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ORIGINAL ARTICLE

Cordyceps militaris Fraction Inhibits Angiogenesis of Hepatocellular Carcinoma *in vitro* and *in vivo*

Zhiwei Li[1], Zhongyi Guo[2], Jianhua Zhu[3], Sixue Bi[2], Yuanyuan Luo[3], Rongmin Yu[2,3], Weijuan Huang[1], Liyan Song[1]

[1]Department of Pharmacology, College of Pharmacy, [2]Biotechnological Institute of Chinese Materia Medica, Jinan University, [3]Department of Natural Product Chemistry, College of Pharmacy, Jinan University, Guangzhou, China

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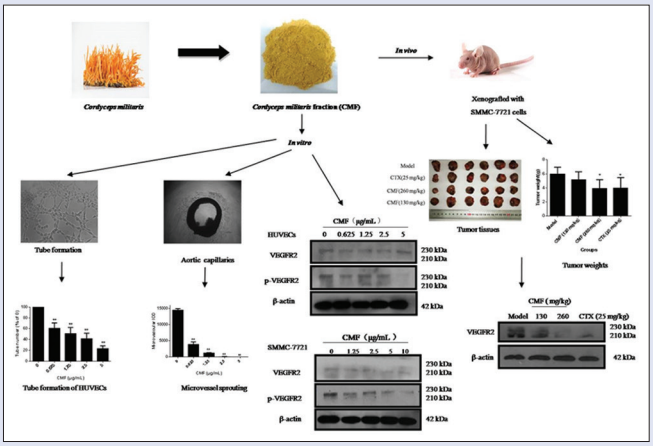
ABSTRACT

Background: *Cordyceps militaris* fraction (CMF) was found to inhibit the proliferation of chronic myeloid leukemia K562 cells, oral squamous carcinoma KB cells, and the metastasis of lung cancer cells. This study focuses on the activity of CMF against angiogenesis of hepatocellular carcinoma (HCC). **Objectives:** The objective of the study is to research the antiangiogenic activity of CMF in HCC cells and the underlying mechanism *in vitro* and *in vivo*. **Materials and Methods:** Transwell migration and invasion assays were used to measure the effects of CMF on migration and invasion of SMMC-7721 cells and human umbilical vein endothelial cells (HUVECs). Tube formation and rat aortic ring assays were used to assess the antiangiogenic potential of CMF. The antiangiogenic mechanism was detected by immunofluorescence and western blot analysis. The nude mice xenografted with SMMC-7721 cells were used to study the antiangiogenic activity of CMF and its underlying mechanism *in vivo*. Immunohistochemistry analysis was used to evaluate the effect of CMF on the expression of CD31, and western blot analysis was also performed to detect the expression of vascular endothelial growth factor (VEGF) and other related proteins in tumor tissues. **Results:** CMF inhibited the migration and invasion of SMMC-7721 cells and HUVECs and suppressed VEGF-induced tube formation of HUVECs and the formation of aortic ring capillaries of rats in a concentration-dependent manner. CMF attenuated the phosphorylation of VEGF receptor 2 (VEGFR2), Akt, and ERK in SMMC-7721 cells and HUVECs. CMF significantly inhibited tumor growth in nude mice xenografted with SMMC-7721 cells. CMF reduced the expression level of CD31, western blot analysis indicated that CMF downregulated the expression of VEGFR2 and attenuated the phosphorylation of Akt and ERK in the tumor tissues, which was consistent with the results obtained from *in vitro* study. **Conclusion:** CMF can inhibit the angiogenesis of HCC and the mechanism is associated with suppression of the VEGF/VEGFR2 signaling pathway.

Key words: Antiangiogenesis, *Cordyceps militaris* fraction, human umbilical vein endothelial cells, SMMC-7721, vascular endothelial growth factor receptor 2

SUMMARY

- *Cordyceps militaris* fraction inhibited the migration and invasion of human umbilical vein endothelial cells and SMMC-7721 cells.
- *Cordyceps militaris* fraction inhibited vascular endothelial growth factor-induced tube formation of human umbilical vein endothelial cells and formation of rat aortic ring capillaries.
- *Cordyceps militaris* fraction significantly inhibited tumor growth in nude mice xenografted with SMMC-7721 cells.
- The antiangiogenic mechanism of *Cordyceps militaris* fraction might be through suppression of the vascular endothelial growth factor/vascular endothelial growth factor receptor 2 signaling pathway.



Abbreviations used: CMF: *Cordyceps militaris* fraction; HCC: Hepatocellular carcinoma; HUVECs: Human umbilical vein endothelial cells; VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2.

Correspondence:

Dr. Liyan Song,
Department of Pharmacology, College of Pharmacy, Jinan University, Guangzhou, 510632, China.
E-mail: tsly@jnu.edu.cn

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INTRODUCTION

Liver cancer is one of the most common cancers in the world, and it is also the third lethal cancer. Liver cancer has maintained a sustained increase in morbidity and mortality. According to the latest report, the mortality rate of liver cancer in male and female were 15.2% and 8.9%, respectively, in 2018.[1]

Hepatocellular carcinoma (HCC) is a vascular malignant tumor, and the process of its occurrence and development is closely related to the formation of tumor blood vessels.[2] Angiogenesis is essential for organ growth, repair, and imbalance in this process can lead to many malignant outcomes such as inflammation, ischemia, infection, and immune disease.[3] Antiangiogenesis can effectively inhibit the occurrence, development, and metastasis of liver cancer and provide important ideas and methods for clinical treatment.

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Cordyceps militaris (L.) Link is an entomopathogenic fungus species. As a valuable Chinese herbal medicine, *C. militaris* has a long history in traditional Chinese medicine. The components of *C. militaris* have a variety of pharmacological activities including immune stimulation, antibacteria, antioxidation, antiaging, antiviral, antitumor, antisteroidogenesis, antihypoglycemia, hypolipidemic, antiangiogenesis, antidiabetes, antifatigue, neuroprotection, liver protection, adrenal protection, and lung protection.[4,5] Cordycepin isolated from *C. militaris* inhibited melanoma in mice and prolonged survival of tumor-bearing mice.[6] *C. militaris* polysaccharide promoted the proliferation of murine splenic lymphocytes,[7] and also played an immunomodulatory role in mice by regulating related genes.[8] Fermented *C. militaris* extract ameliorated hepatosteatosis via activation of fatty acid oxidation.[9] *C. militaris* hot water extract inhibited the lipopolysaccharide-induced inflammatory response in porcine alveolar macrophages by regulating mitogen-activated protein (MAP) kinase signaling pathway.[10] Constituents isolated from *C. militaris* suppressed the inflammatory mediator's production and human cancer cell proliferation, and one of them inhibited the proliferation of HepG2 cells.[11] *C. militaris* aqueous extract was reported to exert the cytotoxic effect on MCF-7 and HepG2 cells through the caspase-dependent mitochondrial pathway.[12]

The vascular endothelial growth factor (VEGF) family plays a crucial role in angiogenesis. As a multifunctional cytokine, VEGF has direct or indirect regulation in angiogenesis and lymphangiogenesis. It can enhance the proliferative capacity of endothelial cells, accelerate the process of angiogenesis, and increase the permeability of blood vessels. VEGF receptor 2 (VEGFR2) is the most important VEGF receptor and mediates VEGF signaling by activating various cellular signaling intermediates. The angiogenesis of liver cancer can be suppressed by inhibiting the phosphorylation of VEGFR2 and regulating the VEGF/VEGFR2 signal transduction.[13] The downstream phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways play the important roles in the growth, proliferation, metastasis, and invasion of tumor cells. It was found that inhibition of the PI3K/Akt/mTOR pathway induced apoptosis and inhibited proliferation of hepatoma cells.[14] It was also reported that suppression of the Ras/Raf/MEK/ERK signaling pathway by sorafenib inhibited tumor angiogenesis and induced tumor cell apoptosis in the HCC model.[15]

Cordyceps militaris fraction (CMF) is the total nucleoside constituents isolated from the artificial culture of *C. militaris*. Previous studies have found that CMF was resistant to lung cancer metastasis,[16] induced apoptosis in oral cancer cells,[17] and inhibited proliferation of multiple liver cancer cells. As a continuous investigation, we currently report the antiangiogenic ability of CMF in liver cancer.

MATERIALS AND METHODS

Fraction preparation and reagents

Cultured *C. militaris* was purchased from Honghao Biological Company of Jiangmen (Guangdong, China). CMF was prepared as per the previous report.[18] CMF was dissolved in DMEM (Gibco, New York, USA) or RPMI-1640 (Gibco, New York, USA) medium with penicillin-streptomycin (Solarbio, Beijing, China) and 10% fetal bovine serum (Gibco, New York, USA) and store at −20°C. The antibodies for Akt, p-Akt, CD31, ERK, p-ERK, VEGFR2, p-VEGFR2, β-actin, HRP-linked goat anti-rabbit antibodies were obtained from Cell Signaling Technology Inc. (Boston, MA, USA). VEGF was purchased from PeproTech (Rocky Hill, USA).

Cell lines and animals

Human HCC SMMC-7721 cells and human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), maintained in DMEM and RPMI-1640 medium with penicillin-streptomycin (Biosharp, China) and 10% fetal bovine serum (FBS). The cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Sprague-Dawley rats (SCXK 2013-0002, 130–150 g, 5–7 weeks old) and BALB/c-nu/nu mice (SCXK 2013-0002, 18–20 g, 6–8 weeks old) were purchased from Guangdong Medical Laboratory Animal Center. All animal programs were approved by the Institutional Animal Care and Use Committee of Jinan University in Guangzhou.

Cell migration and invasion assays

The cell migration and invasion assays were performed according to the reference[2] with minor modifications. The migration of SMMC-7721 cells and HUVECs was evaluated by the use of Transwell chambers with 8-μm pore filters (Corning, New York, USA). Cells (2×10^5/mL) were plated on the upper chambers in serum-free medium with CMF. DMEM or RPMI-1640 medium containing 10% FBS was added to the lower chambers as a chemoattractant. After incubation with the different concentrations of CMF for 24 h at 37°C, non-migrating cells were removed with cotton swabs. Cells that migrated to the bottom of the membrane were fixed with methanol, stained with crystal violet (Aladdin Biotech, Shanghai, China) solution for 30 min, and washed twice with PBS (Dingguo Biotech, Beijing, China). Stained cells were visualized under a microscope (×10), and the numbers of cells counted in three random fields were averaged. To assess the effects of CMF on cell invasion, 2×10^5/mL cells with the different concentrations of CMF were added to upper chambers that had been coated with 30 μL of Matrigel (Corning, New York, USA). DMEM or RPMI-1640 medium containing 10% FBS was added to the lower chambers. Cells were incubated with different concentrations of CMF for 24 h at 37°C and non-invading cells were removed with cotton swabs. Invading cells were fixed, stained, and counted.

Tube formation assay

An assay for the formation of endothelial cell tubes in three-dimensional Matrigel cultures of HUVECs was used to assess angiogenic potential.[2] Matrigel (100 μL) containing 10 ng/mL VEGF with 10% FBS was added into 96-well culture plates. Cells (1×10^5/well) were seeded into these plates and treated with various concentrations of CMF for 6 h. Tube formation was examined in photographs under a microscope.

Rat aortic ring assay

The rat aortas isolated from Sprague-Dawley rats were cleaned of fibroadipose tissue and collateral vessels and cut into approximately 1-mm long rings.[2] The aortic rings were cultured in serum-free DMEM for 12 h, randomized and cultured in Matrigel with VEGF (100 ng/ml) coated in 6-wells, and sealed with a 50 μL overlay of Matrigel with VEGF (100 ng/ml). Medium (150 μL) containing VEGF (100 ng/ml) along with different concentrations of CMF was added. The preparations were incubated at 37°C under 5% CO_2 for 7 days. At the end of incubation, the sprouting microvessels were fixed and photographed. The numbers of sprouts were assessed semi-quantitatively by Image-Pro Plus Version.

Western blot analysis

Western blot assay was performed as described in the literature.[17] The SMMC-7721 cells and HUVECs (2×10^6 cells per well in 6-well plates) were treated with different concentrations of CMF for 24 h. The cultures were washed twice with PBS, the cells were collected and lysed with RIPA lysis buffer on ice for 10 min and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit

(Solarbio, Beijing, China). The tumor tissues were cut into small pieces and lysed with RIPA lysis buffer on ice for 10 min and the protein concentration was determined using a BCA protein assay kit. The proteins were loaded onto a 10% or 8% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Germany). The membranes were blocked in 5% BSA for 3 h and incubated overnight with primary antibodies in 4°C. Then, all of the membranes were subsequently incubated with HRP-linked goat anti-rabbit antibodies. The blots were washed with TBS-T and processed with an ECL detection kit (Tanon Crop, Shanghai, China).

Immunofluorescence

The SMMC-7721 cells or HUVECs (1×10^4 per well) were seeded into the wells and cultured at 37°C in a humidified incubator with 5% CO_2 for 24 h. The different concentrations of CMF were added to the wells and incubated at 37°C for 24 h. The cells were washed twice with PBS. Then, 4% paraformaldehyde was added and incubated for 20 min and finally adding 0.2% Triton X-100 for 5 min. Then, the cells were treated with 5% bovine serum albumin (Solarbio, Beijing, China) for 2 h. After that, p-VEGFR2 antibody was added to Confocal laser overnight at 4°C and then incubated with FITC goat anti-rabbit antibody (Invitrogen, Grand Island, NY, USA) for 2 h. Subsequently, the dishes were washed three times with PBS, incubated with DAPI for 5 min, and then, finally observed using a Leica TCS SP2 microscope (LeicaBiosystems Nussloch GmbH, Heidelberg, Germany).

Antitumor assay in nude mice

A nude mouse xenograft model was established by using 4–6-week-old male BALB/c nude mice. Mice were housed in sterilized cages at a constant temperature and humidity and fed a regular autoclaved chow diet with water *ad libitum*. After 4×10^6 SMMC-7721 cells were inoculated subcutaneously into the flanks of 24 nude mice, six nude mice were orally administered with CMF at a daily dose of 260 mg/kg, six mice were orally administered with CMF at a daily dose of 130 mg/kg, six nude mice were intraperitoneally administered with cyclophosphamide (CTX) at twice a week dose of 25 mg/kg and the last six mice were orally administered with sterile water. The tumor volumes were determined by measuring two dimensions, with tumor volume = length × width × width × 0.5. After 20 days of treatment, mice were sacrificed by cervical dislocation under anesthesia with ether and the tumor tissues were collected. Immunohistochemistry (IHC) analysis was conducted.

Statistical analyses

Data are expressed as mean ± standard deviation of the number of independent experiments shown. The significance of the differences between the groups was evaluated by Student's *t*-test and one-way analysis of variance test. $P < 0.05$ was considered statistically significant.

RESULTS

Cordyceps militaris fraction suppresses migration and invasion of human umbilical vein endothelial cells and SMMC-7721 cells

The process of migration and invasion of tumor cells generally refers to the single tumor cells falling off from the primary tumor and then migrating into the lymphatic vessels or blood vessels, with the blood flow reaching the distant organs and colonizing the organs.[19] The migration and invasion of endothelial cells and tumor cells play the important roles in tumor angiogenesis.[2,20] Therefore, Transwell migration and invasion assays were used to investigate the effect of CMF on migration and invasion of HUVECs and SMMC-7721 cells. The experimental results showed that the cells treated with CMF migrated significantly less than the untreated cells [Figure 1a]. Similarly, the invasion of HUVECs and SMMC-7721 cells were also suppressed by CMF with a concentration-dependent manner [Figure 1b].

Cordyceps militaris fraction inhibits vascular endothelial growth factor-induced tube formation of human umbilical vein endothelial cells *in vitro* and microvessel sprouting *ex vivo*

HUVECs can form a three-dimensional capillary structure spontaneously on Matrigel, so the tube formation experiment is a simple, reliable, and powerful assay to study antiangiogenesis.[21] To investigate whether CMF has the antiangiogenic capability, the tube formation of HUVECs was performed in the experiments. As shown in Figure 2, CMF disrupted the tube formation of HUVECs in a concentration-dependent manner. Due to further determine the antiangiogenic effect of CMF, the rat aortic rings were used in the experiment. The results showed that CMF significantly inhibited the formation of aortic ring capillaries and the inhibitory effect increased with the increase of CMF concentrations. When CMF concentrations were 2.5 and 5 μg/mL, the formation of aortic ring capillaries was almost inhibited completely [Figure 3a and b]. The above results indicated that CMF has the strong antiangiogenic activity.

Cordyceps militaris fraction exerts antiangiogenesis through vascular endothelial growth factor/vascular endothelial growth factor receptor 2 *in vitro*

To explore the anti-HCC angiogenesis mechanism of CMF *in vitro*, western blot analysis was used to detect the expression of proteins in the relevant signaling pathway. The VEGF/VEGFR2 signaling pathway plays an important role in tumor angiogenesis, so the expression of related proteins in this pathway was tested in HUVECs and SMMC-7721 cells. The result showed that CMF attenuated phosphorylation of VEGFR2 in HUVECs and SMMC-7721 cells [Figure 4a]. At the same time, phosphorylation of Akt and ERK in HUVECs and SMMC-7721 cells were also attenuated by CMF [Figure 4b and c].

Western blot assay demonstrated that CMF attenuated the phosphorylation of VEGFR2, followed by immunofluorescence experiments to further examine the effect of CMF on VEGFR2 phosphorylation. The result showed CMF significantly reduced VEGFR2 phosphorylation of HUVECs and SMMC-7721 cells [Figure 5a and b].

Cordyceps militaris fraction suppresses tumor growth in nude mice xenografted with SMMC-7721 cells

The above results indicate that CMF has an antiangiogenic effect *in vitro*. To study the antiangiogenic effect of CMF *in vivo*, a SMMC-7721 nude mouse xenograft model was established. As a result, compared with the model group, CMF significantly inhibited tumor growth and the tumor volume of the CMF group (260 mg/kg) was close to that of CTX positive control group [Figure 6a]. The same result was found in the weight of the tumor [Figure 6b and c]. CD31 is a hallmark protein for tumor angiogenesis, whereas the expression of CD31 indicates neovascularization in IHC experiments. In the study, IHC experiments were performed on the tumor tissues of nude mice to detect the expression of CD31. CD31 expression

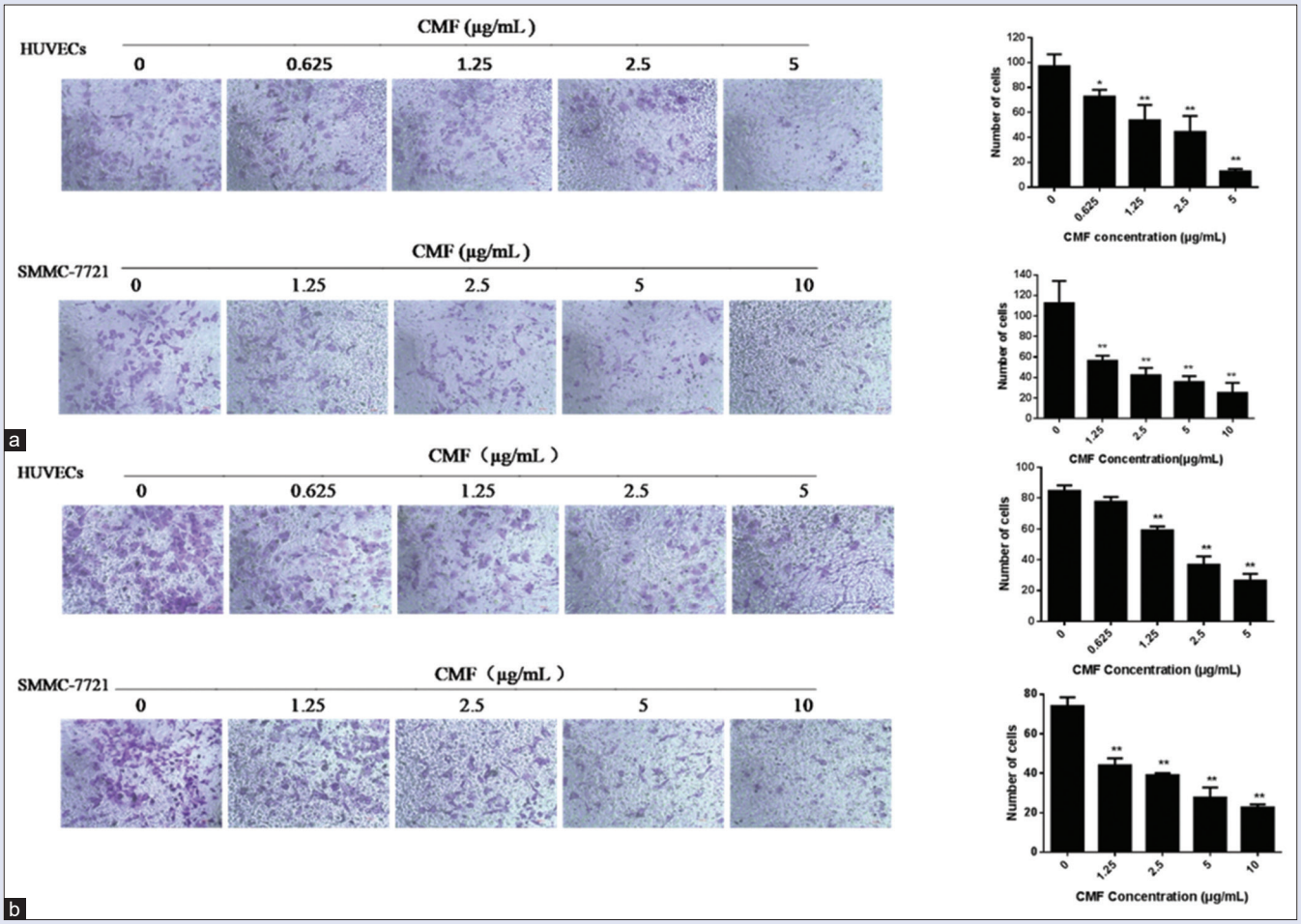

Figure 1: *Cordyceps militaris* fraction inhibits the migration and invasion of human umbilical vein endothelial cells and SMMC-7721 cells. (a) Images and numbers of migrating human umbilical vein endothelial cells and SMMC-7721 cells, and (b) Images and number of invading human umbilical vein endothelial cells and SMMC-7721 cells were assessed by Transwell assay. The cells were exposed to 0, 0.625, 1.25, 2.5, 5 or 0, 1.25, 2.5, 5, 10 μg/mL of *Cordyceps militaris* fraction for 24 h and then stained with crystal violet solution for 30 min. Stained cells were visualized under a microscope (10×) and the numbers of cells counted in three random fields were averaged. **P* < 0.05 and ***P* < 0.01 versus the non-*Cordyceps militaris* fraction group

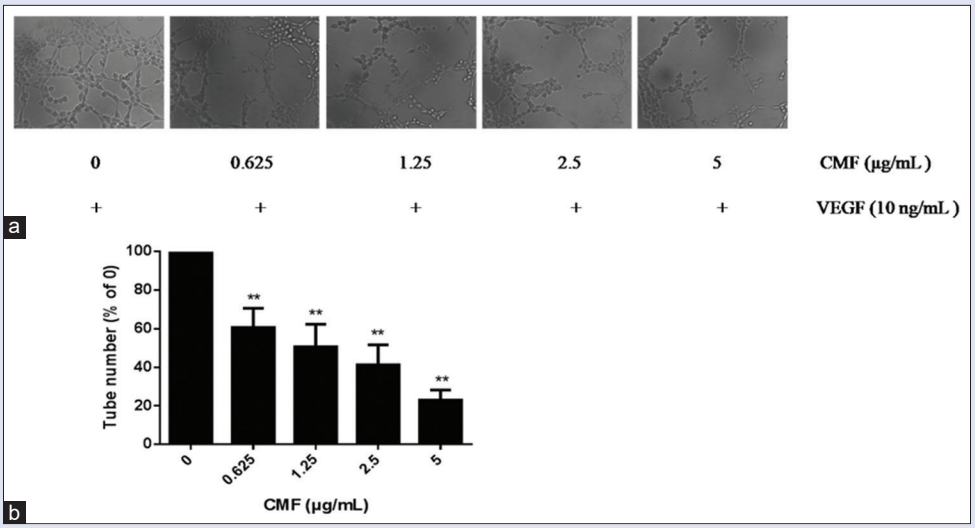

Figure 2: *Cordyceps militaris* fraction inhibits vascular endothelial growth factor-induced tube formation of human umbilical vein endothelial cells *in vitro*. (a) Tubular structures in human umbilical vein endothelial cells were photographed (10×). (b) Numbers of tube in human umbilical vein endothelial cells were measured. The cells were incubated with vascular endothelial growth factor (10 ng/mL) in the presence of 0, 0.625, 1.25, 2.5, or 5 μg/mL of *Cordyceps militaris* fraction for 6 h, and then, the cells were fixed. ***P* < 0.01 versus vascular endothelial growth factor -treated group

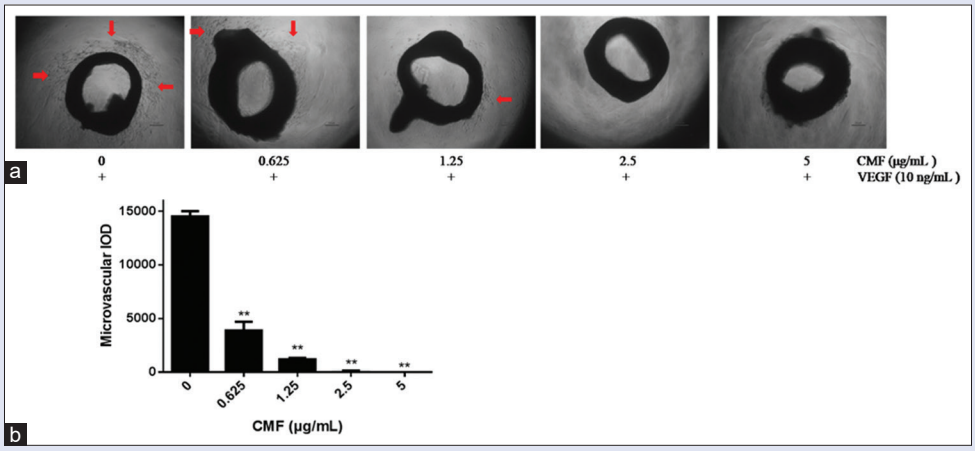

Figure 3: *Cordyceps militaris* fraction inhibits vascular endothelial growth factor-induced microvessel sprouting *ex vivo*. (a) Aortic rings were photographed (4×). Arrows pointed to microvascular sprouting of rat aortic rings. (b) Numbers of sprouts were assessed semi-quantitatively by Image-Pro plus Version (IOD integrated optical density). The aortas isolated from standard deviation rats were placed in the Matrigel-coated plates, overlayed with Matrigel, and then treated with vascular endothelial growth factor (10 ng/mL) in the presence of 0, 0.625, 1.25, 2.5, or 5 μg/mL of *Cordyceps militaris* fraction for 7 days. At the end of incubation, the sprouting microvessels were fixed. $^{**}P < 0.01$ versus vascular endothelial growth factor-treated group

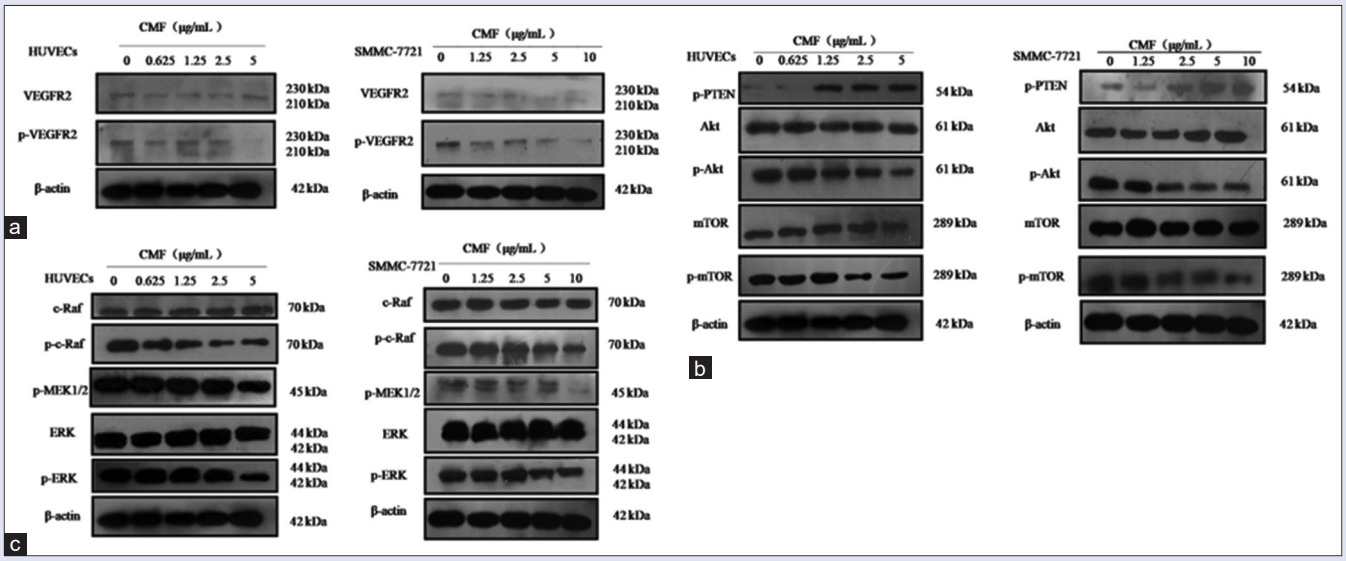

Figure 4: Effects of *Cordyceps militaris* fraction on expression of singling pathway-related proteins in human umbilical vein endothelial cells and SMMC-7721 cells. (a) vascular endothelial growth factor receptor 2/p-vascular endothelial growth factor receptor 2 in human umbilical vein endothelial cells and SMMC-7721 cells, (b) PI3K/Akt/mTOR in human umbilical vein endothelial cells and SMMC-7721 cells and (c) Raf/MEK/ERK in human umbilical vein endothelial cells and SMMC-7721 cells. Human umbilical vein endothelial cells and SMMC-7721 cells were exposed to 0, 0.625, 1.25, 2.5, 5 or 0, 1.25, 2.5, 5, 10 μg/mL of *Cordyceps militaris* fraction for 24 h and cell lysates were analyzed by Western blot analysis

was significantly reduced in the CMF and CTX groups compared with the model group [Figure 6d and e], indicating that CMF could reduce tumor angiogenesis. In addition, the expression of related proteins in tumor tissues was also detected to explore the mechanism of anti-HCC angiogenesis by CMF *in vivo*. The results demonstrated that CMF reduced the expression of VEGFR2 and CD31 and decreased the phosphorylation of Akt and ERK. The results above demonstrated that the anti-HCC angiogenesis mechanism [Figure 6f] of CMF *in vivo* was consistent with *in vitro* study.

DISCUSSION

Liver cancer is one of the five most common cancers in the world. It has maintained a sustained increase in morbidity and mortality. Clinical treatment methods include hepatectomy, liver transplantation, local ablation, hepatic artery embolization, and chemotherapy.[22] With the deepening of antitumor drug research, many oncogenes and signaling pathways have been identified to be closely related to tumorigenesis and development. Our results demonstrated that CMF could suppress the migration and invasion of SMMC-7721 cells and HUVECs inhibit the tube formation of HUVECs and the formation of rat aortic capillaries. Furthermore, CMF significantly inhibited tumor growth in nude mice xenografted with SMMC-7721 cells. The results of western blot analysis suggested that CMF inhibited the angiogenesis of HCC and their possible mechanisms are associated with suppression of the VEGF/VEGFR2 signaling pathway both *in vitro* and *in vivo*.

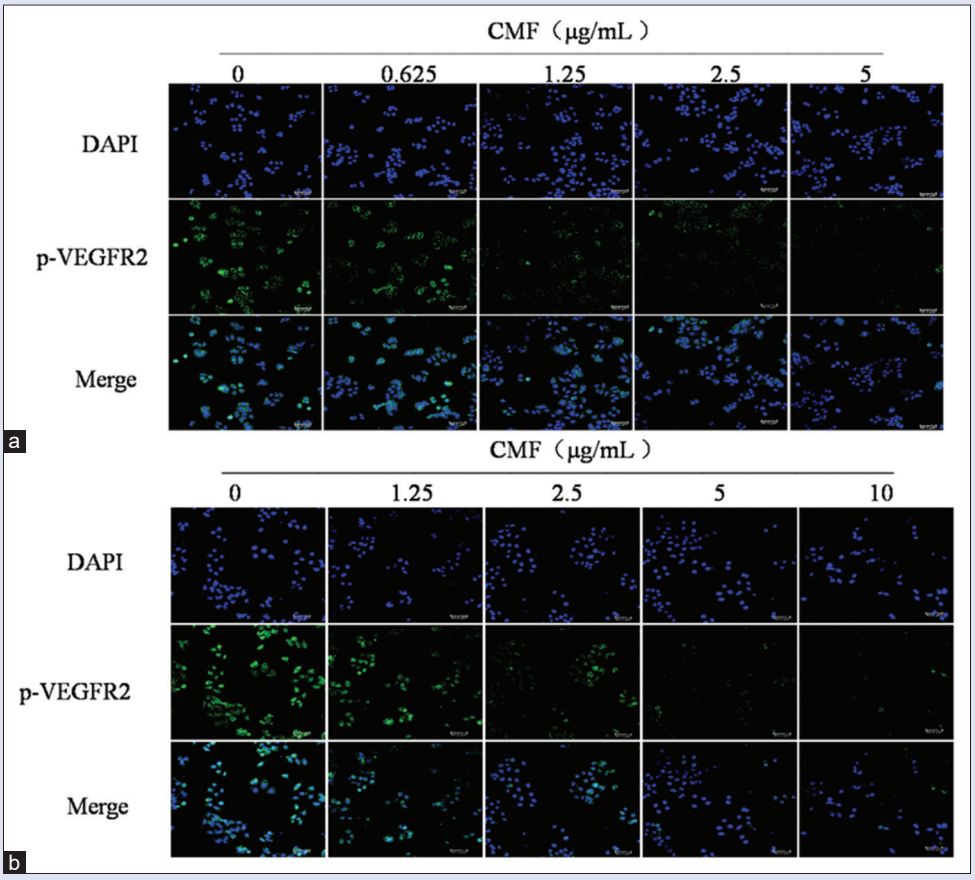

Figure 5: *Cordyceps militaris* fraction reduces phosphorylation of vascular endothelial growth factor receptor 2 in human umbilical vein endothelial cells (a) and SMMC-7721 (b) cells. Human umbilical vein endothelial cells and SMMC-7721 cells were exposed to *Cordyceps militaris* fraction for 24 h and the cells were fixed and immunostained with phosphovascular endothelial growth factor receptor 2 antibody at the end of incubation. Nuclei were stained with 4′,6-diamidino-2-phenylindole and were taken under ×40 objective by laser scanning confocal microscope

The VEGF family plays a crucial role in angiogenesis. VEGF can induce lymphangiogenesis, regulate physiological angiogenesis, and even participate in the development and progression of angiogenic diseases.[23] The lumen formation of HUVECs was often used in antiangiogenesis research, and the VEGF plays a crucial role in this process.[20] We found that VEGF could induce lumen formation of HUVECs and CMF could also inhibit the occurrence of this process. VEGF can induce the sprouting of rats aortic ring capillaries *ex vivo*.[24] Moreover, our experimental result demonstrated that CMF could inhibit VEGF-induced formation of aortic ring capillaries of rats.

The PI3K/Akt/mTOR signal transduction pathway plays an important role in regulating cell survival, proliferation, and migration. Inhibition of PI3K/Akt/mTOR signaling pathway induces apoptosis in liver cancer.[14,25] CMF could reduce phosphorylation of PI3K/Akt/mTOR in both SMMC-7721 cells and HUVECs. The expression levels of Akt and mTOR phosphorylation were reduced in SMMC-7721 cells and HUVECs after treated with CMF for 24 h. Akt, also known as protein kinase B, is a central node for growth signaling, cytokines, and other cellular signaling downstream of cells stimulation. Abnormal or altered activation of Akt is the basis for the pathophysiological properties of a variety of complex diseases, including type 2 diabetes and cancer. The activation of Akt also activates its downstream signaling pathway, which regulates cell survival, growth, proliferation, metabolism, migration, and invasion. In addition, Akt is also associated with tumor angiogenesis.[26] In contrast, the level of phosphorylated PTEN was increased after treated with CMF. As a unique tumor suppressor, PTEN negatively regulates cells and extracellular matrix interactions to maintain cell sensitivity to apoptosis. The cells will be protected from apoptosis without PTEN.[27]

The Ras/Raf/MEK/ERK signaling pathway is associated with the growth, proliferation, metastasis, and invasion of tumor cells. The inhibition of Ras/Raf/MEK/ERK signaling pathway can effectively inhibit the proliferation of hepatoma cells,[15,28] migration, and invasion.[29] Raf-1 has been shown to be a cross-linking point in various signaling pathways and plays an important role. Raf-1 overexpression can be considered as an independent prognostic biomarker in HCC and predicts early tumor recurrence and death in HCC patients. It can be used to stratify the risk of poor patient prognosis and to help the selection of the appropriate HCC treatment regimen.[30] The ERK1/2 MAP kinase plays a key role in the mitogenic signal transduction pathway and is an important component of the MAP kinase cascade, including MEK (MAP kinase kinase) and Raf-1. The ERK1/2 has been observed in almost half of known human tumor cell lines and a large number of human primary tumors.[31] When ERK1/2 is activated, it activates downstream signaling pathways to promote hepatoma cell metastasis and invasion.[32] CMF can attenuate ERK1/2 phosphorylation, and it suggested that antiangiogenic effect of CMF may also be related to the suppression of Ras/Raf/MEK/ERK signaling pathway.

VEGF binds to VEGFR and activates the downstream signal transduction cascade, including ERK and the PI3K/Akt/mTOR signaling pathway.[33] Moreover, VEGF-D induces PI3K-mediated Akt activation

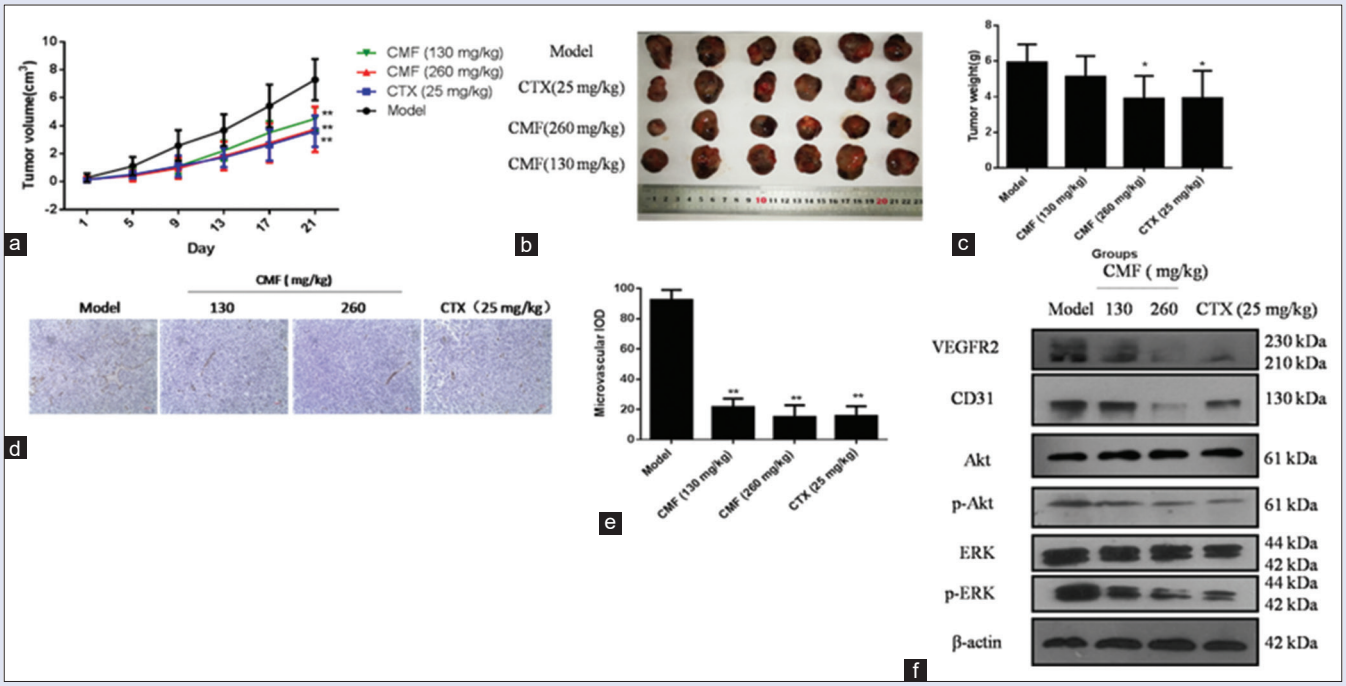

Figure 6: *Cordyceps militaris* fraction inhibits tumor growth in nude mice xenografted with SMMC-7721 cells. (a) Mouse tumor volumes were determined every 4 days during the dosing period. (b) Tumor tissues of nude mice were photographed. (c) Tumor weights of nude mice were measured with an electronic balance. (d) The level of CD31 was measured by IHC analysis (10×). Positive cells were stained brown and negative cells were stained blue.(e) Microvascular IOD was counted by Image-Pro plus Version.(f) Western blots were used to detect protein levels of vascular endothelial growth factor receptor 2, CD31, Akt/p-Akt, and ERK/p-ERK in tumor tissue sections. After 4 × 10^6 SMMC-7721 cells were inoculated subcutaneously into the flanks of 24 nude mice, the mice were dosed intragastrically with *Cordyceps militaris* fraction (130 and 260 mg/kg) daily or intraperitoneally with CTX twice a week for 20 days. Data are presented as means ± standard deviation **P* < 0.05 and ***P* < 0.01 versus the model group, *n* = 6

and stimulates angiogenesis.[34] VEGF could elevate phosphorylation VEGFR2 protein and promoted cholangiocarcinoma cell proliferation, migration, and invasion via downstream Raf/MEK/ERK and PI3K/AKT pathways.[35] Therefore, the antiangiogenic activity of CMF is associated with suppression of VEGF/VEGFR and its downstream signal transduction.

CONCLUSION

In the present study, the antiangiogenic properties and mechanism of CMF were investigated *in vitro* and *in vivo*. CMF inhibited migration and invasion of HUVECs and SMMC-7721 cells as well as tube formation of HUVECs and the formation of rat aortic capillaries. Antiangiogenic effect of CMF was also proved *in vivo* in nude mice xenografted with SMMC-7721 cells. Therefore, we could make a conclusion that CMF can inhibit the angiogenesis of HCC, and its possible mechanism is through the suppression of VEGF/VEGFR2-mediated PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways both *in vitro* and *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Feng RM, Zong YN, Cao SM, Xu RH. Current cancer situation in China: Good or bad news from the 2018 global cancer statistics? Cancer Commun (Lond) 2019;39:22.
2. Shi L, Yang F, Luo F, Liu Y, Zhang F, Zou M, *et al.* Evodiamine exerts anti-tumor effects against hepatocellular carcinoma through inhibiting β-catenin-mediated angiogenesis. Tumour Biol 2016;37:12791-803.
3. Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932-6.
4. Jeong JW, Jin CY, Park C, Hong SH, Kim GY, Jeong YK, *et al.* Induction of apoptosis by cordycepin via reactive oxygen species generation in human leukemia cells. Toxicol In Vitro 2011;25:817-24.
5. Das SK, Masuda M, Sakurai A, Sakakibara M. Medicinal uses of the mushroom *Cordyceps militaris*: Current state and prospects. Fitoterapia 2010;81:961-8.
6. Sato A, Yoshikawa N, Kubo E, Kakuda M, Nishiuchi A, Kimoto Y, *et al.* Inhibitory effect of cordycepin on experimental hepatic metastasis of B16-F0 mouse melanoma cells. *In Vivo* 2013;27:729-32.
7. Luo X, Duan Y, Yang W, Zhang H, Li C, Zhang J. Structural elucidation and immunostimulatory activity of polysaccharide isolated by subcritical water extraction from *Cordyceps militaris*. Carbohydr Polym 2017;157:794-802.
8. Xu GY, Yuan GX, An LP, Du PG, Xie LY, Li HY, *et al.* Immunomodulatory mechanism of *Cordyceps militaris* polypeptide through regulating gene Hist1h2bp, Ctsg and elane in mice. Pharmacogn Mag 2018;14:404-10.
9. Tran NKS, Kim GT, Lee DY, Kim YJ, Park HJ, Park DK, *et al.* Fermented *Cordyceps militaris* extract ameliorates hepatosteatosis via activation of fatty acid oxidation. J Med Food 2019;22:325-36.
10. Hsiao FS, Cheng YH, Wang SK, Yu YH. *Cordyceps militaris* hot water extract inhibits lipopolysaccharide-induced inflammatory response in porcine alveolar macrophages

by the regulation of mitogen-activated protein kinase signaling pathway. Can J Anim Sci 2018;98:44-52.

11. Rao YK, Fang SH, Wu WS, Tzeng YM. Constituents isolated from *Cordyceps militaris* suppress enhanced inflammatory mediator's production and human cancer cell proliferation. J Ethnopharmacol 2010;131:363-7.
12. Song J, Wang Y, Teng M, Zhang S, Yin M, Lu J, *et al. Cordyceps militaris* induces tumor cell death via the caspase-dependent mitochondrial pathway in HepG2 and MCF7 cells. Mol Med Rep 2016;13:5132-40.
13. Wang X, Xiu P, Wang F, Zhong J, Wei H, Xu Z, *et al.* P18 peptide, a functional fragment of pigment epithelial-derived factor, inhibits angiogenesis in hepatocellular carcinoma via modulating VEGF/VEGFR2 signalling pathway. Oncol Rep 2017;38:755-66.
14. Zhu H, Liu Q, Tang J, Xie Y, Xu X, Huang R, *et al.* Alpha1-ACT functions as a tumour suppressor in hepatocellular carcinoma by inhibiting the PI3K/AKT/mTOR signalling pathway via activation of PTEN. Cell Physiol Biochem 2017;41:2289-306.
15. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, *et al.* Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851-8.
16. Zhou Q, Zhang Z, Song L, Huang C, Cheng Q, Bi S, *et al. Cordyceps militaris* fraction inhibits the invasion and metastasis of lung cancer cells through the protein kinase B/glycogen synthase kinase 3β/β-catenin signaling pathway. Oncol Lett 2018;16:6930-9.
17. Xie W, Zhang Z, Song L, Huang C, Guo Z, Hu X, *et al. Cordyceps militaris* fraction induces apoptosis and G2/M arrest via c-Jun N-terminal kinase signaling pathway in oral squamous carcinoma KB cells. Pharmacogn Mag 2018;14:116-23.
18. Tian T, Song L, Zheng Q, Hu X, Yu R. Induction of apoptosis by *Cordyceps militaris* fraction in human chronic myeloid leukemia K562 cells involved with mitochondrial dysfunction. Pharmacogn Mag 2014;10:325-31.
19. Friedl P, Wolf K. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer 2003;3:362-74.
20. Wang F, Wang L, Li Y, Wang N, Wang Y, Cao Q, *et al.* PAC-1 and its derivative WF-210 inhibit angiogenesis by inhibiting VEGF/VEGFR pathway. Eur J Pharmacol 2018;821:29-38.
21. Siveen KS, Ahn KS, Ong TH, Shanmugam MK, Li F, Yap WN, *et al.* Y-tocotrienol inhibits angiogenesis-dependent growth of human hepatocellular carcinoma through abrogation of AKT/mTOR pathway in an orthotopic mouse model. Oncotarget 2014;5:1897-911.
22. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907-17.
23. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, *et al.* Vascular endothelial growth factor C induces angiogenesis *in vivo.* Proc Natl Acad Sci U S A 1998;95:14389-94.
24. Pang X, Zhang L, Wu Y, Lin L, Li J, Qu W, *et al.* Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate (CDODA-me), a derivative of glycyrrhetinic acid, functions as a potent angiogenesis inhibitor. J Pharmacol Exp Ther 2010;335:172-9.
25. Chen XL, Fu JP, Shi J, Wan P, Cao H, Tang ZM. CXC195 induces apoptosis and endoplastic reticulum stress in human hepatocellular carcinoma cells by inhibiting the PI3K/Akt/mTOR signaling pathway. Mol Med Rep 2015;12:8229-36.
26. Manning BD, Cantley LC. AKT/PKB signaling: Navigating downstream. Cell 2007;129:1261-74.
27. Tamura M, Gu J, Tran H, Yamada KM. PTEN gene and integrin signaling in cancer. J Natl Cancer Inst 1999;91:1820-8.
28. Wang Z, Luo S, Wan Z, Chen C, Zhang X, Li B, *et al.* Glabridin arrests cell cycle and inhibits proliferation of hepatocellular carcinoma by suppressing braf/MEK signaling pathway. Tumour Biol 2016;37:5837-46.
29. Guo C, Zhao D, Zhang Q, Liu S, Sun MZ. MiR-429 suppresses tumor migration and invasion by targeting CRKL in hepatocellular carcinoma via inhibiting raf/MEK/ERK pathway and epithelial-mesenchymal transition. Sci Rep 2018;8:2375.
30. Chen L, Shi Y, Jiang CY, Wei LX, Wang YL, Dai GH. Expression and prognostic role of pan-ras, raf-1, pMEK1 and pERK1/2 in patients with hepatocellular carcinoma. Eur J Surg Oncol 2011;37:513-20.
31. Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, *et al.* Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 1999;18:813-22.
32. Chen K, Zhang S, Ji Y, Li J, An P, Ren H, *et al.* Baicalein inhibits the invasion and metastatic capabilities of hepatocellular carcinoma cells via down-regulation of the ERK pathway. PLoS One 2013;8:e72927.
33. Tchaikovski V, Fellbrich G, Waltenberger J. The molecular basis of VEGFR-1 signal transduction pathways in primary human monocytes. Arterioscler Thromb Vasc Biol 2008;28:322-8.
34. Jia H, Bagherzadeh A, Bicknell R, Duchen MR, Liu D, Zachary I. Vascular endothelial growth factor (VEGF)-D and VEGF-A differentially regulate KDR-mediated signaling and biological function in vascular endothelial cells. J Biol Chem 2004;279:36148-57.
35. Huang M, Huang B, Li G, Zeng S. Apatinib affect VEGF-mediated cell proliferation, migration, invasion via blocking VEGFR2/RAF/MEK/ERK and PI3K/AKT pathways in cholangiocarcinoma cell. BMC Gastroenterol 2018;18:169.