Inhibitory effects of *Physalis angulata* on tumor metastasis and angiogenesis


a Department of Cosmeceutics, College of Pharmacy, China Medical University, Taichung, Taiwan
b School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan
c Department of Biomedical Science and Environmental Biology, Graduate Institute of Natural Products, Center of Excellence for Environmental Medicine, Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
d Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan
e School of Pharmacy, China Medical University, Taichung, Taiwan
f Department of Pharmacology, China Medical University, Taichung, Taiwan
g Department of Biological Science and Technology, China Medical University, Taichung, Taiwan
h Department of Fragrance and Cosmetic Science, Kaohsiung Medical University, Kaohsiung, Taiwan
i Institute of Nutrition, China Medical University, Taichung, Taiwan

**A B S T R A C T**

**Ethnopharmacological relevance:** *Physalis angulata* is well-known in traditional Chinese medicine as a ingredient for various herbal formulation; also, it has been shown to exhibit anti-cancer and anti-inflammatory effects. In this study, the ability of *P. angulata* to inhibit tumor metastasis and angiogenesis was investigated.

**Materials and methods:** Anti-proliferative activity of ethyl acetate extracts of *P. angulata* (PA extracts), was determined against human oral squamous carcinoma (HSC-3) and human umbilical vein endothelial cells (HUVECs) by trypan blue exclusion method. Wound-healing migration, trans-well invasion, Western blotting and chick chorioallantoic membrane assay were carried out to determine the anti-metastatic and anti-angiogenic effects of PA extracts in vitro and in vivo.

**Results:** We demonstrated that at sub-cytotoxic concentrations of PA extracts (5–15 μg/mL) markedly inhibited the migration and invasion of highly metastatic HSC-3 cells as shown by wound-healing repair assay and trans-well assay. Gelatin zymography assay showed that PA extracts suppressed the activity of matrix metalloproteinase (MMP)-9 and -2, and urokinase plasminogen activator (u-PA) in HSC-3 cells. In addition, Western blot analysis confirmed that PA extracts significantly decreased MMP-2 and u-PA protein expression in HSC-3 cells. Notably, PA extracts significantly augmented the expression of their endogenous inhibitors, including tissue inhibitors of MMP (TIMP-1 and -2), and plasminogen activator inhibitors (PAI-1 and -2). Further investigations revealed that non-cytotoxic concentration of PA extracts (5–15 μg/mL) inhibited vascular endothelial growth factor (VEGF)-induced proliferation, and migration/invasion of HUVECs in vitro. PA extracts also suppressed the activity of MMP-9, but not MMP-2, in HUVECs. Further, we observed, PA extracts strongly suppressed neovessel formation in the chorioallantoic membrane of chick embryos in vivo.

**Conclusions:** These results strongly support an anti-metastatic and anti-angiogenic activity of *P. angulata* that may contribute to the development of better chemopreventive agent for cancer and inflammation.

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1. Introduction

According to the world cancer report from the international agency for research on cancer (IARC-WHO) categorized that oral cancer is the eighth most common cancer in world wide (Petersen, 2005). World cancer report also classified that betel quid/areca nut (*Areca catechu*) chewing with or without tobacco as a human carcinogen and the oral cancer incidence is most common in parts of the world where betel quid/areca nut chewing as a social or cultural activities (IARC, 1985; Priebe et al., 2008). Chewing of betel quid/areca nut has been shown to be associated with the high incidence of oral cancer that observed in Taiwan (Ko et al., 1992). Since there are two million people who have betel quid chewing habit in Taiwan alone and approximately more than 80% of oral cancer...
Cancer metastasis, the spread of tumor cells from the primary neoplasm to distant sites and their growth there, is the most common cause of death in cancer patients (Yang et al., 2007). Metastasis of tumor cells involves multiple processes and various cytophysiological changes, including changes in adhesion between cells and the extracellular matrix that leads to abnormal intercellular interactions (Hseu et al., 2009). Thus, the degradation of ECM and components of the basement membrane, caused by the concerted action of proteinases, such as PA and MMPs, plays a critical role in tumor invasion and metastasis (Hseu et al., 2009; Kessenbrock et al., 2010). Among these enzymes, MMP-9, MMP-2, and u-PA that degrade the basal membrane components and are known to be deeply involved in cancer metastasis (Yang et al., 2005; Hseu et al., 2009). Therefore, the inhibition of migration/invasion mediated by MMPs and/or u-PA could be a potential method to prevent or inhibit cancer metastasis (Bjorklund and Koivunen, 2005).

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is an essential process in a variety of physiological and pathological conditions, including wound-healing, embryonic development, chronic inflammation, cancer and metastasis (Risau, 1997; Folkman, 2002). Angiogenesis occurs by complex sequential steps, such as basement membrane degradation by proteinases, endothelial cell proliferation and migration/invasion, formation of capillary tubes and survival of newly formed blood vessels (Bussolino et al., 1997; Hseu et al., 2010). Since the tumor epithelial cells in vivo depend on angiogenesis to provide nutrients for their newly proliferated cell growth and survival; it is plausible that the agent possess an anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity (Felmeden et al., 2003; Hseu et al., 2010). Angiogenesis is tightly regulated by an intricate balance between stimulators and inhibitors (Bussolino et al., 1997). Among them, VEGF, a soluble angiogenic factor produced by many tumors as well as normal cell lines, plays a key role in regulating normal and pathologic angiogenesis (Inser and Asahara, 1999; Ferrara, 2004). Moreover, VEGF a potent mitogen for endothelial cells that inducing endothelial cell proliferation, migration/invasion, as well as subsequent organization of cells to form a capillary tube (Kwak et al., 2006). In particular, a number of studies have shown that VEGF is the most important angiogenic factor closely associated with neovascularization in human tumors. The level of VEGF is an important prognostic marker of tumor angiogenesis (Ferrara, 2004; Podar and Anderson, 2005). Therefore, therapeutic strategies have been developed that target various aspects of the anti-angiogenic processes, with promising results, because of the critical dependence of tumor growth and metastasis on angiogenesis.

Many medicinal herbs have been shown to be rich sources of phytochemicals with chemoprevention potential for various types of human cancer and inflammatory diseases. Metastasis and angiogenesis have been a major challenge for the successful treatment of cancer and inflammatory diseases. To date, the inhibition of metastasis and angiogenesis by P. angulata is still limited to a few discoveries. Our previous study has revealed that P. angulata induced apoptosis through the involvement of G2/M phase growth arrest in human oral squamous carcinoma HSC-3 cells (Hsieh et al., 2006; Lee et al., 2009). Therefore, the ability of the ethyl acetate extracts of P. angulata (PA extracts) to inhibit metastasis in HSC-3 cells and to suppress VEGF-induced angiogenesis in human umbilical vein endothelial cells (HUVECs) in vitro, and neo-angiogenesis of the chick chorioallantoic membrane (CAM) in vivo was investigated. The levels of metastatic or angiogenic control and related molecules were assayed to determine the PA extracts mediated anti-metastatic and anti-angiogenic mechanism.

2. Materials and methods

2.1. Chemicals

Dulbecco’s modified Eagle’s Medium (DMEM), M-199 medium, fetal bovine serum (FBS), glutamine and penicillin/streptomycin/neomycin were obtained from GIBCO BRL (Grand Island, NY, USA). Antibodies against MMP-9, MMP-2, u-PA, TIMP-1, TIMP-2, PAI-1, PAI-2 and VEGF were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Antibody against β-actin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available, and supplied either by Merck (Darmstadt, Germany) or Sigma.

2.2. Preparation of PA extracts

The whole plant of P. angulata were collected in Taichung County, Taiwan and identified by Prof. Chao-Lin Kou. A voucher specimen (CMU PA 08012) was stored in the School of Chinese Pharmaceutical Science and Chinese Medicine Resources, China Medical University, Taichung, Taiwan (Lee et al., 2009). Air-dried whole plants of P. angulata were extracted with methanol at room temperature for 3 days. The crude P. angulata extracts were then filtered, collected, and concentrated under reduced pressure at 40 °C. The extracts of P. angulata was subsequently dissolved in water, and separated by n-hexane, ethyl acetate and butanol as described previously (Lee et al., 2009). The ethyl acetate extracts of P. angulata (PA extracts) were concentrated in a vacuum and freeze dried to form powder. For preparation of the stock solution, the powder samples (10 mg) were dissolved in 0.1% DMSO at 25 °C. The stock solution was stored at −20 °C before analysis for anti-metastatic and anti-angiogenic properties.

2.3. Cell culture and assessment of cell viability

The human oral squamous cell carcinoma HSC-3 cell line was kindly provided by Prof. Jing-Gung Chung, Department of Biologi- cal Science and Technology, China Medical University. HSC-3 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1% penicillin–streptomycin–neomycin in a humidified incubator (5% CO₂ in air at 37 °C). HUVECs were prepared from human umbilical veins as described previously (Hseu et al., 2008). In brief, the umbilical cord was infused with 0.05% collagenase solution containing the ECs and flushed from the cord by perfusion with cord buffer and centrifuged. The resulting cell suspension was divided equally between several 10-cm Petri dishes, and grown to confluence in M-199 medium and supplemented with...
20% FBS at 37 °C in 5% CO₂. Upon confluence, the cells of primary cultures were detached using trypsin–EDTA, and sub-cultured to confluence in tissue culture wells at 37 °C. All experiments were carried out using HUVECs after only one passage, and at least 4 days after passage. Cultures were harvested and the cell number was determined using a hemocytometer. Cell viability (3.0 × 10⁵ cells/12-well dish of HSC-3 cells, and 4.0 × 10⁵ cells/12-well dish of HUVEC cells) before and after treatment with PA extracts was checked by phase-contrast microscopy using trypan blue exclusion method. For all experiments, HSC-3 cells were incubated with various concentrations of PA extracts (0–20 μg/mL) for 12–24 h and HUVECs (4 × 10⁵ cells/well) were pre-incubated with various concentrations of PA extracts (0–20 μg/mL) for 1 h followed by incubation with or without VEGF (20 ng/mL) for 24 h.

2.4. In vitro wound-healing repair assay

To determine the effects of PA extracts on cell migration, an in vitro wound-healing repair assay was performed. For the cell migration assay, HSC-3 cells (3 × 10⁵ cells/well) or HUVECs (4 × 10⁵ cells/well) were seeded into 12-well culture dishes and grown in medium containing 10% or 20% FBS to a nearly confluent cell monolayer. At confluence, monolayers were wounded using a 200 μL micropipette tip, washed twice with PBS, and incubated with various concentrations of PA extracts (5–15 μg/mL) in 1% FBS medium with or without VEGF (20 ng/mL) for 12–24 h. Next, cells were washed twice with PBS, then fixed in 100% methanol and stained with Giemsa stain solution. The cultures were photographed (200× magnification) to monitor the migration of cells into the wounded area and the closure of wounded area was calculated.

2.5. Cell invasion assay

Invasion assays were performed using BD Matrigel™ invasion chambers (Bedford, MA, USA). Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Briefly, for the invasion assay, 10 μL Matrigel (0.5 mg/mL) was applied to 8 μm polycarbonate membrane filters, and the bottom chamber of the apparatus contained standard medium (750 μL) with 10% or 20% FBS. Top chambers were seeded with 1 × 10⁵ cells in 500 μL serum-free medium, and the cells were incubated with various concentrations of PA extracts (5–15 μg/mL) with or without VEGF (20 ng/mL). Cells were allowed to migrate for 24 h at 37 °C. After the incubation, the non-migrated cells on the top surface of the membrane were removed with a cotton swab. The migrated cells on the bottom side of the membrane were fixed in cold 75% methanol for 15 min and washed three times with PBS. Further, cells were stained with Giemsa stain solution and then stained with Giemsa stain solution. The cultures were photographed (200× magnification) to quantitate the number of blood vessel branch points.

2.6. Gelatin zymography assay

The activities of MMP-9, MMP-2, or u-PA in the medium released from the cells were measured by gelatin zymography protease assays. HSC-3 cells (3 × 10⁵ cells/well) or HUVECs (4 × 10⁵ cells/well) were seeded into 12-well culture dishes and grown in medium with 10% or 20% FBS to a nearly confluent monolayer. The cells were resuspended in 1% FBS medium, and then incubated with various concentrations of PA extracts and with or without VEGF (20 ng/mL) for 24 h. After treatment, the collected medium were adjusted of an appropriate volume (by vital cell number) were prepared with SDS sample buffer without boiling or reduction, and 1 mg/mL gelatin and casein for MMPs and for uPA, respectively, was added and subjected to 8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in the developing buffer (50 mM Tris-base, 200 mM NaCl, 5 mM CaCl₂ and 0.02% Brij 35) at 37 °C for 24 h. Then, the gels were stained with Coomassie brilliant blue R-250. The relative MMPs and u-PA activities were quantified by Matrix Inspector 2.1 software.

2.7. Preparation of total cell extract and immunoblot analysis

HSC-3 cells (7.5 × 10⁵ cells/10 mm dish) were incubated with various concentrations of PA extracts (5–15 μg/mL) for 24 h. Cells were washed once in PBS and detached. The collected cells were then suspended in 100 μL lysis buffer (10 mM Tris–HCl, pH 8, 320 mM sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM PMSF). Suspensions were kept on ice for 20 min, and then centrifuged at 13,000 × g for 30 min at 4 °C. Total protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) using BSA as a standard. Protein extracts were reconstituted in sample buffer (62 mM Tris–HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and the mixture was boiled at 97 °C for 5 min. Equal amounts (50 μg) of denatured protein samples were loaded into each lane, separated by 8–15% SDS-PAGE and then transferred into PVDF membranes overnight. Membranes were blocked with 5% non-fat dried milk in PBS, which contains 1% Tween-20 for 1 h at room temperature followed by incubated with primary antibodies (MMP-9, MMP-2, u-PA, TIMP-1, TIMP-2, PAI-1, PAI-2, and VEGF) for 2 h, and either horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies for overnight. The blots were detected by ImageQuant™ LAS 4000 mini (Fujifilm) with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, IL, USA).

2.8. VEGF release assay

To determine the effects of PA extracts on VEGF levels, the HSC-3 cells grown to 85% confluence were treated with 0–15 μg/mL of PA extracts for 24 h. Then, the medium was aspirated from the flasks and centrifuged at 500 × g (10 min) to remove cells from the medium. The level of VEGF released into the incubation medium was estimated using an ELISA kit (Chemicon International Inc., Temecula, CA).

2.9. In vivo chick chorioallantoic membrane (CAM) assay

For the CAM assay, fertilized chick embryos were pre-incubated for 8 days at 37.5 °C in 85% humidity. A hole was drilled over the air sac at the end of the egg and an avascular zone was identified on the CAM. A 1 × 1 cm window in the shell was sectioned to expose the CAM. Filter-paper disks were sterilized and loaded with 0, 10, or 20 μg/mL of PA extracts and the disks were applied to the CAM surface. Windows were sealed with clear tape and eggs were incubated for 48 h. Blood vessels were viewed and photographed. Anti-angiogenic effect of PA extracts on CAMs was quantified by counting the number of blood vessel branch points.

2.10. Statistical analyses

Results are presented as mean ± SD. All study data were analyzed using analysis of variance, followed by Dunnett’s test for pair-wise comparison. Statistical significance was defined as p < 0.05 for all tests.

3. Results

In this study, HSC-3 human oral epidermoid carcinoma cells and human umbilical vein endothelial cells (HUVECs) were used to
investigate the capability of PA extracts to inhibit metastasis and angiogenesis, and to elaborate the molecular mechanisms involved.

3.1. Effect of PA extracts on HSC-3 cell viability

To investigate the potential effects of PA extracts on HSC-3 cell viability, HSC-3 cells were exposed to 5–20 μg/mL of PA extracts for 12–24 h. Result shows that at 12 h, concentrations of 5–15 μg/mL did not affect the number of cells; however, 20 μg/mL proved to be cytotoxic (p < 0.05). Up to the concentrations of 10 μg/mL of PA extracts for 24 h were non-cytotoxic to HSC-3 cells, whereas, sub-cytotoxic concentrations of PA extracts (10–20 μg/mL) significantly (p < 0.05) reduced cell viability as evidence of cytotoxicity (Fig. 1). This data suggested that non- or sub-cytotoxic concentrations (5–15 μg/mL) of PA extracts could be used to evaluate its anti-metastatic properties in HSC-3 cells.

3.2. PA extracts inhibit in vitro migration/invasion of HSC-3 cells

To determine the effects of PA extracts on in vitro HSC-3 cell migration, confluent monolayers of HSC-3 cells were scraped to remove a section of monolayer and cultured for 12–24 h with con-
control buffer plus various concentrations of PA extracts (5–15 μg/mL).

Since, the wound-healing assay is a classical and commonly subjecting method for studying cell migration and the biology underlying it (Yarrow et al., 2004). Fig. 2A showed that PA extracts significantly (p < 0.05) decreased migration of HSC-3 cells in a dose- and time-dependent manner. In addition, matrigel trans-well assay has been used by various groups to determine the invasive activity of tumor cells across the basement membrane (Simeone et al., 2008). Therefore, a trans-well assay was used to investigate the invasion potential of HSC-3 cells for 12–24 h after treatment with 5–15 μg/mL PA extracts. According to the result, we observed PA extracts significantly (p < 0.05) decreased the invasion potential of HSC-3 cells in a dose- and time-dependent manner (Fig. 2B).

3.3. PA extracts mediate the down-regulation of MMP-9, MMP-2, and u-PA activity in HSC-3 cells

MMPs and u-PA, which are involved in the degradation of the basement membrane, are essential to the invasive process (Yang et al., 2011). To examine the effect of PA extracts on MMPs and u-PA, HSC-3 cells were treated with PA extracts (5–15 μg/mL) for 24 h. The supernatant was collected and examined for MMP-9, MMP-2, and u-PA activity using gelatin zymography assays. As shown in Fig. 3A and B, treatment with PA extracts significantly (p < 0.05) decreased the MMP-9, MMP-2, and u-PA activity in HSC-3 cells in a dose-dependent manner.

3.4. PA extracts decrease the protein levels of MMP-2 and u-PA protein expression in HSC-3 cells

Western blot analysis was performed to monitor the effect of PA extracts on MMPs and u-PA protein expression in HSC-3 cells. As shown in Fig. 4A, cells treated with PA extracts (5–15 μg/mL) for 24 h remarkably reduced MMP-2 and u-PA protein expression in a dose-dependent manner. However, the experimental treatment did not seem to change the amount of detectable MMP-9 protein expression in HSC-3 cells (Fig. 4A).

3.5. PA extracts increase the protein levels of TIMP-1, TIMP-2, PAI-1, and PAI-2 in HSC-3 cells

Physiological activity of MMPs and u-PA were greatly related to that of TIMPs and PAIs, respectively, the specific endogenous inhibitors of MMPs and u-PA (Yang et al., 2011). Therefore, we
Fig. 5. PA extracts reduced VEGF release (A) and protein expression (B) in HSC-3 cells. Cells were treated with 0–15 µg/mL of PA extracts for 24 h. Concentration of VEGF released into the medium was determined by ELISA. Proteins (50 µg) from each sample were resolved on 15% SDS-PAGE, and Western blot was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Results are presented as mean ± SD of three assays. (*) Significant difference in comparison to the control group (p < 0.05).

3.6. PA extracts inhibit VEGF expression in HSC-3 cells

As shown in Fig. 5A, HSC-3 cells without PA extracts treatment released detectable levels of VEGF into the serum-free media at approximately 130 pg/10⁵ HSC-3 cells. Treatment of cells with PA extracts (5–15 µg/mL for 24 h) resulted in a significant (p < 0.05), dose-dependent decrease in the release of VEGF (Fig. 5A). Further, we performed Western blotting to confirm the effect of PA extracts (5–15 µg/mL for 24 h) on VEGF protein expression in HSC-3 cells. As shown in Fig. 5B, PA extracts significantly down-regulated VEGF protein expression in HSC-3 cells.

3.7. Inhibitory effect of PA extracts on VEGF-induced proliferation of HUVECs

Angiogenesis involves the local proliferation of endothelial cells (Wang et al., 2007) and VEGF, a growth factor that stimulates HUVECs proliferation (Izuta et al., 2009). Therefore, we initially monitored the effect of PA extracts on VEGF-induced HUVECs proliferation. HUVECs were incubated with VEGF (20 ng/mL) in the presence or absence of PA extracts for 24 h. We observed, cells treated with VEGF alone, the proliferation of HUVECs was remarkably increased (147%) as demonstrated by trypan blue exclusion (Fig. 6A). PA extracts, significantly (p < 0.05) suppressed this VEGF-induced HUVECs proliferation in a dose-dependent manner (Fig. 6A). We also found that up to the concentration of 15 µg/mL of PA extracts were not cytotoxic to HUVECs without VEGF stimulation, whereas, over the concentration of 15 µg/mL showed cytotoxic to HUVECs (Fig. 6B). No distinct cellular or morphological changes that are typically associated with apoptosis, such as cell detachment, rounding or chromosomal fragmentation, were detected after a 24 h incubation with PA extracts at a concentration below 15 µg/mL, respectively. Therefore, for all subsequent experiments, we employed the non-cytotoxic concentration of PA extracts (i.e., ≤15 µg/mL), and focused on the effect of PA extracts on VEGF-induced angiogenesis.

3.8. PA extracts inhibit migration/invasion of HUVECs

The migration of endothelial cells through the basement membrane is a crucial step for the establishment of new blood vessels (Bussolino et al., 1997). To determine the effects of PA extracts on endothelial cell migration in vitro, confluent monolayers of HUVECs were wounded and cultured with control buffer plus 5–15 µg/mL PA extracts. We found that treatment with VEGF significantly (p < 0.05) induced the migration of HUVECs and the addition of PA extracts significantly (p < 0.05) decreased VEGF-induced migration of HUVECs (Fig. 7A, Next, the effect of PA extracts on the invasiveness of HUVECs was evaluated using the trans-well assay, which allowed us to determine the ability of cells to pass through a layer of extracellular matrix on to a Matrigel-coated filter. As shown in Fig. 7B, VEGF significantly induced the invasiveness of HUVECs. However, addition of
PA extracts (5–15 μg/mL) significantly (p < 0.05) decreased VEGF-induced invasion of HUVECs.

3.9. PA extracts reduce MMP-9 activity in HUVECs

Since gelatinase and collagenase MMPs are also involved in the angiogenic process (Egeblad and Werb, 2002; Kim et al., 2010). Therefore, we examined the effect of exposure to PA extracts on VEGF-induced gelatinase MMPs, HUVECs were treated with PA extracts (5–15 μg/mL) for 24 h in serum-free medium. The conditioned medium was collected and examined for MMP-9 and MMP-2 activity using gelatin zymography assays. Results from the zymography assay revealed that HUVECs constitutively secreted MMP-9 and MMP-2 (Fig. 8). Moreover, VEGF treatment significantly (p < 0.05) increased the level of MMP-9 and MMP-2 secretion in HUVECs (Fig. 8). However, treatment with PA extracts dose-dependently inhibited MMP-9 secretion in VEGF-induced HUVECs, whereas, PA extracts do not altered VEGF-induced MMP-2 level in HUVECs (Fig. 8).

3.10. PA extracts inhibit in vivo angiogenesis

To determine if treatment with PA extracts could suppress blood vessel formation in vivo, we employed an in vivo angiogenesis model, the chick chorioallantoic membrane (CAM) assay. As shown in Fig. 9A and B, a filter-paper coverslip containing PA extracts (10–20 μg/mL) for 48 h effectively (p < 0.05) inhibited the formation of capillary vessels in the CAM compared with the coverslip
Fig. 8. Effect of PA extracts on the activities of MMP-9 and MMP-2, respectively, in VEGF-stimulated HUVECs. (A) Cells were treated with 0–15 μg/mL of PA extracts or 1 h and then stimulated with VEGF (20 ng/mL) for 24 h. The conditioned media and cells were subjected to gelatin zymography to analyze the activities of MMP-9 and MMP-2. (B) Determined activities of these proteins were subsequently quantified by densitometric analysis. Results are presented as mean ± SD of three assays. (*, #) Indicates significant difference in comparison to control or the VEGF alone group (p < 0.05).

containing 0.1% DMSO alone, which had no visible effect on the preexisting blood vessels. The results indicate that PA extracts are capable of inhibiting neovessel formation in vivo under natural conditions.

4. Discussion

From Ancient times to the modern day herbs have been used for aid health and cure diseases. Especially in traditional Chinese medicine, herbs play a vital role for their great treasure. In the present study, we demonstrated that PA extracts prepared from the whole plant of *P. angulata* could significantly inhibit the metastasis (migration/invasion) ability of HSC-3 cancer cells. This study also revealed that PA extracts potentially inhibit the proliferation, and migration/invasion, an in vitro marker of angiogenesis, by VEGF-activated HUVECs. Moreover, we show that PA extracts exert a potent anti-angiogenic effect in vivo, as verified from its inhibition of naturally induced neovascularization by capillary vessel formation in the CAM. According to our literature search, this is the first scientific report examining the inhibitory effects of *P. angulata* on oral cancer metastasis and vascular endothelial angiogenesis activated by growth factor VEGF, and these observations confirm and extend the anti-metastatic and anti-angiogenic action of *P. angulata*.

The most important indicator of the prognosis of oral squamous cell carcinoma is metastasis to cervical lymph nodes or distant organs (Kudo et al., 2003). Furthermore, nearly 50% of oral squamous cell carcinoma patients present with pathological or clinical evidence of nodal metastases, and the 5-year survival rate are less than 50% for patients with lymph node metastasis (Chen et al., 2006). Thus, we examined HSC-3 cell migration using wound-healing repair assay. Results from the wound-healing assay, we observed that PA extract significantly inhibits HSC-3 cell migration. In other hand, measurement of the inhibition of cancer cell invasion using Boyden-chamber matrigel invasion assay is a widely accepted in vitro assay for screening compound that can inhibit metastasis (Stetler-Stevenson et al., 1993). Invasion assay also strongly supports that PA extracts exerted HSC-3 metastasis in vitro.

To further explore this mechanism, the levels of metastatic control and associated molecules were assayed. It is well described that MMPs form a family of highly homologous, zinc- and calcium-dependent endopeptidases plays a crucial role for the activation of gelatinase A followed by the degradation of gelatin and collagen extracellular matrix, which eventually leads tumor metastasis, including migration and invasion (Polette and Birembaut, 1998). It is noteworthy that PA extract significantly inhibits MMP-2 (gelatinase A) not MMP-9 (gelatinase B) in HSC-3 cells. In addition, PA extracts also significantly inhibits u-PA expression in HSC-3 cells, which is an upstream activator of MMPs plays a crucial role during metastases (Rabbani and Xing, 1998). Therefore, the inhibition of migration/invasion mediated by the suppression of MMP-2 and/or uPA could play a key feature in the prevention of cancer metastasis. Physiological activities of MMPs and uPA are regulated by their specific endogenous inhibitors, TIMPs and PAIs, respectively (Yang et al., 2011). Tissue inhibitors of MMPs such as TIMP-1, TIMP-2, TIMP-3 and TIMP-4 play a key role in determining the proteolytic activity of tumor tissues by regulating the activity of MMPs (Ramer et al., 2007). In addition, PAIs (serine protease inhibitors) regulate urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) to control plasmin generation (Ohki et al., 2010). This study revealed that PA extracts not only inhibits the activity of MMP-9, MMP-2 and u-PA, but also down-regulate the

Fig. 9. PA extracts inhibited angiogenesis in vivo. A filter-paper disk with 0, 10, or 20 μg/mL PA extracts was loaded on chick CAMs. After 48 h, CAMs were peeled off and blood vessels were viewed and photographed. (A) Representative photographs of chick CAM assays. (B) Quantitative analysis of neovascularization from the photographs. Results are presented as mean ± SD of three assays. (*) Significant difference in comparison to control group (p < 0.05).
activity of MMP-9, MMP-2 and u-PA through the up-regulation of their inhibitors TIMP-1, TIMP-2, PAI-1 and PAI-2 in HSC-3 cells. Therefore, strongly supports that P. angulata could be a potential candidate for the prevention of oral cancer metastasis.

In addition, we also found that PA extracts inhibited the expression of a primary angiogenic cytokine, VEGF in HSC-3 cells. Transformed epithelial cells have been shown to be the major source of VEGF expression in many types of solid cancers (Guidi et al., 1997; Abu-Jawdeh et al., 1996). Over-expression of VEGF is linked to increase angiogenesis and more aggressive tumor behavior (McLeskey et al., 1998), anti-angiogenic interventions based on VEGF antibodies or disruption of signal transduction through its receptors have been shown to inhibit tumor growth (Benjamin et al., 1999). The inhibitory effect of PA extracts on VEGF expression in oral cancer cells may be an important mechanism in the regulation of neovascularization of human oral tumors, further contributing to the overall control of tumor growth and progression.

Angiogenesis is an essential processes involved in the normal growth and differentiation. In other hand, angiogenesis also a crucial event in carcinogenesis and its onset has been associated with premalignant tumor stages (Raica et al., 2009). These vessels provide the principle route by which tumor cells exist in the primary site and enter the circulation (Zetter, 1998). Thus, angiogenesis has been a major challenge for the successful treatment of cancer. Accumulating evidences indicates that angiogenic inhibitors are potential candidate for the development of chemopreventive or chemotherapeutic drug for cancer and inflammation (Felmeden et al., 2003; Folkman, 2002). In addition, the pharmacology of many anti-inflammatory drugs has revealed that at least part of their efficacy is attributable to their anti-angiogenic effects (Alex et al., 2010). Hence, we hypothesized that the anti-cancer and anti-inflammatory activity of PA extracts could be partly attributable to its anti-angiogenic activity, and that P. angulata could be a promising candidate drug for the treatment of diseases with impaired angiogenesis. Furthermore, our results demonstrate that PA extracts inhibits VEGF-stimulated activities of MMP-9 in HUVECs. MMPs are playing a major regulatory role in matrix reorganization and are clearly implicated in the initiation of angiogenesis, endothelial cell differentiation and spreading during angiogenesis (Egeblad and Werb, 2002; Kahari and Saarialho-Kere, 1999). In particular, the release of MMP-9 and MMP-2 from endothelial cells represents an important step in neovascularization, because this major extracellular matrix proteolytic enzyme is secreted when endothelial sprouting takes place, thus enhancing cell migration across the extracellular matrix (Mignatti and Rifkin, 1993). Because increased MMPs activity is closely associated with the angiogenic pathway, thereby, we believed that the inhibition of HUVECs migration and invasion by PA extracts due to decreased activity MMPs.

Recent scientific literatures confirmed that aqueous/ethanolic/methanolic extracts of P. angulata possessed variety of biological activities including anti-inflammation (Choi and Hwang, 2003; Soares et al., 2003), anti-cancer (Hsieh et al., 2006) and anti-proliferation (Ismail and Alam, 2001). In the present study, we found that PA extracts could significantly inhibit the metastatic and angiogenic ability of HSC-3 and endothelial cells. The effective and powerful components in this extracted compound, however, still need to be characterized further. Phytochemical studies of P. angulata have demonstrated the presence of steroids, known as physalins (D, I, G, K, B, F, E), phyasulins (E, F and G), anolides and flavonoids (Chiang et al., 1992a,b; Ismail and Alam, 2001; Shingu et al., 1992). Furthermore, purified compounds isolated from the organic fractions of P. angulata, namely physalins (physalins A, B, D and F) and glycosides (myricetin-3-O-neohesperidoside) have antitumor activity (Ismail and Alam, 2001; Soares et al., 2003). They were shown to exert anti-cancer activity on MDA-MB-231 (breast cancer), MCF-7 (breast cancer), HepG2 (hepatoma), HA 22T (hepatoma), Hela (cervical carcinoma), leukemia, lung adenocarcinoma, and KB-16 cells (epidermoid carcinoma of the nasopharynx) (Chiang et al., 1992a,b; Hsieh et al., 2006; Ismail and Alam, 2001; Lee et al., 2009; Wu et al., 2004). Further bioassay-directed fractionation leading to the identification and purification of the compounds of PA extracts responsible for anti-metastatic or anti-angiogenic activities are warranted.

In conclusion, our observations indicate that ethyl acetate extracts of P. angulata exerts an inhibitory effect on several essential steps of metastasis, including migration and invasion of HSC-3 cells. To the best of our knowledge, this is the first scientific report examining the inhibitory effects of P. angulata on oral cancer metastasis and vascular endothelial angiogenesis activated by growth factor VEGF. Our findings revealed the mode of action of this extract through the down-regulation of migration/invasion-associated proteinases such as MMP-2, MMP-9 and u-PA and the up-regulation of their natural inhibitors TIMP-1, TIMP-2, PAI-1 and PAI-2 in HSC-3 cells. This study also documents the inhibitory actions of P. angulata on several angiogenic responses, including proliferation, migration, and invasion of vascular endothelial cells in vitro, and neo-angiogenesis of the chick choioallantoic membrane in vivo. Thereby, we propose that P. angulata is a potential anti-metastatic and anti-angiogenic agent for the treatment of inflammation or cancer. However, further investigations are required to elicit the responsible component(s) and establish the beneficial role of P. angulata for the treatment of oral cancer through the inhibition of metastasis and angiogenesis.

**Conflict of interest**

The authors have declared no conflict of interest.

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