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Antiangiogenic Effects and Mechanisms of *trans*-Ethyl *p*-Methoxycinnamate from *Kaempferia galanga* L.

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ABSTRACT: *Kaempferia galanga* L. (Zingiberaceae) is an aromatic herb and a popular spice used as a condiment in Asian cuisine. The ethanol extract of the dried plant and its successive four subfractions were investigated on zebrafish model by quantitative endogenous alkaline phosphatase assay. Both *n*-hexane and ethyl acetate fractions had antiangiogenic activity, and two major active components (*trans*-ethyl *p*-methoxycinnamate and kaempferol) showed potent antiangiogenic effects on wild-type zebrafish. Because of its much stronger effect and no antiangiogenic activity reported, *trans*-ethyl *p*-methoxycinnamate was further investigated for its action mechanism. It dose dependently inhibited vessel formation on both wild- and *Tg(fli1a:EGFP)y1*-type zebrafish embryos. The semiquantitative reverse transcription polymerase chain reaction assay suggested that *trans*-ethyl *p*-methoxycinnamate affects multiple molecular targets related to angiogenesis. In vitro, it specifically inhibited the migration and tube formation of human umbilical vein endothelial cells. In vivo, it could block bFGF-induced vessel formation on Matrigel plug assay.

KEYWORDS: *Kaempferia galanga, spice, antiangiogenesis, zebrafish, trans-ethyl p-methoxycinnamate*

INTRODUCTION

Spices are exotic food adjuncts used widely as flavoring agents, colorants, and preservatives in the East; five centuries ago, they were introduced to the Western world. Many spices are noted to possess medicinal properties. With advances in modern technologies, their health benefits have been appreciated more thoroughly in recent decades. Various spice derivatives including 1′-acetoxychavicol acetate, anethole, capsaicin, cardamonin, curcumin, dibenzoylmethane, diosgenin, eugenol, gambogic acid, gingerol, thymoquinone, ursolic acid, xanthohumol, and zerumbone have been isolated from galangal, anise, red chili, black cardamom, turmeric, licorice, fenugreek, clove, kokum, ginger, black cumin, rosemary, hop, and pinecone ginger, respectively.[1,2] They show digestive stimulant actions, hypolipidemic effects, antidiabetic influences, antilithogenic properties, antioxidant potentials, anti-inflammatory functions, and antimutagenic, antimicrobial, and anticarcinogenic activities.[3] Extensive research over the past few years has indicated that spice derivatives, such as capsaicin, *trans*-anethole, thymoquinone, diosgenin, and allicin, can not only reduce the risk of acquiring cancer but also modulate different stages of tumorigenesis, including tumor cell survival, proliferation, invasion, and angiogenesis.[2] Most spices lack toxicity and can be safely used as flavoring foods and household remedies. Some spices and their derivatives have been regarded as safe by the U.S. Food and Drug Administration (FDA) for use in cereals, chips, cheese, butter, and other food products. So far, only a few spices and their active components have been tested for their potential in cancer treatment; the most noteworthy one is curcumin, a common ingredient in curries, which is to be investigated in a phase I/II trial for the treatment of bowel cancer.[2,4] Further studies on spices may reveal their efficacy in the treatment and prevention of human cancer and other diseases.

Kaempferia galanga L. is a herb with very aromatic rhizomes. It is widely cultivated in tropical and subtropical Asia but little known in the West. Its rhizome has been a popular condiment in Asian cuisine. In China, it is popularly used in food as a spice and in folk medicine for treating hypertension, pectoral and abdominal pains, headache, toothache, rheumatism, dyspepsia, coughs, inflammations, and tumors. Its aroma has also been used for a long history in relieving restlessness, stress, anxiety, and depression.[5−9] Its rhizome contains about 2.5−4% essential oil. The main components are ethyl cinnamate, *trans*-ethyl *p*-methoxycinnamate, *cis*-ethyl *p*-methoxycinnamate, *p*-methoxycinnamic acid, and a monoterpene ketone compound, 3-carene-5-one. The other constituents are borneol, 1,8-cineole, 3-carene, (*E*)-cinnamaldehyde, eucalyptol, kaempferol, methyl *p*-coumaric acid ethyl ester, and pentadecane.[10−13] The ethyl *p*-methoxycinnamate derived from this plant could be widely used in food, cosmetics, and medicines for its sedative, insecticidal, and antibacterial activities.[14,15] According to the hippocratic screening test, acute and subacute toxicities in rats, the ethanolic rhizome extract and hexane fraction of *K. galanga* caused neither gross abnormalities nor histopathological changes. Also, no sign of irritation was observed during the dermal irritation test of the hexane fraction.[7] Because of its antirheumatism, anti-inflammatory, and antitumor effects, *K. galanga* warrants further investigation of its potential antiangiogenic properties.

MATERIALS AND METHODS

Animals. Zebrafish embryos used for screening were obtained from natural spawning of wild-type fish bought from local pet stores in Hong

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Kong, and the *Tg(fli1a:EGFP)y1* line was obtained from the Zebrafish International Resource Center (5274, University of Oregon, Eugene). The zebrafish were maintained in flow-through aquaria (36 L) at 28.5 °C on a 14/10 h (light/dark) photoperiod. Male C57BL/6 mice (6 weeks old) were supplied and maintained by Laboratory Animal Service Center, the Chinese University of Hong Kong. The handling of fish and mice was licensed by the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Herbal Materials. *K. galanga* was purchased from a herb shop in Shenzhen, Guangdong Province, People's Republic of China, in February, 2008. Authentication of the raw herb, both morphological and chemical authentication, followed specifications in the Pharmacopoeia of the People's Republic of China (2010). The chemical profiles were confirmed qualitatively by thin-layer chromatography (TLC) with the reference herb provided by National Institute for the Control of Pharmaceutical and Biological Products. A voucher specimen (2010-3263) was deposited in the Museum of Chinese Medicine, Institute of Chinese Medicine, The Chinese University of Hong Kong.

Cell Culture. Primary human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA). HUVECs at early passages (passages 10−15) were used in all of the experiments and were cultured in gelatin-coated plates with Complete Growth Medium [Dulbecco's modified Eagle's medium: nutrient mixture F-12 (D-MEM/F-12), 10% fetal bovine serum (FBS)] (GIBCO, Gaithersburg, MD) containing 0.1 mg/mL heparin, 0.03−0.05% mg/mL endothelial cell growth supplement, and 1% penicillin−-streptomycin (P/S) (Sigma, St. Louis, MO). Cells were incubated at 37 °C in 5% CO_2 in air.

Chemical Reagents. Endogenous alkaline phosphatase (EAP) staining was assayed with phosphatase substrate kit (Pierce, Rockford, IL) and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) ready-to-use tablets (Roche Diagnostics GmbH, Germany). *trans*-Ethyl *p*-methoxycinnamate (KG-1) and kaempferol (KG-2) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SU5416 (1,3-dihydro-3-[(3,5-dimethyl-1*H*-pyrrol-2-yl)methylene]-2*H*-indol-2-one), basic human fibroblast growth factor (bFGF), heparin, and Drabkin's reagent were obtained from Sigma. Basement membrane matrix Matrigel (with reduced growth factor) was from BD Biosciences (Bedford, MA).

Preparation of Ethanol Extracts and Subfractions. The pulverized herbs (200 g) were extracted with 95% ethanol three times (reflux, 2 h each time), and then, 22 g of ethanol extract (EE) was obtained after evaporation under reduced pressure. For solvent fractionation, EE was suspended in distilled water and extracted successively with equal volumes of *n*-hexane (Hex), ethyl acetate (EA), and *n*-butanol (BuOH), leaving a residual aqueous fraction (Aq). Each subfraction was evaporated under reduced pressure to yield the extracts of Hex (13.4 g), EA (0.9 g), BuOH (2.9 g), and Aq (3.8 g) fractions, respectively.

Embryo Handling. Zebrafish embryos were generated by natural pairwise mating. The embryos were maintained in embryo water (0.2 g/L Instant Ocean Salt, Spectrum Brands, Madison, WI) at 28.5 °C incubator. Any dead or unfertilized embryos should be cleaned out in the first few hours of development and periodically thereafter to prevent developmental delay. They were manually dechorionated with forceps at 24 h postfertilization (hpf) immediately prior to drug treatment.

Drug Administration. Twenty-four hpf zebrafish embryos were arrayed in a 96-well plate, one embryo per well, and incubated with 100 μL of embryo water per well containing various concentrations of an extract or compound at 28.5 °C for 48 h. Dimethylsulfoxide (DMSO, Sigma) was used as a carrier control (0.2%).

EAP Assay on Zebrafish Embryo. During zebrafish development, the stage between 24 and 72 hpf has the highest angiogenic activity, and quantitative EAP assay was performed. Drug-treated embryos at 72 hpf were dehydrated by increasing the concentration of ethanol. Then, the embryos were washed three times with diethanolamine buffer (Pierce). Next, the embryos were stained according to the protocol described in phosphatase substrate kit. After staining, 50 μL of 2 M NaOH was added to stop the reaction. The optical density of soluble end product was measured at 405 nm using a microplate reader. Vessel growth was presented as a percentage in optical density as compared with control [% vessel formation = (OD treated day 3 − OD control day 1)/(OD control day 3 − OD control day 1) × 100%]. Each assay was repeated at least three times. No additional maintenance was required as the embryo yolk sac supplies nutrients during the process of the experiment.

Microscopic Imaging. For better documentation of the blood vessels in embryos, *Tg(fli1a:EGFP)y1* zebrafish embryos were used. An observation was made on enhanced green fluorescent proteins (EGFP), which are expressed in all endothelial cells of the vasculature in intersegmental blood vessels (ISVs) and subintestinal vessel plexus (SIVs) at 48 and 72 hpf, respectively, after drug treatment at 24 hpf. The embryos at 24 hpf were incubated with embryo water containing 1-phenyl-2-thiourea (PTU, Sigma; final concentration, 0.2 mM) before drug administration to prevent pigment development to facilitate blood vessel observation. The embryos were examined for blood vessel development using an Olympus IX71S8F-2 inverted microscope (Olympus, Tokyo, Japan). Dead or unhealthy embryos were removed.

Total RNA Isolation, Reverse Transcription (RT), and Semi-quantitative Polymerase Chain Reaction (PCR). Total RNA samples were isolated from three embryos using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RT was performed at 37 °C for 2 h in a total volume of 10 μL of reaction solution containing 1 × moloney murine leukemia virus (MMLV) reverse transcriptase buffer, 0.5 mM each dNTP, 0.5 μg of oligo(dT), and 100 U of MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). The primers were synthesized by Invitrogen, and the cycle numbers of half maximal amplification were used for subsequent semiquantitative RT-PCR.[16] The PCR was carried out in a volume of 15 μL containing 1:15 diluted RT reaction product, 1× PCR buffer, 0.1 μM each primer, and 0.375 U *Taq* polymerase, using the iCycler Thermal Cycler (Bio-Rad, Hercules, CA) with an annealing temperature of 61 °C . All of the PCR products were visualized in 1.6% agarose gel stained with ethidium bromide.

MTT Assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. HUVECs (4000 cells/well) were seeded in a 96-well plate in the Complete Growth Medium for 12 h for attachment. Then, cells were treated for 48 h with growth medium containing vehicle 0.1% DMSO and indicated compounds. Thirty microliters per well of 5 mg/mL MTT in phosphate-buffered saline (PBS, Invitrogen) was added, and incubation continued for another 2 h at 37 °C. The culture medium was removed, and 100 μL of DMSO per well was added. Then, after complete dissolution, absorbance was detected at 540 nm by a Benchmark microtiter plate reader (Bio-Rad). Each point represents the mean ± standard error of the mean (SEM) (n = 5) from a representative experiment.

[Methyl-^{3}H]-Thymidine Incorporation Assay. HUVECs (4000 cells/well) were seeded in a 96-well plate in the Complete Growth Medium for 12 h for attachment. Then, cells were treated for 48 h with growth medium containing vehicle 0.1% DMSO and indicated compounds. At the end of 48 h of incubation, 10 μCi of tritiated thymidine (GE Healthcare, Piscataway, NJ) in 20 μL of the growth medium was added to each well for incubation at −20 °C overnight and then thawed at room temperature before the cells were harvested onto unifilter-96 GF/C plates (PerkinElmer, Waltham, MA) with a unifilter-96 harvester (PerkinElmer). Thirty microliters per well of MicroScint TM 40 scintillation cocktail (PerkinElmer) was added, and the plates were subjected to measurement using the TopCount 2 detector 96-format liquid scintillation analyzer (PerkinElmer). Each point represents the mean ± SEM (n = 5) from a representative experiment.

Migration Assay. HUVEC migration assay was performed in a modified Boyden chamber (Transwell, Corning, NY). Two hundred microliters of HUVEC suspension (5 × 10^4 cells/well) in Complete Growth Medium containing 1% FBS and indicated compounds together with 0.1% DMSO (vehicle) was added to the upper compartment of each chamber. Five hundred microliters of Complete Growth Medium containing 10% FBS was added to the lower compartments. SU5416, a selective vascular endothelial growth factor (VEGF) receptor-2 inhibitor, was used as a positive control. After 3 h of incubation in an

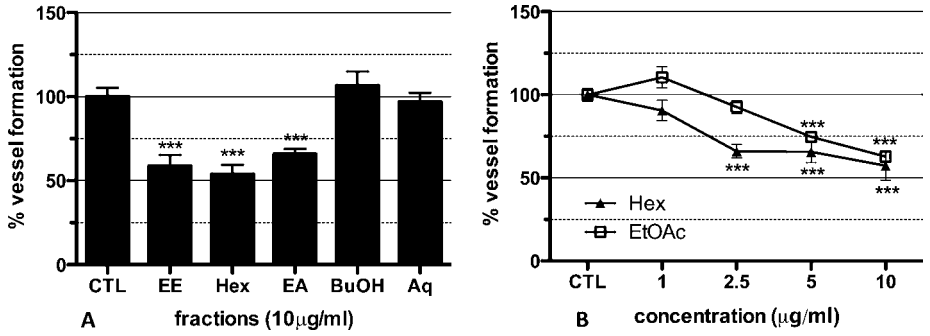

Figure 1. Antiangiogenic activity of the fractions of *K. galanga*. Fractions were dissolved in DMSO and then added directly to the embryo water. DMSO (0.2%) was used as the carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± SEM (n = 10) from a representative experiment. *** Represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

incubator, the nonmigrated cells on the upper side of the membrane were removed by cotton swab. The migrated cells on the bottom side of the membrane were fixed with 1% paraformaldehyde for 3 min and then stained with hematoxylin (Merck, Germany) for 5 min. Each treatment was done in triplicate, and four randomly selected fields under 100× magnification of each well were scored for migrated cells.

Tube Formation Assay. HUVECs (1.5×10^4 cells/well) in Complete Growth Medium were seeded in 96-well plate precoated with growth factor-reduced Matrigel (60 μL/well) and exposed to 0.1% DMSO and indicated compounds for 17 h. SU5416 was used as a positive control. The tube formation was visualized and imaged under inverted microscope (Nikon, Tokyo, Japan) at 100× magnification, and each treatment was done four times. The tubule length was quantified by Image-Pro Plus software (Media Cybermetics, Silver Spring, MD).

Mouse Matrigel Plug Assay. Matrigel (500 μL, BD) was mixed with heparin (64 U/mL), bFGF 200 ng/mL, and KG-1 (40 or 200 μM) prior to subcutaneous injections into the flanks of male C57BL/6 mice (6 weeks old). Negative controls were obtained by injecting mice with Matrigel in the absence of bFGF and KG-1. After 7 days, the Matrigel plugs were removed and photographed. The hemoglobin content of the Matrigel plugs was quantified using Drabkin's reagent kit (Sigma). The hemoglobin content was expressed as mg/mg of wet Matrigel plug.

Statistical Analysis. All experiments were repeated at least three times. Values are given as means ± the SEM. Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed by one-way analysis of variance (ANOVA) or Student's t test. P values less than 0.05 were considered statistically significant.

RESULTS

Quantitative EAP Assay on Zebrafish Embryo. The 95% ethanol crude extract and Hex, EA, BuOH, and Aq fractions obtained from the 95% EE of *K. galanga* were examined with zebrafish angiogenic assay. As shown in Figure 1A, the EE inhibited vessel formation by 42% at 10 μg/mL. Successive fractionation and the subsequent bioassay showed that the Hex and EA fractions at 10 μg/mL potently inhibited vessel formation in the embryos by 47 and 35%, respectively, and both fractions showed dose-dependent manner (Figure 1B), which indicates the presence of antiangiogenic components in the two fractions. The existence of compounds KG-1 and KG-2 was confirmed by comparing with authentic chemical markers on TLC (data was not shown).

The antiangiogenic activity of compounds KG-1 and KG-2 were evaluated with zebrafish angiogenic assay. As shown in Figure 2, both compounds displayed antiangiogenic activity in the zebrafish model, starting to inhibit vessel formation by about 20% at 2.5 μM. At the highest concentration (20 μM), the inhibition of KG-1 could reach about 60%. However, the effect of KG-2 was less than 25% at all doses tested. The inhibitory effect

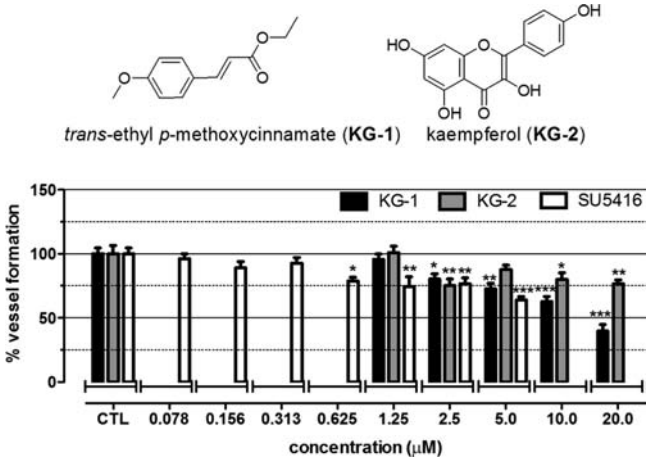

Figure 2. Dose response of the antiangiogenic activity of compounds *trans*-ethyl *p*-methoxycinnamate (KG-1) and kaempferol (KG-2). DMSO (0.2%) was used as the carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± SEM (n = 10) from a representative experiment. * Represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

of KG-1 showed a dose-dependent manner, which was a more potent candidate than KG-2 for further antiangiogenic investigation.

Microscopic Imaging. The loss of vessel formation in the embryos treated with 20 μM compound KG-1 was further confirmed using microscopic imaging on *Tg(fli1a:EGFP)y1* zebrafish embryos. The ISVs were the most easily observed angiogenic vessels in the embryos at 48 hpf, and treatment with 0.2% DMSO had no effect on the vessel formation and served as a vehicle control (Figure 3A, CTL). After treatment, KG-1 could block ISVs formation at 48 and 72 hpf (Figure 3A,B). The SIVs, appearing as a smooth basketlike structure with 5−6 arcades, is also easily observed on *Tg(fli1a:EGFP)y1* zebrafish at 72 hpf (Figure 3B, CTL). At 20 μM, KG-1 could completely inhibit SIVs formation as compared to control group after 72 h of treatment (Figure 3B). Like SU5416, KG-1 caused pericardinal edema both at 48 and 72 hpf (Figure 3A,B, dot-shaded line), and the similar phenomenon was also found in the embryos treated by PTK787/ZK222584, which is a novel anilinophthalazine compound, a high affinity inhibitor of VEGF receptors, and currently under phase II clinical trial for the treatment of metastatic gastrointestinal stromal tumors.[17,18] Meanwhile, the overall morphology and structure of treated embryos were

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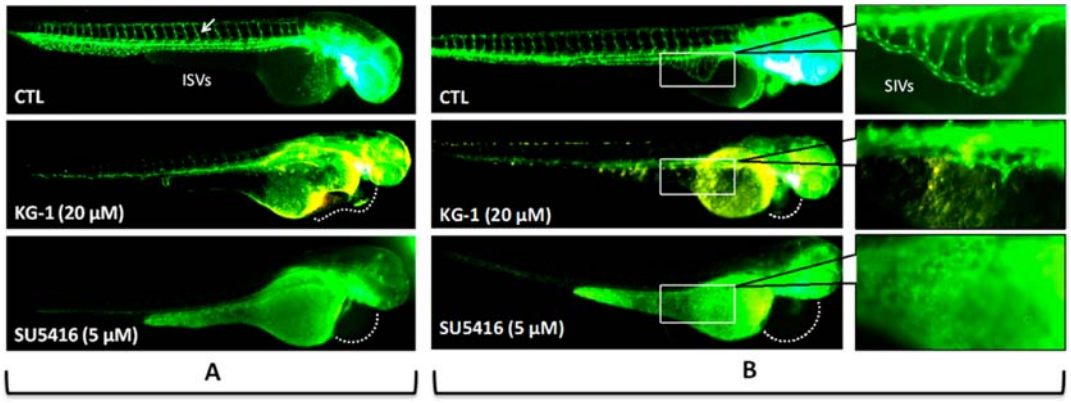

Figure 3. Lateral view of *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 hpf (A) and 72 hpf (B) treated with compound KG-1 at 20 μM. (CTL) Control, live fluorescence microscopy highlights EGFP expressing ISVs (arrowhead) and the SIVs, which appears as a smooth basketlike structure with 5−6 arcades; SU5416 (5 μM), positive control.

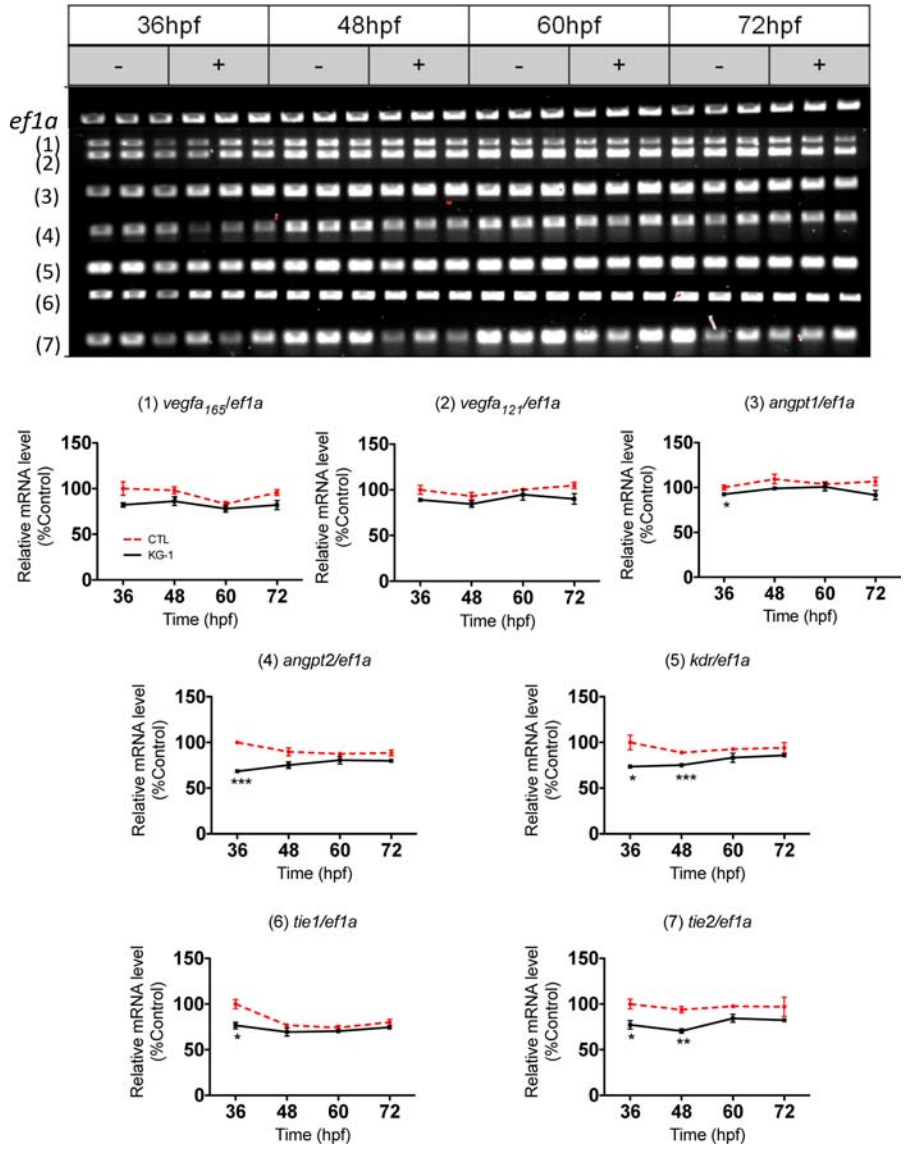

Figure 4. Time−course effects of KG-1 on the expressions of *vegfa* (1 and 2), *angpt1* (3), *angpt2* (4), *kdr* (5), *tie1* (6), and *tie2* (7) genes in zebrafish embryos after drug treatment at 24 hpf (10 μM). *ef1a* was used as an internal control to normalize the expression levels. Each value represents the mean ± SEM (n = 3) from a representative experiment. * Represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$ in Student's t test.

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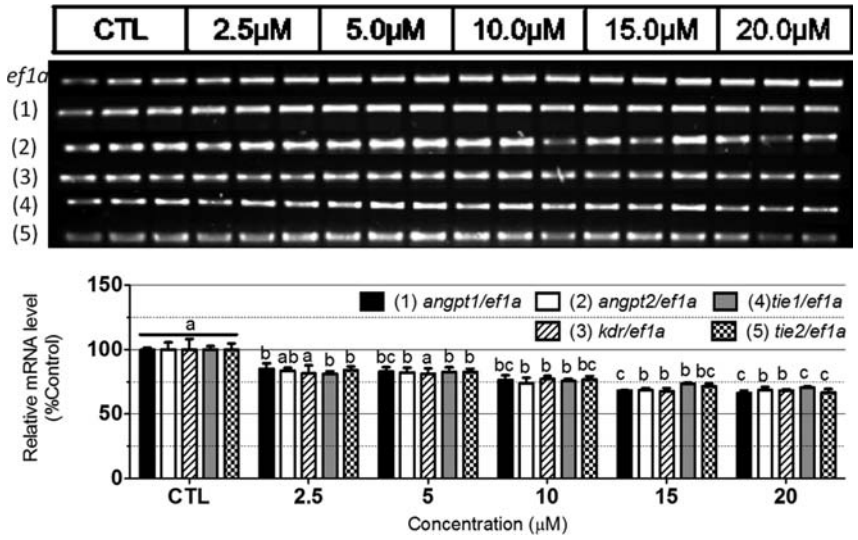

Figure 5. Dose−response effects of KG-1 on the expressions of *angpt1* (1), *angpt2* (2), *kdr* (3), *tie1* (4), and *tie2* (5) genes in zebrafish embryos at 42 hpf. *ef1a* was used as an internal control to normalize the expression levels. Each value represents the mean ± SEM ($n = 3$) from a representative experiment. The data were analyzed by one-way ANOVA followed by the Newman−Keuls test for comparisons of all pairs of groups. Different letters indicate statistical significance among groups ($P < 0.05$).

generally normal. All of these results suggested the necessity of further investigation on KG-1 of its action mechanism.

Molecular Mechanism of KG-1 on Zebrafish Angiogenesis. KG-1 showed potent antiangiogenic activities on zebrafish model; however, its action mechanism is unknown. VEGFA-VEGFR and ANGIOPOIETIN (ANGPT)-TIE are two major studied signaling pathways involved in angiogenesis.[19,20] VEGFA (commonly referred to as VEGF) is a potent angiogenic factor that mediates most biological functions via KDR (VEGFR2) and is expressed as various isoforms due to alternative splicing, which leads to mature 121-, 165-, 189-, and 206-amino acid proteins. $VEGFA_{165}$ is the predominant isoform and is commonly overexpressed in many human solid tumors.[19,21] The ANGPT-TIE system acts as vascular specific ligand/receptor system essential for blood vessel formation, and it consists of four ligands (ANGPT1−4) and two corresponding tyrosine kinase receptors (TIE1 and TIE2). The best-characterized ligands are ANGPT1 and ANGPT2.[20,22] So, the major molecules participating in these two signaling pathways, including $VEGFA_{165}$, KDR, ANGPT1, ANGPT2, TIE1, and TIE2, were thus investigated. Because zebrafish embryos were treated with drugs at 24 hpf and evaluated at 72 hpf, the time frame between 24 and 72 hpf was selected for the mechanism study.

In the time−course study after treatment with 10 μM KG-1 at 24 hpf (Figure 4), the mRNA expression of five genes (*angpt1*, *angpt2*, *kdr*, *tie1*, and *tie2*) was down-regulated at the early developmental stage at 36 or 48 hpf after 12 or 24 h of treatment. By comparison, the expression of $vegfa_{165}$ and $vegfa_{121}$ remained unchanged. In the dose−response study after 18 h of treatment at 42 hpf (Figure 5), the mRNA expression encoded by *angpt1*, *angpt2*, *kdr*, *tie1*, and *tie2* was reduced in a dose-dependent manner. At the highest concentration of 20 μM, the mRNA expression of all five genes could be reduced significantly by about 30%.

In Vitro Effects of KG-1 on HUVEC Proliferation, Migration, and Tube Formation. To further confirm the antiangiogenic effect of KG-1, HUVECs were used in the MTT-based cytotoxicity test and the tritiated thymidine incorporation assay. In MTT assay (Figure 6I,A), KG-1 showed no obvious cytotoxicity on HUVECs at the range from 2.5 to 40 μM. Using tritiated thymidine incorporation assay, it also had no inhibitory effect on the proliferation of HUVECs at the same concentration range (Figure 6I,B). Migration is a key step for angiogenesis, and KG-1 could inhibit HUVEC migration induced by the FBS concentration gradient, achieving about 30−40% inhibition at 20 and 40 μM (Figure 6II). Tube formation is another key step usually associated with cell migration and differentiation, and it showed an inhibitory effect by about 30% at 20 and 40 μM on HUVECs (Figure 6III). All of the results revealed that KG-1 could specifically inhibit the migration and tube formation steps of HUVECs to prevent the angiogenesis progress without obvious cytotoxicity.

In Vivo Antiangiogenic Effect of KG-1 on Matrigel Plug Model. KG-1 was a potent angiogenesis inhibitor in vitro. We further assessed the in vivo antiangiogenic effect of KG-1 using Matrigel plug assay. We tested two doses of KG-1, 40 and 200 μM. bFGF (200 ng/mL) together with heparin (64 U/mL) induces new blood vessel formations in plug from nearby tissues at 7 days postinjection (positive control, Figure 6B). KG-1 at 40 μM potently inhibited angiogenesis in the plug induced by bFGF and heparin. KG-1 at 200 μM almost completely blocked angiogenesis as compared to the negative control (Figure 7A).

DISCUSSION

For thousands of years, spices have been known for their flavor-enhancement characteristics and medicinal values. Because of the rise of health care costs related to the prevalence of chronic diseases, researchers have become interested in those foods with multiple health benefits, including a reduction in cancer risk and modification of tumor behavior. Within the last two decades, extensive research has shown that these chemical components derived from spices may prevent different kinds of chronic illnesses, such as cancerous, diabetic, cardiovascular, pulmonary, neurological, and autoimmune diseases. In addition, the increase of epidemiological and preclinical evidence reveals the feasibility of culinary herbs and spices as minor dietary constituents with multiple anticancer properties. Lots of spice-derived agents, such

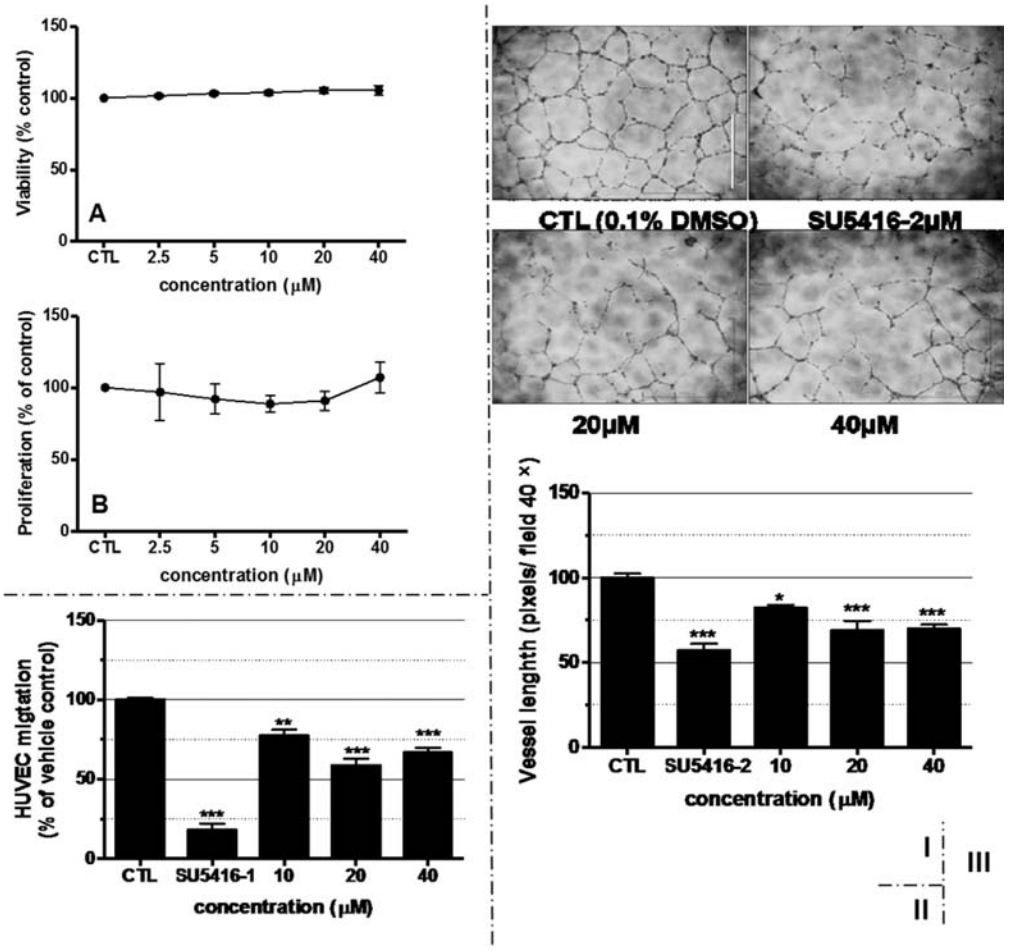

Figure 6. HUVEC proliferation (I), migration (II), and tube formation (III) assays. (I) MTT (A) and tritiated thymidine (B) assay of KG-1 on HUVECs. Each point represents the mean ± SEM (n = 5) from a representative experiment. (II) The percentage of HUVEC migration following a 3 h exposure to the indicated concentrations of KG-1 and SU5416 (1 μM, positive control) relative to control. Each point represents the mean ± SEM (n = 3) from a representative experiment. (III) Representative images and diagram-depicting formation of capillary-like tube structures by HUVECs following 17 h of treatment with indicated concentrations of KG-1 and SU5416 (2 μM, positive control). Each point represents the mean ± SEM (n = 4) from a representative experiment. Scale bar = 1000 μm. * Represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with respect to the control group.

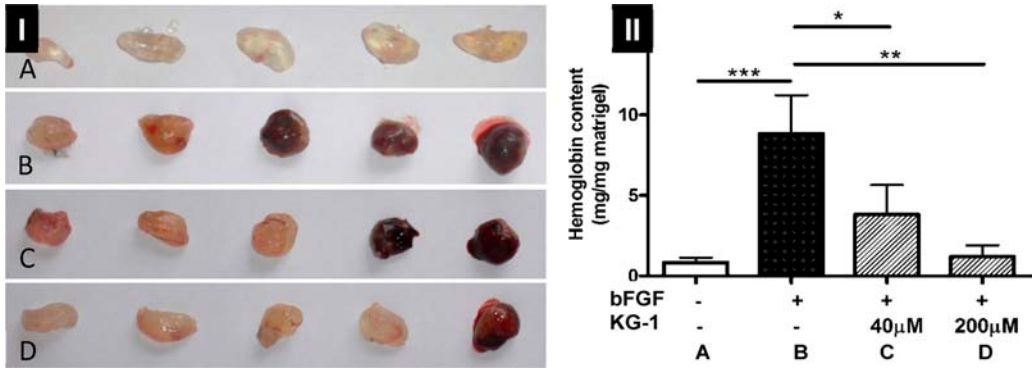

Figure 7. Inhibitory effects of KG-1 in in vivo mouse Matrigel Plug assay. Animals were injected with Matrigel only (A, negative control); Matrigel plus 64 U/mL heparin and 200 ng/mL bFGF (B, positive control); Matrigel plus 64 U/mL heparin, 200 ng/mL bFGF, and 40 μM KG-1 (C); and Matrigel plus 64 U/mL heparin, 200 ng/mL bFGF, and 200 μM KG-1 (D). (I) Images of Matrigel plug removed from respective animals. (II) Each point represents the mean ± SEM (n = 5) from a representative experiment. * Represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with respect to the control group.

as curcumin, capsaicin, eugenol, zerumbone, anethole, gambogic acid, diosgenin, and thymoquinone, have shown effects in cancer prevention. Moreover, the mechanisms of anticancer effects by these agents are also becoming increasingly evident.[23,24]

K. galanga can be used as food seasoning and medicinal herb with various effects, especially antirheumatism, anti-inflammatory, and antitumor.[9] Rheumatoid arthritis is closely related to angiogenesis, which is characterized by destruction of peripheral

joints, and the cartilage and bone are destroyed by proliferative synovitis, involving inflammatory cells infiltration and new vessel formation. Since the early stage of the disease, angiogenesis occurs and supports the arthritis progression.[25] Our work has demonstrated that spice food with anti-inflammatory property may also possess the antiangiogenic effect, which would provide a good indicator to look for antiangiogenic agents. Our work also has successfully used an in vivo zebrafish model for both the antiangiogenic agents discovering and the mechanism exploring.

As shown in Figure 1A, both Hex and EA fractions had antiangiogenic activity, and the Hex fraction had a much stronger effect than the EA fraction according to the dose-dependent result (Figure 1B). In rhizomes, KG-1 (*trans*-ethyl *p*-methoxycinnamate) is the major bioactive component with an anticancer effect. Results showed that both *cis*- and *trans*-ethyl *p*-methoxycinnamate in *K. galanga* had a relatively strong anticarcinogenic potential with IC_{50} of 5.5 and 9.5 mol/L, respectively.[26,27] KG-1 had inhibitory activity against HeLa cells and showed promise as a potential chemopreventive agent due to its induction of increased glutathione *S*-transferase activity, which is believed to be a major mechanism for chemical carcinogen detoxification and has been recognized as one of the characteristics of the action of anticarcinogens.[28,29] Recently, Liu et al. reported that KG-1 could inhibit the proliferation of HepG2 cells in a dose-dependent manner by inducing cells to enter into apoptosis.[8] KG-1 (51.6−59.24%) in the rhizome of *K. galanga* is the major component.[13,30,31] Because of its low polarity, the essential oil should be extracted mostly in Hex solvent; therefore, KG-1 may be the most potential component to inhibit vessel formation. The TLC identification result proved our hypothesis that KG-1 mainly existed in Hex fraction and also partially in EA fraction, suggesting that this compound should be the major bioactive component for Hex fraction. The assay on zebrafish model revealed that its antiangiogenic effect was mainly mediated through blocking ISVs and SIVs formation (Figure 3). KG-2 (kaempferol) is another major bioactive component existing in EA fraction with various biological activities including inhibition of lipoxygenase and cyclooxygenase, antiaggregatory, antibacterial, and anticancer.[32] KG-2 could inhibit tumor necrosis factor-α-induced endothelial cell selectin (E-selectin) expression on HUVECs.[33] KG-2 also had an anti-inflammatory property through modulating pro-inflammatory genes and blocking signaling molecules NF-κ and activator protein-1 (AP-1) induced by a cytokine mixture in HUVECs.[34] With low cytotoxicity, KG-2 could suppress the VEGF-stimulated HUVEC tubular structure formation by 15% and reduced VEGF secretion from breast cancer cells at 0.1 μM.[32,35] Its inhibition of angiogenesis and VEGF expression in human ovarian cancer cells was mediated through both HIF-dependent (Akt/HIF) and HIF-independent (ESRRA) pathways and also through a novel ERK-NFκB-cMyc-p21 pathway.[36−38] Our results further confirmed its potential application as an antiangiogenic agent on zebrafish model.

Because of no antiangiogenic activity reported on KG-1 and its stronger effect than KG-2, we decided to use zebrafish to elucidate its molecular targets. KG-1 could reduce multitarget expression, including *kdr*, *angpt1*, *angpt2*, *tie1*, and *tie2*, in time- and dose-dependent manners during zebrafish angiogenesis (Figures 4 and 5). Although the expression reduction of these five genes occurred only at the early stage (36 or 48 hpf), the inhibition of vessel formation could last up to 72 hpf (Figure 3B). This drug target pathway has not been reported before. As a multigenetic disease, the process of cancer also requires a permissive microenvironment to facilitate its development and maintenance. In this perspective, the use of multiple-targeting compounds able to target both the tumor and the microenvironment cells might represent a more efficient strategy in fighting cancer. Moreover, multitargeted antiangiogenic drugs like Sorafenib and Sunitinib have been recently approved by the U.S. FDA to treat advanced cancer. In VEGFA-VEGFR pathway, the ligand of predominant isoform-$VEGFA_{165}$ binds to and activates tyrosine kinase-VEGFR2 (KDR) to regulate the angiogenesis. KDR expression is restricted primarily to the vasculature and is the key mediator of VEGF-induced angiogenesis.[19] In the ANGPT1-TIE2 pathway, ANGPT1 and ANGPT2 are specific ligands of TIE2 with similar affinity. TIE2 activation promotes vessel assembly and maturation. ANGPT2 is produced by endothelial cells and acts as an autocrine antagonist of ANGPT1-mediated TIE2 activation, which is required to maintain the quiescent resting state of the endothelium. ANGPT2 induces vascular destabilization at higher concentrations through antagonizing ANGPT1-TIE2 signaling. Also, ANGPT2 is strongly expressed in the vasculature of many tumors and may act synergistically with other cytokines such as VEGF to promote the tumor-associated angiogenesis and tumor progression. However, the function of ANGPT1 in tumor-associated angiogenesis remains controversial. The promoting or inhibiting functions are dependent on the tumor cell type, the dosage, and possibly on the amount of ANGPT2 in the tumors. TIE1 is almost exclusively expressed by endothelial cells and critical for vascular development; nevertheless, it is still largely considered as an orphan receptor, and the function remains enigmatic.[20] However, given that VEGF-KDR and ANGPT2-TIE2 cooperatively increase angiogenesis, coblockade of these two pathways is superior in inhibiting tumor angiogenesis, metastasis, and leakage.[39,40]

Zebrafish homologues of mammalian genes have been cloned and found to have similar functions. Its value as a model organism for drug screening and target discovery has begun to be recognized. Increasing evidence showed that antiangiogenic compounds effective in mammals elicit similar effects in zebrafish. Further tests on in vitro HUVEC model (Figure 6) and in vivo Matrigel plug model (Figure 7) confirmed the potential antiangiogenic effect of KG-1.

In conclusion, as a spice, *K. galanga* and its components, *trans*-ethyl *p*-methoxycinnamate and kaempferol, are potent angiogenesis inhibitors with potential application in cancer treatment, and the antiangiogenic effect of this herb may partially contribute to its anticancer effect. Both *vegfa-vegfr* and *angpt-tie* signaling pathways are potential targets for *trans*-ethyl *p*-methoxycinnamate-induced antiangiogenesis. In addition, zebrafish is an excellent model not only for discovering potential antiangiogenic agents but also for exploring molecular targets.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ANGPT, ANGIOPOIETIN; AP-1, activator protein-1; Aq, aqueous fraction; bFGF, basic human fibroblast growth factor; BuOH, *n*-butanol; D-MEM/F-12, nutrient mixture F-12; DMSO, dimethylsulfoxide; EA, ethyl acetate; EAP, endogenous

alkaline phosphatase; EE, ethanol extract; EGFP, enhanced green fluorescent proteins; FBS, fetal bovine serum; Hex, *n*-hexane; FDA, Food and Drug Administration; HUVECs, human umbilical vein endothelial cells; hpf, hours postfertilization; ISVs, intersegmental blood vessels; MMLV, moloney murine leukemia virus; NBT/BCIP, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt; P/S, penicillin–streptomycin; PTU, 1-phenyl-2-thiourea; PCR, polymerase chain reaction; RT, reverse transcription; SEM, standard error of the mean; SIVs, subintestinal vessel plexus; TLC, thin-layer chromatography; VEGF, vascular endothelial growth factor

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