Tumor suppression by Chinese cordyceps extract via anti-angiogenesis

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Research Article

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Abstract

Development of pharmacological agents for anti-angiogenesis to disrupt the vascular supply and starve tumor of nutrients and oxygen is a novel strategy for tumor therapy. Chinese cordyceps (*Ophiocordyceps sinensis*), a traditional Chinese herb, has been widely reported to exhibit antitumor effects. In the present study, we tested suppression of Chinese cordyceps extract (CCE) on tumor angiogenesis in tube formation assay using human umbilical vascular endothelial cells (HUVECs) in vitro and Lewis carcinoma xenografts in mice. In addition, CCE was also investigated in an *ex vivo* angiogenesis assay using rat aortic rings. CCE could dose-dependently suppress capillary tube formation in HUVECs as well as in rat aortic ring. The antiangiogenesis activity of CCE was further verified in Lewis xenografted mice models where tumor volume was significantly decreased in the treatment of CCE at 100 mg/kg. The mean vascular density (MVD) in the tumor, measured by platelet endothelial cell adhesion molecule-1 (CD31) immune-histochemical staining, was also markedly inhibited by CCE at 100 mg/kg treatment. These results indicated that Chinese cordyceps may have antitumor activity through antiangiogenesis activity and could be used in prevention and/or therapy of angiogenesis related cancers.

Introduction

The Chinese cordyceps, a parasitic Thitarodes insect-*Ophiocordyceps sinensis* fungus complex, is a traditional Chinese herb that has been used as medicine for a long time in Asia, especially in China. Since 1964, Chinese cordyceps has been recorded as an herbal drug in the Chinese pharmacopoeia. Accumulated evidences from both animal and human studies prove that Chinese cordyceps extract (CCE) has efficient anticancer activity (Zhang et al. 2005; Niwa et al. 2013; Mei et al. 2014; Chiu et al. 2016). Meanwhile, antitumor mechanism of CCE has also been investigated (Niwa et al. 2013; Yoshikawa et al. 2007; Jordan et al. 2010; Ji et al. 2011; Asmitananda et al. 2011; Huo et al. 2011).

Angiogenesis, the formation of new blood vessels from pre-existing vessels, has been characterized as an essential process for tumor cell proliferation and viability (Ramjiawan et al., 2017). Angiogenesis has become an effective target for cancer therapy (Zhao et al. 2015; Marme et al. 2018) and several angiogenic inhibitors such as Bevacizumab for metastatic colorectal cancer has been approved by FDA because their effectiveness (Chu et al. 2004; Yadav et al. 2015). We have found the anticancer effects of CCE in Lewis xenografted mice and CCE antitumor activity could be through anti-angiogenesis. Therefore, we designed a series of experiments with CCE to investigate angiogenesis inhibition activity in an *in vitro* tube formation assay and a rat aortic ring assay.

Materials And Methods

Reagents, kit, cell culture and animals

Reagents

Dulbecco's modified eagle medium (DMEM) medium, fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco invitrogen cell culture (Carlsbad, USA), cis-Platinum was obtained from Sigma (Saint Louis, USA).

4',6-Diamidino-2-Phenylindole (DAPI) staining kit

First rinsed off the medium with PBS (Beijing Dingguo Changsheng Biotechnology Co.Ltd.,Beijing, China), the membranes were fixed with 4% PFA for 30 min. Rinse three times with PBS each for 5 minutes. After stain with DAPI (Beyotime Biotechnology, Shanghai, China) for 5 minutes, rinsed again three times with PBS each for 5 minutes. Finally, cells were photographed by microscope at 200-fold magnification.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Allcells (Shanghai, China) and cultured in special medium for HUVEC cells at 37°C with 5% CO_2. CCE was dissolved in cell cultivated medium to prepare different concentrations at 10, 3, 1, 0.3, 0.1, 0.03, and 0.01mg/mL.

Animals

Male C57BL/6 mice at 7–8 weeks of age were obtained from Slaking Experimental Animal Co. LTD. (Changsha, China). Mice were kept in a specific pathogen free (SPF) animal lab and were fed with sterile food and water freely. Animal care and experiments were performed according to the Institutional Animal Care and Use Guidelines.

Preparation of Chinese cordyceps extracts

Chinese cordyceps extraction (CCE) was prepared from the cordyceps produced by Sunshine Lake Pharma Co., Ltd (Dongguan, China). Briefly, 500 g artificial cultivated Chinese cordyceps (including sclerotia that the caterpillar of *Thitarodes xiaojinensis* infected by *Ophiocordyceps sinensis* and the stroma) were cut into small pieces, and soaked in 5000 mL distilled water. The mixture was homogenized in ice bath with a homogenizer (IKA, Staufen ,Germany) at 16,400 rpm for 3 min, and then sonicated for 30 min. The matrix was cooled at − 20°C for 40 min, and sonicated again for another 30 min to obtain a uniform matrix. After extraction, the residues were removed by using suction filtration, and the filtrate was lyophilized to obtain Chinese cordyceps extract (CCE). The nucleoside content of CCE was analyzed by high performance liquid chromatography (HPLC) as previous reported (Huo et al. 2017). Briefly, the HPLC analysis was performed using a Waters HPLC system (Waters Corporation, Milford, USA). The extracted solution was separated in a C18 column (Agilent ZORBAX SB-AQ, Palo Alto, USA) by maintaining the isocratic low rate (0.8 mL/min) of the mobile phase (0.4 M KH_2PO_4, pH 3.7: methanol 90: 10) and detected at 260 nm absorbance. Peaks of various compounds in the extraction were identified by their retention times and co-injection tests with corresponding standard compounds.

Bioassay

Matrigel Tube Formation Assay

Matrigel (Corning, Inc., Corning City, USA) was thawed at 4°C overnight. Each well of a pre-cooling 96-well plate was coated with 50 μL matrigel and then allowed to gel at 37°C for 30 min. The HUVECs were suspended in 50 μL culture medium (5×10^3 cells per well) with designated concentrations of CCE and seeded into the coated plates in triple. After incubating at 37°C and 5% CO_2 for 6 h, the capillary tubes formed were photographed under a phase contrast microscope at magnification of 100× (Olympus IX51, Tokyo, Japan). The total branching length and the total segments length were quantified by using Image J software. The inhibition rate was calculated using untreated wells as control.

The ex vivo angiogenesis assay

The Rat aortic ring assay was slightly modified method as described previously (Ernens et al. 2015). In short, a 6-weeks old male SD rat was sacrificed under anesthesia with pentobarbital sodium (3%). The thoracic aorta was stripped out and washed twice with cold DMEM medium. Then the artery was cut into segment rings of about 1.0 mm. The aortic rings were then sandwiched into a 48-well plate with 300 μL matrigel per well. After the gel solidifies, 200 μL DMEM medium with penicillin-streptomycin (100 IU/mL-100 μg/mL), and fetal bovine serum (1%) were added into each well. After 24 h, the aortic rings were treated with various concentrations of CCE. Incubation was carried out for another 5 days in a humidified atmosphere containing 5% CO_2 at 37°C, then aortic rings were photographed using a phase contrast microscope (Nikon TS2R-FL, Tokyo, Japan) with a magnification of 40×. The total branching length and the total segments length were quantified and analyzed by Image J software.

Bioassay with Lewis xenografts mice model

The subcutaneous tumor mouse model was established by inoculating 2×10^6 of Lewis lung cancer cells in the right armpit region of C57BL/6J mice. When the tumor volume increased to approximately 200 mm^3, the mice were randomly divided into 4 groups (n = 10) and treated with intraperitoneally injected with physiological saline, 3 mg/kg cis-platinum (DDP), 25 mg/kg CCE and 100 mg/kg CCE, respectively. CCE were administrated once daily for 12 consecutive days, while DDP was administrated every 2nd day. Tumor size was measured three times per week during the treatment. Tumor volumes were calculated using the formula $V = a * b^2 * 0.5$.[19] Mice were sacrificed by cervical dislocation at the end of the experiment, and the tumor tissues were collected for immunohistochemical analysis.

Immunohistochemical analysis

Tumor tissues harvested from the sacrificed mice were fixed in 10% formalin, paraffin-embedded and sectioned. After deparaffinization and rehydration, 3-μm thick tumor tissue sections were pre-treated in 0.01 mM sodium citrate (pH 6.0) solution at 100°C for 3 min, followed by staining with rabbit anti-human CD31 antibody (Cell Signalling, Boston, USA) at final dilution of 1:1000. Positive staining was carried out by using Detection System HRP Polymer Kit (Lab Vision, Fremont, USA). The slides were then rinsed in

distilled water, counterstained with Gill's haematoxylin (Fisher Scientific, Fair Lawn, USA), and mounted. For angiogenesis analysis, all morphological structures with a lumen interspersed by CD31-positive endothelial cells were identified as blood vessels. Images were photographed at high magnification (200×) using Leica DM6 microscopy system with DFC7000T digital camera (Leica, Munich, Germany). Quantification of microvessels was carried out as previously described. Images were quantified by means of computer assisted image analyzer for mean vascular density (MVD) (Gao et al. 2011).

Statistical analysis

Statistical analysis was carried out using SPSS 16.0 software (SPSS Inc., Chicago, USA), with Student's two tailed t-test for two groups or one-way ANOVA followed by Dunnett's post hoc test for multiple groups. The results were presented as mean ± SEM and $p < 0.05$ was considered statistically significant.

Results

Chromatographic profile of CCE

Nucleosides had been reported to be good marker for quality control of Chinese cordyceps (Xiao et al. 2013). In this experiment, the chromatographic profiles and the percentage contents of the nucleosides of CCE were shown in Fig. 1. The adenosine quality is 0.044% that is higher than the required standard (≥ 0.010%) in the Chinese Pharmacopeia.

Effects of CCE on HUVECs tube formation

To investigate whether different concentrations of CCE are cytotoxic to HUVEC, cell morphology and DAPI stain were performed. Little abnormal morphological changes of HUVECs were observed after 24 h exposure at the concentration of 1, 0.3, 0.1, 0.03, 0.01 mg/mL of CCE. However, after HUVECs were exposed to CCE at the concentration of 10 and 3mg/mL, more spindly morphology could be observed and nucleus were destroyed (Fig. 2). The results further demonstrated that there was no significant toxicity of CCE at the concentration of 1, 0.3, 0.1, 0.03, 0.01 mg/mL in the cellular level. To investigate whether CCE can inhibit capillary tube formation, a matrigel assay with HUVEC cells was performed. The number of branches of HUVECs were decreased in a CCE dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) calculated from normalized total branching length and normalized total segments length were 0.31 and 0.29 mg/mL respectively (Fig. 3). The data suggests that CCE contains components with activity to inhibit tube formation of HUVECs.

Effects of CCE on angiogenic outgrowth rat aortic ring

To further determine the anti-angiogenic activity of CCE, an *ex vivo* angiogenesis model using a rat aortic ring was performed. CCE can inhibit the sprouting angiogenesis of rat aortic ring in a dose-dependent manner. An extensive microvascular network with an average total branching length of 2.49 cm was observed in control. In contrast, aortic rings treated with CCE exhibited shorter and less sprouts (Fig. 4). In

addition, the number of branches formed was also affected by CCE treatment. At the concentrations of 0.03-3.0 g/mL of CCE, the number of branches was significantly decreased in a dose-dependent manner. The inhibition effect was represented by IC_{50} calculated from the number of sprouts-concentration curve and the normalized total branches length-concentration curve that were 0.25 mg/mL and 0.28 mg/mL, respectively. These results demonstrated that CCE can reduce the angiogenesis in aortic ring *ex vivo*.

In vivo anticancer and antiangiogenic activity of CCE

To confirm the antiangiogenic effects of CCE *in vivo* in an animal model, a Lewis lung carcinoma xenografted mouse model was established. CCE showed a dose-respond manner to inhibit the tumor growth. The mean tumor volumes of treated groups with CCE at 25 and 100 mg/kg were 2.74 ± 0.78 and 2.27 ± 0.83 cm^3 respectively, which was significantly lower than that of the control group (3.51 ± 1.09 cm^3, $p < 0.05$). The DDP group also showed a significant decrease in tumor volume (2.37 ± 0.70 cm^3, $p < 0.05$) (Fig. 5).

The tumor was sectioned and studied for its microvessels by immunohistochemical staining of CD31. The result showed that number of microvessels formed by cells expressing CD31 in the CCE treated tumor clearly decreased compared to untreated tumor. CCE significantly decreased MVD value at 100mg/kg dosage compared with that of control (1.54 vs. 3.56, $p < 0.05$) (Fig. 6), which was consistent with the tumor volume index.

Discussion

Chinese cordyceps has been used as traditional Chinese medicine for a long time (Wang et al. 2017). The antitumor activities of this Chinese herb have been extensively investigated (Nakamura et al. 2015), but its antiangiogenesis effect has not been reported. Angiogenesis plays a vital role in tumor growth, invasion and metastatic spread (Nakamura et al. 2015) and has become an attractive antitumor target. Suppression of angiogenesis is a good strategy to control cancer progression (Bhat et al. 2008). *Cordyceps militaris*, a similar cordycipitoid fungus of Chinese cordyceps, has shown antiangiogenesis activity and its extract has resulted in a dramatic antitumor effect with downregulation of VEGF expression in the mouse model xenografted with human melanoma cells (Ruma et al. 2014). In this study, we demonstrated that the extract of Chinese cordyceps could inhibit tumor growth by the antiangiogenensis effect.

The angiogenesis process consists several steps: dissociation of pericytes from pre-existing vessel, digestion of extracellular matrix with proteases, growth, migration and invasion of endothelial cells, tube formation, and finally remodelling. The in vitro formation of capillary-like tubes by human umbilical veins endothelial cells is a simple, quick and quantitative method *in vitro*. This assay has been widely used to test various factors that inhibit angiogenesis (Shin et al. 2008; Pang et al. 2009). In this study, CCE could suppress tubular formation of human umbilical veins endothelial cells in a dose-dependent manner, indicated that CCE has anti-angiogenesis activity. In order to further explore the role of CCE on anti-

angiogenesis, the rat aortic ring assay, as a bridge between *in vivo* and *in vitro* assay, which can be used to simulate angiogenesis *in vivo* (Bellacen et al. 2009; Baharara et al. 2014), was measured. The result demonstrated that CCE could suppress vessel sprouts and the length of new forming capillaries in a dose-dependent manner, proving that CCE could inhibit angiogenesis. Furthermore, we demonstrated that CCE dose-dependently inhibited tumor growth in Lewis lung carcinoma tumor bearing C57BL/6 mice. At the same time, we examined the microvessel in tumor tissue by immunohistochemistry. Endothelial cells were marked by CD31 in histological tissue sections (Virman et al. 2015). The result suggests that CCE possess a good ability of anti-angiogenesis. These findings indicated that CCE suppressed tumor growth through anti-angiogenesis. Cai et al. (2018) have found the Chinese cordyceps could inhibit the tumor metastasis which has been documented that angiogenesis has a crucial role (Yakes et al. 2011). Therefore, we speculate that the CCE could inhibit the tumor metastasis by inhibiting the angiogenesis.

A critical issue is that what the mechanism of CCE is for its anti-angiogenetic effect. VEGF is a major pro-angiogenetic factor, which may promote vascular endothelial cells proliferation and inhibit angiogenesis in the tumor growth and metastasis (Chang et al.2020). Yang et al. (2005) found that exopolysaccharide fraction from cultivated *O. sinensis* fungus can inhibit VEGF expression in the lungs and livers of B16 melanoma-bearing mice. Ji et al. (2011) found that polysaccharide of O. *sinensis* combined with cisplatin can significantly decrease the expression levels of VEGF and bFGF, another potent angiogenic factor, in non-small cell lung cancer H157 cell line(Ji et al. 2011). These results suggest that regulating the VEGF signalling pathway maybe the way for CCE inhibiting angiogenesis and anti-tumor. The main active ingredient of CCE may be Cordyceps polysaccharide. We will test our conjecture in future research.

Chinese cordyceps, as a traditional Chinese medicine for both food and medicine, has been regarded as a good tonic for a long time. Chinese cordyceps and its fungal cultures have exhibited strong anti-tumor activity in pharmacodynamics researches. Considering the complex active ingredients of Chinese cordyceps, its antitumor mechanism remains unknown. The exactly anti-angiogenesis active components of Chinese cordyceps should be further investigated. The large mass-scale production of Chinese cordyceps has made it availability to study its active components and supply for market demands.

Declarations

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Authors’ contributions

Wenjia Li, Jiangchun Wei and Xingzhong Liu participated in the design of this study. Wenjia Li carried out the experiment. Data collection and analysis were performed by Jing Li, Xuefeng Hu, Linghua Xu and Xiaoyu Liu. Material preparation was performed by Yimin Zhang. Literature search and manuscript preparation were performed by Zhengming Qian and Liling Jin. The first draft of the manuscript was

written by the first author Wenjia Li and edited by profs Jiangchun Wei and Xingzhong Liu. All authors read and approved the final manuscript.

Funing

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Data availability

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of Interest.

Ethical approval The protocols for animal experiments have been approved by local Institutional Animal Care and Use Committee (IACUC) of HEC Pharma. Co. Ltd., Dongguang, Guangdong, China. The accreditation number is IAEC -K-200720-03.

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Figures

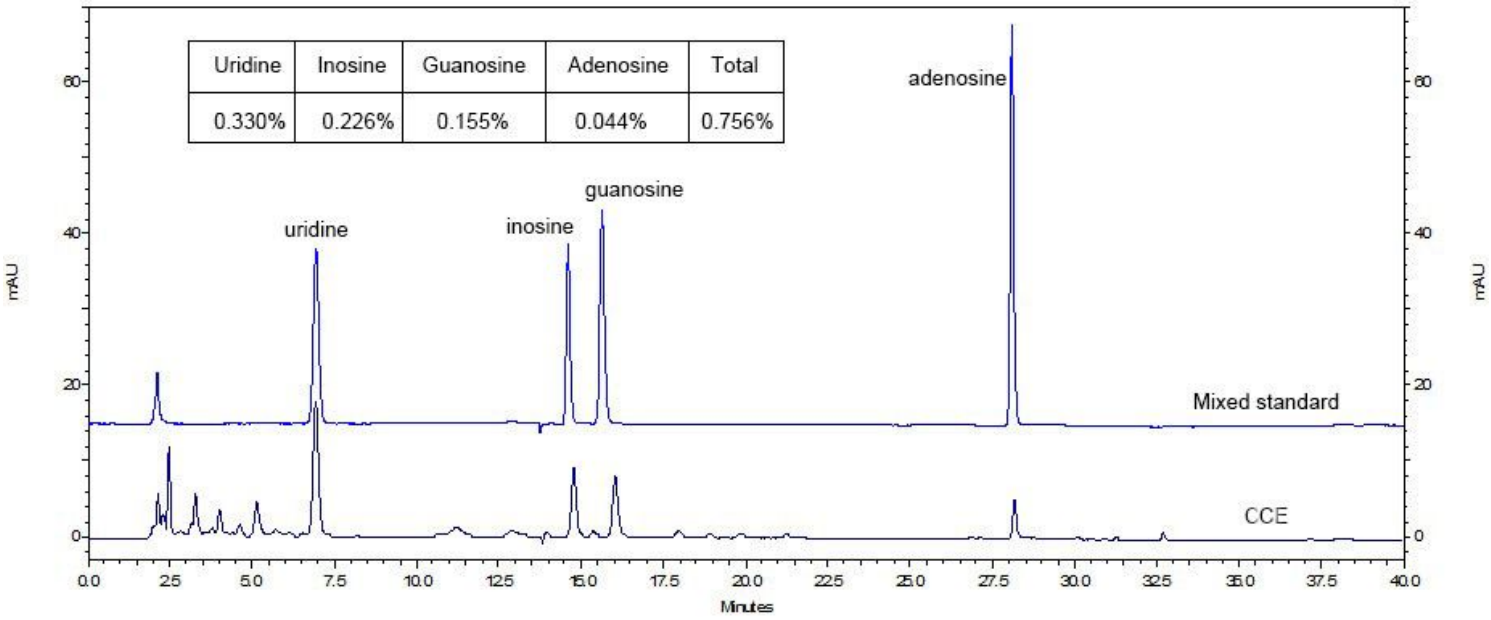

Figure 1

Fingerprint map of the CCE detected with HPLC. The nucleoside contained in Chinese *cordyceps* extract (CCE) was analyzed by HPLC with a C18 column for quality control of CCE. Peaks were detected at 260 nm. The percentage contents of different nucleosides were calculated via corresponding standard compounds.

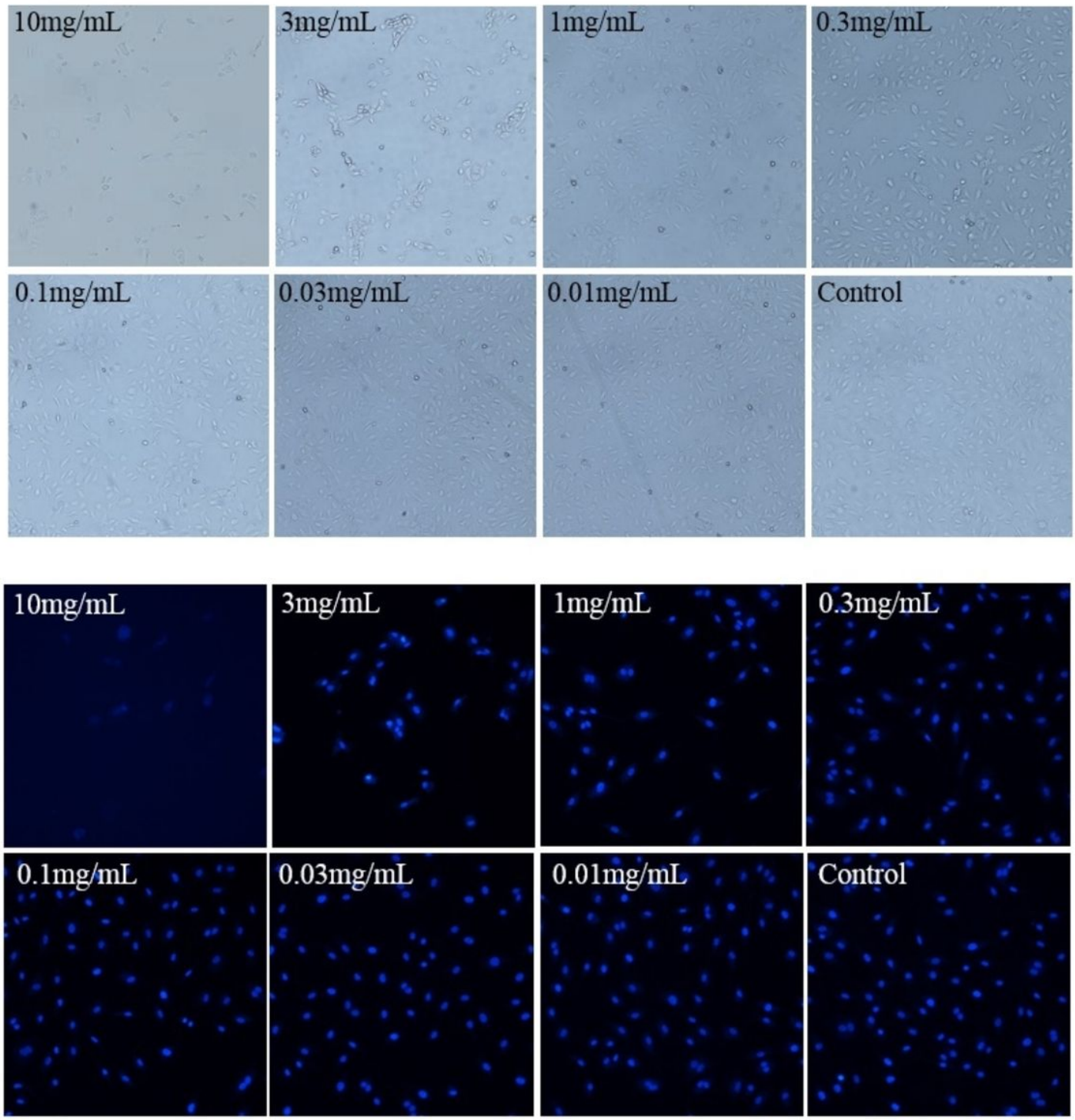

Figure 2

Effects of CCE on morphology and nucleus of HUVECs. HUVECs were treated with CCE at different concentrations for 24 h. (A) Cells were photographed by microscope at 100-fold magnification for the detection of morphological changes of HUVECs. (B) Nuclei were stained with DAPI, and then photographed by microscope at 200-fold magnification for the detection of nuclear changes of HUVECs.

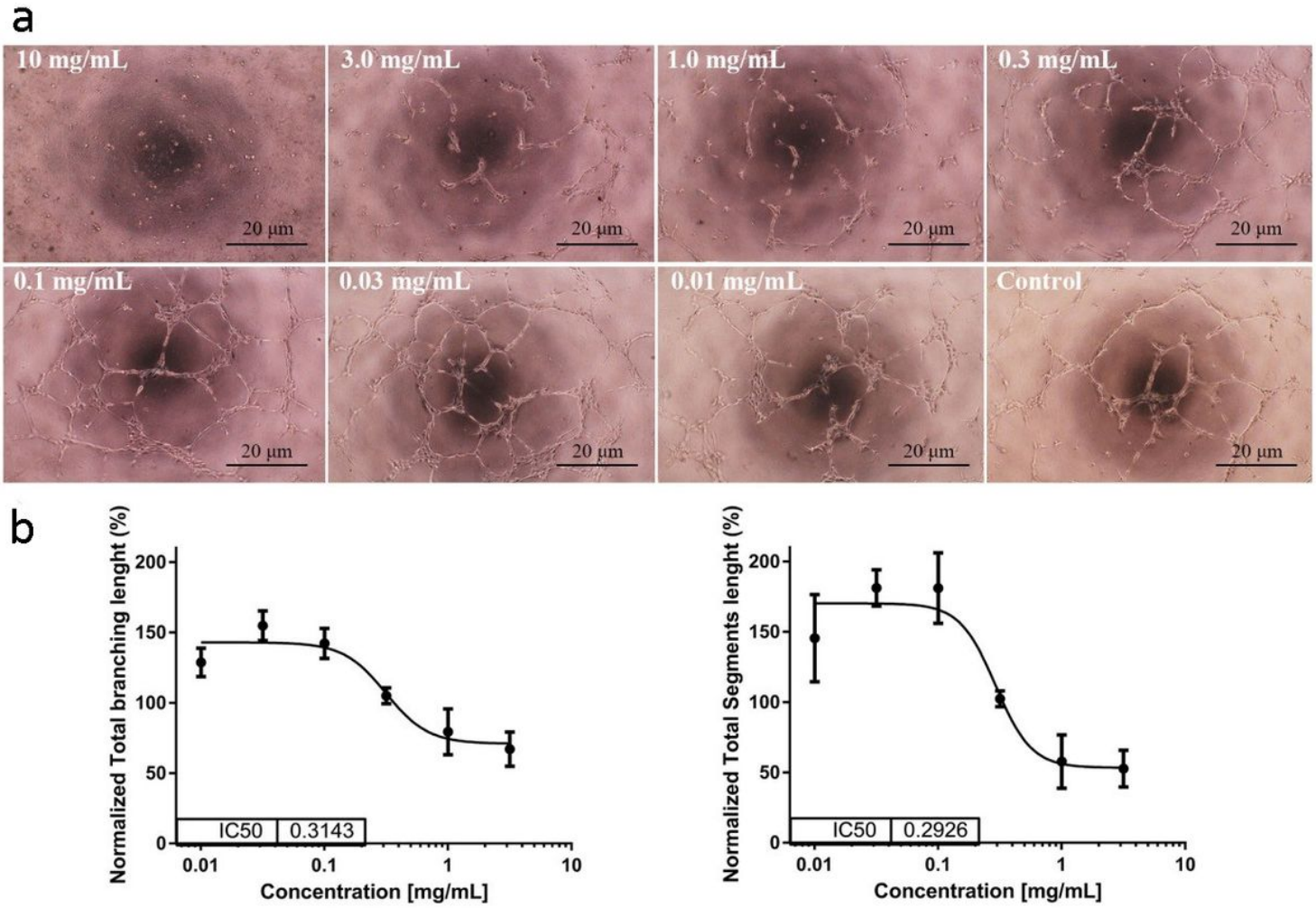

Figure 3

Effect of CCE on HUVEC cell tube formation. (A) HUVEC cells were seeded into 96-well plates pre-coated with matrigel and incubated with the indicated concentrations of CCE for 6 h. The microtubes formed were captured by a phase contrast microscope (100×). (B) Tube formation on 3-D matrigel was quantified by Image J. The data was represented as mean ± SEM. The total branching length and total segment length were normalized and plotted against CCE concentration. IC_{50} values were calculated and shown.

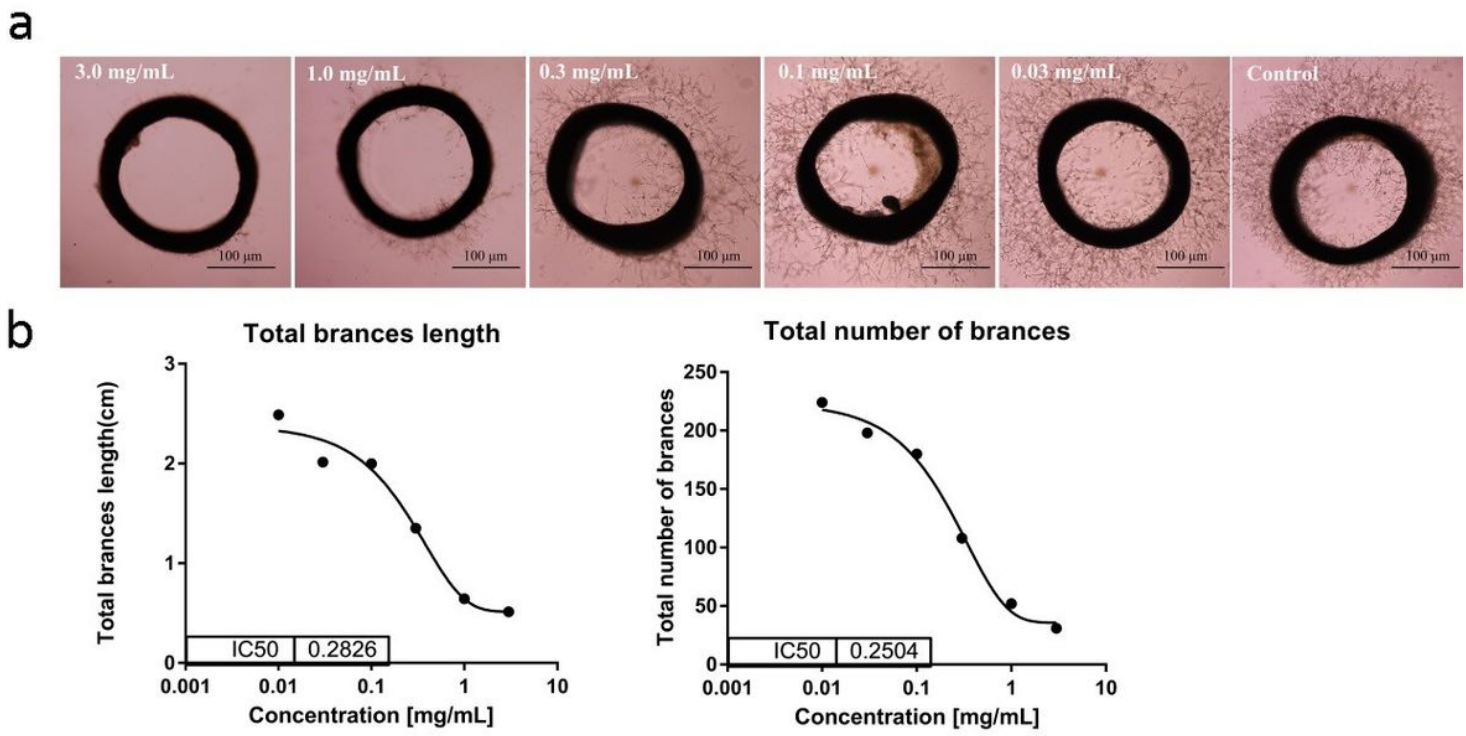

Figure 4

Effects of CCE on sprouting angiogenesis from rat aortic ring. (A) Rat aortic ring were incubated in matrigel in the presence of different concentrations of CCE and photographed. Representative images of aortic rings were shown. (B) The total number of branches and the total branch length were plotted against concentrations of CCE. The IC_{50} were calculated and shown.

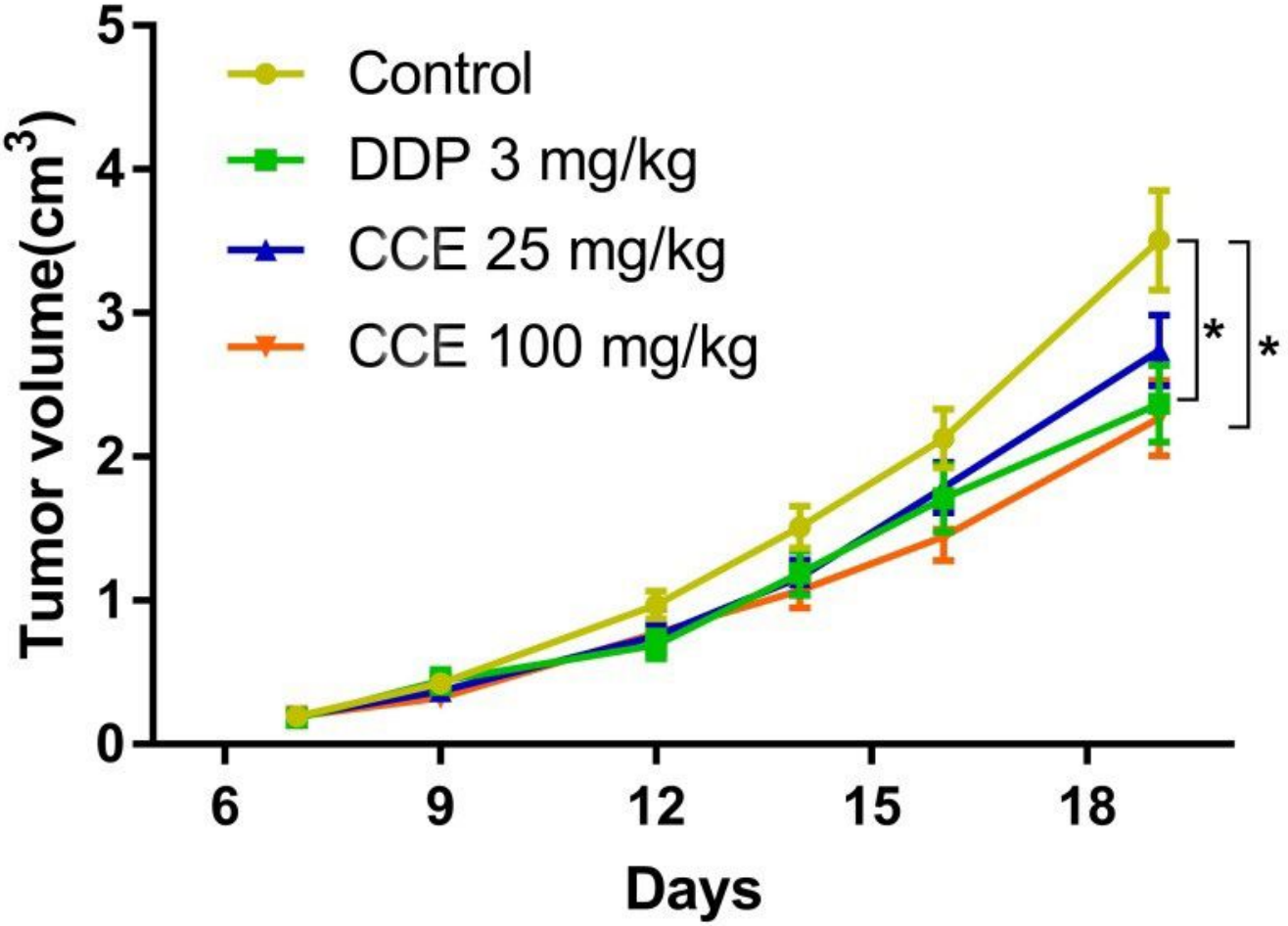

Figure 5

Anti-tumor and anti-angiogenesis effects of CCE in Lewis homograft mouse model. The mice were treated with or without CCE. Tumor volumes were measured and calculated using the formula $V = a * b^2 * 0.5$. Data was expressed as mean ± SEM (n = 10). *$p < 0.05$ vs. control groups.

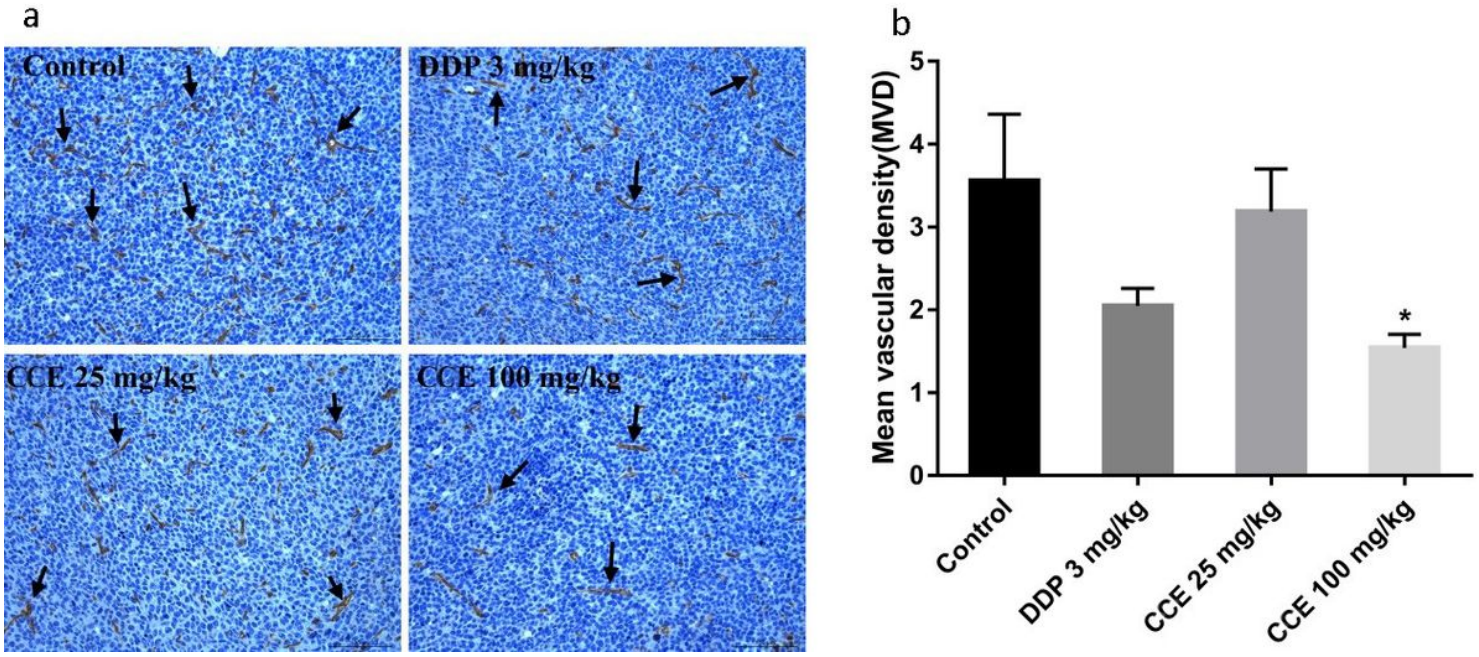

Figure 6

Immunohistochemical analysis of microvessels in Lewis lung tumor stained by CD31 antibodies. Tumors from mice treated with or without CCE *in vivo* from Fig. 3 were sectioned and stained for CD31. (A) Representative images of CD31 stained pathological sections from the control or CCE groups (200×). The microvessels staining positive for CD31 was marked by black arrows. (B) Statistical analysis of the microvessel density in control or CCE treated groups were shown. *$p < 0.05$ vs. control groups.

Supplementary Files

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