheless, treatment with the fermented extracts, particularly the extracts fermented by mixed cultures of *L. plantarum* and *L. casei* (LPLC), resulted in marked reduction in crucial VEGF-induced angiogenic events namely cell motility, migration, invasion, and tubular formation. Similarly, in a study done by Liu et al. (2010), grape extracts did not significantly affect the proliferation and morphology of HUVEC but inhibited capillary tube-formation. Meanwhile, endothelial cell migration is an important
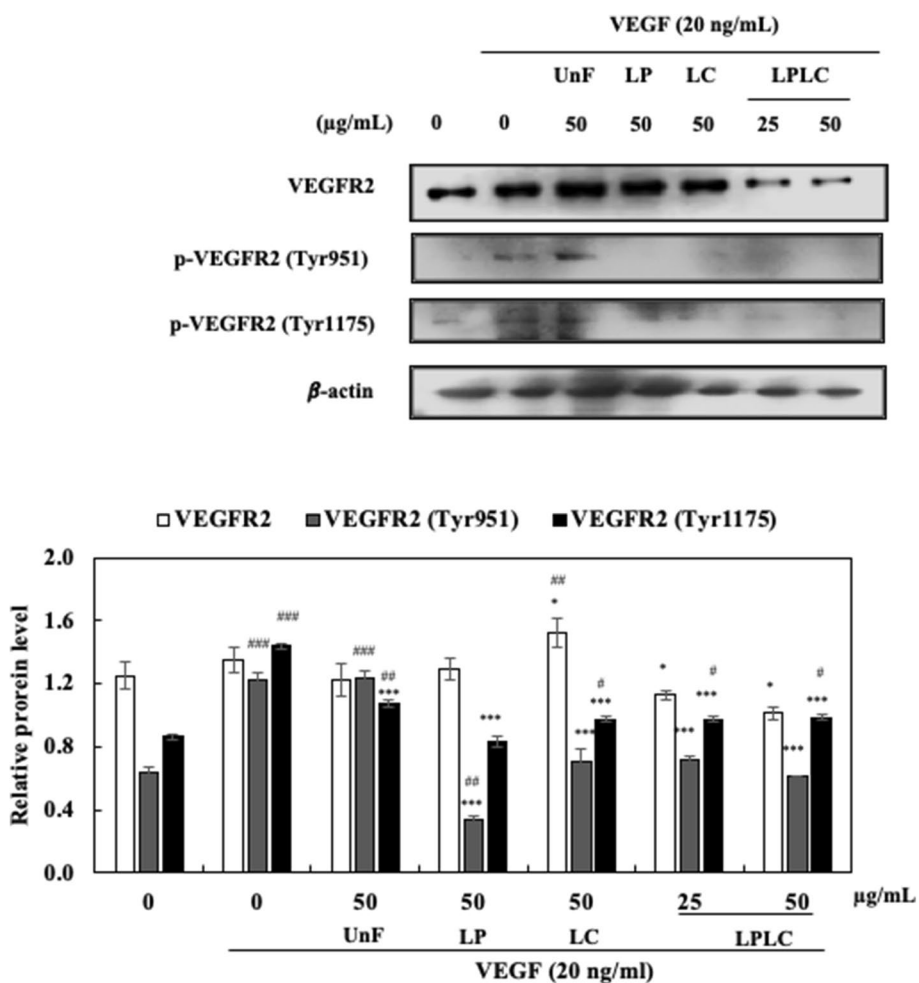
Fig. 5 Inhibition of the activation of VEGFR2 in HUVEC treated with the extracts of fermented cherry silverberry fruits. Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and with the VEGF group at #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. VEGF, vascular endothelial growth factor; UnF, unfermented cherry silverberry; LP, *Lactobacillus plantarum* fermented extract; LC, *L. casei* fermented extract; LPLC, extract of fruits co-fermented w/ *L. plantarum* and *L. casei*

step during vascularization and angiogenesis and is involved in pathological processes including cancer and metastasis (Justus et al. 2014; Michaelis 2014). Chemotactic cell migration is induced by stimuli and VEGF has been considered as one of the most important stimulus in cell migration (Carmeliet 2005a, b; Michaelis 2014). Sprouting and outgrowth of new blood vessels can also be induced by chemical stimuli, such as VEGF, which may be overexpressed and released by tumorigenic cells (Carmeliet 2005a, b). Hence, inhibition of these events is important in anti-angiogenesis therapy in cancer progression.

Our previous study showed that the CS fruit contain an array of polyphenols which exhibit potential chemopreventive properties. Particularly, the extracts of the fruits fermented by co-culture of *L. plantarum* and *L. casei* contain higher concentration of flavonoids namely

epigallocatechin, catechin, epigallocatechin gallate, rutin, naringin, and quercetin. The combination and interaction among these polyphenols have been found to affect and even directly alter several pathways that are involved in cancer progression and angiogenesis (Diniz et al. 2017; Rajasekar et al. 2019). These dietary polyphenols and fruit extracts rich in these compounds were also proven to mediate angiogenic processes including migration, invasion and capillary tube-like formation (Morbidelli 2016; Sarkar & Thirumurugan 2019). For instance, pomegranate fractions (Toi et al. 2003) as well as crowberry extracts (Bae et al. 2016) containing a variety of polyphenols displayed anti-angiogenesis potential by suppression of endothelial cells proliferation and tube formation. In a study done by Sudha et al. (2021), pomegranate fruit extract *(Punica granatum)* containing a variety of polyphenols and
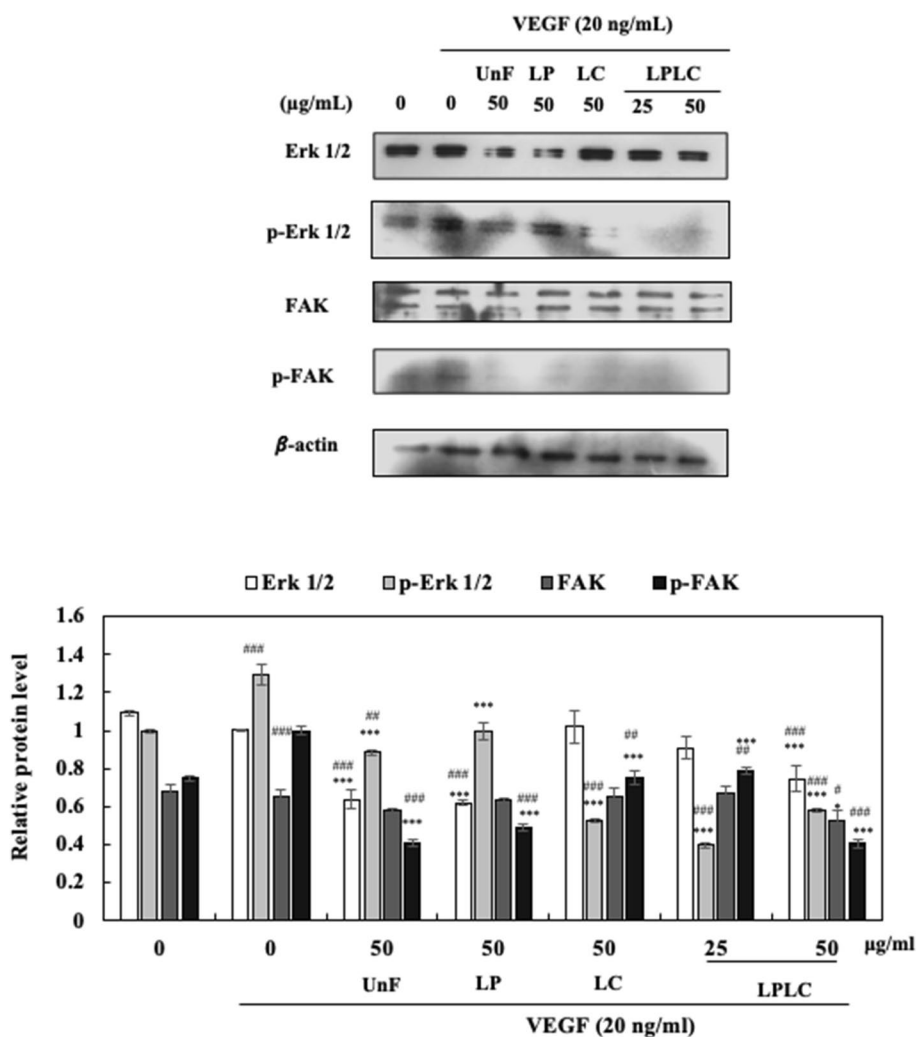
Fig. 6 Expression of pro-angiogenic kinases in HUVEC treated with the extracts of fermented cherry silverberry fruits. Data are expressed as mean ± SD of three independent measurements. Significant differences were compared to the control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and with the VEGF group at #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. VEGF, vascular endothelial growth factor; UnF, unfermented cherry silverberry; LP, *Lactobacillus plantarum* fermented extract; LC, *L. casei* fermented extract; LPLC, extract of fruits co-fermented w/ *L. plantarum* and *L. casei*

flavonoids also exhibited anti-angiogenic effect against pancreatic and colon cancer cell lines. Phenolic compounds can also interfere in the activation of VEGFR2 thereby modulating a cascade of signaling pathways and downstream effectors (Diniz et al. 2017; Morbidelli 2016). In a study done by Chin et al. (2018), kaempferol effectively inhibited VEGF-induced angiogenic events through modulation of downstream kinases linked with the VEGFR activation namely AKT, mTOR and MEK1/2-ERK1/2. Specific phenolic acid and flavonoids found in edible berries such as ferulic acid, catechin and rutin were also found to display anti-angiogenic property by suppressing the inducible expression of VEGF (Roy et al. 2002).

VEGF is the most important and relevant angiogenesis-inducing growth factor that is released by tumor cells and stimulate primarily the endothelial cells (Marmé 2018). It also stimulates chemotaxis in endothelial cells making it a key activator of angiogenic events (Carmeliet 2005a, b; Prager & Zielinski 2013). Moreover, VEGF and its receptors have been found to be upregulated in most human cancers. Their expression has also been correlated with disease-linked angiogenesis and progression of cancer (Holmes, Roberts, Thomas, & Cross 2007; Prager and Zielinski 2013). Because of its role in mediating several angiogenic events such as cell survival, permeability, migration, invasion, and tube formation, VEGFR2 is considered as a significant therapeutic target in pathological

angiogenesis (Holmes et al. 2007; Ramjiawan et al. 2017). In the findings of the present study, the extracts of fermented CS fruits effectively downregulated the activation of VEGFR2 both at the main autophosphorylation sites Tyr951 and Tyr1175. In a similar study, phenolic compound-rich extract of red raspberry exhibited anti-angiogenesis property in human endothelial cells by altering the VEGFR2 signaling pathway (Sousa et al. 2016). Another study also reported the ability of ellagic acid to suppress the proliferation, migration and tube formation in endothelial cells by directly downregulating VEGFR2 tyrosine kinase activity and a series of signaling proteins related to MAPK and PI3K/Akt pathways (Wang et al. 2012). Flavonoids found in berries and their extracts were also shown to have the ability to inhibit angiogenesis and other events related to this process by regulating several pathways mainly the VEGF as well as PI3K/Akt and ERK 1/2 (Raffa, Maggio, Raimondi, Plescia, & Daidone 2017). Several groups of phenolic compounds isolated from natural sources have exhibited their potential influence and alter cancer development, progression, and other related activities by regulating various cell signaling pathway (Abbaszadeh, Keikhaei, & Mottaghi 2019). Along with the present research, these studies have demonstrated the effective application of berry extracts and some specific compounds found in the extracts in the inhibition of angiogenesis by targeting the VEGFR2 and the linked signaling pathways.

Several downstream signaling pathways and molecules have been implicated with the activation of VEGFR2 pathway. Targeting these intracellular signaling molecules may offer wide range and long-term anti-angiogenesis treatment. In the result of the present study, it was established that the CS extracts fermented by the lactic acid bacteria *L. plantarum* and *L. casei* effectively downregulated p-ERK 1/2. The ERK pathway is one of the essential pathways that regulates the angiogenic activities in endothelial cells. In particular, ERK 1 and 2 facilitate the proliferation and migration of endothelial cells during angiogenesis (Srinivasan et al. 2009). Moreover, ERK 1/2 activation is also involved in vessel sprouting and tubular formation (Mavria et al. 2006). In a study done by Matsunaga et al. (2010), inhibition of angiogenesis was demonstrated by bilberry (*Vaccinium myrtillus*) extracts by downregulation of ERK 1/2 phosphorylation thereby reducing cell growth and migration (Matsunaga, Chikaraishi, Shimazawa, Yokota, & Hara 2010).

VEGF-stimulated activation of FAK is also fundamental in the VEGFR-2 related cell migration (Jian Hua Qi & Claesson-Welsh 2001). FAK has been shown to mediate endothelial cell migration, proliferation and survival. FAK and its kinase activity also regulate transcription of VEGFR2 (Sun, Wu, & Guan 2018). The

upregulation of these pathways can mediate VEGF-induced vascular permeability and migration and may eventually influence metastatic processes (Ramjiawan et al. 2017). Our findings indicated the significant effect of the extracts of CS fermented by *L. plantarum* and *L. casei* on the reduction in the expression of phosphorylated FAK. As validated by the findings of Zhao et al. (2017) and Shiau et al. (2021), inhibition of cell proliferation, migration and angiogenesis was modulated by FAK activity mediated by the VEGFR2 signaling pathway .

This study showed the mechanism behind the ability of the fermented CS extracts to effectively reduce cell migration, invasion, tube formation and blood vessel sprouting by modulating the VEGFR2 pathway and the related downstream targets. Specifically, the extract of CS fruits fermented by the co-culture of *L. plantarum* and *L. casei* downregulated the expression and inhibited the phosphorylation of the proteins namely ERK ½ and FAK.

Conclusion
In this study, the anti-angiogenesis activity of aqueous extracts of CS fruits fermented by *L. plantarum* and *L. casei* has been established. The findings showed that the fermented extracts of CS fruits containing enhanced concentrations of bioactive flavonoids exhibited anti-angiogenesis by inhibiting cell motility, migration, invasion and capillary-like tube formation in HUVECs induced by VEGF in vitro. The fermented extract, particularly LPLC, also suppressed microvessel sprouting in rat aortic ring ex vivo. The suppression of these angiogenic events was found to be associated with the modulation of the VEGFR2 signaling pathway as well as the direct inhibition of the phosphorylation of intracellular downstream molecules namely ERK ½ and FAK. Determination of the anti-angiogenesis property of the extracts of lactic acid fermented CS in vivo may be conducted in further studies for validation of the findings. Moreover, studies on the possible toxicity, determination of dose or concentration acceptable for human consumption, and clinical trials are recommended to be done in further research to fully establish the potential of fermented CS fruit extracts as a functional food ingredient with angiogenesis inhibitory properties.

Authors' contributions
Conceptualization and validation: HDC; Methodology, formal analysis and investigation: RCML; Writing - original draft preparation: RCML; Writing - review and editing: HDC and KIS; Funding acquisition, resources and supervision: KIS. All authors reviewed and approved the final manuscript.

Declarations

Ethics approval and consent to participate
The procedures carried out in mice were approved by the Dong-A University Committee for the Care and Use of Laboratory Animals (DIACUC-16-12).

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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