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Evaluation of Antiangiogenic Efficacy of *Emilia sonchifolia* (L.) DC on Tumor-Specific Neovessel Formation by Regulating MMPs, VEGF, and Proinflammatory Cytokines

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Abstract

Formation of new blood vessels from preexisting vasculature is an indispensable process in tumor initiation, invasion, and metastasis. Novel therapeutic approaches target endothelial cells involved in the process of angiogenesis, due to their genetic stability relative to the rapidly mutating drug-resistant cancer cells. In the present study, we investigated the effect of an active fraction from *Emilia sonchifolia*, belonging to the family Asteraceae, a plant well known for its anti-inflammatory and antitumor effects, on the inhibition of tumor-specific angiogenesis. Administration of the active fraction from *E sonchifolia* (AFES; 5 mg/kg, body weight, intraperitoneally) containing the major compound γ-humulene significantly inhibited B16F10 melanoma-induced capillary formation in C57BL/6 mice. The level of serum vascular endothelial growth factor and serum proinflammatory cytokines such as interleukin-1β, interleukin-6, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor were also reduced significantly. At the same time, administration of AFES significantly enhanced the production of antiangiogenic factors such as tissue inhibitor of matrix metalloproteinase-1. Dose-dependent reduction can be seen in the budding and expansion of microvessels from rat thoracic aorta by AFES treatment. Inhibition of the activation of proenzyme to active enzyme of matrix metalloproteinase along with a successful reduction of proliferation, invasion, and migration of human umbilical vein endothelial cells demonstrated the antiangiogenic effect of AFES in vitro. To date, no study has examined the antiangiogenic activity of this plant with already well-known anti-inflammatory and antitumor effects. Results obtained in the present study by using both in vivo and in vitro angiogenic models altogether proved the inhibitory effect of AFES on tumor-specific neovessel formation.

Keywords

angiogenesis, vascular endothelial growth factor, matrix metalloproteinase, *Emilia sonchifolia*, γ-humulene, endothelial cell migration, invasion

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Introduction

The requirement of tumor cells for the supply of oxygen and other nutrients is fulfilled by the formation of new blood vessels. The induction of neovessel formation starts with the release of various molecules by the tumor cells. These molecules will send signals to the surrounding normal tissue and activate genes for making proteins involved in the process of angiogenesis.[1] Tumor cell dissemination in the process of metastasis requires the development of angiogenic blood vessels. Neovasculature should possess certain structural characteristics to sustain active intravasation of tumor cells and their dissemination to secondary sites.[2] The relatively delicate process of neovessel formation can be a target for antiangiogenic therapy in cancer treatment because of the genomic stability of endothelial cells compared to cancer cells. Cancer cells require less generation time and have more genetic instability, whereas endothelial cells require long generation time and have higher genomic stability. The rapidly proliferating cancer cells often undergo mutations and would likely acquire drug resistance.[3]

Neovessel formation is initiated by the secretion of various growth factors, especially vascular endothelial growth factor (VEGF), which can pursue capillary growth into the

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tumor. VEGF is a powerful inducer of angiogenesis, stimulates growth and proliferation of endothelial cells, acts as a survival factor of endothelial cells, prevents their apoptosis, and also regulates vascular permeability. Matrix metalloproteinases (MMPs) induce angiogenesis by degrading extracellular matrix (ECM) along with the release of angiogenic mitogens stored in the matrix.[1] The degradation of ECM components surrounding the tumor tissue is mainly brought about by the gelatinases MMP-2 and MMP-9.[2] The family of natural inhibitors of metalloproteinase are TIMPs (tissue inhibitors of metalloproteinases), and TIMP expression is related to tumor metastasis and angiogenesis.[4] Interactions between malignant and inflammatory cells present in the stroma could be closely associated with angiogenesis and tumor progression.[5] The same molecular events trigger inflammation and angiogenesis[6]; pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) can be prometastatic or proangiogenic, and their deregulated expression directly correlates with the metastatic potential of several human carcinomas.[7] The proinflammatory response can be directed toward tumor promotion via stimulation of the angiogenic process.[8]

Several traditionally used natural compounds and herbal extracts have been investigated as antiangiogenic agents to prevent neovascularization of developing tumors. These include some of the works on *Andrographis paniculata*,[9] (+)-catechin,[10] vernolide-A,[11] and ursolic acid.[12] Various biological activities of *Emilia sonchifolia* covering a gamut of beneficial properties have been reported. Preliminary studies conducted on this plant showed its antitumor[13] and anti-inflammatory activity along with reduction of inducible nitric oxide production.[14,15] Further studies revealed its protective effect on oxidative stress,[16] modulation of selenite cataract,[17] apoptotic activity on cancer cells,[18,19] antinociceptive effects,[20] and immunomodulatory effects.[21] Recently, we have done a complete phytochemical screening of the plant, and its antimetastatic effect was analyzed using the most active solvent fraction containing the major active principle γ-humulene ($C_{15}H_{24}$).[22] The present study was designed to explore the inhibitory effect of the active fraction from *E sonchifolia* (AFES) on tumor-specific neovessel formation, using both in vivo and in vitro models.

Materials and Methods

Animals

Healthy adult male C57BL/6 mice (4-6 weeks old) were purchased from the National Institute of Nutrition, Hyderabad, India. They were accommodated in individual ventilated cages, fed with normal mice chow, and given water ad libitum. All the animal experiments were carried out with the prior approval of the Institutional Animal Ethics Committee and were conducted strictly adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India (Sanction No. 149/1999/CPCSEA).

Cell Lines and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord vein according to the method of Jaffe et al.[23] The cells were grown in medium 199, supplemented with 20% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 ng/mL VEGF and fibroblast growth factor (FGF) at 37°C in 5% CO_2 atmosphere. B16F10 melanoma cells were obtained from National Centre for Cell Science, Pune, India. Highly specific quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-1β, IL-6, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Pierce Biotechnology (Rockford, IL). ELISA kits for VEGF and TIMP-1was purchased from R&D Systems (Minneapolis, MN). Radioactive ^{3}H-thymidine was obtained from the Board of Radiation and Isotope Technology, Mumbai, India. Silica gel, collagen, FGF, MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and TNP 470 were purchased from Sigma Aldrich (Bangalore, India). *n*-Hexane, ethyl acetate, and dimethyl sulfoxide were obtained from Merck Specialities Private Ltd, Mumbai, India. All other chemicals used were of analytical reagent grade.

Plant Material, Isolation of Active Principle, and Experimental Design

The whole plants of *E sonchifolia* were obtained from Amala Ayurveda Pharmacy, Thrissur, India, after authentication, the voucher specimen is deposited at the herbarium of Amala Cancer Research Centre (Voucher No. 108/ACRC). The whole plants of *E sonchifolia* were dried at 45°C and powdered. The powder was then extracted with 70% methanol in Soxhlet apparatus for 24 hours, and the solvent was evaporated to dryness at 42°C under reduced pressure using a rotary evaporator. The extract obtained was then subjected to silica gel column chromatography (200-400 mesh size, *n*-hexane–ethyl acetate, gradient). Different fractions were collected, and the fraction with least polarity was again subjected to purification using *n*-hexane–ethyl acetate (99:1) to yield the active fraction that contains γ-humulene. Gas chromatography mass spectrometry analysis of the isolate revealed the presence of 71% of the major sesquiterpene γ-humulene (Figure 1A) present in the plant *E sonchifolia.* For animal experiments the AFES, 5 mg/kg body weight, was administered intraperitoneally (ip).

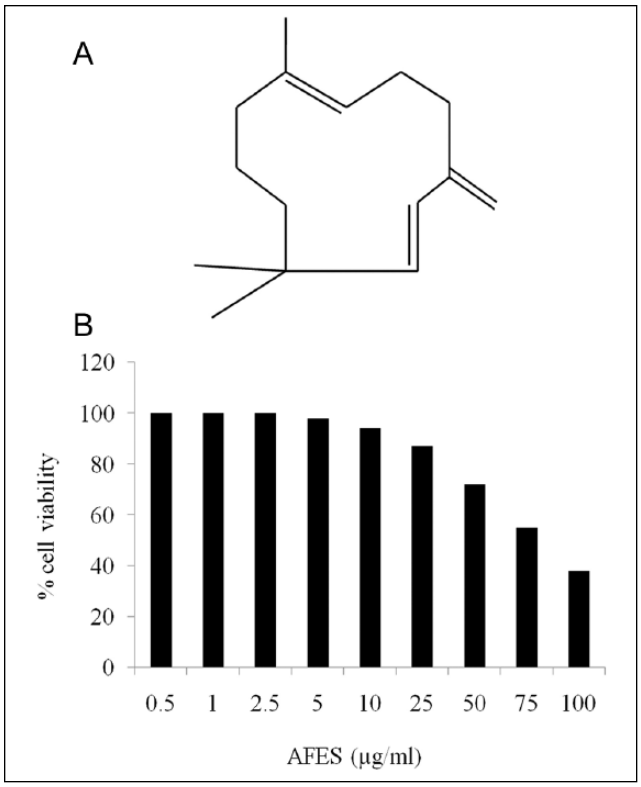

Figure 1. (A) Structure of γ-humulene. (B) MTT assay showing viability of HUVECs following treatment with the indicated concentrations of AFES.

Three groups of C57BL/6 mice (8 animals per group) were used for the study. Angiogenesis was induced in all groups of animals by injecting B16F10 melanoma (10^6 cells/animal) intradermally on the shaven ventral skin of each mouse. Group I animals were kept as control. Group II and Group III animals were treated with 5 consecutive dose of AFES (5 mg/kg body weight) and TNP-470 (30 mg/kg body weight), respectively, starting simultaneously with tumor challenge. For in vitro experiments 3 nontoxic concentrations of AFES (2.5, 1, and 0.5 µg/mL) were resuspended in 0.1% dimethyl sulfoxide. Dimethyl sulfoxide alone when used as vehicle in 0.1% and lesser concentrations showed no inhibitory effect on neovessel formation in the experimental conditions.

Toxicological Evaluation of AFES

AFES in different concentrations (5, 10, 15, 20 mg/kg body weight) was administered intraperitoneally to Balb/c mice (n = 8) for 14 days. Animals were observed for mortality, behavioral changes, and change in body weight. On 15th day, all the animals were sacrificed and selected organs such as liver, spleen, thymus, kidney, and lungs were dissected and weights were recorded. Blood was collected by heart puncture; the serum was separated and used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP),[24] glutamate pyruvate transaminase (GPT),[25] and kidney function markers such as creatinine[26] and blood urea nitrogen[27] were determined.

Determination of the Effect of AFES on Tumor-Specific Capillary Formation

The angiogenesis-induced animals were sacrificed on the ninth day after tumor challenge. The skin from the ventral side was dissected out, washed with phosphate-buffered saline, and the number of tumor-directed capillaries were counted using a dissection microscope at 20× magnification.

Determination of Serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF, and TIMP-1 Levels

Blood was collected from the caudal vein of all groups of angiogenesis-induced animals at 2 time intervals—days 2 and 9 after tumor induction. Serum was separated and used for the estimation of IL-1β, IL-6, TNF-α, GM-CSF, VEGF, and TIMP-1 levels using ELISA kits according to the manufacturer's instructions, and the readings were taken using an ELISA plate reader (Thermo LabSystems, Beverly, MA).

Cell Viability by MTT Assay

The viability of cultured cells was determined by assaying for the reduction of MTT to formazan.[28,29] HUVECs were seeded (5000 cells/well) in a 96-well culture plate and incubated for 24 hours at 37°C in 5% CO_2 atmosphere. Different concentrations of AFES (0.5-100 µg/mL) were added and incubated further for 48 hours. Four hours before the completion of incubation, 20 µL of MTT (5 mg/mL) was added. The percentage of viable cells was determined using an ELISA plate reader.

^{3}H-Thymidine Incorporation Assay

HUVECs (5000 cells/well) were seeded on a 96-well culture plate and incubated at 37°C in 5% CO_2 atmosphere. After 24 hours, various concentrations of AFES (2.5, 1, and 0.5 µg/mL) were added along with 2 ng/mL of VEGF and further incubated for 48 hours. ^{3}H-thymidine was added to each well (1 µCi/well) and incubation was continued for an additional 18 hours. After completing incubation DNA was precipitated using 10% ice-cold perchloric acid, and pellets were dissolved in 0.5 mL of 6 N NaOH and transferred to 5 mL scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter (Wallac 1209; Pharmacia, Uppsala, Sweden).

Table 1. Toxicity Profile of AFES[a].

		Concentrations of AFES (mg/kg Body Weight)			
	Control	5	10	15	20
Mortality (D/T)	None[b]	None	None	None	None
Behavioral change	None	None	None	None	None
Change in body weight (g)	+2.13 ± 1.39	+2.12 ± 0.69	+2.06 ± 0.42	+2.01 ± 0.21	+1.92 ± 1.56
Relative organ weights (g/100 g body weight)					
Liver	5.32 ± 0.32	5.38 ± 0.16	5.23 ± 0.18	5.19 ± 0.18	4.97 ± 0.15
Spleen	0.38 ± 0.03	0.41 ± 0.02	0.36 ± 0.02	0.39 ± 0.02	0.40 ± 0.02
Thymus	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
Kidney	1.36 ± 0.24	1.34 ± 0.13	1.34 ± 0.16	1.34 ± 0.15	1.31 ± 0.12
Lungs	0.60 ± 0.03	0.61 ± 0.02	0.60 ± 0.03	0.61 ± 0.02	0.57 ± 0.03
Serum ALP (U/mL)	13.5 ± 0.40	13.4 ± 0.42	14.21 ± 0.8	15.8 ± 1.08	14.5 ± 2.01
Serum GPT (U/mL)	58.35 ± 3.7	55.77 ± 3.0	59.97 ± 4.6	64.17 ± 7.0	63.91 ± 6.1
Blood urea (mg/dL)	43.38 ± 1.5	42.81 ± 1.6	44.31 ± 0.8	43.55 ± 2.7	43.22 ± 1.0
Serum creatinine (mg/dL)	0.91 ± 0.01	0.9 ± 0.02	0.93 ± 0.02	0.99 ± 0.05	0.98 ± 0.13

Abbreviations: AFES, active fraction from *Emilia sonchifolia*; D/T, dead/treated mice; ALP, alkaline phosphatase; GPT, glutamate pyruvate transaminase.
[a]Values are the mean ± standard deviation. All the treated animals were carefully examined for 14 days for any signs of toxicity (behavioral changes and mortality).
[b]"None" means that no toxic symptoms were seen during the observation period.

Rat Aortic Ring Assay

The rat aortic ring assay is used as an in vitro angiogenesis experimental model. Dorsal aorta from a freshly sacrificed rat was taken out in a sterile manner and rinsed in ice-cold phosphate-buffered saline. It was then cut into approximately 1-mm-thick sections, and each ring was placed in a collagen-precoated 96-well plate. The rings were incubated for 24 hours at 37°C in complete medium and then replaced with conditioned medium from B16F10 melanoma cells and incubated with different concentrations of AFES (2.5, 1, and 0.5 µg/mL). Controls were kept without AFES treatment. The rings were analyzed by phase-contrast microscopy for microvessel outgrowth on the sixth day and the sections were photographed.

Determination of the Effect of AFES on Endothelial Cell Migration

HUVECs were seeded on a collagen-precoated 96-well plate at a density of 2×10^5 cells/well and incubated for 24 hours at 37°C in 5% CO_2 atmosphere. The monolayers of cells were scraped with a narrow tip by applying suction. The scraped-out cells were removed by washing with serum-free medium. Different concentrations of AFES (2.5, 1, and 0.5 µg/mL) were added along with 2 ng/mL VEGF and further incubated for 24 hours. After incubation the cells were fixed in formalin and stained with crystal violet and photographed.[30]

Determination of the Effect of AFES on Endothelial Cell Invasion

The invasion assay was carried out in modified Boyden chambers as described by Albini et al.[31] The lower compartment of the chamber was filled with serum-free medium and a polycarbonate filter membrane coated with 25 µg type I collagen was placed above this. HUVECs (10^5 cells/150 µL medium 199) were then seeded on to the upper chamber in the presence and absence of AFES (2.5, 1, and 0.5 µg/mL) along with 2 ng/mL VEGF and FGF and incubated at 37°C in 5% CO_2 atmosphere for 10 hours. After incubation, the membranes were removed, fixed with methanol, and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate membrane were counted under a microscope. The results were expressed as percentage inhibition of invasion.

Gelatin Zymography

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed by incorporating 0.1% gelatin in the separating gel.[32] Subconfluent HUVECs were incubated with serum-free medium for 24 hours at 37°C in 5% CO_2 atmosphere. The conditioned medium was then collected and subjected to zymographic analysis. Samples equivalent to 100 µg proteins were activated with 5 µL trypsin solution (75 µg/mL) in the presence and absence of AFES (2.5, 1, and 0.5 µg/mL) in 0.1 M Tris-HCl, 10 mM $CaCl_2$ buffer (pH 8.0), and incubated for 1 hour at room temperature. Samples were mixed with an equal volume of 2× sample buffer and loaded on to 11% polyacrylamide gels containing 0.1% gelatin. Electrophoresis was carried out at 4°C with a constant current of 2 mA/tube until the tracking dye reached the periphery. The gels were then washed with 2% Triton X-100 in 0.1 M Tris-HCl, 10 mM $CaCl_2$ at 37°C for 18 hours followed by staining with Gelcode Blue stain reagent for 2 hours. The clear areas on the gels were then visualized.

Statistical Analysis

The data were analyzed using Graphpad InStat software (San Diego, CA) and expressed as mean ± SD. Statistically significant differences between groups were calculated by the application of an analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Values of $P < .05$ were considered as significant.

Results

Toxicological Evaluation of AFES

The toxicity study (Table 1) revealed the no observed-adverse-effect level of AFES as 15 mg/kg body weight. The doses of 5, 10, and 15 mg/kg body weight, administered for 14 days, did not produce any mortality, change in behavior, body weight, relative organ weight, and hepatic and renal functions when compared with untreated animals. Administration of AFES at 20 mg/kg body weight produced slight weight loss. Based on these results and some preliminary screening on biological activity, we selected the nontoxic lowest dose of 5 mg/kg body weight for further studies.

Effect of AFES on Tumor-Specific Capillary Formation

The effect of AFES on tumor-directed capillary formation is shown in Figure 2. AFES treatment produced significant ($P > .001$) inhibition (57%) of tumor-directed capillary formation along with a noticeable reduction in tumor size. The result obtained as shown in the figure was comparable to the well-known antiangiogenic compound TNP-470 used as reference in this study.

Effect of AFES on the Level of Proinflammatory Cytokines, VEGF, and TIMP

AFES treatment significantly inhibited the elevated levels of serum IL-1β, IL-6, TNF-α, and GM-CSF, as shown in Figure 3. The elevated level of angiogenesis-promoting VEGF in tumor-bearing animals was also lowered significantly by AFES treatment compared to control animals. At the same time the level of tissue inhibitor of MMPs was increased by the treatment (Figure 4).

Effect of AFES on Cell Viability

The percentage viability of HUVECs after treatment with AFES is shown in Figure 1B. The concentration needed for 50% growth inhibition (IC50) of HUVECs was found to be 80.8 µg/mL. The cells were 100% alive at 0.5, 1, and 2.5 µg/mL concentrations of AFES, and these concentrations were used for further in vitro experiments.

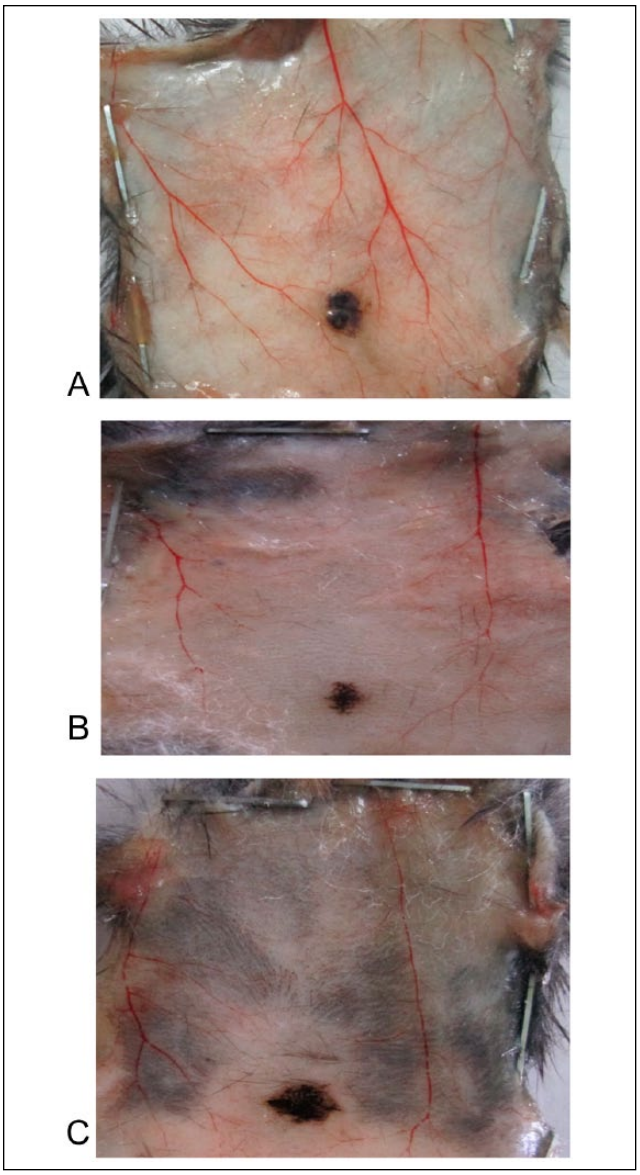

Figure 2. Effect of AFES on in vivo angiogenesis. Tumor angiogenesis was induced by subcutaneous injection of B16F10 melanoma cells (106 cells/mice) on shaven ventral side of C57BL/6 mice treated simultaneously with AFES or TNP-470: (A) Control; (B) AFES; (C) TNP-470.

Effect of AFES on Endothelial Cell Proliferation

Rate of proliferation of HUVECs was determined by ^{3}H-thymidine incorporation into the cellular DNA. Proliferation was expressed as radioactive counts per minute (cpm). HUVECs showed very high rates of proliferation (4313 ± 110 cpm) when stimulated with VEGF. Administration of AFES at a concentration of 2.5 µg/mL showed a significant inhibition (55%, 1940 ± 40 cpm) of VEGF-induced proliferation of HUVECs. Considerable inhibition of proliferation was also observed when AFES

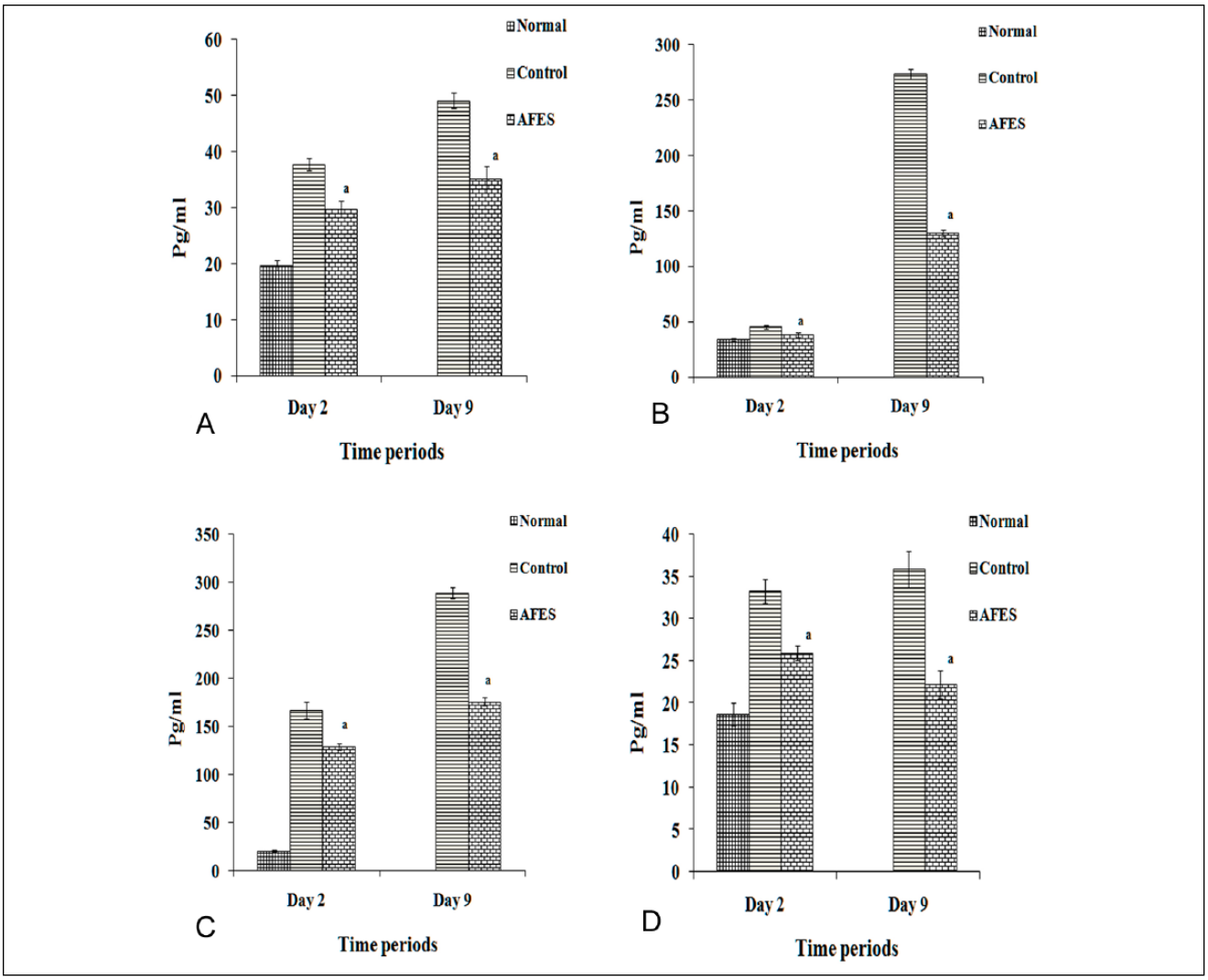

Figure 3. Effect of AFES on the serum cytokine levels: (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) GM-CSF. The serum was collected from the caudal vein of C57BL/6 mice on the second and ninth days after induction of B16F10 melanoma cells (106 cells/mice). Values are mean ± SD. $^{a}P < .001$ significantly different from untreated control.

was administered at concentrations of 1 µg/mL (48%, 2236 ± 95 cpm) and 0.5 µg/mL (22%, 3349 ± 105 cpm).

Effect of AFES on the Microvessel Outgrowth From the Rat Aortic Ring

The microvessel outgrowth from the rat aortic ring was induced by treatment with conditioned medium from B16F10 melanoma cells. Treatment with different concentrations of AFES significantly inhibited the microvessel outgrowth in a dose-dependent manner compared to the control group without any treatment (Figure 5).

Effect of AFES on Endothelial Cell Migration

Effect of AFES on the migration of HUVECs is shown in Figure 6. HUVECs migrated into the scraped clear area when stimulated with VEGF as shown in the control without any treatment. AFES significantly inhibited the VEGF-induced migration of endothelial cells in a dose-dependent manner and maximum inhibition of endothelial cell migration was observed at 2.5 µg/mL as evident from the figures.

Effect of AFES on Endothelial Cell Invasion Assay

The invasive property of HUVECs through the collagen matrix is very high when stimulated with VEGF and FGF. The administration of AFES produced significant inhibition in the invasion of cells in a dose-dependent manner. The number of cells found on the lower surface of the polycarbonate membrane at a concentration of 2.5 µg/mL AFES was very low. The treatment of HUVECs with higher concentration of AFES produced an inhibition of invasion by

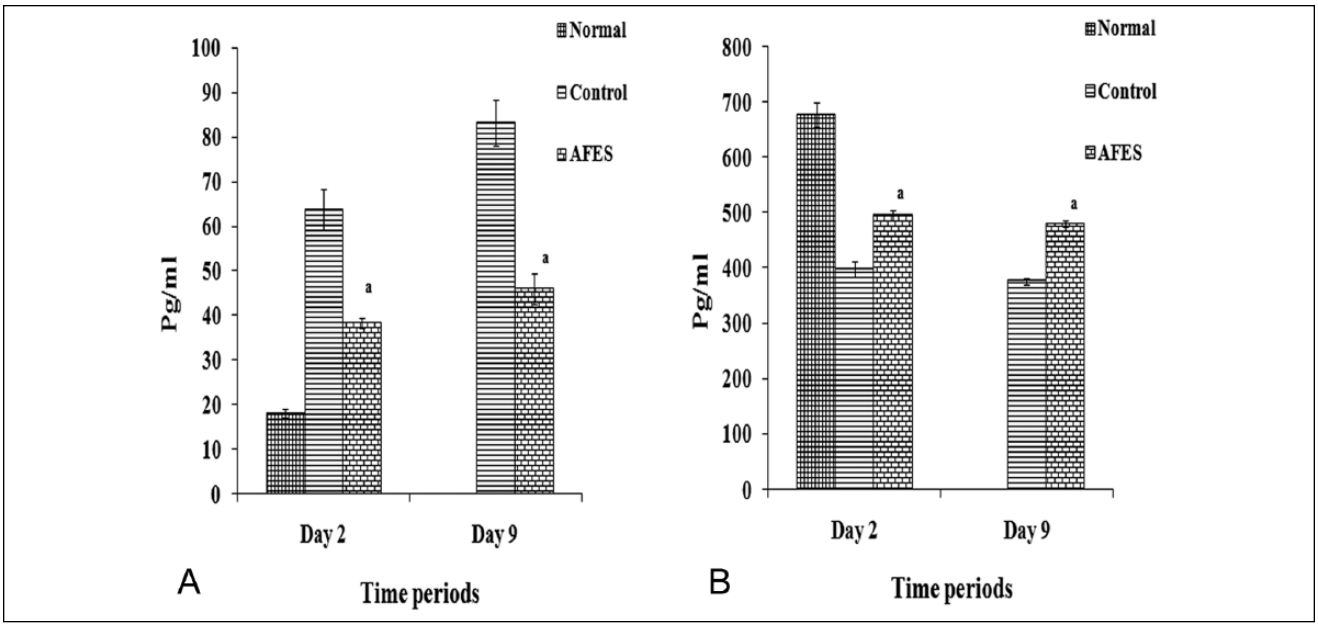

Figure 4. Effect of AFES on the serum (A) VEGF and (B) TIMP levels. The serum was collected from the caudal vein of C57BL/6 mice on the second and ninth days after induction of B16F10 melanoma cells (106 cells/mice). Values are mean ± SD. [a]*P* < .001 significantly different from untreated control.

61%, whereas at 1 μg/mL and 0.5 μg/mL, the inhibition of invasion was found to be 52% and 39%, respectively (Figure 7).

Gelatin Zymography

Type IV collagenases MMP-2 and MMP-9 secreted by the cells into the conditioned medium are mainly proenzymes that require activation. Conditioned medium after trypsin activation showed digested clear areas at 92 kD and 72 kD, which were identical to MMP-9 and MMP-2 activity. The inactive form of the enzyme did not show clear degradative areas when the gels were loaded by conditioned medium without trypsin activation. EDTA inhibits the proteolytic activity by chelating Ca^{+}, and the enzyme responsible for degradation is metalloproteinase, which is evidenced when gels loaded with trypsin-activated conditioned medium were incubated with 10 mM EDTA, which did not produced clear degradative areas. When conditioned medium was treated with AFES at concentrations 2.5 and 1 μg/mL during trypsin activation, it did not show any clear bands suggesting that AFES inhibited the activation of proenzyme to active enzyme of metalloproteinases. But AFES at 0.5 μg/mL showed an activation of metalloproteinases as shown in Figure 8.

Discussion

Tumor vasculature is considered as a prime prognostic marker of tumor grading.[33] Antiangiogenic therapy is a highly effective strategy for destroying tumor development because it directly affects the vascular supply, the basic requirement for tumor growth. The agents that target angiogenesis can be effectively used in therapy because of its more specific nature compared to chemotherapy and radiotherapy. These agents are also less toxic and can be used for long term without the development of drug resistance in the target cells. Most blood vessels in the adult organism remain quiescent but have the capability to divide in response to proper stimulus and results in angiogenesis.[1] Plants constitute a major source of highly effective conventional drugs for the treatment of different types of cancer. A large number of the sesquiterpenes obtained from medicinal plants that are used in traditional medicine show anticancer activity by inhibition of inflammatory responses, prevention of metastasis, and angiogenesis. In this study, an active fraction containing the major sesquiterpene γ-humulene from the plant *E sonchifolia*, with renowned anti-inflammatory and antitumor effects, showed significant reduction in tumor-directed capillary formation, with a simultaneous inhibition in the proliferation, migration, and invasion of endothelial cells, the crucial processes of neovessel formation.

The inhibition of capillary formation toward the developing tumor by AFES treatment revealed its antiangiogenic effect in an in vivo experimental condition. AFES significantly halted the endothelial cell proliferation, invasion, and migration, further proving its targeted activity toward the cells involved in neovascularization. The in vitro

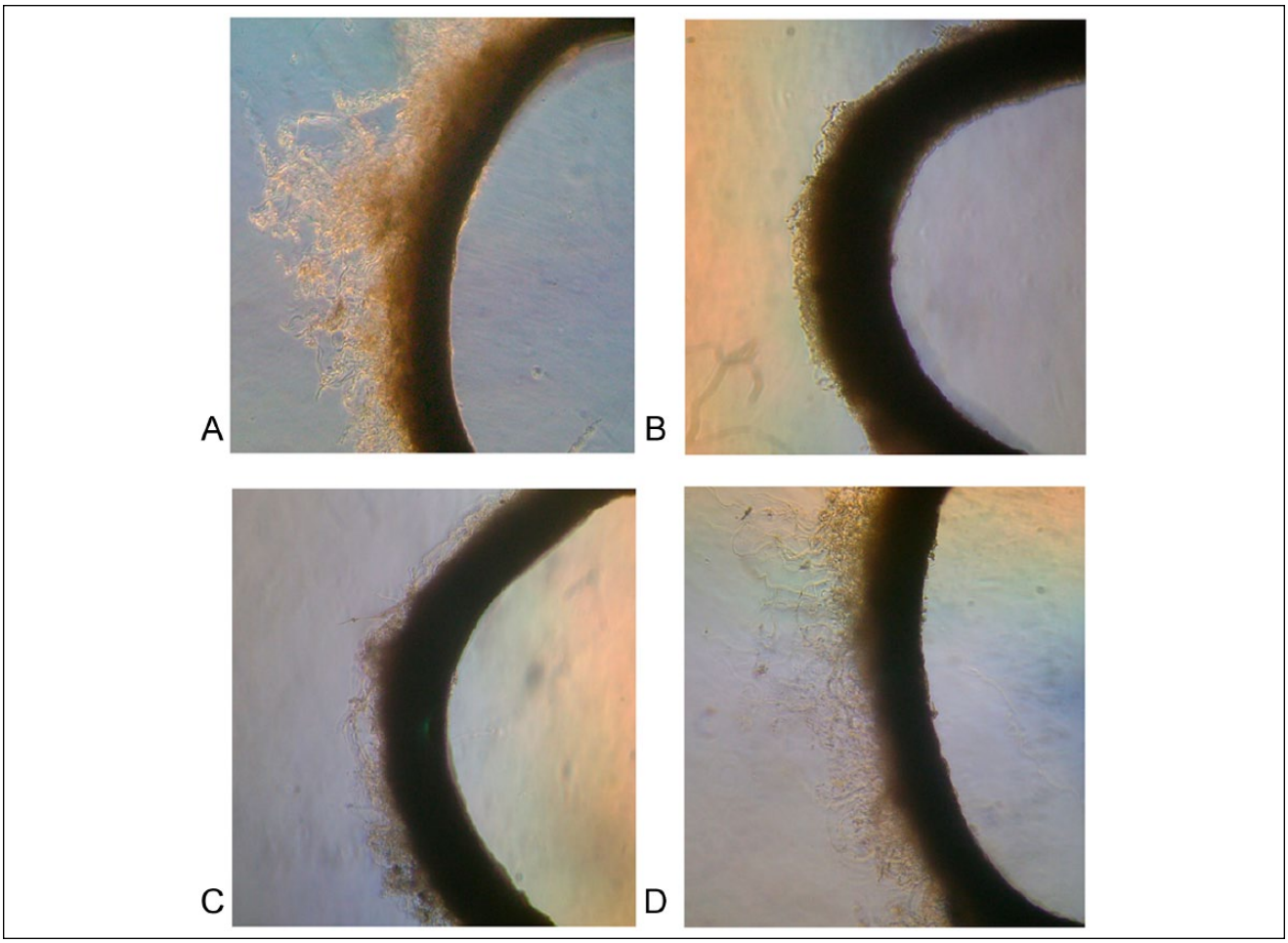

Figure 5. Effect of AFES on in vitro angiogenesis. The conditioned medium from normal semiconfluent bottles of B16F10 cells acts as the control: (A) Control with conditioned medium alone; (B) Conditioned medium + treatment with AFES (2.5 µg/mL); (C) Conditioned medium + treatment with AFES (1 µg/mL); (D) Conditioned medium + treatment with AFES (0.5 µg/mL).

experiment of microvessel outgrowth from the aortic ring is strong direct evidence for the inhibitory effect of AFES on the endothelial cell-dependent angiogenic process. Endothelial cell invasion during angiogenesis is a key process that involves degradation of the basement ECM barriers to allow free mobility of the cells required for the formation of new blood vessels. This is accomplished by the production of lytic enzymes that are able to digest the specific matrix components and permit cell invasion.[2] There is a correlation between MMP expression and tumor invasion. The metastasis-supporting vasculature is shaped by continuous proteolytic modification of ECM and MMPs. MMP-mediated regulation of VEGF-induced tumor vascularization was completely inhibited by MMP blockage and reduced the volume of angiogenic vasculature.[34] The matrix degrading enzymes are produced in inactive proenzymatic forms, and the enzyme trypsin can activate pro-MMPs to active MMPs. The gelatinases A and B (MMP-2 and MMP-9) were found to be overexpressed in invasive tumor cells.[4] MMP-9 not only readily digests denatured collagens and gelatins but also plays its particular role in angiogenesis since it increases the bioavailability of proangiogenic factors. AFES treatment showed a significant inhibition in the invasion and migration of the collagen matrix by HUVECs in a dose-dependent manner, and zymography analysis showed an inhibition in the activation of the proenzyme form to the active form of metalloproteinases; both these processes are mediated by the involvement of matrix-degrading enzymes. These results denote the regulatory effect of AFES on MMPs. TIMPs regulate MMPs through endogenous protease inhibition; high levels of TIMP were always associated with inhibition of endothelial cell migration. Progression of invasive and metastatic tumors showed a decreased level of TIMPs.[35] The TIMP-free status of pro-MMP-9 is an important biochemical characteristic required for unencumbered and rapid activation and high angiogenic capacity of naturally produced pro-MMP-9.[36] The enhancement in the level of TIMP-1 by AFES again pronounces its

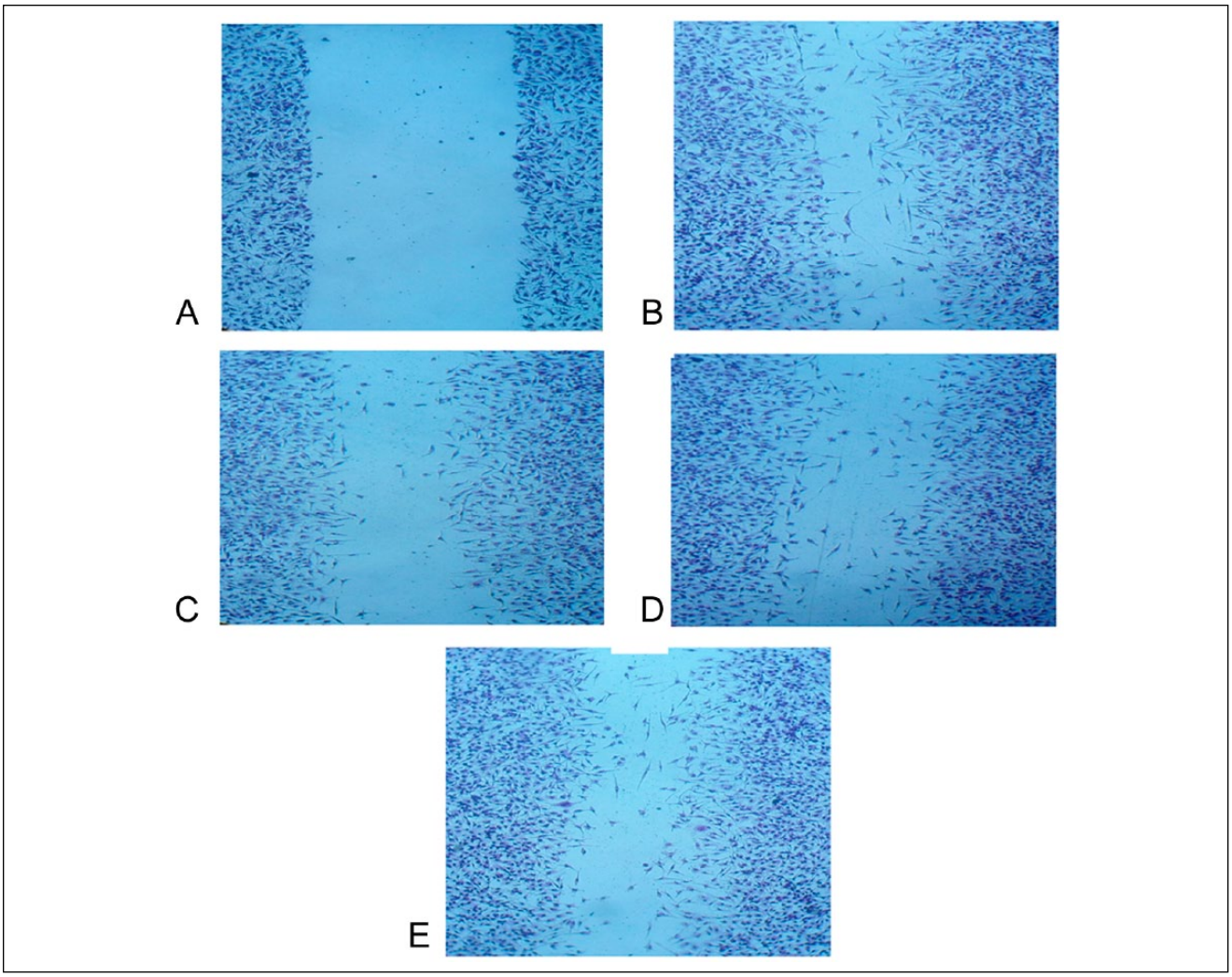

Figure 6. Inhibitory effect of AFES on HUVECs migration. The HUVECs (2 × 105 cells/well) were seeded on type I collagen coated 96-well titer plate and incubated overnight at 37°C. A clear area was made with a narrow tip in the monolayer and further incubated for 24 hours in the presence and absence of AFES (2.5, 1, and 0.5 µg/mL) along with VEGF (2 ng/mL). After incubation, the cells were fixed and stained using crystal violet and photographed. (A) Control "0" hour incubation; (B) Control after 24-hour incubation in medium without AFES; (C) AFES (2.5 µg/mL); (D) AFES (1 µg/mL); (E) AFES (0.5 µg/mL).

antiangiogenic effect and the ability to block MMPs via positively modulating the level of its tissue inhibitors.

VEGF is a potent mitogen, acts as a survival factor for endothelial cells, and also mediates vessel permeability and migration of endothelial progenitor cells from the bone marrow. VEGF is a rational therapeutic target because it has a limited role in adults. Often, VEGF is secreted by tumors.[1] The prime target of VEGF is endothelial cells, and cancer cells release VEGF to induce tumor angiogenesis. The VEGF stimulates VEGF receptors on the tumor endothelium or in the tumor cell itself. VEGF therapy supports tumor growth by inducing angiogenesis and also by direct action via VEGF receptor expression by tumor cells.[37] Administration of AFES reduced the elevated level of serum VEGF in angiogenesis-induced animals, indicating the efficiency of AFES in targeting VEGF and thereby reducing the VEGF-assisted migration and survival of endothelial cells. By lowering the level of VEGF, AFES can also inhibit the neovessel formation induced by tumor cells for their growth and development. The role played by TNF-α can be linked to almost all steps involved in tumorigenesis, including proliferation, invasion, angiogenesis, and metastasis.[38] TNF-α acts as a macrophage-activating factor and activates these cells to secrete angiogenic factors.[1] IL-1β is required for invasion and metastasis of cancer cells and promotes tumor growth and angiogenesis.[39] IL-6 produced by endothelial and tumor cells is a potent proangiogenic cytokine and promotes MMP-9 activation and VEGF

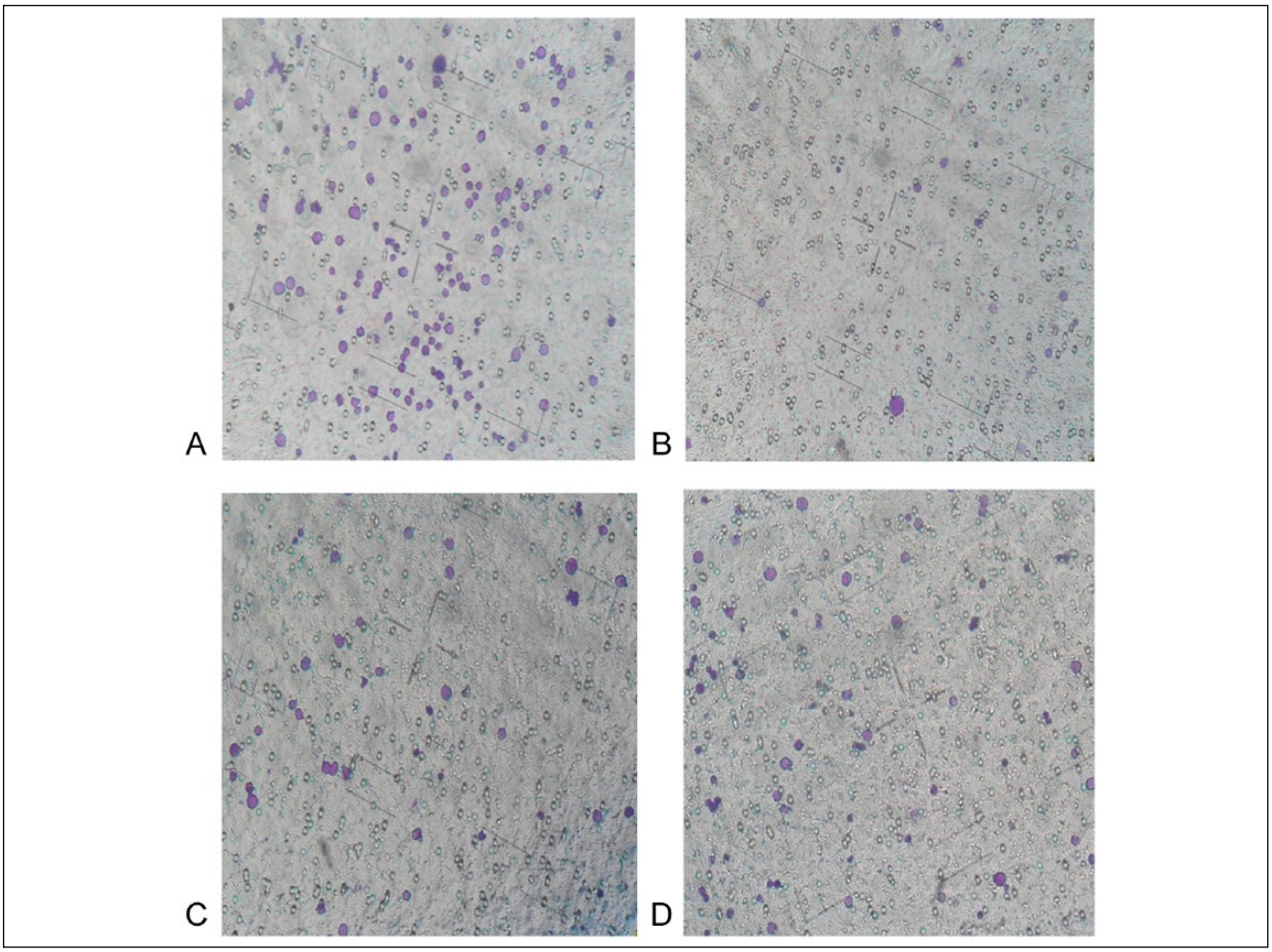

Figure 7. Inhibitory effect of AFES on HUVECs invasion through collagen matrix. HUVECs (105 cells/150 µL medium 199) were seeded on to the upper chamber of Boyden chamber in the presence and absence of AFES (2.5, 1, and 0.5 µg/mL) along with 2 ng/mL VEGF and incubated at 37°C in 5% CO_2 atmosphere for 10 hours. After incubation, the cells migrating to the lower surface of the polycarbonate membrane were fixed with methanol and stained with crystal violet and photographed. (A) Untreated control; (B) Treatment with AFES (2.5 µg/mL); (C) Treatment with AFES (1 µg/mL); (D) Treatment with AFES (0.5 µg/mL).

release from these 2 types of cells.[40] IL-6 is also capable of increasing endothelial permeability and stimulating proliferation of endothelial cells.[41] GM-CSF enhances tumor cell proliferation, migration, and angiogenesis. It stimulates angiogenesis by endothelial cell proliferation and migration.[42] Lowering of the elevated levels of TNF-α, IL-1β, IL-6, and GM-CSF further confirmed the antiangiogenic effect of the active fraction from *E sonchifolia* with already proven anti-inflammatory effects. By lowering the level of these cytokines, AFES was involved directly or indirectly in the inhibition of tumor-directed neovessel formation by blocking the main culprits like MMP and VEGF that actively take part in the process.

In conclusion, antiangiogenic therapy that targets vascular growth within the tumor is now widely accepted to treat various tumors, because the agents used in this treatment modality have fewer side effects due to the quiescent nature of the blood vessels in adults. The genetic instability of tumor cells is the main cause of the failure of systemic chemotherapies. But the endothelial cells of the tumor stroma are genetically stable and believed to respond to antivascular therapy because they are unable to become drug resistant. The present study clearly proved the inhibitory effect of the active fraction containing the major active principle γ-humulene on tumor angiogenesis by efficiently decreasing MMPs, VEGF, and proinflammatory cytokines while at the same time increasing the level of TIMP. The retarding effect of AFES was clear-cut from the decreased capillary formation and prevention of microvessel outgrowth from the aorta. Additional evidence for the inhibitory effect of AFES is the impediment on endothelial cell proliferation, invasion, migration, and also hampering of the activation of

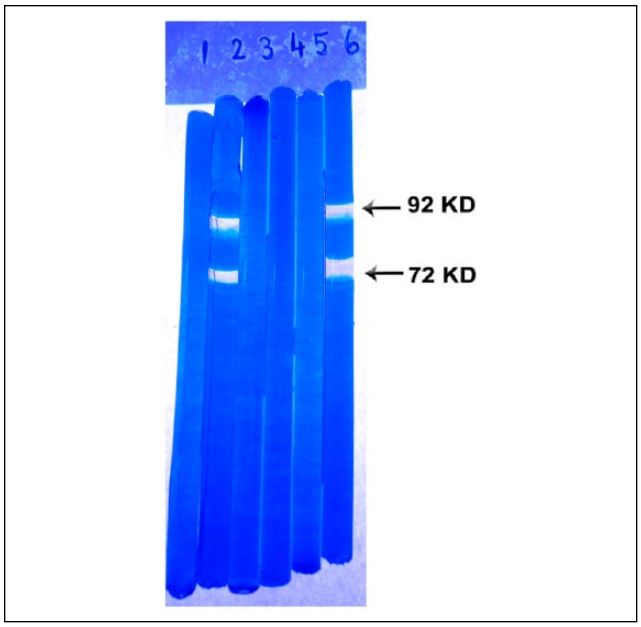

Figure 8. Effect of AFES on MMP-2 and MMP-9 production by HUVECs. (1) Condition medium from untreated HUVECs without trypsin activation; (2) Condition medium from untreated HUVECs after trypsin activation; (3) Condition medium from untreated HUVECs after trypsin activation + EDTA; (4) Condition medium from pretreated HUVECs (2.5 µg/mL AFES) after trypsin activation; (5) Condition medium from pretreated HUVECs (1 µg/mL AFES) after trypsin activation; (6) Condition medium from pretreated HUVECs (0.5 µg/mL AFES) after trypsin activation.

proenzyme to active enzyme as evident by the gelatin zymographic analysis.

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Declaration of Conflicting Interests

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