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RESEARCH ARTICLE

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Kochia scoparia seed extract suppresses VEGF-induced angiogenesis via modulating VEGF receptor 2 and PI3K/AKT/mTOR pathways

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ABSTRACT

Context: *Kochia scoparia* (L.) Schrad (Amaranthaceae), known as a traditional medicine in China, Japan and Korea, is reported to have various biological activities. However, *K. scoparia* seed extract (KSE) functional roles on angiogenesis and prostate cancer inhibition have not been elucidated.

Objective: This study elucidates the effects of KSE on vascular endothelial growth factor (VEGF)-induced angiogenesis in human umbilical vein endothelial cells (HUVECs) and inhibition of proliferation in prostate cancer cells.

Materials and methods: HUVECs were treated with 10–20 µg/mL of KSE and 20–50 ng/mL of VEGF for 12–72 h. Anti-angiogenesis properties of KSE were determined by wound healing, trans-well, tube formation, rat aortic ring assay and western blotting. Prostate cancer and normal cells were incubated with 10–250 µg/mL of KSE for 24 h, and cell viability was measured by SRB assay. Phenolic compounds in KSE were analyzed using a HPLC-PDA system.

Results: IC_{50} for cell viability of HUVECs, LNCaP, PC-3, RC-58T and RWPE-1 by KSE were 30.64, 89.25, 123.41, 141.62 and >250 µg/mL, respectively. Treatment with KSE (20 µg/mL) significantly suppressed VEGF-induced migration, invasion and capillary-like structure formation of HUVECs and microvessel sprouting from rat aortic rings. In addition, KSE down-regulated PI3K/AKT/mTOR levels and phosphorylation of VEGF receptor 2 in HUVECs. 3-OH-tyrosol (1.63 mg/g) and morin hydrate (0.17 mg/g) were identified in KSE.

Conclusions: KSE inhibits angiogenesis in HUVECs as well as proliferation in human prostate cancer cells, suggesting KSE may be useful herbal medicine for preventing progression of prostate cancer and angiogenesis.

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KEYWORDS
Herbal medicine; antiangiogenesis; anticancer; VEGF

Introduction

Traditional medicines, which are composed of various herbal drugs, are considered to have great potential for modern drug development, and their molecular mechanisms have been precisely studied (Corson and Crews 2007). *Kochia scoparia* (L.) Schrad (Amaranthaceae) is a large annual broadleaf species and is a native plant to Eurasia (Beckie et al. 2013). It grows throughout in China, Japan and Korea; its mature fruit is traditionally used as a dietary food supplement and herbal remedy for treatment of skin diseases, malignant tumours in the head and neck regions, inflammation and allergic diseases (Matsuda et al. 1997; Han et al. 2016). Previous studies reported that *K. scoparia* fruit contains abundant saponins (Xia et al. 2002), momordin IC, triterpenoid glycosides and flavone glycosides (Wen et al. 1995). It also potentiates proliferative inhibition against immortal neuroblastoma cells (Mazzio and Soliman 2009), human hepatocellular carcinoma (Wang et al. 2013, 2014) and oral squamous cell carcinoma (Han et al. 2016). Although *K. scoparia* has shown promising cancer prevention activity, whether or not *K. scoparia* can modulate angiogenesis and proliferation of prostate cancer has not been determined.

Angiogenesis is the formation of new capillaries from preexisting vessels, and it is used by various organs to transport oxygen and nutrients (Tahergorabi and Khazaei 2012). It is estimated that most cancer deaths are due to tumour angiogenesis, invasion and metastasis of cancer to vital organs. Furthermore, Gimbrone et al. (1972) reported that solid tumours show highly limited growth (2–3 mm diameter) without inducing their own blood supply. Vascular endothelial growth factor (VEGF), a glycoprotein expressed in most cancer cells, is known as one of the most critical angiogenesis factors modulating the mitogenic activity of vascular endothelial cells (Lu et al. 2010). VEGF family members, including VEGF-A, -B, -C, -D and -E, exert their biological actions through interactions with tyrosine kinase receptors, VEGF receptors-1, -2 and -3 (Tahergorabi and Khazaei 2012). Specifically, VEGFR2 activation is involved in the angiogenic activity of VEGF through a cascade of downstream signalling pathways that regulate endothelial cell proliferation, migration, differentiation and tube formation. Dimerization of VEGF to extracellular VEGFR2 induces activation of phosphatidylinositol 3-kinase (PI3K)/AKT kinase, mammalian target of rapamycin (mTOR) kinase, focal adhesion kinase (FAK),

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extracellular signal-related kinase 1/2 (Erk1/2) and p38 kinase following autophosphorylation of intracellular domains in endothelial cells (Pang et al. 2010; Leelahavanichkul et al. 2014).

Prostate cancer, the second most commonly diagnosed cancer in the USA, is a leading cause of death in men worldwide. Standard treatment options include androgen deprivation therapy, immunotherapy, gene therapy and utilization of chemotherapy drugs to improve the efficacy of prostate cancer treatment, but significant adverse effects and resistance to chemotherapy can result in continued increases in metastatic prostate cancer progression (Ost et al. 2015; Sweeney et al. 2015). These detrimental effects of prostate cancer treatment on general health and quality of life have led to a search for alternative treatments, such as natural products and food ingredients.

Since adequate development of new blood vessels is essential for the proliferation and metastasis of solid tumours, VEGF plays a critical and specific role as an angiogenesis factor (Otrock et al. 2007). Although effective antiangiogenic agents are currently used for treating tumours, it is difficult to achieve complete tumour suppression via an individual modality. In addition, due to intrinsic cytotoxicity against non-tumour-associated endothelial cells, long-term use of angiogenesis inhibitors usually causes various side effects such as hypertension, thrombosis, reversible posterior leukoencephalopathy, cardiac toxicity and endocrine dysfunction (Chen and Cleck 2009; Österlund et al. 2011). Currently, the US Food and Drug Administration has approved a variety of antiangiogenic drugs targeting VEGF or VEGFRs, such as bevacizumab (Avastin®), sunitinib malate (Sutent®) and sorafenib (Nexavar®), for the treatment of specific types of cancer (Kamba and McDonald 2007). However, these antiangiogenic agents induce serious side effects such as hypertension, proteinuria, impaired wound healing, gastrointestinal perforation, haemorrhaging, thrombosis, reversible posterior leukoencephalopathy, cardiac toxicity and endocrine dysfunction (Chen and Cleck 2009; Österlund et al. 2011). Therefore, the identification of natural antiangiogenic agents that are safer and more efficient has attracted significant interest for cancer therapy (Ferrara and Kerbel 2005; Varinska et al. 2017).

In the present study, we evaluated the role of *K. scoparia* seed extract (KSE) for inhibition of angiogenesis and prostate cancer *in vitro*. In addition, the involvement of signalling pathways on KSE-induced anti-angiogenesis in human umbilical vein endothelial cells (HUVECs) and anticancer in prostate cancer cells were investigated for the first time. Therefore, the aim of this study was to determine how KSE inhibits angiogenesis in HUVECs, and promotes cell death in prostate cancer cells in an effort to facilitate the development of promising cancer therapeutics using natural product-derived ingredients.

Materials and methods

Chemicals

Endothelial cell basal medium, foetal bovine serum (FBS), trypsin-EDTA, penicillin and antibiotic-antimycotic were purchased from LONZA Inc. (Basel, Switzerland). Recombinant human VEGF ($VEGF_{165}$) was obtained from the Peprotech (Rocky Hill, NJ, USA). Growth factor-reduced Matrigel was purchased from BD Bioscience (Franklin Lakes, NJ, USA). Antibodies against anti-Bcl-2, anti-VEGF, anti-PI3K, anti-AKT, anti-mTOR, anti-Bax, anti-caspase-3, anti-caspase-9, anti-β-actin, phosphorylated-specific anti-AKT (Ser^{473}) and anti-mTOR (Ser^{2448}) were purchased from Santa Cruz Biotechnology (Dallas, CA, USA). Anti-VEGFR2 and phosphorylated-specific anti-VEGFR2 (Tyr^{1175}) were obtained from Cell Signaling Technology (Danvers, MA, USA).

Preparation of K. scoparia methanol extract

The seeds of *K. scoparia* were provided from Bioresources Lab., Department of Life Resources Industry, Dong-A University, Busan, Republic of Korea. *K. scoparia* species authentication was performed by Dr. Du-Hyun Kim, Professor of College of Natural Resources and Life Science, Dong-A University. Dried *K. scoparia* seeds (10 g) were extracted three times with MeOH (100 mL) at room temperature for 12 h. The extracts were filtered through Whatman no. 2 filter paper, and the filtrate was concentrated by rotary evaporation at 30 °C. Then, obtained KSE (0.81 g) was stored at −20 °C and used to examine its potential anti-angiogenesis and anticancer properties.

Cell lines and cell culture

Primary HUVECs were purchased from Lonza Inc. (Basel, Switzerland). HUVECs were cultured in endothelial cell growth medium (ECGM) supplemented with growth factors and 2% FBS (Lonza Inc., Basel, Switzerland). HUVECs were incubated at 37 °C under a humidified 95%:5% (v/v) mixture of air and CO_2.

PC-3 (prostate cancer derived from bone), LNCaP-FGC (prostate cancer derived from lymph node) and RWPE-1 (human prostate epithelial) cells were purchased from the American Type Culture Collection (ATCC, Rockville, ND, USA). RC-58T/h/SA#4 primary prostate cancer cells were obtained from the Center for Prostate Disease Research (Washington, DC, USA). Cells were cultured in DMEM (PC-3, LNCaP and RC-58T/h/SA#4) and keratinocyte-SFM (RWPE-1) medium supplemented with 10% FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Gibco BRL, Life Technologies, Grand Island, NY, USA) in an incubator containing a humidified atmosphere of 5% CO_2 at 37 °C.

Sulforhodamine B (SRB) assay

Cell proliferation was determined by SRB (Sigma, St. Louis, MO, USA) assay. The prostate endothelial, prostate cancer cells and HUVECs were seeded at a concentration of 3×10^4 cells/well in 48-well tissue culture plates and incubated with various concentrations of KSE for 24 h. After treatment, the medium was aspirated and 10% trichloroacetic acid was added. After 1 h incubation at 4 °C, the plate was washed five times with D.W and air-dried. The cells were stained with 0.4% (w/v) SRB at room temperature for 1 h and then washed five times using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris, and the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA).

Wound healing migration assay

Wound healing migration assay was performed as described previously (Cho et al. 2018). HUVECs were seeded onto 24-well plates at a density of 1×10^5 cells/well and cultured to confluence. The monolayer cells were wounded using 1-mL pipette tips to produce cross scratches and washed with PBS to remove non-adherent cells. ECGM containing 0.5% FBS was added with or without 20 ng/mL of VEGF and different concentrations of KSE

(5–20 µg/mL). Images of cells were taken after 12 h of incubation through an inverted microscope (200×).

Trans-well migration assay

Trans-well migration assay was performed as described previously (Cho et al. 2018). The 24-well trans-well insert culture unit (8 µm pore size, Trans-well, Corning, NY, USA) was used to evaluate the effects of KSE on invasion of HUVECs. Cells were seeded on the upper plate surface at a density of 4×10^4 cells per well along with the indicated concentrations of KSE, and the bottom chambers were filled with 1 mL of ECGM supplemented with 20 ng/mL of VEGF. After 16 h of incubation, migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (200×). The percentages of migrated cell numbers in treated groups were recorded using untreated wells at 100%.

Capillary-like tube formation assay

Capillary-like tube formation was assessed as described previously (Cho et al. 2018). To prepare the thin collagen layer, 250 µL of Matrigel solution was poured into 48-well culture plates, which were incubated at 37 °C for 1 h to solidify gels. HUVECs were plated in 24-well plates at a density of 1×10^5 cells per well and cultured for 24 h in ECGM containing 2% FBS. After culturing, cells were incubated in ECGM containing 0.5% FBS for 6 h and then treated with various concentrations of KSE for 1 h before seeding in a Matrigel-coated well plate. HUVECs were collected and placed onto the Matrigel layer (1×10^5 per well) in 1 mL of ECGM containing 0.5% FBS, followed by the addition of 20 ng/mL of VEGF. After 12 h of incubation at 37 °C under a humidified 95%:5% (v/v) mixture of air and CO_2, cells were photographed using an inverted microscope (200×).

Rat aortic ring assay

Rat aortic ring assay was performed as described previously (Cho et al. 2018). For this, 48-well plates were coated with 150 µL of Matrigel solution per well and polymerized at 37 °C for 1 h. Periadventitial fat and connective tissues were removed from aortas isolated from 4-week-old male Sprague-Dawley rats, placed in cold PBS, and cut into rings with a circumference of 1 to 1.5 mm. Aortic rings were placed into Matrigel-coated wells and sealed with a 100-µL overlay of Matrigel. VEGF in 500 µL of ECGM (0.5% FBS) with or without KSE was added into wells. Fresh medium was replaced every 2 days. After 3 days, microvessel sprouting was fixed and photographed using an inverted microscope (200×). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed six times.

Western blot analysis

HUVECs were seeded at a density of 1×10^6 cells in a 100-mm dish and then cultured for 24 h in ECGM (2% FBS). After culturing, HUVECs were first starved in serum-free ECGM for 6 h and then pretreated with or without various concentrations of KSE for 2 h, followed by stimulation with 50 ng/mL of VEGF for 1 h (VEGFR2 activation) or 2 h (for PI3K/AKT/mTOR pathway kinase activation). The resulting pellets were lysed by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM PMSF and 2 µg/mL of aprotinin) for 30 min on ice. The protein content of the supernatant was measured using a BCA protein kit (Pierce, Rockford, IL, USA). The protein samples were then loaded at 10 µg of protein/lane and then separated by 12% SDS-PAGE at 100 V of constant voltage/slab for 1.5 h. Following electrophoresis, the membranes were treated with primary and secondary antibodies and then washed with T-TBS. Detection of each protein was performed using an ECL kit (Santa Cruz, CA, USA).

Measurement of phosphorylated VEGFR2 concentration

The concentration of phosphorylated VEGFR2 (tyr1175) was assessed using a PathScan p-VEGFR2 sandwich ELISA kit (Cell Signaling Technology). Briefly, HUVECs were seeded at a density of 5×10^4 cells in a 24-well plate and then cultured for 24 h in ECGM (2% FBS). After culturing, cells were first starved in serum-free ECGM for 6 h and then pretreated with or without various concentrations of KSE for 2 h, followed by stimulation with 50 ng/mL of VEGF for 1 h. Whole-cell extracts were prepared in cell lysis buffer supplemented with protease inhibitor. A total of 100 µL of cell lysate was incubated with p-VEGFR2 antibodies coated onto micro-well plates for 2 h at 37 °C. After 2 h of incubation, p-VEGFR2 proteins were detected by using anti-rabbit IgG and HRP-linked antibodies. HRP substrate, TMB, was used for colour development, and the magnitude of optical density for this developed colour was proportional to the quantity of p-VEGFR2 proteins.

HPLC analysis

Analyses were performed on an HPLC-PDA (Waters Alliance e2695 separation module, MA, USA) with solvent delivery unit LC-20A, auto-sampler SIL-20A, photo-diode array detector SPD-M20A, and UV-VIS detector SPD-20A. The column was maintained at 30 °C in a CTO-20A column oven. After injecting 10 mL of sample, the separation was performed in a Waters SunFire C18 4.6 × 5 µm guard column. For detection and quantification of compounds, chromatograms were recorded at 220 nm in the photo-diode detector. The mobile phase consisted of 0.1% trifluoroacetic acid in water (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 1 mL/min. As shown in the HPLC chromatograms (Figure 7), 3-OH-tyrosol (MCE MedChemExpress Co., Princeton, NJ, USA, purity >99%) and morin hydrate (Aktin Chemicals Inc., Chengdu, China, purity >98%) were eluted at retention times of 7.172 and 35.606 min, respectively.

Statistical analysis

Data are expressed as means ± SD. We employed simple pairwise comparison with one-way ANOVA, followed by the Tukey–Kramer multiple comparisons test. A significant value was defined as $^*p < 0.05$, $^{**}p < 0.01$ and $^*p < 0.001$.

Results

KSE inhibits proliferation of HUVECs

Since angiogenesis has been shown to be involved in the proliferation of fibroblasts and endothelial cells, we investigated the effects of various concentrations of KSE on the proliferation of HUVECs using SRB assay. The half maximal inhibitory concentration (IC_{50}) for cell viability of HUVECs by KSE treatment was 30.64 µg/mL.

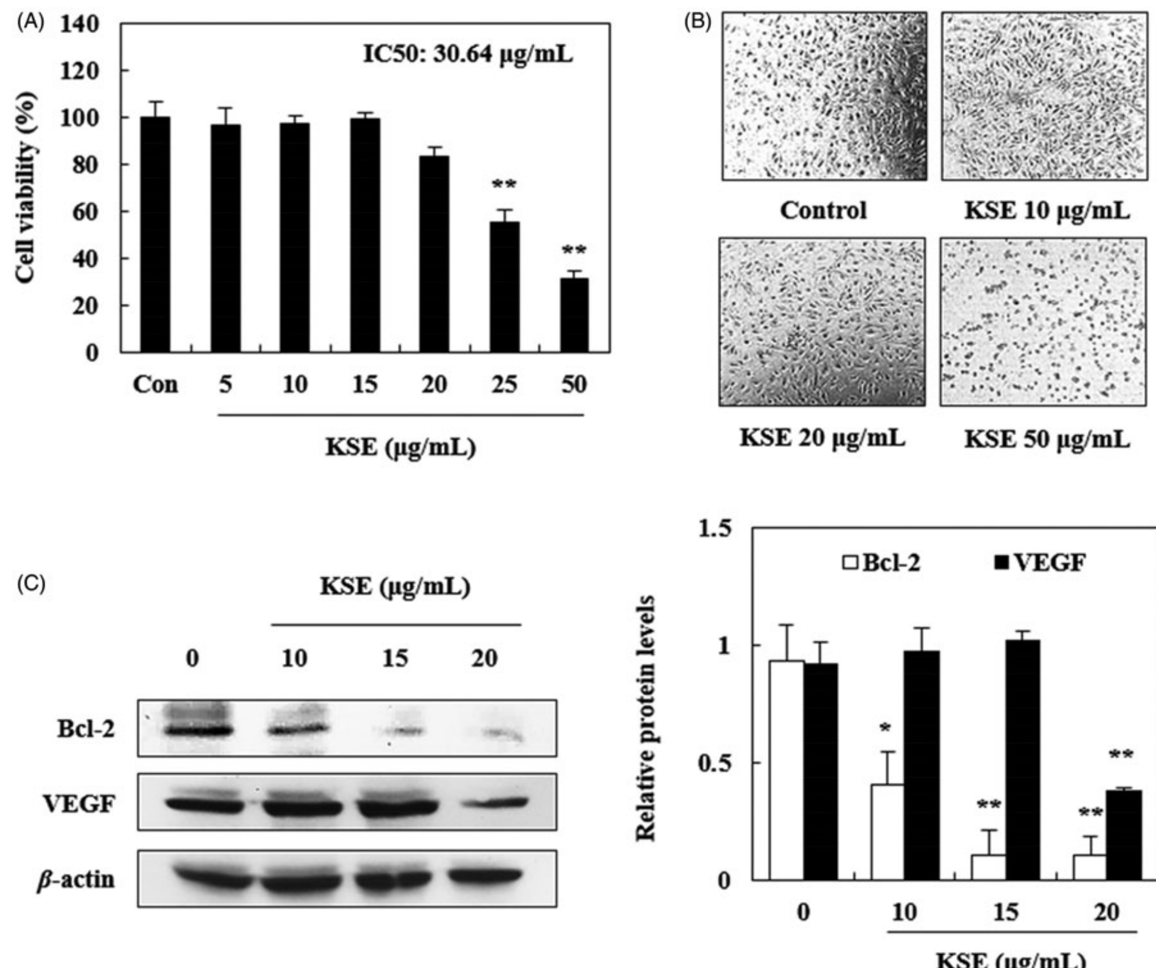


Figure 1. Effects of KSE on proliferation of HUVECs. Cells were treated with KSE for 24 h. (A) Cell growth was determined by SRB assay. (B) After 24 h incubation with KSE, cell morphology was visualized by inverted microscopy (200×). (C) KSE suppressed the expression of Bcl-2 and VEGF. Data values were expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA and Tukey's multiple comparison.

Exposing HUVECs to 1–20 μg/mL of KSE for 24 h did not affect the viability of HUVECs; however, the viability of cells treated with 25 μg/mL of KSE for 24 h was reduced to 43% compared with that of the control group (Figure 1(A,B)). Further, KSE treatment resulted in down-regulation of anti-apoptotic Bcl-2 and VEGF protein expression in HUVECs (Figure 1(C)). These data show that KSE suppressed the proliferation of HUVECs, indicating its potential in clinical applications to mediate angiogenesis.

KSE suppresses motility, invasion and capillary-like tube formation of endothelial cells induced by VEGF

To evaluate the anti-angiogenic properties of KSE *in vitro*, the inhibitory activities of KSE on the chemotactic motility of HUVECs were examined by wound healing assay. As shown in Figure 2(A), the proliferation of HUVECs was significantly stimulated by 20 ng/mL of VEGF treatment. However, KSE treatment for 12 h had a dose-dependent inhibitory effect on migration of HUVECs in the presence of VEGF. To determine the effects of KSE on VEGF-induced chemotactic angiogenesis in endothelial cells, we performed trans-well assay and capillary-like tube formation assay, respectively. Similar to the effects on VEGF-induced motility of HUVECs, VEGF significantly increased invasion and capillary-like networks in HUVECs (Figure 2(B,C)). KSE treatment significantly reduced VEGF-induced invasion in HUVEC monolayers as well as impaired the number, length and area of capillary-like structures seeded on growth factor-reduced two-dimensional Matrigel. Together, the above data suggest that KSE markedly suppressed VEGF-induced migration, invasion as well as capillary-like structure formation of HUVECs.

KSE inhibits VEGF-induced microvessel sprouting *ex vivo*

Since angiogenesis of preexisting vascular tubes is a complex procedure *in vivo*, we performed rat aortic ring assay, which can represent several stages of angiogenesis such as endothelial cell proliferation, migration and tube formation (Huang et al. 2017). As shown in Figure 3(A,B), 50 ng/mL of VEGF markedly facilitated microvessel sprouting and stereotypical branching of vascular networks around the rat aortic rings. However, the addition of KSE (10–20 μg/mL) significantly suppressed VEGF-induced sprouting in a dose-dependent manner. These results indicate that KSE inhibited VEGF-induced angiogenesis *in vitro* and *ex vivo*.

KSE inhibits activation of the PI3K/AKT/mTOR pathway in HUVECs

The PI3K/AKT/mTOR signalling pathway can be stimulated by attachment of VEGF-VEGFR2 and is a known regulator of cell

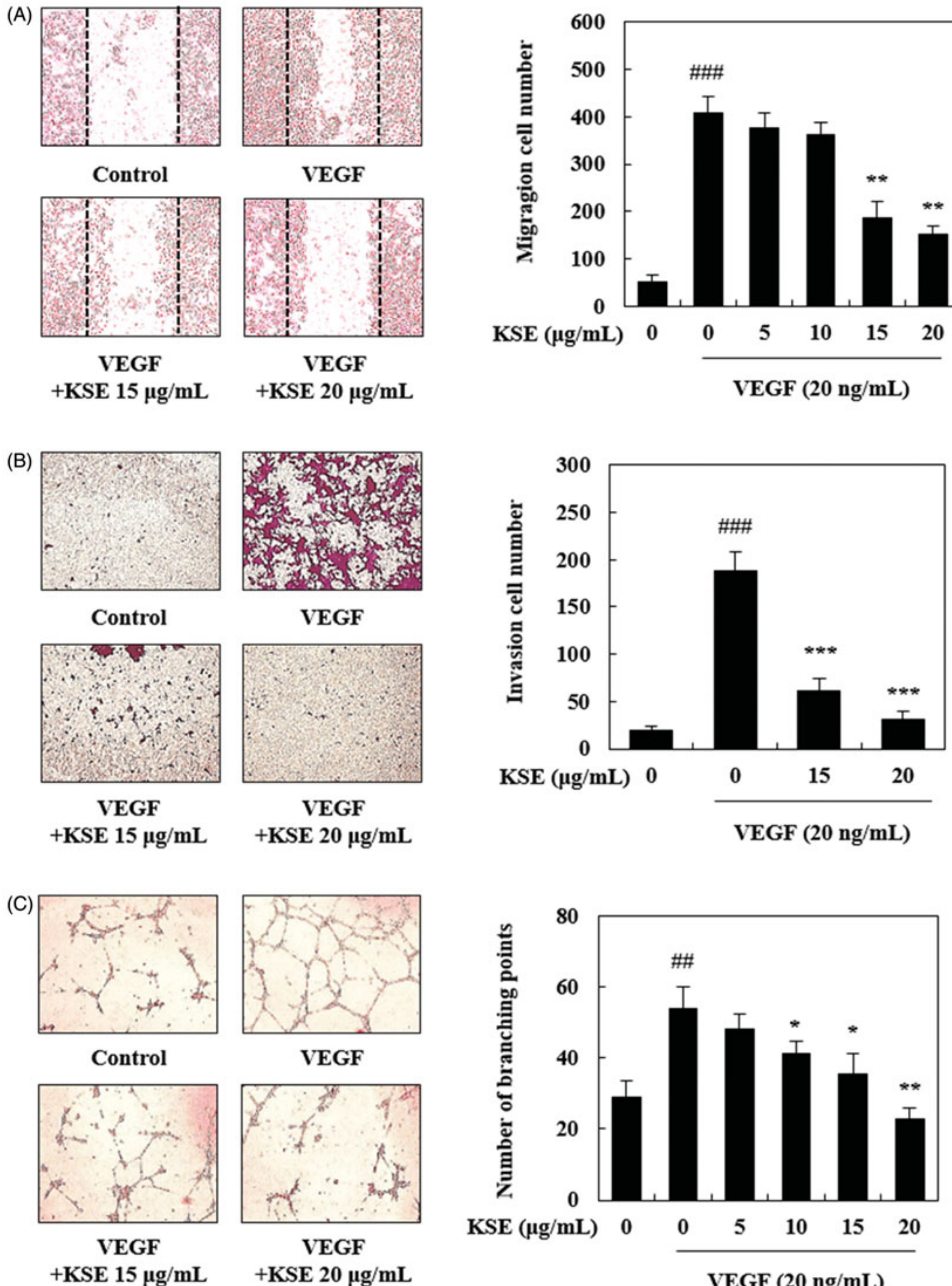


Figure 2. The effects of KSE on VEGF-induced chemotactic motility, invasion and capillary structure formation in HUVECs. (A) HUVECs were scratched by pipette and treated with or without 20 ng/mL of VEGF and 0–20 μg/mL of KSE. After incubation for 12 h, the migrated cells were photographed (magnification, 200×). Migrated cells were quantified by cell counting. Data values were expressed as mean ± SD of triplicate determinations. (B) After incubation for 16 h, migrated cells through the trans-well membrane was photographed (magnification, 200×) and quantified by cell counting. (C) After incubation on the matrigel for 12 h, capillary structure formation of endothelial cells was photographed (magnification, 200×) and quantified by counting of branching point. Significance of difference was compared with the control at $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, and with VEGF group at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA and Tukey's multiple comparison.

survival, proliferation, vascular development and angiogenesis (Shiojima and Walsh 2002; Fearnley et al. 2015). To determine whether or not KSE inhibits anti-angiogenic mechanisms in HUVECs, we analyzed the expression levels of PI3K/AKT/mTOR kinases by western blot assay. As shown in Figure 4, treatment with KSE down-regulated VEGF-induced phosphorylation of phosphoinositide 3-kinase, phosphorylated AKT and phosphorylated mTOR in a dose-dependent manner. These results indicate that the PI3K/AKT/mTOR signalling pathway mediates KSE-induced inhibition of proliferation and angiogenesis in HUVECs.

KSE suppresses activation of VEGFR2

To gain more insight into the molecular basis of KSE-mediated anti-angiogenesis, activation of VEGFR2 was examined in

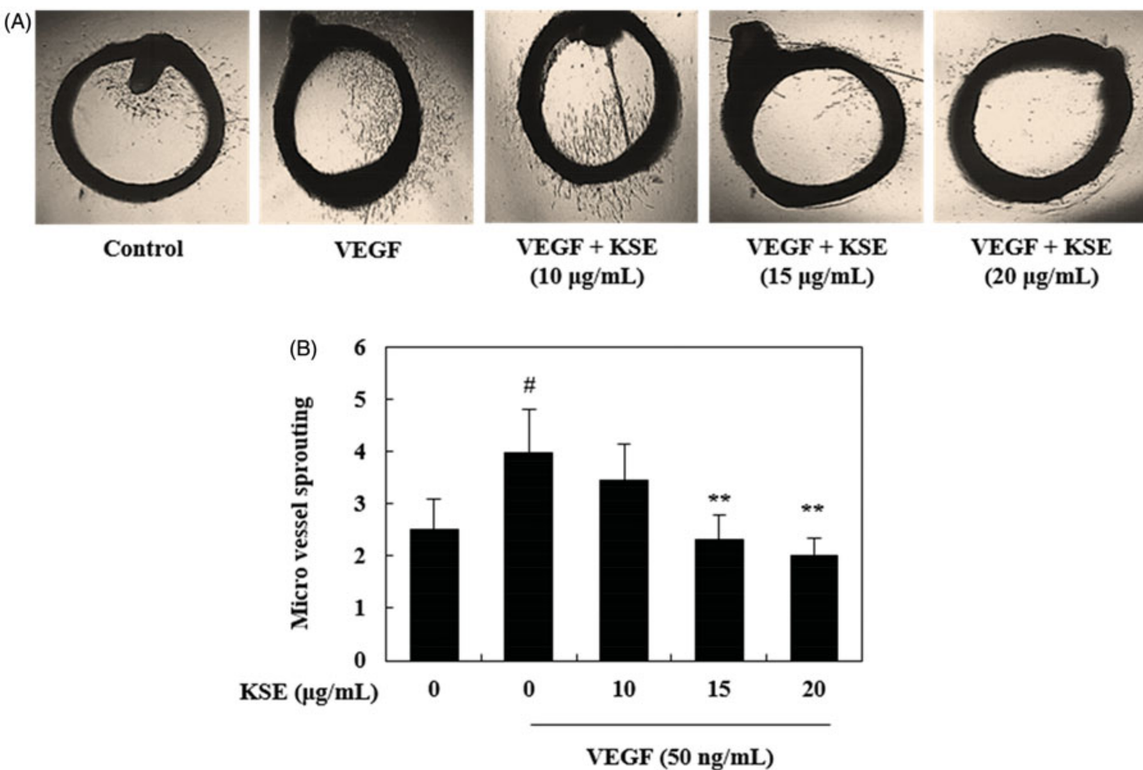


Figure 3. The effects of KSE on VEGF-induced microvessel sprouting *ex vivo*. (A) Aortic rings isolated from Sprague–Dawley rats were incubated on the matrigel-coated 96-well plates and treated with VEGF in the presence or absence of KSE. After incubation for 6 days, representative aortic rings were photographed (magnification, 200×). (B) Sprouts from rings were scored from 0 to 5 in double-blinded manner. Data values were expressed as mean ± SD of triplicate determinations. Significance of difference was compared with the control at $^{\#}p < 0.05$, and with VEGF group at $^{**}p < 0.01$ by one-way ANOVA and Tukey's multiple comparison.

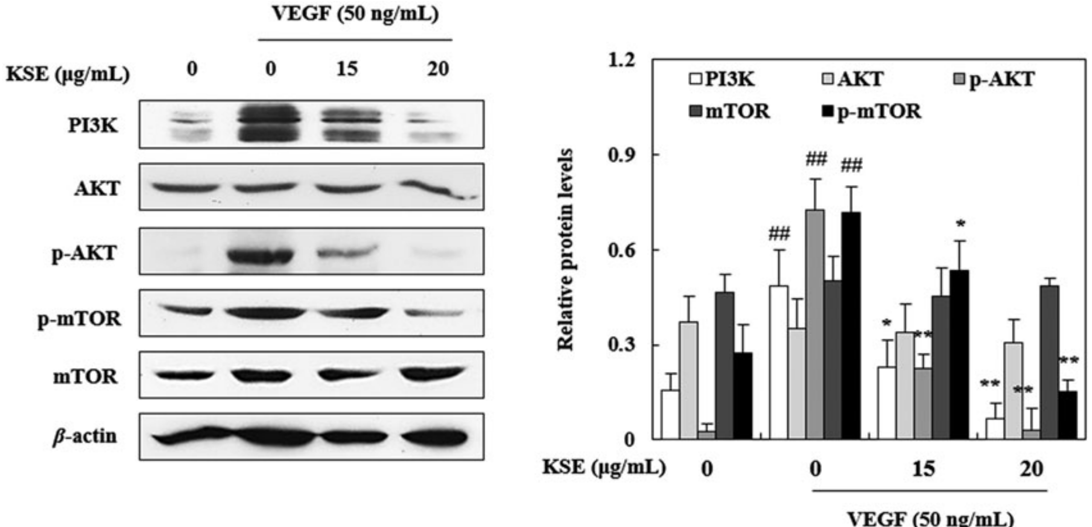


Figure 4. The effects of KSE on inhibition of PI3K/AKT/mTOR signalling pathway in HUVECs. HUVECs were treated with or without KSE for 2 h followed by VEGF treatment for 2 h and total cell lysates were subjected to western blotting to detect expression levels of proteins. Significance of difference was compared with the control at $^{\#\#}p < 0.01$, and with VEGF group at $^{*}p < 0.05$ and $^{**}p < 0.01$ by one-way ANOVA and Tukey's multiple comparison.

HUVECs treated with VEGF. As shown in Figure 5(A), treatment with VEGF caused up-regulation of phosphor-VEGFR2 proteins (Tyr1175 and Tyr996) whereas co-treatment with VEGF and KSE resulted in down-regulation of phosphor-VEGFR2 proteins. Furthermore, VEGFR2 activity in HUVECs was significantly reduced in the KSE-treated group compared with the VEGF control group (Figure 5(B)). These results show that KSE at a dose of 15–20 μg/mL dramatically blocked VEGF-induced VEGFR2 activation in HUVECs.

KSE more sensitively inhibits prostate cancer cell viability compared with human prostate normal cells

Since angiogenesis plays a critical role in the progression and metastasis of solid tumours, anti-angiogenic chemotherapy has become an attractive target for cancer treatment (Lin et al. 2013). To elucidate the effects of KSE on prostate cancer cell lines, we used SRB assays to examine cell proliferation. As shown in Figure 6(A), KSE had direct and dose-dependent cytotoxic

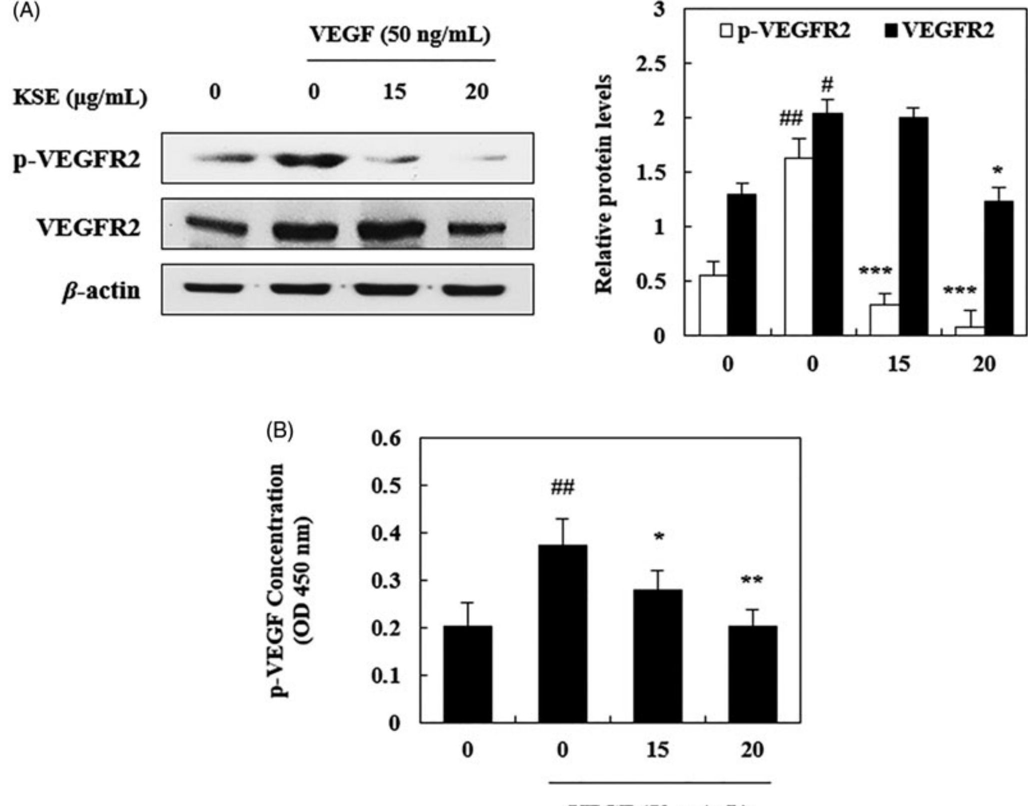
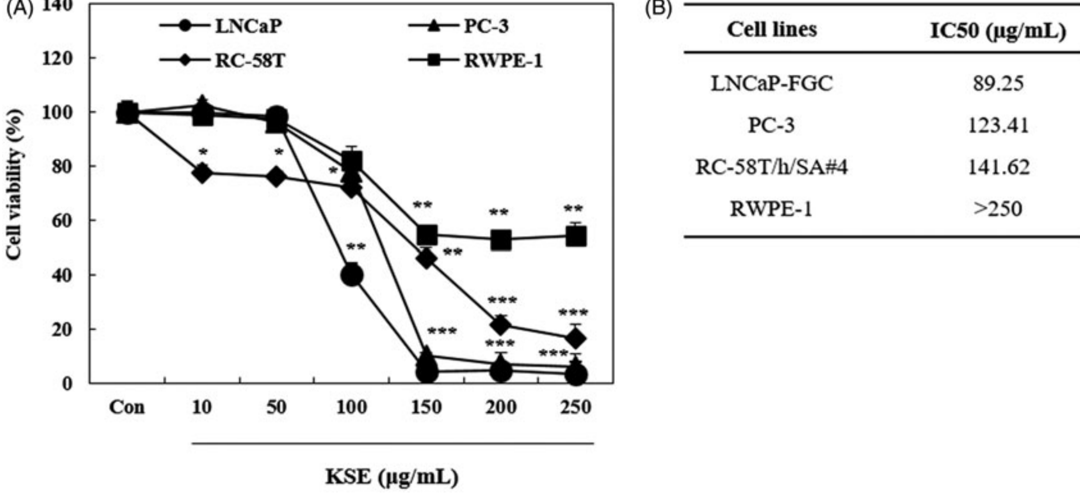


Figure 5. The effects of KSE on inhibition of VEGFR2 in HUVECs. (A) HUVECs were treated with or without KSE for 2 h followed by VEGF treatment for 1 h and total cell lysates were subjected to western blotting to detect expression levels of proteins. (B) The concentration of VEGFR2 (tyr1175) was assessed using a PathScan p-VEGFR2 sandwich ELISA kit. Data values were expressed as mean ± SD of triplicate determinations. Significance of difference was compared with the control at $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, and with VEGF group at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA and Tukey's multiple comparison.



Cell lines	IC50 (μg/mL)
LNCaP-FGC	89.25
PC-3	123.41
RC-58T/h/SA#4	141.62
RWPE-1	>250

Figure 6. Effects of KSE on proliferation of human prostate cancer (LNCaP, PC-3 and RC-58T), and human prostate endothelial (RWPE-1) cells. (A) After incubation with KSE for 24 h, cell growth was determined by SRB assay. (B) IC_{50} values of KSE were estimated from a plot of the percentage of viable cells. Data values were expressed as mean ± SD of triplicate determinations. Significance of difference was compared with the control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA and Tukey's multiple comparison.

effects on prostate cancer cells (LNCaP-FGC, PC-3 and RC-58T/h/SA#4), whereas it showed relatively low cytotoxicity in prostate endothelial cells (RWPE-1). IC_{50} values of KSE on LNCaP-FGC, PC-3, RC-58T/h/SA#4 and RWPE-1 cells were 89.25, 123.41, 141.62 and >250 μg/mL, respectively (Figure 6(B)). Cells undergoing apoptosis show biological changes, including membrane blebbing, cytoplasmic and nuclear condensation, nucleosomal fragmentation and related protein expression changes (Walker et al. 2016). Incubation with 50–100 μg/mL of KSE for 24 h induced chromatin condensation and DNA fragmentation in LNCaP prostate cancer cells. Furthermore, treatment of KSE for 24 h resulted in a dose-dependent increase in the late apoptotic cell population. In addition, pro-apoptotic proteins, including Bax, caspases-3 and -9, were up-regulated while anti-apoptotic

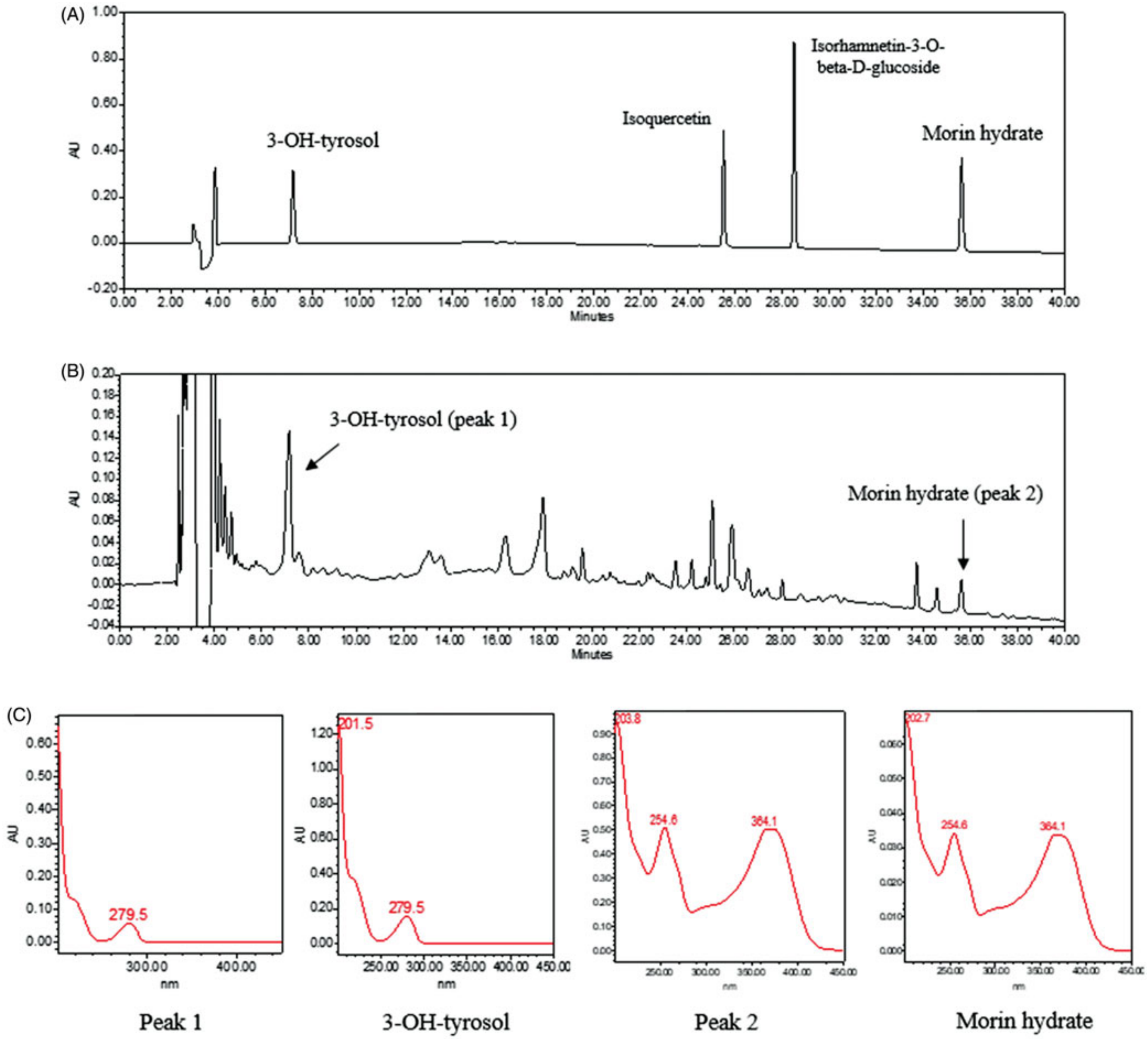


Figure 7. Chromatography of KSE (A) and phenolic compound standards (B) at 220 nm in HPLC. (C) UV spectra curves of phenolic compounds from KSE and standards.

proteins such as Bcl-2 were down-regulated upon KSE treatment (Supplementary Figure S1). These data indicate that KSE selectively reduced prostate cancer cell viability via activation of mitochondrial apoptosis pathway proteins.

Content of phenolic compounds in KSE

Chromatograms of compounds in KSE corresponded with standards at 220 nm (Figure 7(A)), and two major phenolic compounds, including 3-OH-tyrosol and morin hydrate, were quantified in KSE (Figure 7(B)). Furthermore, UV spectra absorptions and calibration curves of peak of KSE and standard compounds are shown in Figure 7(C). As shown in Table 1, 1.63 mg/g of 3-OH-tyrosol and 0.17 mg/g of morin hydrate were found in KSE. These results indicate that the antiangiogenic and anticancer activity of KSE may be related to the content of numerous phenolic compounds.

Discussion

In the current study, we showed that KSE, a traditional Korean medicine, can be a potent angiogenesis inhibitor and anticancer agent. KSE inhibited multiple steps of VEGF-mediated angiogenesis in HUVECs, including cell proliferation, migration, invasion and tube formation. One of the major mechanisms by which KSE mediated its activities against HUVECs seems to be through suppression of the PI3K/AKT/mTOR signalling pathway and VEGFR2 phosphorylation. Furthermore, KSE effectively induced apoptotic cell death in human prostate cancer cells without any toxicity in human prostate endothelial cells.

Phenolic compounds in natural products have been reported to suppress not only proliferation and metastasis of cancer but also angiogenesis of endothelial cells via multiple molecular targets (Oyenihi and Smith 2019). *Kochia scoparia* fruits contain various phenolic compounds, such as saponins, glycosides and steroids, that are indicative of anticancer activity (Mazzio and

Table 1. Contents of 3-OH-tyrosol, isoquercetin, isorhamnetin-3-*O*-β-D-glucoside and morin hydrate in KSE.

Phenolic compounds	Concentration in KSE (mg/g)
3-OH-tyrosol	1.63 ± 0.19
Isoquercetin	–
Isorhamnetin-3-*O*-β-D-glucoside	–
Morin hydrate	0.17 ± 0.03

Soliman 2009; Lu et al. 2012; Wang et al. 2013, 2014). In this study, two phenolic compounds, 3-OH-tyrosol and morin hydrate, were found in KSE at concentrations of 1.63 and 0.17 mg/g, respectively. These two compounds have significant inhibitory effects on angiogenesis of endothelial cells (Fortes et al. 2012) and proliferation of cancer cells (Kuo et al. 2007; Fuggetta et al. 2012) as demonstrated in previous studies. Although further study is needed to confirm unknown compounds at $t_R = 16.387$, 17.925, 25.083, 25.907 and 33.572 min, these results indicate that bioactive phenolic compounds in KSE contribute to its antiangiogenic and anticancer activities.

Previous studies have shown that KSE suppresses the growth of human tumours in *in vitro* and *in vivo* models. Han et al. (2014, 2016) showed that methanol extract of *K. scoparia* fruit at a dose of 25–40 μg/mL could inhibit growth of breast and oral cancer cells through caspase-dependent apoptosis. This suppression of cancer cell proliferation is in agreement with our data that KSE induced apoptotic cell death in prostate cancer cells. Interestingly, our study demonstrated that working concentrations of KSE (75–100 μg/mL) against LNCaP prostate cancer cells did not affect proliferation of RWPE-1 prostate epithelial cells. Wang and Yi (2008) reported that the effective and selective killing of tumour cells is one of the important considerations for therapeutic approaches in clinical stages. Although further studies are needed to evaluate the selective toxicity of KSE for prostate cancer, these results suggest that KSE might be a potent cancer therapeutic.

Crucial roles for VEGF and its receptors in both normal and tumour-associated vascular formation have been well established. VEGF, the most potent inducer of angiogenesis, binds to VEGFR2, resulting in activation of diverse intracellular signalling molecules (Niu et al. 2002; Wei et al. 2003; Yadav et al. 2015). In the present study, treatment with KSE resulted in a sharp decrease in the phosphorylation and activation of VEGFR2 at Tyr^{1175} sites. Further, VEGF-induced proliferation, migration, invasion and tubular-like formation in HUVECs were significantly inhibited by KSE treatment. Since VEGF receptor inhibitors are considered as promising agents for treating cancer, more than 20 agents with anti-angiogenic activities are already undergoing clinical studies for cancer treatment (Ferrara and Kerbel 2005; Pourgholami and Morris 2008; Pang et al. 2010). Interestingly, KSE was more effective in inhibiting HUVEC proliferation, migration, invasion and tubular-like formation (15–20 μg/mL) than inducing significant cell death in HUVECs (50 μg/mL) and prostate cancer cells (50–100 μg/mL), suggesting KSE can inhibit tumour-related angiogenesis of HUVECs without causing significant cell death in HUVECs and prostate cancer cells. These findings may provide a new therapeutic strategy for tumour-related angiogenesis with low chemotoxicity during chemotherapy.

Several signalling pathways are implicated in blood vessel formation, including VEGF synthesis, VEGFR2 autophosphorylation and the PI3K/AKT/mTOR pathway. In endothelial cells, binding of VEGF to VEGFR2 activates AKT phosphorylation (Ser^{473} and Thr^{308} sites) and constitutively promotes mTOR expression, resulting in endothelial cell survival, migration, invasion and tube formation (Shiojima and Walsh 2002; Dazert and Hall 2011). Thus, the current study demonstrated that PI3K/AKT/mTOR and its upstream kinases are functional mediators in angiogenesis (Fearnley et al. 2015). Interestingly, some phytochemicals suppressed xenograft mice tumour growth through inhibition of VEGF and AKT/mTOR phosphorylation (Lu et al. 2010; Pang et al. 2010). In this study, KSE suppressed VEGF-induced PI3K/AKT/mTOR activation in HUVECs, suggesting that KSE could function as a direct AKT/mTOR inhibitor. Therefore, these findings indicate that KSE can be applied to treat cancer invasion, migration and metastasis.

To conclude, this study identified that KSE suppresses angiogenesis through down-regulation of VEGFR2 phosphorylation and PI3K/AKT/mTOR pathways in HUVECs. Moreover, the proliferation of prostate cancer cells was decreased by KSE. Importantly, KSE inhibited angiogenesis of HUVECs and proliferation of cancer cells at a lower concentration compared with RWPE-1 prostate endothelial cells. These results expand our understanding of KSE, suggesting its potential in clinical applications as an effective herbal medicine to prevent prostate cancer and angiogenesis.

Disclosure statement

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