

Using of animal models of angiogenesis to confirm a *Bidens pilosa*-sourced polyacetylenic glucoside inhibits angiogenesis targeting hypoxia through VEGF and PDIA4 suppression

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Simply Summary: Translation of new cancer treatments between pets and human were noticed in comparative oncological investigation. The current study aims at evaluating a polyacetylenic glucoside purified from an edible herb, *Bidens pilosa*, to present its anti-angiogenic effects. We innovatively find this polyacetylenic glucoside, cytopiloyne, shows anti-angiogenic effect on different *in vitro* assays and various *in vivo* animal models under hypoxia. Based on results of this study, cytopiloyne will be a prospective herb angiogenesis inhibitor candidate to control animal or human cancer formation as adjuvant therapy.

Abstract: Anti-angiogenesis is a pivotal combination treatment approach in cancer therapy but rare using on companion animals. This study aimed at evaluating the anti-angiogenic effect of a *B. pilosa* sourced polyacetylenic glucoside, cytopiloyne, on various *in vitro* assays and *in vivo* models. We provide evidences showing that CP has anti-angiogenic activities. Firstly, CP inhibited sponge/ Matrigel plug angiogenesis from tumor cells and decreased the survival of tumor cells on hypoxic conditions. Besides, CP declined PKC α protein expression which a protein leads to the growth and spread of tumors under hypoxia. Secondly, inhibitory effects of CP on endothelial angiogenesis were confirmed by chick chorioallantoic membrane assay, tube formation of SVEC4-10 cells and Matrigel plug assay. A dose-dependent CP treatment inhibited 4T1 cells proliferation under hypoxia and migration. It also suppresses VEGF transcription under hypoxia. Finally, we found that CP decreased

PDIA4, a novel regulator of cancer growth, expression in endothelial cells. This effect was confirmed by PDIA4 knockout mice with reduced angiogenesis in Matrigel plug assay. Taken together, these results suggest that CP might act as a promising anti-angiogenic herbal agent candidate to be used in animal hypervascularized cancer of veterinary medicine or in combination to control human cancer as adjuvant therapy.

Keywords: angiogenesis, *Bidens pilosa*, cancer, hypoxia, PDIA4, VEGF

1. Introduction

Translation of cancer therapeutic strategy between pets and humans became an important target on comparative oncological investigations [1]. A web information (Cancer in Dogs and Cats; Pet Care, March 10, 2020; <https://www.prudentpet.com/blog/cancer-dogs-and-cats/>) demonstrated that prevalence and mortality of cancer on pets. With one in five cats were diagnosed with cancer. Cancer is often more common in dogs than cats. 50% of dogs over ten years of age develop cancer. Cancer is the leading cause of death in dogs and cats, mortality of cancer dogs and cats were about 47% and 32%, respectively. As for treatments, common treatment methods include surgery, chemotherapy, and radiation therapy. Anti-angiogenesis is a pivotal combination treatment approaches in cancer therapy but rare using on companion animals. Current methods using anti-vascular endothelial growth factor (VEGF) antibodies or inhibitors targeting VEGF receptors after surgeon on cancer patients [2]. Due to their low blocking VEGF efficiencies on signaling transfer, toxicity and high risk of adverse effects in clinic [3], a mild and auxiliary method co effectively block cancer angiogenesis during treatment is necessary in veterinary clinic. Recent studies in pet dogs with cancer were undertaken to assist in the evaluation of anti-angiogenic peptide mimetics of thrombospondin 1 (TSP1) [1]. Follow-up study for cooperative activity between cytotoxic chemotherapy and TSP1 anti-angiogenic treatment in dogs with lymphoma has now supported these potential combinational therapies [4].

Based on above-mentioned issues, finding less toxic angiogenesis inhibitors from herbal phytochemicals, such as flavonoids, sulphated carbohydrates, or triterpenoids are being studied [5, 6]. In advance, phytochemical targeting VEGF and related multifactor as anticancer therapy was also investigated [7-11]. But less active and pure phytocompounds were found, especially worked on hypoxia *in vitro* and *in vivo* systems. Many experiments tried to screen functional herbal sources acted on angiogenesis. Among these, members of the *B. pilosa* (Asteraceae family) are listed as a food staple and herbal medicine by Food and Agriculture Organization of the United Nations and the Taiwan government [12]. It was also used as composition of

herbal teas in different areas or countries, including native Amazon, Himalaya, Jammu, Kashmir, China, India and Taiwan [13-15]. We previously reported that a polyacetylene glucoside, 2- β -D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetraene (named cytopiloyne; CP) manifests anti-diabetic, anti-coccidial, anti-bacterial, anti-candidal and immunomodulatory effects [16-21]. In addition, *B. pilosa* was used as the investigation on anti-cancer [22-24] and anti-angiogenesis [25, 26]. In these related studies on *B. pilosa* demonstrated that polyacetylenes acted as anti-angiogenic agents [25] through activation of CDK inhibitors and caspase-7 [26]. However, the anti-angiogenic mechanism with hypoxia underlying CP is not clear. Further, over 200 compounds were identified in *B. pilosa* [12], the using angiogenic models to confirm its anti-angiogenic compounds are deficient, which limits the clinical use in the veterinary medicine.

In this study, we first evaluate the anti-angiogenic effect of CP, on various *in vitro* systems and *in vivo* models. Next, using hypoxia system to confirm CP owned anti-angiogenic mechanism through protein kinase C (PKC) and VEGF modulation. Based on the background, CP docking and modulate gene protein disulfide isomerase $\alpha 4$ (PDIA4) which acts as a novel regulator of cancer growth. Finally, we also used PDIA4 KO mice verify angiogenesis inhibition effect of VEGF under Matrigel plug assay.

2. Materials and Methods

2.1. Chemicals and Reagents

Agarose, amiloride, bFGF, dimethyl sulfoxide (DMSO), ethanol, heparin, isopropanol, methanol, MTT assay reagents, SDS, sodium bicarbonate, phosphatase inhibitor cocktail 2, Tris-base and VEGF were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI1640 (Cat.31800-022), fetal bovine serum (FBS), 1 % glutamax, TRIzol, 1 mM sodium pyruvate, penicillin/streptomycin/glutamine, 1% nonessential amino acid, trypsin, 5X first-strand buffer and TEMED were obtained from Invitrogen Corp. (Carlsbad, CA, USA). High glucose DMEM (Dulbecco's modified Eagle's medium; 4.5 mg/mL) was purchased from Gibco (NY, USA). Monoclonal anti- β -actin (1:5,000; Abcam, MA, USA), PKC α (1:5,000; Y124, Abcam, MA, USA) and PDIA4 (1:5000; Stressgen, MI, USA) were used. *B. pilosa* plants were collected from the Farm of Changhua County, Taiwan. CP was prepared to 98% purity from whole plant of *B. pilosa* as previously described [16]. Briefly, CP was isolated on an RP-18HPLC column by methanol extraction and ethyl acetate partition of whole *B. pilosa* plants. Structure and purity were confirmed by NMR spectra using a Bruker DMX-500 spectrometer and nuclear magnetic resonance determination, respectively.

2.2. Cell Culture

Murine 4T1 mammary carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% non-essential amino acids (NEAA), 1% glutaMAX, 1 mM sodium pyruvate, 1% nonessential amino acid and 1% penicillin/streptomycin/glutamine.

SVEC4–10 cells is an endothelial cell line derived by SV40 (strain 4A) transformation of endothelial cells from axillary lymph node vessels; these fully transformed small vessel murine immortal endothelial cells, were purchased from BCRC (Bioresource Collection and Research Center, Taiwan). These cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine and 1.5 g/L sodium bicarbonate.

2.3. Laboratory Animal

C57BL/6JNarl mice were obtained from the National Laboratory Animal Center (NLAC; Taipei, Taiwan). PDIA4 KO mice were provided by Dr. W.C. Yang (Agricultural Biotechnology Research Center, Academia Sinica, Taiwan). Animals were housed in a 12 h light/dark cycle in controlled temperature (22 ± 2 °C) and humidity ($55 \pm 10\%$) in a specific pathogen-free animal facility. All mice gained *ad libitum* access to rodent diet (5010, LabDiet, MO, USA) and water for 1 week prior to the study and beyond. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Utilization Committee (IACUC) of National Chung-Hsing University (Taichung, Taiwan)(permit number: 101-83).

2.4. MTT Assay

MTT assay was used to determine cell viability of 4T1 cells. Briefly, the cells were grown in the presence of CP at 6×10^3 cells/well in a 96-well plate for 24–48 hr. After removing medium, MTT dye at 0.5 mg/mL was added to each well and incubated at 37 °C. After 4 h, DMSO (150 μ L/well) was added to each well and incubated at 37 °C for additional 1 h. The plate was measured at 570 nm (signal) and 650 nm (reference) using a microplate reader (UVM340, Biochrom, UK).

2.5. The Sponge/Matrigel Angiogenesis Assay

We used a sponge angiogenesis assay modified from the method described by previous studies [27, 28]. Matrigel (500 μ L) was injected subcutaneously in the midventral abdominal region of C57BL/6 mice (10–12 weeks of age) and permitted to solidify. Subsequently (after 20–30 min), mice were anesthetized with ketamine 100 mg/kg and xylazine 7.0 mg/kg. The skin overlying the Matrigel plug was gently

shaved, after which a small (0.5 cm) nick was made in the skin using a #15 surgical blade. Using the same blade, a smaller nick was made in the Matrigel plug. A sterilized polyvinyl sponge (appx. $3 \times 2 \times 1.5$ mm) containing with/without 0.1×10^6 4T1 cells to induce angiogenesis was introduced through the nick in the Matrigel and advanced to the center of the plug with the help of tweezers. The wound was then closed with a suture. Mice were observed after 24 h to monitor condition of the wound. Plugs could be recovered for several weeks but typically tumor-induced angiogenesis was measured after 7 and 14 days. Mice were killed after 3–5 min; the Matrigel plug with sponge was removed, separated from the abdominal muscle, fixed in 10% formalin and stained following sections with H&E. Light microscopy was used to visualize these sections for blood vessels formation and metastasis potential.

2.6. Chick chorioallantoic membrane (CAM) assay [29]

One-day-old fertilized eggs were incubated for 3 days in the water-jacketed incubator (38°C, 85% humidity). Next, the chick embryos with intact yolks were placed in plastic Petri dishes after cracked and incubated with 0 mL of RPMI-1640 medium (38°C, 85% humidity, 3% of CO₂). After 3 days of incubation, the methylcellulose disk containing with BSA (0.1% 100 nM), 100 nM VEGF, 100 nM bFGF, 30 µg amiloride, 2.5 µg and 5 µg CP/embryo was implanted on the CAMs of the individual embryos. After 48 hr of incubation, CAM of individual embryo was photographed for avascular zones formation analysis. The angiostatic effect was determined and normalized as a percentage of the area of blood vessels under the methylcellulose disks (3-5 eggs per treatment) in relation to the non-treated areas.

2.7. Mouse Matrigel Plug Assay

Flanks of C57BL/6 mice (8 weeks of age) were injected subcutaneously 500 µL of Matrigel (BD Biosciences, MA, USA) with an ice-cold syringe containing bFGF (100 ng/ mL) and heparin (50 U/ 500 µL) with or without CP (2.5 or 5 µg). After seven days, the skin of the mouse was pulled back to expose the Matrigel plug, which remained intact. The Matrigel plugs were weighted and photographed. To quantitate the formation of functional blood vessels, the amount of hemoglobin (Hb) was measured using the Drabkin hemoglobin assay with Drabkin reagent kit 525 (Sigma, MO, USA) as described previously [30]. Plugs were fixed in 10% formalin and stained following sections with H&E. Moreover, same experiment and operation was also completed on PDIA4 knockout mice. Another angiogenic factor VEGF (500 ng/mL) was used in Matrigel (500 µL).

2.8. Tube Formation Assay [31]

Matrigel (160 μ L) was loaded into 48-well plate at 37°C for 1 hr for polymerization.

SVEC4-10 endothelial cells (2.5×10^4 cells/ well) were added with serum-free DMEM with/without CP (5 μ g/mL) for 24-48 hr pretreatment. Tubes will begin to form within 2-4 hr. The final tube phenomenon will be examined under light microscopy after 6 hr.

2.9. *In Vitro Migration Assay*[32]

4T1 cells in medium containing 10% FBS were seeded into wells of 24-well plates. After the cells grew to confluence, wounds were made by sterile pipette tips. Cells were washed with PBS and refreshed with medium with/without 10% FBS and various concentrations of CP (1, 2.5 and 5 μ g/mL). After overnight incubation at 37°C, the cells were fixed and photographed.

2.10. *Hypoxia Induction and Western Blot*

In vitro, hypoxia is modelled in hypoxic incubators set at low oxygen levels (e.g. 1%) or by treating cells with a chemical mimetic, such as cobalt chloride (200 μ M CoCl₂, 24-48 hr) [33]. 4T1 cells (8×10^5 cells) in medium containing 10% FBS with/without CP (5 μ g/mL) were seeded into wells of 24-well plates under incubators of normoxia and hypoxia (1% O₂, 5% CO₂ and 94% N₂) in a humidified environment at 37 °C for 18-24 hr. For PDIA4 expression of SVEC4-10 endothelial cells after CP (2.5 or 5 μ g/mL) treatment, the incubation time are 12, 24 and 48 hr. Total lysates from 4T1 cells were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and, subsequently, transferred to nitrocellulose membrane (Schleicher and Scheull, Keene, NH, USA), immunoblotted with the antibodies against PKC α (1:5,000; Y124, Abcam, MA, USA) and/or β -actin (1:5,000; Abcam, MA, USA) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG as secondary antibody. After developing with ECL substrate (GE Healthcare, Little Chalfont, UK), the membranes were detected using FluorChem HD2 system (Bio-Techne, Minneapolis, MN, USA). Moreover, the PDIA4 expression between CP and SVEC4-10 endothelial cells under various times (12-48 hr) treatment was also detected by western blot. Immunoblotted with the antibodies against PDIA4 (1:5000; Stressgen, MI, USA) and/or β -actin (1:5,000; Abcam, MA, USA) were utilized.

2.11. *Reverse Transcription-polymerase Chain Reaction (RT-PCR)*[34]

Total RNA was isolated using the Trizol Reagent (Invitrogen, Carlsbad, CA). RT-PCR was carried out with the Superscript II One-Step RT-PCR system with Taq polymerase (Invitrogen) according to the manufacture's protocol using primer sets

specific for VEGF (5'-GTACCTCCACCATGCCAAGT-3' and 5'-GCGAGTCTGTGTTTTTGCAG-3'), GAPDH (5'-ACAGTCTTCTGAGTGGCA-3' and 5'-CCCATCACCATCTTCCAG-3'). The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Relative band intensities were quantified by ImageJ software (NIH, Bethesda, MD).

2.11. Statistical Analysis

Data from three independent experiments or more are presented as mean \pm SEM. Two-tailed Student's t test, Kruskal-Wallis test and ANOVA test were used for statistical analysis of differences between Groups according to the data type, and a P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. *In vivo Effects of CP on Tumor-induced Angiogenesis in Sponge/Matrigel Assay*

There are many murine *in vivo* tumor models for investigating angiogenesis, especially work on oxygen supply and metastasis [35, 36]. These tumor dissemination methods include tumor cells injection (subcutaneous, intravenous, intraperitoneal and intrahepatic etc.) and graft transplantation but without direct interaction data between angiogenesis and metastasis. For this reason, we use sponge/Matrigel angiogenesis assay [27] was used to check anti-angiogenic effect of CP on hypoxia condition. We found that blood vessel formation was rare in the part of Matrigel around control sponge without 4T1 cells (Fig. 1A. and 1B.; left panels). Apparent angiogenesis was observed in the H&E section in matrigel with 4T1 cell loaden sponge. Because erythrocytes increased in the new formed blood vessels, redness level was apparent elevated around sponge contained 4T1 cells (Fig. 1A. and 1B.; middle panels). Comparing with 4T1 cells group, redness level of Matrigel around 4T1 cells-loaden sponge were apparently observed in CP-cotreated one because inhibition on angiogenesis (Fig. 1A. and 1B.; right panels).

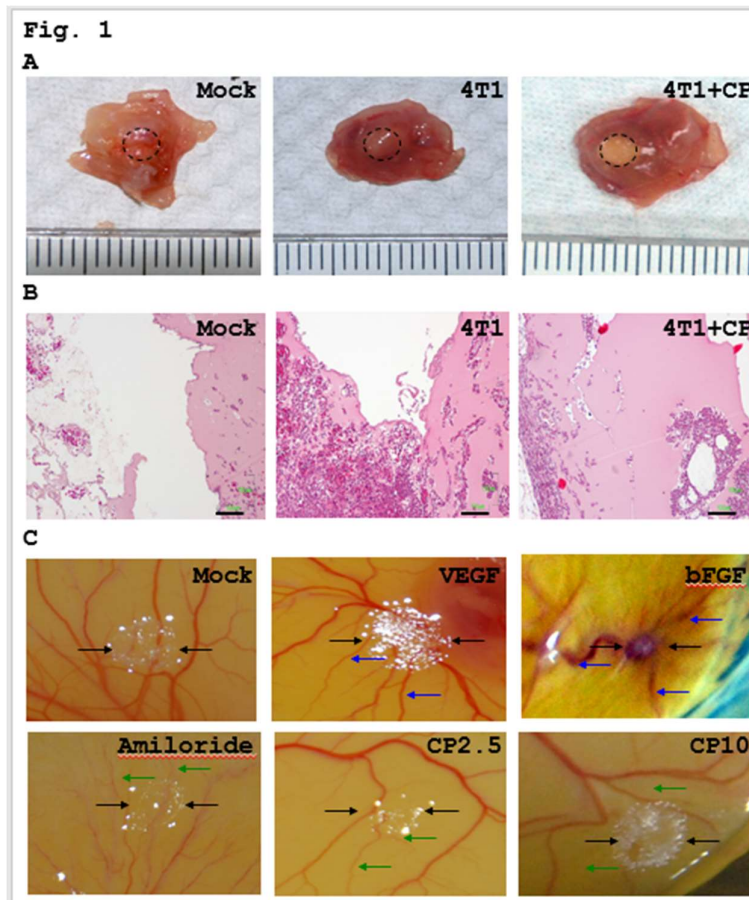


Fig. 1 Anti-angiogenic effects of CP in sponge/Matrigel Assay (A, B) and chick chorioallantoic membrane (CAM) assay (C). CP inhibited 4T1 cancer cells-induced angiogenesis under sponge/Matrigel assay in C57BL/6 mice. Gross (A) and section with H&E stain (B) photos of removed Matrigel with 4T1 cell loaded sponge were detected after 7-14 days. Dot lines indicated the location of sponge. CP lowered redness in Matrigels (A) and erythrocyte distribution in Matrigel sections (B). CP directly inhibited blood vessel formation in CAM assay (C). After 3 days incubation of fertilized eggs, methylcellulose disk containing with BSA (0.1% 100 nM), 100 nM VEGF, 100 nM bFGF, 30 μ g amiloride, 2.5 μ g and 5 μ g CP/embryo (CP2.5 and CP5) was implanted on the CAMs of the individual embryos. After 48 hr of incubation, CAM of individual embryo was photographed for avascular zones formation analysis. Black, blue and green arrows indicated the location of methylcellulose disk, new formation of blood vessels and inhibition of angiogenesis, respectively.

3.2. Influence of CP on Angiogenesis in Chick Chorioallantoic Membrane Assay

The CAM has served as an *in vivo* platform for advantages to research and manipulate vascular functions near 60 years. This system has been used for the study of vascular

development and angiogenesis, especially on tumor growth and metastasis [37]. It can also be advanced studies on respiratory properties, ion transport [34,35], selective vascular occlusion therapies, biocompatibility of engineered materials, drug distribution and toxicology [38-40]. Control CAMs implanted on the empty methylcellulose disks without angiogenic inhibitor did not develop avascular zones as determined by visual examination (Fig. 1C; Mock group). We found that an inhibitor of urokinase plasminogen activator, amiloride, cause a significant reduction of angiogenesis (Fig. 1C; Amiloride group). A larger avascular zone outside of area covered by disk containing this inhibitor was observed. As the positive controls, we used VEGF and bFGF disks, dense areas or increased newly formed vessels were developed (Fig. 1C; VEGF and bFGF groups). In contrast, CP-contained disks apparently showed inhibition of angiogenesis (Fig. 1C; CP2.5 and CP10 groups) under concentrations (2.5 and 10 $\mu\text{g}/\text{disk}$) lower than amiloride group (30 $\mu\text{g}/\text{disk}$).

3.3. CP Inhibits Angiogenesis in *in vivo* Murine Matrigel Plug Assay

We used matrix gel plug assay, an efficient method, to assess angiogenic and antiangiogenic compounds. First, bFGF was used as positive control. Removed Matrigel plugs were photographed. An increased redness level indicated that angiogenesis was induced by angiogenic factor, bFGF (Fig. 2A). More than 30 folds of the content of hemoglobin of the plugs provided a synchronous data on bFGF-induced angiogenesis (Fig. 2B). This significant data was confirmed by higher erythrocytes distribution in Matrigel section with H&E stain (Fig. 2C). In contrast, CP significantly inhibited angiogenic effect of bFGF in Matrigel plug. The redness level of plugs was decreased, especially on 5 μg CP/mL plug (Fig. 2A) and confirmed by significant lower content of hemoglobin with almost same as control ones (Fig. 2B). Almost same erythrocytes distributions in plug sections between Mock group and CP groups were observed (Fig. 2C).

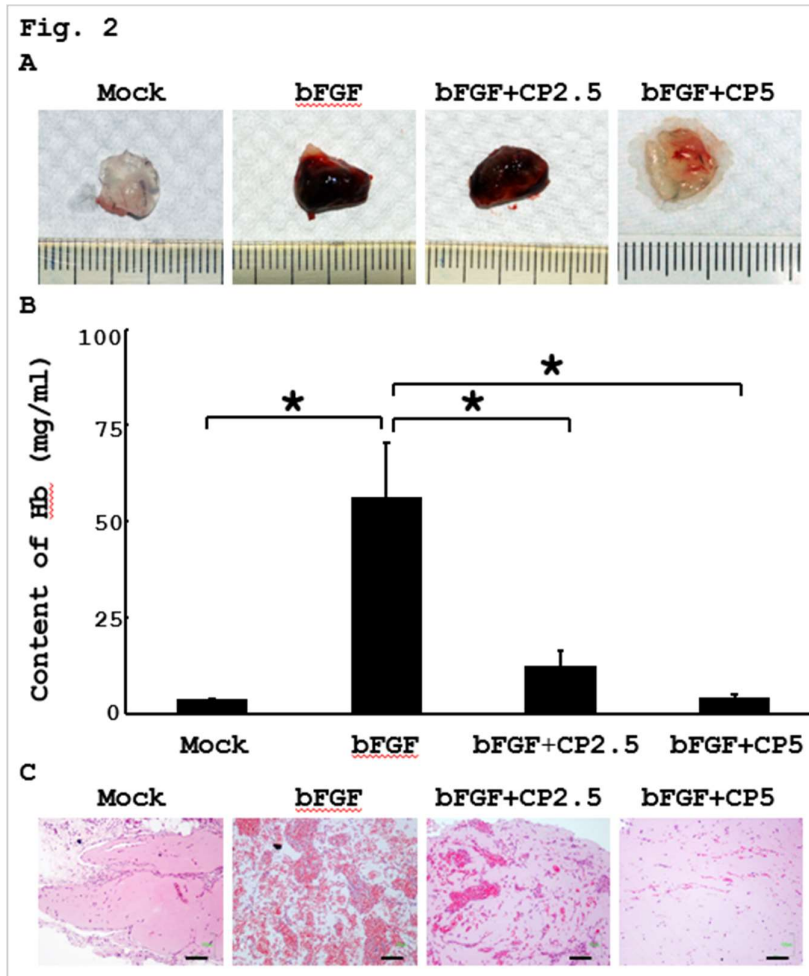


Fig. 2 Inhibitory effect of CP in bFGF-induced angiogenesis in Matrigel plug assay. Matrigel (500 μ L) containing bFGF (100 ng/ mL) and heparin (50 U/ 500 μ L) with or without CP (2.5 or 5 μ g) were subcutaneously injected into C57BL/6 mice (8 weeks of age). After 7 days, the Matrigel plugs were removed for weight and photograph (A). The concentration of hemoglobin (Hb) was measured using the Drabkin hemoglobin assay (B). Sampling part of plugs were fixed and stained with 10% formalin and H&E, respectively. These sections were photographed by light microscope observations (C).

3.4. CP Inhibits the Tube Formation of SVEC4-10 Endothelial Cells

To estimate the effect of CP on the differentiation of SVEC4-10 cells, we carried out tube formation assay [31]. SVEC4-10 cells were placed on a growth factor-reduced Matrigel-coated plate and were incubated for 24-48 h. As shown in Figure 3A, SVEC4-10 cells on Matrigel formed blood vessel network in the absence of CP, whereas the treatment of CP strongly inhibited the formation of tube-like structure. Moreover, CP did not show toxic effect on SVEC4-10 cells for 24-48 hr (data not

shown).

3.5. 4T1 Cell Viability after CP Treatments under Normoxia and Hypoxia

As other investigation description, tolerated cancer cells in the hypoxia region will show faster proliferation. But as tumor size increased, the part located away from blood vessels will enhance deterioration on oxygen deficiency and form necrotic zone [41]. This vicious effect on hypoxia will induce advanced angiogenesis [42]. Therefore, screening toxic effect of compounds on cancer cells with angiogenesis under different oxygen levels, normoxia and hypoxia, is a pivotal step. We found that CP (1 and 5 $\mu\text{g}/\text{mL}$) did not induce any toxic response on 4T1 cells under normoxia by microscopic examination and MTT test (Fig. 3B and 3C). A significant concentration-dependent of increment of dead 4T1 cells after CP treatment under hypoxia was notice (Fig. 3B and 3C).

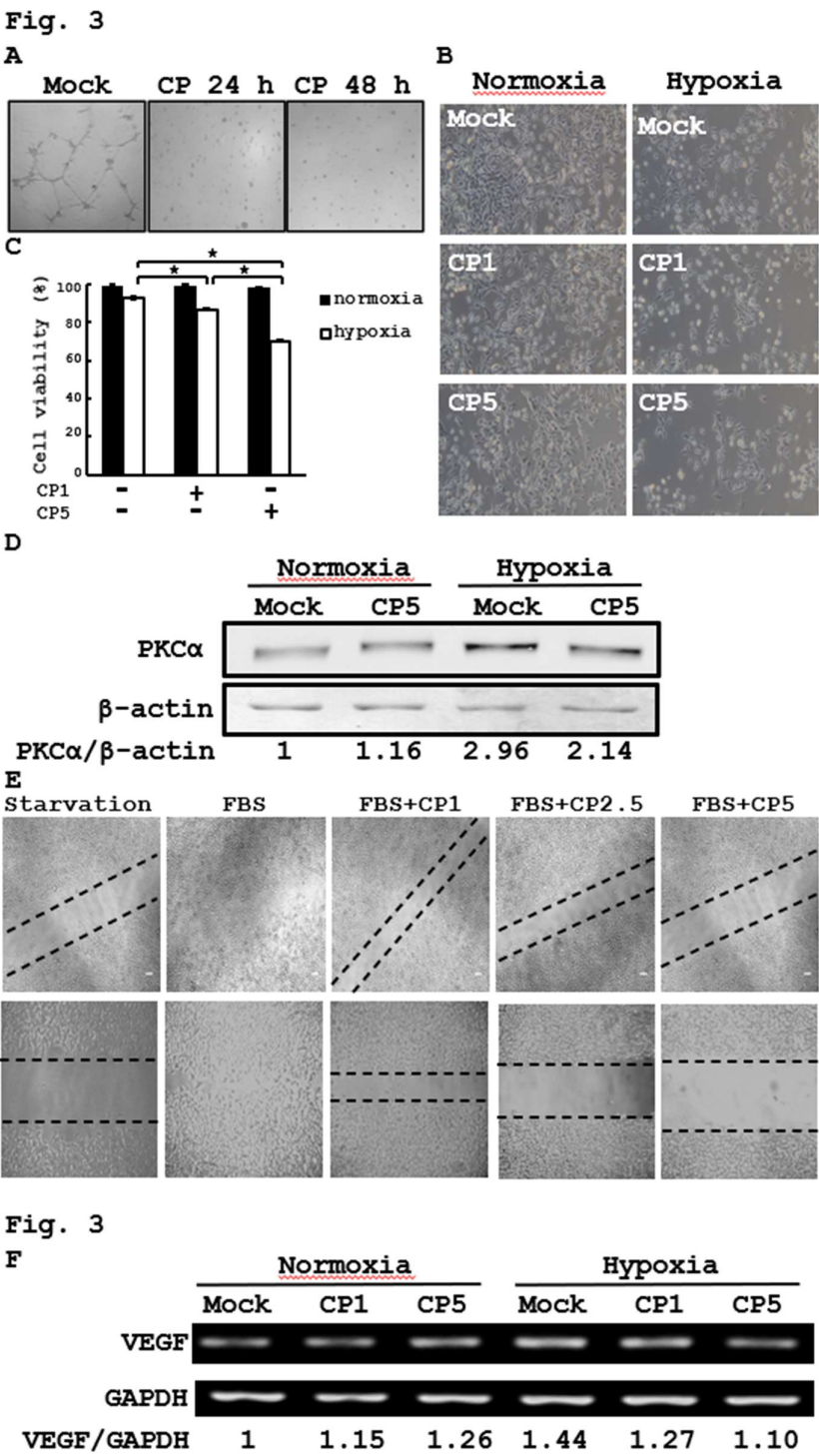


Fig. 3 CP inhibits endothelial cells tube formation, 4T1 cancer cells proliferation and migration under hypoxia state with related mechanisms. SVEC4-10 endothelial cells with/without CP (5 μ g/mL) for 24-48 hr pretreatment were added into Matrigel (160 μ L/ well) coated 48-well plate. The final tube phenomenon will be

examined under light microscopy after 6 hr (A). 4T1 cells were incubated with/without CP (1 and 5 $\mu\text{g}/\text{mL}$) in a 96-well plate under normoxia or hypoxia (1% O_2 , 5% CO_2 and 94% N_2) for 24-48 hr. These cells were photographed by light microscope observations (B). MTT assay was used to determine cell viability of 4T1 cells (C). Proteins from 4T1 cells lysate under control and CP treatment (5 $\mu\text{g}/\text{mL}$) with normoxia or hypoxia were detected the levels of PKC α and β -actin by western blot (C). Same cell sources were also analyzed by RT-PCR to detect VEGF transcription levels (F). Except hypoxia state, 4T1 cells migration activity were observed with/without starvation state. A dose-dependent CP treatment (1, 2.5 AND 5 $\mu\text{g}/\text{mL}$) showed apparently inhibition of FBS-incubated 4T1 cells migration (E).

3.6. CP Decreased PKC α Expression VEGF Transcription of 4T1 cells under Hypoxia

In the early 1980s, PKC was identified as the target of phorbol esters of natural products [43]. Therefore, many dietary phytochemicals were screened for cancer chemoprevention based on PKC pathway related with tumor-promoting activity [44]. PKC α , one of PKC isoforms, which can accelerate tumor cells proliferation/metastasis and inhibit cancer cells apoptosis [45]. Hypoxia will also induce PKC α expression to promote tumor VEGF production and malignancy [46-48]. We found that CP did not modify 4T1 cells PKC α expression on normoxia. But a decreased PKC α level in 4T1 cells with CP treatment was noticed in hypoxia (Fig. 3D). Hypoxia can significantly increase VEGF transcription and production [49]. We used RT-PCR confirmed that 4T1 cells under dose-dependent CP treatment showed lower VEGF transcription under hypoxia, but not normoxia (Fig. 4F).

3.7. Inhibition of 4T1 Cells Migration by CP

Apart from oxygen deficiency, fast growing tumor presented nutrition demands. Therefore, FBS enhanced 4T1 cells migration by overcome starvation [32] (Fig. 3E). A dose-dependent CP treatment showed apparently inhibition of FBS-incubated 4T1 cells migration (Fig. 3E).

3.8. CP Decreased PDIA4 Expression of SVEC4-10 Endothelial cells

Abnormal PDIA4 expression combined endoplasmic reticulum stress is related with a self-protection to various diseases, including angiogenesis related to survival and progression of different cancer types [50-52]. A dose- and time-dependent CP treatment on SVEC4-10 endothelial cells will apparently decrease 30-70 % PDIA4 expression by western blot detection (Fig. 4A).

3.9. Angiogenesis in in vivo Murine Matrigel Plug Assay on PDIA4 Knockout Mice

Previous study indicated that PDIA4 is not an essential protein because mice with PDIA4 knockout survive without obvious phenotypes [51]. But no investigation worked on the relationship among angiogenesis, angiogenic factor and PDIA4. VEGF can also induce angiogenesis (Fig. 4B and 4C) which is same as our data in this study of bFGF-induced angiogenesis (Fig. 2) in in Matrigel plug in C57BL/6 mice. An increased redness level in Matrigel plug indicated that angiogenesis was induced by angioenic factor, VEGF (Fig. 4B). About 5 folds of the content of hemoglobin of the plugs provided a synchronous data on VEGF-induced angiogenesis (Fig. 4C). The VEGF-induced angiogenesis in Matrigel plug on PDIA4 KO mice were significantly decreased (Fig. 4B and 4C).

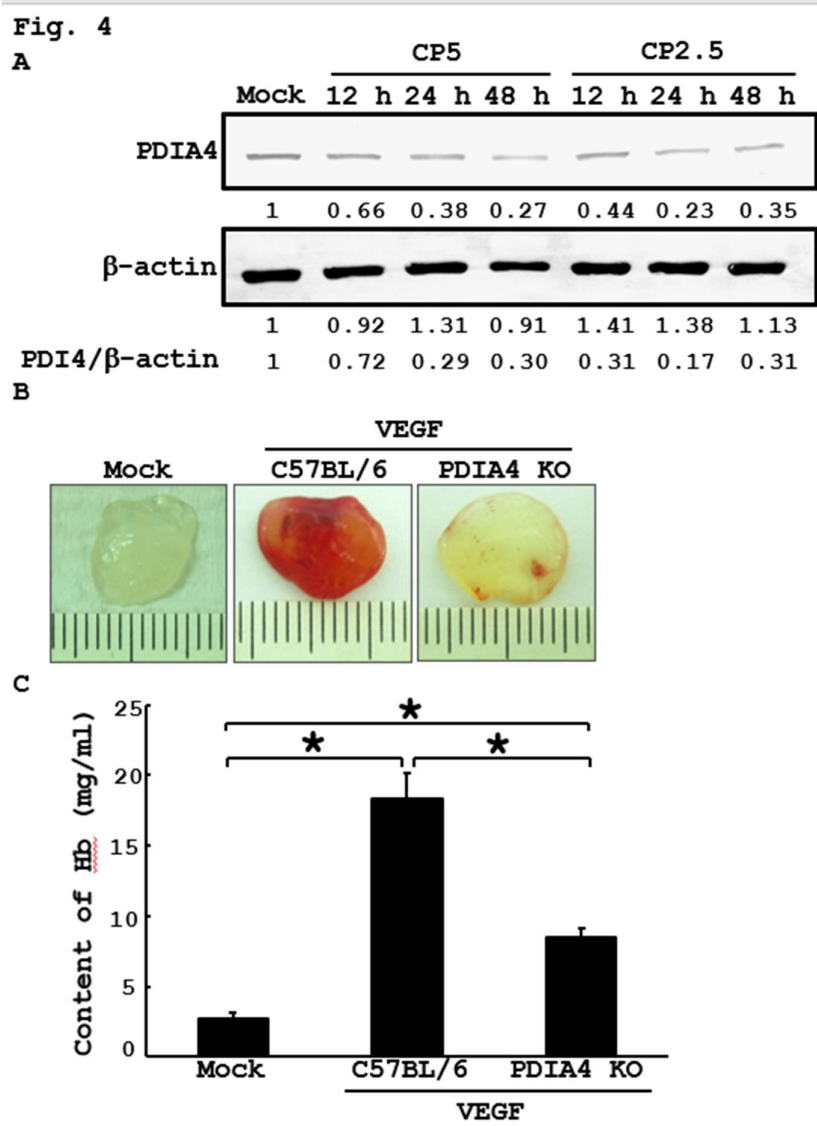


Fig.4 CP inhibits PDIA4 expression in endothelial cells and significant lower angiogenesis in PDIA4 knockout mice. CP (2.5、5 μg/mL) decreased PDIA4

expression of SVEC4-10 endothelial cells under various time treatment (12-48 hr). PDIA4 expression from these cell lysates were analyzed by western blot (A). Matrigel (500 μ L) containing VEGF (500 ng/mL) and heparin (50 U/ 500 μ L) were subcutaneously injected into PDIA4 knockout mice (8 weeks of age). After 7 days, the Matrigel plugs were removed for weight and photograph (B). The concentration of hemoglobin (Hb) was measured using the Drabkin hemoglobin assay (C).

4. Discussion

Arthur T. Hertig first name the term angiogenesis in 1935 [53]. Judah Folkman found a revolutionary novel way to consider about cancer and advanced therapy on tumor angiogenesis more than 40 years [54-57]. Based on anti-angiogenic mechanism worked on blocking nutrition and oxygen supply to tumors, more than 10 angiogenesis inhibitors were discover from 1980 to 2005 [57]. After that phytochemicals targeting angiogenic factor and other related inflammatory pathway (eg. upstream PKC activate a distinct set of transcription factors, including NF- κ B) were screened for cancer prevention and adjuvant therapy [7, 44, 58]. There are many consistencies on tumor types and progression between bet animals and human. Therefore, translational drug development studies became an optimal choice on integrated approach to link the pet dog with cancer and conventional preclinical models (mouse, research-bred dog and non-human primate) and the human clinical trial [1]. Tolerated cancer cells in the hypoxia region will show faster proliferation. But as tumor size increased, the part located away from blood vessels will enhance deterioration on oxygen deficiency and form necrotic zone [41]. This vicious effect on hypoxia will induce advanced angiogenesis [42]. Here, we report a study on a polyacetylene glucoside, cytopiloyne (CP), purified from an edible herbal medicine *B. pilosa* in inhibiting angiogenesis targeting hypoxia through VEGF and PDIA4 suppression through various *in vitro* and *in vivo* angiogenic models. Amazingly, our data revealed that CP can inhibit angiogenesis in different animal models researched on angiogenesis (Fig. 1 and 2). In line with the *in vivo* reduced angiogenesis data induced by CP treatments, CP inhibited mouse 4T1 mammary carcinoma cells proliferation under hypoxia and migration with rich nutrition (FBS) supplement (Fig. 3B, 3C and 3E).

Normal tissues with acute and chronic diseases will present hypoxia under inflammation and toxic response. Cancer cells can tolerate and proliferate in hypoxia but as tumor cells located > 180 μ m from the blood vessels were observed to become necrotic [59]. Therefore, metastatic tumor cells or new blood vessels formation will prevent the progressive damage on cancer mass. The relationship between hypoxia and angiogenesis on tumor tissue organization were investigated by previous studies. Results of these investigation confirmed that hypoxia-induced angiogenesis will be

occurred once the tumor size exceeds 1000 μm diameter and/or the distance to the nearest blood vessel exceeds 180 μm [60]. Direct cancer cells injection or tumor graft transplantation cannot fully provide a mimic animal model link hypoxia, angiogenesis and metastasis. So, we used a cancer cells-loaded sponge/Matrigel assay to confirm that CP can inhibit 4T1 cells migrate from sponge into Matrigel and form new blood vessel from implanted mice (Fig. 1A and 1B) to overcome oxygen and nutrient deficiency. CAM assay and bovine aortic endothelial cells (BAECs) model are easy and quick ways to screen out drugs or phytochemicals influence angiogenesis. Based on these assays, there are 3 herbs enhanced and 7 herbs significantly inhibited angiogenesis which were screened from 24 traditional Chinese medicines used as curing ischemic heart disease in clinic [61]. Comparing positive angiogenic factors, VEGF and bFGF, CP can direct suppress chick embryo blood vessels development as amiloride, an inhibitor of urokinase plasminogen activator, inhibiting angiogenesis (Fig. 1C). Besides the direct inhibitory effect of angiogenesis of CP, we also check whether or not CP can lower the angiogenic effect on bFGF stimulation. A dose-dependent CP treatment significantly curbs bFGF-induced angiogenesis used *in vivo* murine Matrigel plug assay (Fig. 2). At least 10-30 folds lower concentrations of hemoglobin extract from erythrocytes in plugs were analyzed in CP groups (Fig. 2B). Moreover, the matrix gel plug assay has proven to be a convenient and powerful method to evaluate gene regulation in angiogenesis, angiogenic and antiangiogenic compounds *in vivo*, and to supplement *in vitro* tests [62]. As above mention, PDIA4 is gene related with regulating cancer growth [50-52]. Similar as Fig. 2, another angiogenic factor VEGF can induce angiogenesis in Matrigel plug in implanted mice (Fig. 4). Significantly, PDIA4 KO mice did not show VEGF-induced angiogenesis in Matrigel plug (Fig. 4B and 4C). The hemoglobin levels decreased about 5 folds in PDIA4 mice implanted with VEGF plug (Fig. 4C). It meant that PDIA4 is related with angiogenesis on tumor progression.

As other investigation description, polyacetylenes isolated from *B. pilosa* possess significant anti-angiogenic effects and regulate the expression of cell cycle mediators, p27(Kip1), p21(Cip1), or cyclin E, on human umbilical vein endothelium cells (HUVEC) [25]. Advanced data following this study indicated that a novel polyacetylene, 1,2-dihydroxy-5(E)-tridecene-7,9,11-triyn, structure similar with CP can promote apoptosis of HUVEC through overexpress death ligand FasL, activate caspase-7 and CDK inhibitors to inhibit angiogenesis [26]. These pilot studies worked on anti-angiogenesis of polyacetylenes from *B. pilosa* did not provide direct data on angiogenesis animal models and responses of cancer cells under hypoxia. But they suggest that phytocompounds such as polyacetylenes owned potential as candidates for anti-angiogenic therapeutics [26]. In our study, we provided different *in vivo* data

to confirm that CP can inhibit angiogenesis and probably suppress metastasis (Fig. 1, and 2). Correlated *in vitro* data also prove that CP can slow 4T1 cells migration and endothelial cell tube formation (Fig. 3A and 3E). More important, the toxic effect of CP on 4T1 cells only presented in hypoxia (Fig. 3B and 3C). It meant that polyacetylenes with anti-angiogenic effects, especially CP, showed safe characteristic and owned potential in combinational therapy with other cancer treatment. Apart from these *in vitro* and *in vivo* data of CP used to evaluate anti-angiogenic effects, this is the first report to demonstrate that polyacetylene worked on angiogenic factors under hypoxia. First, we checked PKC α expression in 4T1 cell under hypoxia. CP can suppress PKC α expression in 4T1 cells under hypoxia but not normoxia (Fig. 3D). Because elevated PKC α expression will increase VEGF levels in tumors under hypoxia and enhance malignancy progression [46-48]. Next, we found that higher VEGF transcription in 4T1 cells under hypoxia was decreased by CP co-incubation (Fig. 3F).

Of note, the anti-angiogenic mode of action of CP needs to be further addressed and is under way. At the outset, the data in this study indicated that CP will be a safe phytocompound to be used in cancer patient and pet animals, especially on early stage. CP did not enhance angiogenesis and toxic effect under normoxia (Fig. 3B-D and 3F). Second, the data in previous publications [16, 21, 63] and this work suggest that CP owned immune modulatory effects, especially on anti-inflammation. Approximately 15% of all cancers are linked to inflammation which contributes to the development of neoplasms. Related intracellular signaling mechanisms involved in NF- κ B activation and the induction of iNOS response [64]. Our other investigation found that CP inhibited inflammatory cytokines production by lymphocyte [16, 21] and suppressed NF- κ B pathway and iNOS activity (data not shown). Usually, anti-inflammatory drugs will be used in cancer patients or pets, but non-steroidal anti-inflammatory drugs (NSAIDS) will raise the risk of cardiovascular incidence and gastrointestinal bleeding, make herbs and spices potentially appealing alternatives [65]. Extract from *B. pilosa* can improve gastric ulcer [66, 67] and cardiovascular function [68]. Taken together with our earlier publications [16, 21, 63], our data illustrate the applicability of CP for prophylactic use or combination therapy to control abnormal angiogenesis in progressive tumor development.

5. Conclusions

Finding anti-angiogenic drugs and/or alternative herbal drugs is a key and difficult works to overcome. For these reasons, we tried to evaluate the anti-angiogenic effect and related mechanism of CP purified from *B. pilosa*. We found this polyacetylenic glucoside from a safe and edible herbal medicine can significantly inhibit

angiogenesis in various animal models (sponge/Matrigel angiogenesis assay, CAM and Matrigel plug assay) and in vitro angiogenic systems (tube formation assay and migration assay). The anti-angiogenic effect of CP is especially significantly presented under hypoxia state through inhibiting PKC α expression and VEGF expression. We also confirmed that a CP docking gene PDIA4 on a knockout mouse model which inhibit angiogenesis by Matrigel plug assay. Finally, further research screening anti-angiogenic polyacetylenic phytochemicals to achieve clinical usage on human and pet cancer patients will be need for translation medicine investigation,

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