

Anti-angiogenic effect of *Livistona chinensis* seed extract *in vitro* and *in vivo*

ZHIYUN CAO^{1,2}, LIANGPU ZHENG^{1,2}, JINYAN ZHAO^{1,2},
QUNCHUAN ZHUANG^{1,2}, ZHENFENG HONG¹ and WEI LIN^{1,2}

¹Academy of Integrative Medicine Biomedical Research Center; ²Fujian Key Laboratory of Integrative Medicine on Geriatrics, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China

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Abstract. The present study aimed to detect the impact of the ethanol extract of the *Livistona chinensis* seed (EELC) on angiogenesis in human umbilical vein endothelial cells (HUVECs). A chorioallantoic membrane (CAM) assay was used to detect the anti-angiogenic activity of EELC *in vivo*. *In vitro*, the effect of EELC on the proliferation, migration and angiogenesis of HUVECs was determined by an MTT assay, a wound healing assay and a tube formation assay, respectively. The vascular endothelial growth factor (VEGF)-A and VEGF receptor (VEGFR)-2 protein and mRNA level were measured with ELISA and reverse transcription-semi-quantitative polymerase chain reaction. It was observed that EELC significantly decreased the formation of new vessels in the CAM assay. EELC inhibited the proliferation and migration of HUVECs. The extent of tube formation by HUVECs was also reduced by EELC. In addition, EELC treatment reduced the level of VEGF-A and VEGFR-2 mRNA and protein. The results suggest that EELC inhibits tumor angiogenesis through inhibiting the proliferation and migration of HUVECs, and by downregulating VEGF and VEGFR.

Introduction

Through screening for naturally occurring antitumor medicines, an increasing number of natural products have been

identified for use in clinical tumor therapy. A number of these natural products were originally used in traditional Chinese medicine and other forms of folk medicine (1).

Angiogenesis is associated with tumor recurrence and metastasis. In the 1970s, Folkman advanced the theory of inhibiting the formation of new blood vessels as a strategy against cancer (2). Since then, anti-angiogenesis has been a key subject in anticancer research. At present, the FDA has approved a range of drugs with anti-angiogenic activity, including sorafenib, sunitinib, pazopanib and everolimus (3-5). Anti-angiogenic natural extracts are also being considered in anticancer drug screening.

Livistona chinensis, the Chinese fan palm or fountain palm, which belongs to the monocotyledonous *Palmaceae* family, has been used for centuries as a medicinal herb in eastern Asia. It is native to southern Japan, Taiwan, the Ryukyu Islands and the Guangdong region of southern China (6). Extracts from the seeds of *Livistona chinensis* have been used to treat a range of types of cancer in traditional Chinese medicine including HCC and colon cancer (7,8). Previous studies screening for naturally occurring angiogenesis inhibitors have identified that the extracts from the shells of *Livistona chinensis* seeds were candidates for use in anti-angiogenic and antitumor therapy (8,9). Extracts of the *Livistona chinensis* seed have been demonstrated to suppress cancer cell growth (10). However, the precise mechanisms for its antitumor activity have yet to be characterized. In the present study, the anti-angiogenic effect of the extract from *Livistona chinensis* seeds will be considered.

Materials and methods

Reagents. RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and SuperScript II reverse transcriptase were obtained from Promega Corporation (Madison, WI, USA); trypsin-EDTA and TRIzol® reagent were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An angiogenesis assay kit was purchased from EMD Millipore (Billerica, MA, USA). Human vascular endothelial growth factor (VEGF)-A and VEGF receptor 2 (VEGFR)-2 Quantikine ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). All other chemicals were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Correspondence to: Dr Wei Lin, Academy of Integrative Medicine Biomedical Research Center, Fujian University of Traditional Chinese Medicine, 1 Huatuo Road, Minhou Shangjie, Fuzhou, Fujian 350122, P.R. China
E-mail: linwei311616@126.com

Abbreviations: EELC, ethanol extract of *Livistona chinensis* seed; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; CAM, chorioallantoic membrane; HUVEC, human umbilical vein endothelial cell

Key words: angiogenesis, vascular endothelial growth factor, ethanol extract of *Livistona chinensis* seed

Preparation of ethanol extract from *Livistona chinensis* seeds. A refluxing process was used to extract the *Livistona chinensis* seeds (500 g) with 85% ethanol (5,000 ml) and the extract was filtered. The extract was concentrated to a relative density of 1.05. The extraction liquid was condensed by a rotary spray dryer (Model B-290; Buchi, Flawil, Switzerland) to produce solid powder. The solid powder of EELC was initially diluted to 300 mg/ml in saline; further dilutions were performed with dimethyl sulfoxide (DMSO). DMSO was also used as the control treatment.

***In vivo* angiogenesis assay with a chorioallantoic membrane (CAM) model.** The anti-angiogenic activity of EELC was detected *in vivo* with a CAM assay. Briefly, 5-day old chicken embryos in eggs were purchased from Dabeinong Biotech Co., Ltd. (Fuzhou, China) and incubated for 2 days at 37°C with a relative humidity of 80%. Subsequent to the incubation, holes were cut in the shells to expose the CAMs and the embryos were placed in plastic culture dishes (Merck KGaA) according to an established shell-less culture technique: A chick embryo was removed from an eggshell and cultured in an automatic biochemical incubator (XiHeng Biological Co., Ltd., Shanghai, China) at a temperature of 38°C and constant air humidity of 70% after which time angiogenesis could be quantified via image analysis. This is an important technique for the generation of transgenic chickens that produce useful substances in their eggs, and for various embryonic manipulations. A 5-mm diameter Whatman filter paper circle sterilized by high pressure was loaded with 10 μ l EELC (10 mg/ml). The filter paper was then placed on the CAMs. Images were captured (using a phase-contrast microscope at a magnification of x50) of the treated areas and the extent of angiogenesis was evaluated at 24 h. The total number of vessels that had sprouted from the primary vessel of the CAM was counted in the area within 2.5 mm from the edge of the filter paper, and the total length of neoangiogenesis was evaluated with Saisam software (version 2.0; Microvision Instruments, Lisses, France). Ten replicates of the experiment were performed.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Evaluation of cell viability by MTT assay. An MTT colorimetric assay was used to detect cell viability. HUVECs (1x10⁴ cells/well) were seeded into 96-well plates and treated with different concentrations of EELC (0, 0.125, 0.25 and 0.5 mg/ml) for 24 h in the previously described cell culture conditions. MTT solution (20 μ l, 5 mg/ml) was added to each well of the plate. The MTT solution was discarded following 4 h of incubation in the previously described cell culture conditions, and 100 μ l DMSO was added to each well. An ELISA plate reader (EXL800; BioTek Instruments, Inc., Winooski, VT, USA) was used to measure the relative absorbance of the samples at 570 nm. Experiments were repeated in triplicate.

Quantification of cell migration by a wound-healing assay. HUVECs were grown to confluence in 6-well plates, washed with serum-free medium and wounded with a 200 μ l pipette tip. The wounded monolayers were then incubated, as previously described, for 24 h. The cells were observed with phase-contrast microscopy (Olympus Corporation, Tokyo, Japan) and images were captured with x100 magnification. The experiment was performed in triplicate.

Capillary-like tube formation assay. Tube formation by HUVECs was detected *in vitro* with the previously described Angiogenesis Assay kit, used according to the manufacturer's protocol. HUVECs were treated with different concentrations of EELC (0, 0.125, 0.25 and 0.5 mg/ml) in starvation RPMI-1640 medium for 24 h in the previously described incubation conditions. The cells were harvested with trypsin, resuspended with fresh assay medium from the angiogenesis assay kit and seeded (5x10³/well) into 24-well plates coated with ECMatrix gel (EMD Millipore). They were then cultured at 37°C for 3 h.

Cellular morphology and the development of tube formation were evaluated with phase-contrast microscopy at x100 magnification. The length of capillary tube formation was measured in three randomly chosen fields from each well with Image-Pro Plus software (version 5.0; Media Cybernetics, Inc., Rockville, MD, USA). The treated groups were compared with the untreated groups. The experiment was performed in triplicate.

RNA extraction and reverse transcription-semi-quantitative polymerase chain reaction (PCR) analysis. HUVECs (2x10⁵) were seeded onto 6-well plates and treated with different concentrations of EELC (0, 0.125, 0.25 and 0.5 mg/ml) for 24 h in the previously described cell culture conditions. Total RNA was then extracted from the HUVECs with TRIzol reagent, used according to the manufacturer's protocol. A total of 1 μ g RNA was reverse transcribed according to the manufacturer's protocol with the previously described SuperScript II reverse transcriptase. The produced cDNA was used for the evaluation of VEGF-A and VEGFR-2 mRNA by semi-quantitative PCR with Taq DNA polymerase (Fermentas; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: VEGF-A forward, 5'-GCCTTGCCTTGCTGCTCTA-3' and reverse, 5'-GATGTCCACCAGGGTCTCG-3'; VEGFR2 forward, 5'-ACGCCGATTATGTGAGA-3' and reverse, 5'-AGGCAGGAGTTGAGTATGT-3'; GAPDH forward, 5'-GTCATCCATGACAACTTTGG-3' and reverse, 5'-GAGCTTGACAAAGTGGTCGT-3'. The thermocycler conditions were as follows: 1 cycle of denaturation at 95°C for 5 min, then denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, elongation at 72°C for 45 sec, for 35 cycles, and 1 cycle of extension at 72°C for 10 min. For quantification, samples were electrophoresed in 1.5% agarose gel containing GoldView™ in Tris-acetate/EDTA buffer (Sigma-Aldrich; Merck KGaA), and PCR product images were analyzed using the ChemiDoc™ system and Quantity One 4.62 software (Bio-Rad Laboratories, Hercules, CA, USA). All cDNA samples were synthesized in parallel, and PCR reactions were run in triplicate. mRNA levels of each gene were normalized to GAPDH mRNA levels using the 2^{- $\Delta\Delta$ Cq} methods (11).

ELISA analysis. HUVECs cells (2×10^5) were seeded into 6-well plates and treated with different concentrations of EELC (0, 0.125, 0.25 and 0.5 mg/ml) for 24 h. Cells were lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) on ice for 10-15 min. Then, the supernatant from each well was collected by centrifugation at $1,000 \times g$ for 25 min at 4°C . The instructions from the Quantikine ELISA kit were followed and the VEGF-A and VEGFR-2 levels were measured with the ELISA plate reader. Experiments were repeated in triplicate.

Statistical analysis. A Student's t-test was used for comparisons between two groups; an analysis of variance followed by Tukey's post-hoc test was used for more than two groups. The data were analyzed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EELC inhibits the angiogenesis of CAMs *in vivo*. A CAM assay was used to determine the *in vivo* anti-angiogenic activity of EELC. Following 24 h of EELC treatment, the total number of blood vessels sprouting from the primary vessels in the CAMs was significantly reduced compared with the untreated control (Fig. 1; $P < 0.05$). This result indicated that angiogenesis is suppressed by EELC *in vivo*.

EELC inhibits the proliferation of HUVECs. The effect of EELC on the growth of HUVECs was evaluated. HUVECs were incubated with 0, 0.125, 0.25 or 0.5 mg/ml EELC for 24 h. As presented in Fig. 2, the growth of HUVECs treated with EELC was significantly inhibited compared with untreated control cells, in a dose-dependent manner ($P < 0.05$).

EELC inhibits the migration of HUVECs. As it had been demonstrated that EELC reduced cell viability, the effect of EELC on the migration abilities of HUVECs were assessed at 0, 0.125, 0.25 or 0.5 mg/ml EELC with a wound healing assay. As presented in Fig. 3, the migration of HUVECs was significantly inhibited following treatment with EELC at doses above 0.25 mg/ml ($P < 0.05$).

EELC inhibits the tube formation of HUVECs. To determine the effect of EELC on endothelial capillary tube formation, HUVECs were seeded on a solid ECMatrix gel containing mouse basement membrane proteins, which allowed endothelial cells to rapidly align to form hollow tube-like structures. HUVECs treated with EELC exhibited a dose-dependent decreased capacity for capillary tube formation compared with untreated HUVECs (Fig. 4; $P < 0.05$).

Effects of EELC on VEGF-A and VEGFR-2 mRNA and protein expression. The effects of EELC on VEGF-A secretion and mRNA, and VEGFR-2 protein and mRNA expression in HUVECs were determined by ELISA and semi-quantitative PCR. The ELISA revealed that VEGF-A secretion was dose-dependently reduced in HUVECs following treatment with EELC (Fig. 5A, $P < 0.05$). EELC treatment also suppressed VEGFR-2 protein expression in HUVECs (Fig. 5B, $P < 0.05$).

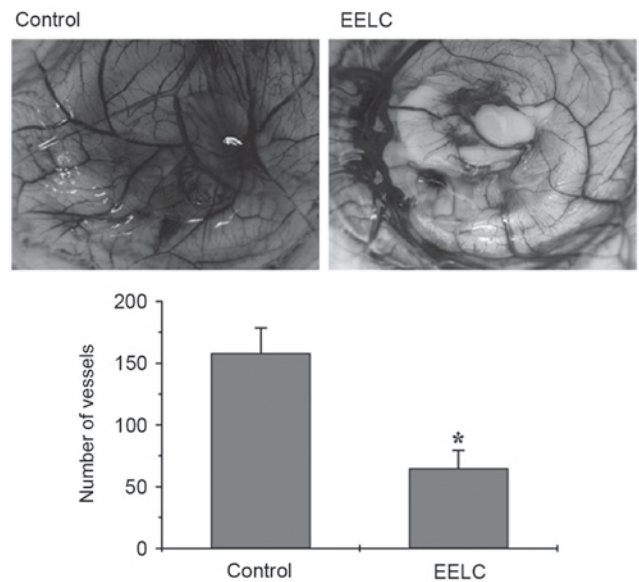


Figure 1. Effect of EELC on the angiogenesis of CAMs *in vivo*. Filter paper loaded with EELC was applied to the CAM of a 7 day-old chicken embryo and incubated for 24 h. The treated area was photographed and the level of angiogenesis was evaluated. The total number of vessels that had sprouted from the primary vessels of the CAM was counted. Data are means \pm standard deviation from 10 replicates. * $P < 0.05$ vs. control. Magnification, $\times 50$. EELC, ethanol extract of *Livistona chinensis* seed; CAM, chorioallantoic membrane.

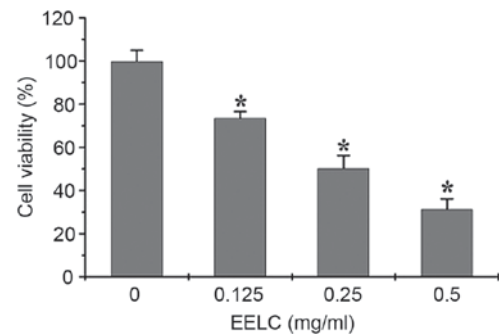


Figure 2. Effect of EELC on HUVEC cell viability. HUVEC cells were treated with EELC for 24 h. The viability of the treated cells was compared with untreated cells by an MTT assay. Data are means \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. control. EELC, ethanol extract of *Livistona chinensis* seed; HUVEC, human umbilical vein endothelial cell.

The mRNA levels of VEGFR-2 and VEGF-A were evaluated by semi-quantitative PCR. The results revealed that the relative mRNA levels of VEGFR-2 and VEGF-A were reduced following treatment with EELC (Fig. 6, $P < 0.05$).

Discussion

Anti-angiogenesis research began >35 years ago with the study of the late Folkman (12-14). Though tumors may stimulate the growth of new vessels with inflammation, mutation/over-expression or mechanical stress, the principal stimulus for angiogenesis is oxygen deprivation (15). Once angiogenesis has been activated, tumors express pro-angiogenic factors and tumor vascularization increases. Pro-angiogenic paracrine factors, including angiogenin, VEGF, fibroblast growth factor,

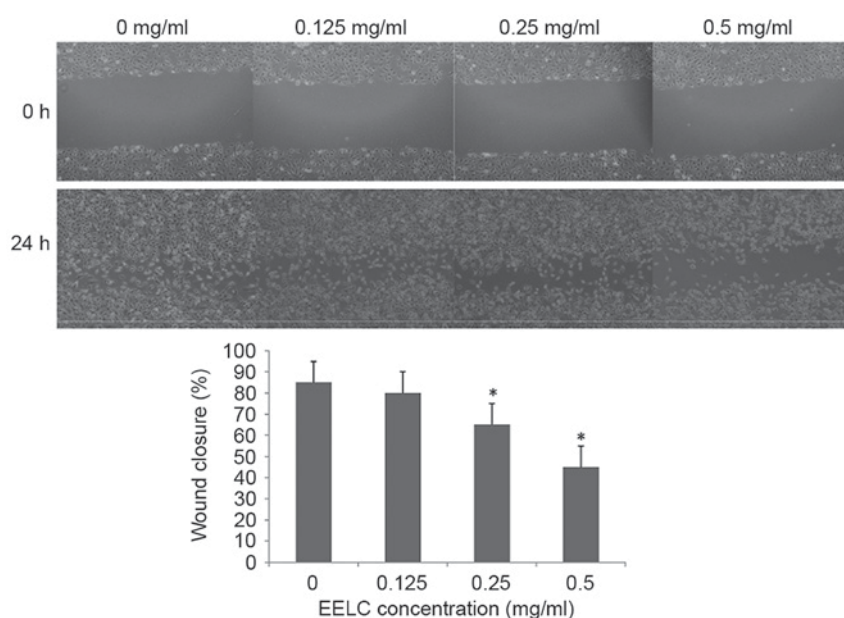


Figure 3. Effect of EELC on HUVEC migration. Following treatment with EELC for 24 h, the extent of the migration by HUVECs was determined with images from phase contrast microscopy (magnification, x100). Images are representative of three independent experiments. Data are means \pm standard deviation. * P <0.05 vs. control. EELC, ethanol extract of *Livistona chinensis* seed; HUVEC, human umbilical vein endothelial cell.

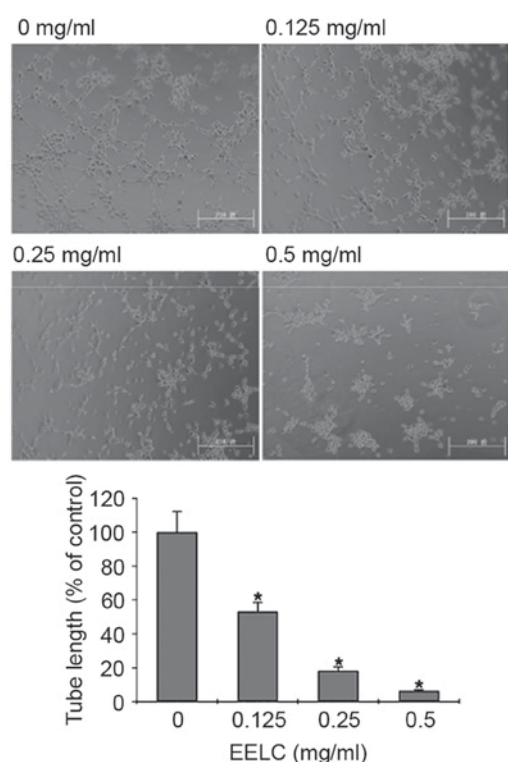


Figure 4. Effect of EELC on capillary-like tube formation. Following treatment with EELC, HUVECs were harvested and seeded into a plate coated with basement membrane protein gel and incubated for 9 h. Images of the extent of tube formation were examined with phase-contrast microscopy (magnification, x100). The total length of the capillary-like tubes was measured and compared with the untreated control. Images are representative of three independent experiments. Data are means \pm standard deviation. * P <0.05 vs. control. EELC, ethanol extract of *Livistona chinensis* seed; HUVEC, human umbilical vein endothelial cell.

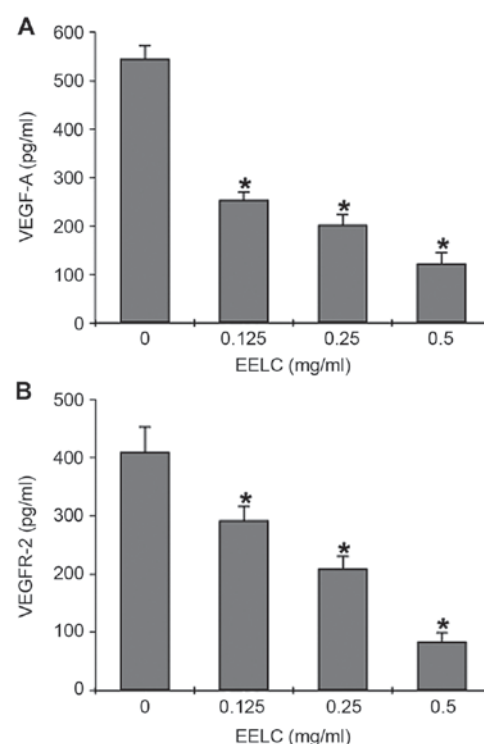


Figure 5. Effect of EELC on (A) VEGF-A and (B) VEGFR-2 protein expression. HUVECs were treated with EELC for 24 h. The protein levels of VEGF-A in the supernatant of each group and VEGFR-2 from lysed HUVECs were determined by ELISA. Data are the means \pm standard deviation of triplicate experiments. * P <0.05 vs. control. EELC, ethanol extract of *Livistona chinensis* seed; VEGF-A, vascular endothelial growth factor-A; VEGFR-2, vascular endothelial growth factor receptor-2; HUVEC, human umbilical vein endothelial cell.

and transforming growth factor- β , are released by tumor cells to stimulate the growth of new vessels as a response to the

stimulus (16). These factors activate endothelial cell proliferation, migration and invasion, resulting in new vessel formation from neighboring blood vessels.

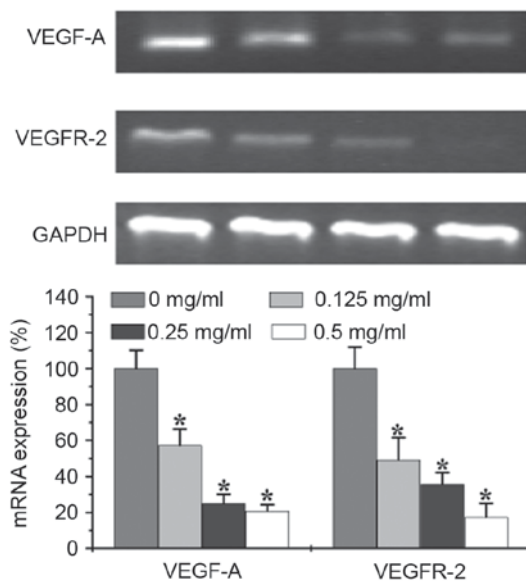


Figure 6. Effect of EELC on mRNA expression. The mRNA levels of VEGF-A and VEGFR-2 were detected by reverse transcription-semi-quantitative polymerase chain reaction with an internal control of GAPDH. The treated group data were compared with an untreated control. Data are means \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. control. EELC, ethanol extract of *Livistona chinensis* seed; VEGF-A, vascular endothelial growth factor-A; VEGFR-2, vascular endothelial growth factor receptor-2.

Livistona chinensis is used as a folk medicine in China to treat various types of tumor (6). The crude aqueous extract of *Livistona chinensis* seeds can inhibit the growth of HUVEC, breast cancer and colon adenocarcinoma (Ht-29) cells (9). Our previous study demonstrated that the ethanol extract of EELC inhibited tumor growth in an HCC mouse model *in vivo*, and induced the apoptosis of HepG2 cells *in vitro*. The apoptosis following EELC treatment was associated with the loss of mitochondrial membrane potential, the activation of caspase-9 and caspase-3, and an increase in the Bax/Bcl-2 ratio (10). In the present study, EELC inhibited angiogenesis *in vivo* and *in vitro*, and inhibited the growth and migration of HUVECs. EELC treatment also reduced the VEGF-A secretion and VEGFR-2 expression of HUVECs. Our previous study identified that EELC may affect angiogenesis via the inhibition of VEGFR-2 through the Notch signaling pathway (17). Other previous studies have indicated that the activated Notch pathway reduced the level of VEGFR-2 by affecting transcriptional regulation in mouse endothelial cells (18,19). EELC may therefore inhibit the VEGFR-2 through notch signaling pathway. It has been demonstrated that the proliferation, migration and invasion of endothelial cells are highly dependent on VEGF and VEGFRs, including in mice and zebrafish (20,21). EELC may limit the angiogenic behavior of endothelial cells by suppressing blood vessel formation via the regulation of the VEGF signal pathway (22,23). In our future study, we will aim to further characterize the effects of EELC on the Notch signaling pathway in endothelial cells.

A modern clinical strategy against cancer is the combination of anti-angiogenesis drugs with other treatments, including chemotherapy, radiation or other targeted drugs, to obtain improved therapeutic effects compared with using treatments individually. For example, monotherapy with bevacizumab failed to prolong the survival time of patients with cancer,

whereas bevacizumab combined with cytotoxic chemotherapy may increase the survival time (24,25). The effect of EELC combined with 5-FU on HCC cells will also be investigated in future study.

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