Polyacetylenes Function as Anti-angiogenic Agents

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Purpose. To investigate the antiangiogenic effects of plant extracts and polyacetylenes isolated from *Bidens pilosa* Linn., which is a popular nutraceutical herbal tea and folk medicine in anti-inflammatory, antitumor, and other medications worldwide.

Methods. Anti-cell proliferation, anti-tube formation, and cell migration assays were used for the valuation of bioactivities of target plant extracts and phytocompounds against angiogenesis. Bioactivity-guided fractionation, HPLC, and various spectral analyses were used to identify active fraction and phytocompounds for anti-angiogenesis.

Results. We show that an ethyl acetate (EA) fraction of *B. pilosa* exhibited significant anti-cell proliferation and anti-tube formation activities against human umbilical vein endothelium cells (HUVEC). Bioassay-guided fractionation led to isolation of one new and one known polyacetylenes, 1,2-dihydroxytrideca-5,7,9,11-tetrayne (**1**) and 1,3-dihydroxy-6(*E*)- tetradecene-8,10,12-triyne (**2**), respectively, from the EA fraction. Compounds **1** and **2** manifested highly specific and significant activities against HUVEC proliferation with IC_{50} values of 2.5 and 0.375 μg/ml, respectively, however, compound **1** had a more potent effect on preventing tube formation of HUVEC than compound **2** at a dose of 2.5 μg/ml. Western blot analysis showed that both compounds upregulated p27(Kip) or p21(Cip1), cyclin-dependent kinase inhibitors, in HUVEC.

Conclusions. This is the first report to demonstrate that polyacetylenes possess significant anti-angiogenic activities and the ability to regulate the expression of cell cycle mediators, for example, p27(Kip1), p21(Cip1), or cyclin E.

KEY WORDS: anti-angiogenic activities; *Bidens pilosa* Linn.; cell cycle mediators; human umbilical vein endothelium cells (HUVEC); polyacetylenes.

INTRODUCTION

Angiogenesis, formation of new blood vessels from pre-existing endothelium, has been shown to play an important role both in animal development and pathologic conditions like tumor growth and metastasis or cardiovascular diseases (1,2). Angiogenesis is a complex process involved in migration, proliferation, and capillary tube formation of endothelial cells (3), which depends on both positive and negative regulators such as basic fibroblast growth factor (bFGF) and thrombospondin 1. An imbalance between these two regulators often results in deregulation of angiogenic activity (4). Preclinical studies have shown that anti-angiogenic therapy, targeting tumor endothelium, offers great promise for the treatment of cancer (5–8). Recognition of the potential benefits of controlling angiogenesis has led to a search for angiogenesis inhibitors, including investigation of natural compounds existing in medicinal herbs. These research activities have contributed to new or renewed public interests worldwide in herbal medicines, health foods, and nutritional supplements.

Bidens pilosa Linn. var. *radiata* is a tropical weed widely distributed in the countryside of Taiwan. This plant was originally found in tropical America and later introduced into the Pacific region and parts of Asia. The whole plant or its aerial parts are used in various folk medicines and as a popular ingredient in herbal tea for its anti-inflammatory, antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects (9–13). Topical application of this herbal extract is also used for skin disorders (14). This plant is eaten as food in Africa and is believed to contribute to the etiology of human esophageal cancer (15,16). A variant of *B. pilosa* has been also identified to have an antitumor effect on a leukemia cell line (17). Although diverse bioactivities have been identified in *B. pilosa*, to date, there are still no studies pertaining to the chemopreventive benefits of *B. pilosa* via angiogenic modulation.

In this study, we investigated the anti-angiogenic effect of plant extracts and phytocompounds of *B. pilosa* using one primary HUVEC and several transformed human cell lines, including oral cancer KB cell, lung carcinoma A549 cells, and HACAT keratinocytes. Although various phytocompounds (e.g., glucosides, polyacetylenes, a diterpene, flavonoids, and flavone glycosides) have been isolated from *B. pilosa* (18–23), we identified here two active polyacetylenes, including one new compound (**1**) and one known compound (**2**), which have not been reported from the plant. These two compounds exhibited significant but differential ability to block endothelial cell proliferation, migration, and tube formation, while having only minimal or no detectable effect on cell proliferation of the tested cancer cell lines and immortalized keratinocytes. The mechanism of action in anti-angiogenic activity of compounds **1** and **2** identified from *B. pilosa* are discussed.

MATERIALS AND METHODS

Materials

All chemicals and solvents used for herbal extraction and compound purification were of reagent or HPLC grade. Human basic fibroblast growth factor (bFGF) was purchased from Pepro Tech (Rocky Hill, NJ, USA). EGM2 medium for the growth of human umbilical vein endothelial cells (HUVEC) was from BioWittaker (Walkersville, MD, USA). Bovine serum albumin (BSA), gelatin, phorbol myristate acetate (PMA), and all other chemicals were ordered from Sigma Chemical Co. (St. Louis, MO, USA). Celltiter 96 One AQueous One Solution Cell Proliferation Assay (MTS) kit was from Promega (Madison, WI, USA). Renaissance

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelium cells; IR, infrared absorption; NMR, nuclear magnetic resonance; UV, ultraviolet and visible absorption.

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Chemiluminescence Reagent Plus was obtained from NEN Life Science Products (Boston, MA, USA). Antibodies against p21(Cip1) and cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and those against cyclin D1 and α-tubulin were from Neo Markers (Fremont, CA, USA). Antibody to p27(Kip) was from BD Transduction Laboratories (San Diego, CA, USA), whereas that to p53 was from Oncogene Research Products (Boston, MA, USA). Annexin V-FITC apoptosis detection kit was from Oncogene Research Products (San Diego, CA, USA). Endothelial Cell Growth Supplement (ECGS), heparin sulfate, and type I collagenase for HUVEC isolation were from Life Technologies (Rockville, Maryland, USA). Geimsa stain was from Merck (Darmstadt, Germany).

Preparation of Plant Extracts

Bidens pilosa Linn. var. *radiata* (Compositae) was collected on the campus of Academia Sinica, Taiwan, in 2001. A voucher specimen (no. 0211943) has been deposited at the Herbarium of the Department of Botany, National Taiwan University, Taipei, Taiwan. Approximately 2.5 kg of raw plant materials, crushed from fresh whole plants, was extracted at room temperature with 25 l of 70% (v/v) ethanol in water for 7 days. The extract was evaporated *in vacuum* to yield a residue (ca. 130.0 g). The dry ethanolic extract was suspended in 1.0 l of water and then successively partitioned with ethyl acetate (1.0 l × 3 times) and *n*-butanol (1.0 l × 3 times) yielding three fractions designated as EA, BuOH, and water fractions. Each fraction was evaporated on a vacuum rotary evaporator under reduced pressure to remove organic solvent and then lyophilized. The yield percentage of EA, BuOH, and water fractions from the total ethanolic extract were 14.1%, 15.2%, and 69.0% (w/w dry weight), respectively.

Compound Isolation

The EA fraction (18.0 g) was subsequently chromatographed over a silica gel (347 g) column with a MeOH/CH_2Cl_2 gradient solvent system to give subfractions 1–8. Fraction 5 [3.5 g, eluted by 5% (v/v) MeOH in CH_2Cl_2], having the most significant anti-angiogenic activity of all the isolated fractions, was further fractionated using a silica gel (61 g) column with an ethyl acetate/hexane gradient solvent system to give subfractions 9–17. Fraction 16 [0.1 g, eluted by 70% (v/v) ethyl acetate in hexane] displaying a selective toxicity against endothelial cell growth, but not KB cells, was further purified by semipreparative HPLC on a RP-18 column [Phenomenex Luna 5μ C18 (2), 250 mm × 10 mm]. A mixture of compounds **1** and **2** (14.8 mg) was isolated from fraction 16 at retention times of 15.0 min, using a MeOH/H_2O gradient as a solvent system with a flow rate of 5.0 ml/min and measured by a UV detector at 254 nm. The HPLC gradient was MeOH (solvent B) in H_2O (solvent A): 60–70% (v/v) from 0 to 15 min, 70–100% from 15 to 20 min, maintained at 100% from 20 to 23 min, 100–60% from 23 to 25 min, and re-equilibration with 60% from 25 to 30 min. Pure compounds **1** (3.2 mg) and **2** (3.1 mg) were further purified by HPLC [Phenomenex Luna 5μ Silica (2), 250 mm × 10 mm] using 70% (v/v) ethyl acetate/hexane as the solvent system.

General Methods for Compound Structure Elucidation

Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 983G spectrophotometer. ^1H and ^{13}C NMR spectra were run on a Bruker DMX-500 spectrometer. EIMS were obtained on a JEOL JMS-HX 300 mass spectrometer.

1,2-dihydroxytrideca-5,7,9,11-tetrayne (**1**): Amorphous colorless solid; IR ν_{max}: 3364, 2924, 2229, 1052 cm^{-1}; $[\alpha]^{25}_D$ 34.1° (CH_3OH, *c* 0.3); ^1H- and ^{13}C-NMR data: Table I; EIMS *m/z* (relative intensity): 200 $[M]^+$ (16), 182 $[M\text{-}H_2O]^+$ (9), 169 $[M\text{-}CH_2OH]^+$ (46), 152 (54), 139 (61), 127 (100), 115 (66), 99 (39); HREIMS *m/z*: 200.0837 (calculated for $C_{13}H_{12}O_2$, 200.0838).

1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**): Amorphous colorless solid. The spectral data of this compound were confirmed with the results published elsewhere (24).

Isolation and Maintenance of HUVEC

Primary HUVEC were obtained from fresh human umbilical veins with a slight modification as previously described (25). Isolated HUVEC cells were seeded on gelatinized dishes and routinely maintained in EGM2 medium containing 2% (v/v) FBS. To avoid genetic variation, resulting from different individuals, HUVEC from five or more different donors were pooled together. HUVEC at no more than six passages were used for the following experiments.

Growth and Maintenance of Various Cell Lines

Human oral cancer cell line KB and human lung carcinoma cells A549 were obtained from and maintained as described by American Tissue Cell Collection (Rockville, MD, USA). HACAT, immortalized human keratinocytes, were maintained as previously described (26).

Drug Treatment and Cell Proliferation Assay

Subconfluent HUVEC at early passages were used for cell proliferation assays. HUVEC at 5,000 cells/well were

Table I. NMR Data for Compound **1** (125 and 500 MHz in CD_3OD, *J* in Hz)

	1	
Position	δ_H	δ_C, mult
1	3.46 (2H, d, J = 5.6 Hz)	67.0, t
2	3.65 (1H, m)	71.7, d
3	1.60, 1.75 (each 1H, m)	32.8, t
4	2.50 (2H, m)	16.4, t
5	—	66.1,[a] s
6	—	61.8,[a] s
7	—	60.1,[a] s
8	—	60.9,[a] s
9	—	62.4,[a] s
10	—	65.0,[a] s
11	—	81.4,[a] s
12	—	77.9,[a] s
13	1.98 (3H, s)	3.8, q

[a] Data among positions number 5 to 12 may be interchanged.

seeded in a gelatinized 96-well tissue culture plate. After 12 h, cells were cultured in starvation medium containing 1% heat-inactivated FBS and 0.1% BSA in M199 for 12 h. Serum-deprived cells were treated with starvation medium supplemented with 1 ng/ml bFGF together with the indicated concentration of herbal extract, compound or vehicle for 48 h. Likewise, cancer cells and immortalized cell lines, that is, KB, HACAT, and A549, at 3500–5000 cells/well, were starved overnight with serum-free medium containing 0.1% (w/v) BSA. For antiproliferation assay, the individual cell lines were cultured in their respective growth medium containing 2% FBS, and treated for 48 h with phytocompound or plant extract. Cell proliferation assays were performed using the Cell Titer 96^R AQueous One Solution Cell Proliferation Assay kit. All assays were performed in quadruplicate and two to three independent experiments were conducted to confirm the effects of growth factor treatment.

Cell Migration Assay

HUVEC at a density of 2.2×10^4 cells/well in M199 medium with 0.1% (w/v) BSA were plated in triplicate at the bottom of a modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). The chamber was assembled and inverted, and cells were allowed to attach for 2 h at 37°C to polycarbonate membranes (8-μm pore size). The chamber was then re-inverted and the indicated concentrations of plant extracts or vehicle, mixed with 1 ng/ml of bFGF, in treatment medium were added to the wells of the upper chamber. After 4 h incubation at 37°C, the cells that had migrated to the upper chamber were fixed, and stained with a diluted Giemsa solution (1:10). The number of cells per 9 high-power fields (HPF) at 400× magnification was counted. Each experiment was repeated twice. The Student's *t* test was used to determine the significance ($p < 0.05$) of each treatment compared to the control. HUVEC treated with 1 ng/ml of bFGF only served as a positive control.

Western Blot Analysis

Serum-deprived subconfluent HUVEC or various cell lines were treated for 24 h with compounds identified from *B. pilosa* in the treatment medium containing 1 ng/ml of bFGF. Phytocompound-treated or vehicle-treated (control) HUVEC cells were lysed in boiled lysis buffer, containing 1% (w/v) SDS and 10 mM Tris-HCl (pH 7.4). Following centrifugation at 13,000 rpm for 10 min, the protein concentration in each lysate was measured by Bradford protein assay. Equal amounts of total protein were fractionated by SDS-PAGE, and then blotted onto PVDF membrane. The protein blot was hybridized with the primary antibody of interest then with alkaline phosphatase-conjugated secondary antibodies, followed by detection using Renaissance Chemiluminescence Reagent Plus.

Tube Formation Assay of Endothelial Cells in a Collagen Gel

A suspension of HUVEC (3×10^5 cells/well) in M199 medium was mixed with 5.7X M199, 1 mM HEPES, 0.125% (w/v) $NaHCO_3$, 1 mM NaOH, 1.5 mM $CaCl_2$, and 0.2% (w/v) rat tail tendon collagen on ice. The rat tail collagen was isolated as described (27). The mixture (1 ml/well) was added to a 6-well plate. After formation of collagen gel at 37°C, 1 ml/well of M199 supplemented with 1% (w/v) heat-inactivated FBS, 0.5 mg/ml ECGS, 20 U/ml heparin, 0.08 mM PMA, 0.05 mg/ml ascorbic acid, 40 ng/ml VEGF, and 40 ng/ml bFGF together with phytocompound from *B. pilosa* or not, was added to each well. The whole assay was incubated at 37°C for 6 days with drug replenishment every 2 days. These wells during the drug treatment were observed by light microscopy and pictures taken at 200× magnification.

Apoptosis Assay by Flow Cytometry

Annexin V, a calcium-dependent phospholipid-biding protein with high affinity for phophatidyl serine (PS) was used to detect early stage apoptosis. Briefly, serum-depleted HUVEC were treated or not treated with 2.5 μg/ml of compound **1** or **2** together with 1 ng/ml bFGF and 1% (v/v) FBS for the indicated times. UV-treated HUVEC was used as a positive control, whereas EGM2-treated cells served as a negative control. Cells were then washed and incubated with annexin V-FITC followed by propidium iodide (PI) staining. The cells were then analyzed using a Becton Dickson FACstar plus flow cytometer. In each sample, a minimum of 10,000 cells were counted and stored in list mode. Data analysis was performed with standard Cell Quest software (Becton-Dickinson). Each experiment was repeated twice.

RESULTS

EA Fraction of *B. pilosa* Extract Inhibits the Tube Formation of HUVEC in Collagen Gels

Angiogenesis is a process involving proliferation, migration, and tube formation of endothelial cells. We found in preliminary experiments that the total 70% ethanolic extract of *B. pilosa* inhibited tube formation of HUVEC on collagen gels (data not shown). We further examined the effects of EA, BuOH, and water fractions derived from the total 70% ethanolic extract on the tube formation of HUVEC. The EA fraction of *B. pilosa* (25 μg/ml) exhibited a strong inhibitory effect on the tube formation of HUVEC as compared to that of the vehicle-or PBS-treated cells. Conversely, at a dosage of 25 μg/ml the BuOH and water fraction had little or no effect phenotypically on HUVEC seeded in collagen gels (Fig. 1).

EA Fraction of *B. pilosa* Extract Inhibits Proliferation but not Migration of HUVEC

To evaluate whether plant extracts of *B. pilosa* had any inhibitory effect on cell proliferation or migration of HUVEC, bFGF at 1 ng/ml followed by 25 μg/ml of EA, BuOH, or water fractions were added to culture media. The result in Fig. 2A shows that the EA fraction of *B. pilosa* potently attenuated the bFGF-promoted proliferation of HUVEC. Approximately 80% of bFGF-promoted cell proliferation was inhibited by the EA fraction. Unlike the EA fraction, both the BuOH and water fractions of *B. pilosa* had slight stimulatory effects (1.3- to 1.5-fold increase) on bFGF-promoted HUVEC proliferation, as compared to the control cells. These results indicated that EA but not other subfractions from the total ethanolic extract of *B. pilosa* contained certain compounds that could inhibit the proliferation of HUVEC.

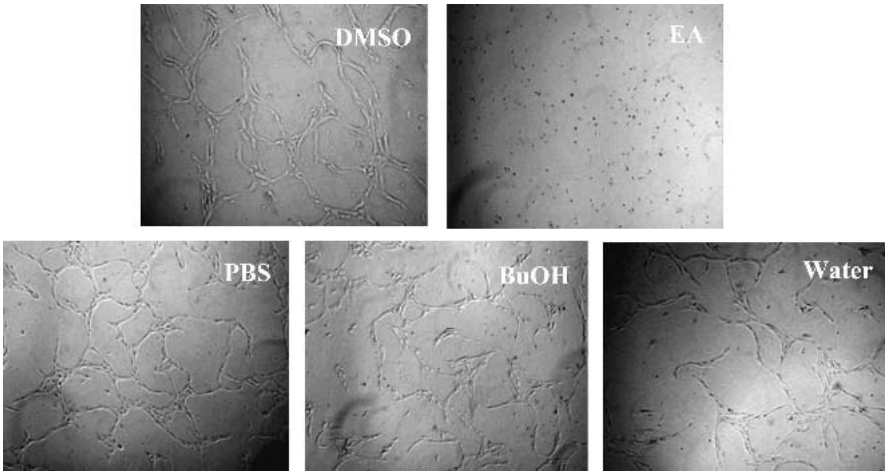

Fig. 1. Inhibitory effect of plant extracts from *B. pilosa* on tube formation of HUVEC. HUVECs seeded in collagen gels were treated for 6 days with 0.1% DMSO (vehicle for EA fraction), 0.1% PBS (vehicle for BuOH and water fractions), EA, BuOH, and water fractions of *B. pilosa* at 25 μg/ml in tube forming medium. The tube-like structures in each well were observed using light microscopy and photographed at 200× magnification.

The EA, BuOH, and water fractions of *B. pilosa* were also investigated for their effect on HUVEC migration. None of the three fractions manifested any negative effect on the migration of HUVEC toward bFGF (Fig. 2B). Instead, all three fractions significantly promoted HUVEC migration with a p value less than 0.05. Among the test extracts, the EA fraction had the greatest stimulatory effect on HUVEC migration by almost 2-fold in comparison to that of the vehicle control (0.1% DMSO-treated) cells.

Structural Elucidation of Compounds 1 and 2

Because the EA fraction of *B. pilosa* possessed the ability to inhibit tube formation and cell proliferation of HUVEC, we then used high-performance liquid chromatography to obtain the active phytocompounds. The structure of compounds **1** and **2** are shown in Fig. 3. Compound **1** had high-resolution mass spectroscopy (HREIMS) and ^{13}C NMR data consistent with the molecular formula $C_{13}H_{12}O_2$, indicating eight indices of hydrogen deficiency (IHD). The IR spectrum showed the presence of hydroxyl groups (3364 cm^{-1}) and acetylenic groups (2229 cm^{-1}). The ^1H NMR spectrum of **1** (Table I) exhibited signals for a singlet methyl group [δ_H 1.98 (s, H_3-13)], four methylene protons [δ_H1.60 and 1.75 (each 1H, m, H_2-3), and 2.50 (2H, m, H_2-4)], two oxymethene protons [δ_H 3.46 (2H, d, J = 5.6 Hz, H_2-1)], and one oxymethine proton [δ_H 3.65 (1H, m, H-2)]. ^{13}C (Table I), and DEPT spectra of **1** indicated one CH_3, three CH_2, one CH, and eight C, including two carbons to which oxygen was attached [δ_C 67.0 (C-1) and 71.7 (C-2)]. From the molecular formula and the above evidence, compound **1** was identified as a tetrayne with a terminal methyl group and a vicinal diol at the other terminus. Therefore, compound **1** is a novel compound: 1,2-dihydroxytrideca-5,7,9,11-tetrayne. Compound **2**, 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne, identified in this report from *B. pilosa* had similar ^1H and ^{13}C NMR data as **1** on its polyyne moiety and the spectral data was previously published (19).

EA Fraction and Its Derived Compounds (1 and 2) Specifically Inhibit Proliferation of HUVEC in a Dose-Dependent Manner While Having a Minor or No Effect on Other Types of Cells

To examine whether the inhibitory effect of the EA fraction of *B. pilosa* on cell proliferation was specific to HUVEC, we treated serum-deprived HUVEC and an oral cancer cell line KB, respectively, with EA fraction in the presence of growth factors for 48 h. We then conducted cell proliferation assays. The result in Fig. 4A shows that the EA fraction at a range of 5–25 μg/ml dose-dependently blocked bFGF-promoted HUVEC proliferation. The proliferation of EA-treated HUVEC was detected as only approximately 20% relative to the vehicle control (0.1% DMSO-treated) cell, while only minor effect for the inhibition of KB cell proliferation was noted at the same EA (25 μg/ml) treatment (Fig. 4A).

1,2-Dihydroxytrideca-5,7,9,11-tetrayne (**1**) and 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**) identified from the active EA fraction of *B. pilosa* were also analyzed for their inhibitory effect on the proliferation of HUVEC, KB, human lung carcinoma cell line A549, and human keratinocytes HACAT. Compounds **1** and **2** inhibited HUVEC proliferation with IC_{50} values of 2.5 μg/ml (= 12.5 μM) and 0.375 μg/ml (= 1.725 μM), respectively (Figs. 4B and 4C). More than 80% of HUVEC proliferation was inhibited with 5 μg/ml of compounds **1** or **2**, while only minor or no detectable inhibition was observed in KB, A549, or HACAT cells. Therefore, compounds **1** and **2** strongly and specifically inhibited HUVEC proliferation but not the growth of other test cell types.

Effects of Compounds 1 and 2 on Cell Cycle Mediators

To examine whether the inhibition of HUVEC proliferation by compounds **1** and **2** was through deregulation of various cell cycle mediators, Western blot analyses of total proteins isolated from HUVEC treated with vehicle (0.1%

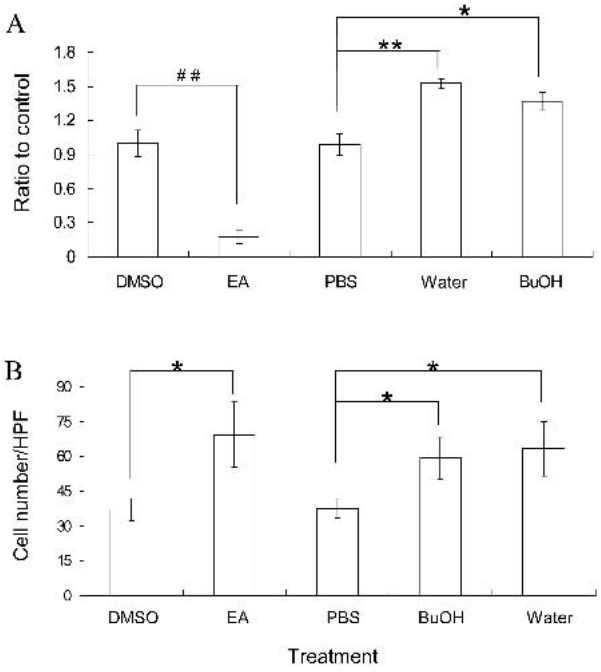

Fig. 2. Attenuation of HUVEC proliferation but not migration by the EA fraction of *B. pilosa*. (A) Serum-starved HUVECs were treated for 48 h with bFGF (1 ng/ml) and either vehicles (0.1% DMSO or PBS) or the EA, BuOH, or water fractions of *B. pilosa* at 25 μg/ml. Each assay was repeated at least in triplicate. Any changes in cell proliferation of the treated samples were normalized using the vehicle-treated cells as a control and expressed as a mean ratio of the control (mean ± SD). Significant inhibition (EA-treated vs. vehicle control) is indicated by ##, with a p value <0.01, whereas significant promotion (Water- or BuOH-treated vs. vehicle control) is indicated by * and **, with p values <0.05 and <0.01, respectively. (B) HUVEC treated with vehicle (DMSO or PBS) or with EA, BuOH, or water fractions from *B. pilosa* were subjected to cell migration assays. The total cell number in 9 high-power fields (HPF) (400× magnification) on the upper side of the porous membrane was used to represent the migratory cells responding to stimulation. Changes in cell numbers of the extract-treated samples were compared to vehicle-treated cells to ascertain their statistical significance. The data are shown as cell number/HPF ± SD. Significant enhancement of cell migration is indicated with one asterisk (*), with a p value less than 0.05.

DMSO) and compound **1** or **2** were performed. In this study, the protein levels of two cyclin-dependent kinase inhibitors (CKIs), p21(Cip1) and p27(Kip1), cyclins D1 and E involved in G1 to S transition, and p53 in HUVEC during a 24-h ex-

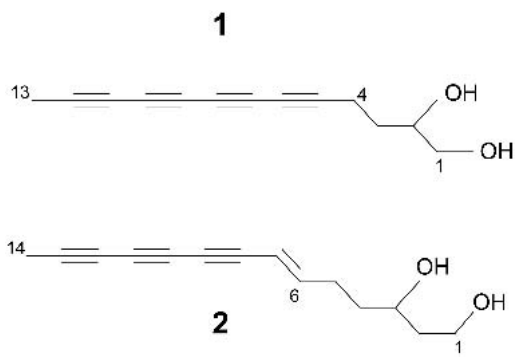

Fig. 3. Chemical characteristics of compounds **1** and **2**.

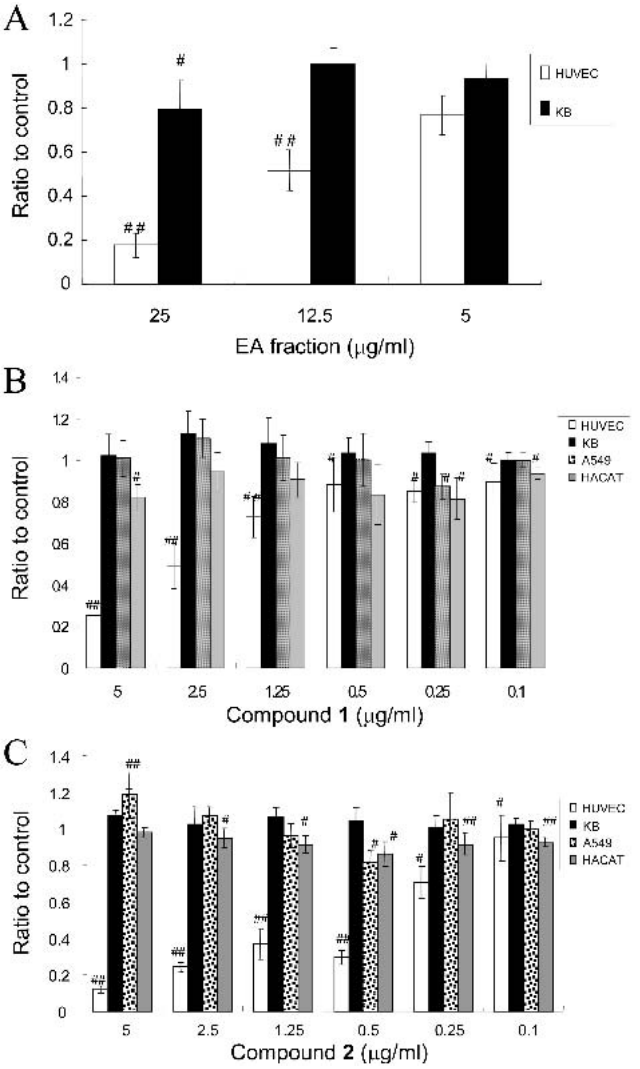

Fig. 4. Dose-dependent repression of HUVEC but not other cell lines by the EA fraction of *B. pilosa* and its derivatives, compounds **1** and **2**. (A) Following treatment with the EA fraction of *B. pilosa* at 12.5 and 25 μg/ml, HUVEC proliferation was drastically inhibited, whereas treatment with the EA fraction at 25 μg/ml had only a slight inhibitory effect on KB cell proliferation. (B) Following treatment with compound **1** at 0.1–5 μg/ml for 48 h, HUVEC proliferation was inhibited, while at the same concentration range HACAT proliferation was only slighted inhibited. Compound **1** had little or no effect on KB or A549 cells. (C) HUVEC, KB, A549 and HACAT were treated with 0.1–5 μg/ml of compound **2** for 48 h followed by cell proliferation assays. Compound **2** significantly attenuated the proliferation of HUVEC but not other cell lines in a dose-dependent manner. The change of cell proliferation in the treated samples was normalized using DMSO-treated cells as a control and expressed as a mean ratio of control. Significant inhibition (compound-treated vs. control) is indicated by # and ##, with p values less than 0.05 and 0.01, respectively. Significant promotion (compound-treated vs. control) is indicated by **, with a p value less than 0.01.

posure to compound **1** or **2** were examined. The results in Fig. 5 show that, compared to the vehicle control, compounds **1** and **2** had a stimulatory effect on the protein level of p27(Kip1) while having little detectable effect on that of cyclin D1 or p53. Approximately 2.2-fold and 3.0-fold increase in protein levels of p27(Kip1) were observed in compound **1**-

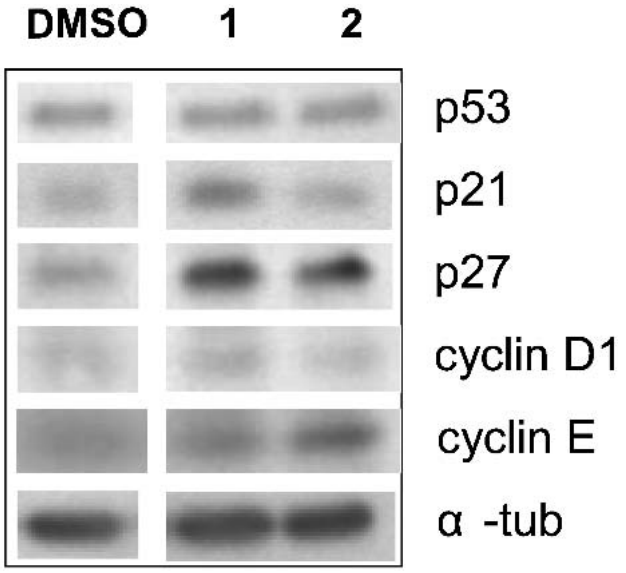

Fig. 5. Deregulation of cell cycle mediators in compound **1**- or **2**-treated HUVEC. Serum-starved HUVECs were treated or not treated for 24 h with 2.5 μg/ml of compounds **1** or **2** in the presence of bFGF (1 ng/ml). Protein lysates were subjected to SDS-PAGE, followed by Western blot analysis using specific antibodies against p53, p21, p27, cyclin D1, and cyclin E. The α-tubulin, indicated in the bottom panel, serves as a loading control. Specific protein contents in control or compound-treated HUVECs were quantified using densitometry.

and compound **2**-treated cells, respectively, as compared to that of the control (0.1% DMSO-treated) cells. Interestingly, compound **2**, but not **1**, had a stimulatory effect on the expression of cyclin E (1.4-fold), whereas only compound **1** increased the expression (2.3-fold) of p21(Cip1) in HUVEC. We have also examined whether compounds **1** and **2** affected the expression of various cell cycle mediators, including p53, p21(Cip1), p27(Kip1), and cyclins D1 and E, in KB, A549, and HACAT cell lines. No detectable effects on these proteins were observed in the three cell lines, as evaluated using Western blot analysis (data not shown). These results demonstrate that the effect of polyacetylene compounds on the expression of cell cycle mediators is highly specific to HUVEC.

Effects of Compounds 1 and 2 on Tube Formation and Migration of HUVEC

Angiogenesis, formation of new blood vessels from preexisting endothelium, consists of distinct processes including endothelial cell migration, proliferation and differentiation, and tube formation. Compounds **1** and **2**, already shown to inhibit HUVEC proliferation, were then characterized for their effect on tube formation of the same cells. Results in Fig. 6 show that compounds **1** or **2** at 2.5 μg/ml (12.5 μM and 11.5 μM for **1** and **2**, respectively) attenuated the ability of HUVEC to form tube-like structures in collagen gels. Unlike the results obtained from cell proliferation assays, compound **1** appeared to have a more potent inhibitory effect than that of the compound **2** on tube forming ability. Although migration is one of many processes involved in tube formation in collagen gel, we found, however, that compound **1** or **2** had no significant effect on HUVEC migration (data not shown),

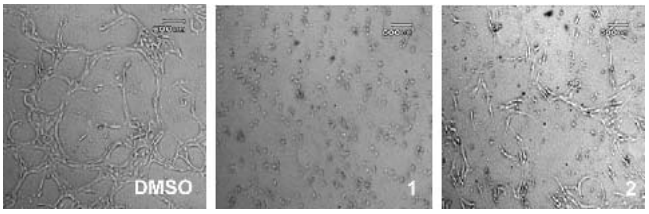

Fig. 6. Differential inhibitory effects of compounds **1** and **2** on tube formation of HUVEC in collagen gels. HUVECs seeded in collagen gel were treated for 6 days with 0.1% DMSO (vehicle), compound **1** or **2** at 2.5 μg/ml in tube forming medium. Treated cells were observed by light microscopy and photographed at 200× magnification.

suggesting that the inhibitory effect of both compounds on tube formation was not through alteration of endothelial cell migration.

Compound 2 Induces Apoptosis in HUVEC

We examined in this study if induction of apoptosis was involved in the angiogenic inhibition mediated by both compounds **1** and **2** in HUVEC. Flow cytometric analysis of control and compound-treated HUVEC stained with annexin-V and propidium iodide (PI) was performed. Less than 10% of cells were found apoptotic in vehicle-treated control (24 h). Significantly increased apoptotic HUVEC by a level of 9–10%, as compared to the vehicle control, was observed when the cells were treated with compound **2** for 24 h, whereas compound **1** exhibited very little apoptotic effect (Fig. 7). Very similar results were obtained in two independent experiments. This result suggests that the anti-angiogenic effect of compound **2** may be partly via induction of apoptosis in HUVEC.

DISCUSSION

Angiogenesis is a process of new capillary formation involving the degradation of the vascular basement membrane, endothelial cell proliferation, migration, and tube formation (3). It is known that tumor growth and metastasis depend on

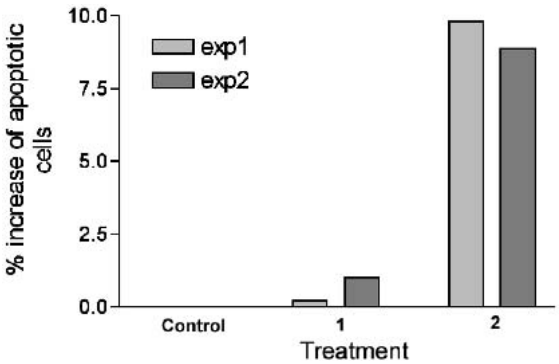

Fig. 7. Apoptotic effect of compounds **1** and **2** on HUVEC. Subconfluent HUVECs were treated with 2.5 μg/ml of compound **1** or **2** in the presence of 1 ng/ml bFGF and 1% FBS for 24 h. HUVEC treated with 0.1% DMSO in the same medium served as a control. Following treatment, cells were harvested for staining with PI and annexin V. Apoptotic cells at early phase were PI-negative but annexin V-positive. Two independent experiments are shown as exp1 (gray bar) and exp2 (black bar). Compared to vehicle control, apoptosis was expressed as % increase of apoptotic cells.

angiogenesis (2). Many plant-derived polyphenols, like resveratrol and quercetin from grapes and other plant sources, have been shown to inhibit tumor growth *in vivo* (28,29) and angiogenesis *in vitro* (30), suggesting that compounds such as these may be useful in the development of anti-angiogenic drugs against cancer. In searching for anti-angiogenic agents from natural products, in this study we demonstrated that an enriched extract (EA fraction) from a medicinal plant, *Bidens pilosa*, can confer a selective, inhibitory effect on tube formation and proliferation of HUVEC. Using bioactivity-guided fractionation and various spectral analyses, we further identified two active polyacetylene compounds (**1** and **2**) that possessed significant anti-angiogenic bioactivities from the active EA fraction of *B. pilosa*. 1,2-Dihydroxytrideca-5,7,9,11-tetrayne (**1**) is a novel compound that has never been reported before. Compound **2**, 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne, is an aglycone derivative of 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne. This acetylenic glucoside was previously isolated from *B. pilosa* var. *radiata* and found to possess anti-hyperglycemic activities via lowering blood glucose levels in type II diabetic mice (13). In this study, we demonstrated both polyacetyelene aglycones (**1** and **2**) manifest specific anti-proliferative and anti-tube forming abilities against human endothelial cells, but not against cancer cells or keratinocytes. This observation is very similar to most anti-angiogenic drugs that endothelial cells are the primary targets for their inhibitory effects on cell proliferation. Although the IC_{50} value of compound **2** for inhibiting HUVEC proliferation was approximately 6.5-fold lower than that of compound **1**, compound **1** appeared to be more potent than compound **2** in attenuating tube formation of HUVEC in collagen gels. The differential effects on HUVEC proliferation and tube formation of both compounds may be due to distinct mechanisms of action on endothelial cells.

Cyclin is one essential component of cyclin-dependent kinase (Cdk) complex and plays a crucial role in regulating the activity of Cdk. D-type cyclins, like cyclin D1, are active in mid-G1 phase, whereas E-type cyclins, like cyclin E, are active in late-G1 phase prior to the transition of cell cycle from G1 to S phase (31). Although deregulation of both forms of cyclins may either arrest cycling cells in G1 or promote their transition into S phase, the slight increase of cyclin E in compound **2**-treated HUVEC had no gross effect on progression of the test cells when analyzed using flow cytometric analysis with annexin V and PI staining (data not shown). We have also examined the protein level of p53, a gatekeeper for cell cycle progression. Deregulation of p53 can induce cell cycle arrest or apoptosis (32). However, we did not observe any alteration in the expression of p53 in compound **1** or **2** treated endothelial cells. These results indicate that the anti-angiogenic effect of compound **1** or **2** may not affect cell cycle progression.

Both cyclin-dependent kinase inhibitors (CKI), that is, p21(Cip1) and p27(Kip1), belong to the Cip/Kip family, which bind to cyclin/Cdk complexes and arrest cell division (33). Overexpression of p27(Kip1) in cultured HUVEC inhibits their ability to form tube-like structures, underscoring a role of p27(Kip1) in regulating angiogenesis in addition to its role as a CKI to arrest the cell cycle (34). Here we found that p27(Kip1) expression in HUVEC was upregulated (2.2- to 3.0-fold increase) by both polyacetylene aglycones (**1** and **2**) (Fig. 6) and this may directly contribute to the inhibition of tube formation in HUVEC. Although p27(Kip1) was a common target for both compounds, a higher level of apoptosis was induced by compound **2** compared to compound **1** (Fig. 7), suggesting that the differential effect of both compounds on HUVEC apoptosis may not be through p27(Kip1). The induction of p21(Cip1) expression can be regulated by either a p53-dependent- and p53-independent mechanism (35, 36). Here, the induction of p21 observed in compound **1**-treated HUVEC was probably via a p53-independent pathway because there was no detectable difference between p53 protein levels in control and compound-treated cells (Fig. 5). However, the exact role of p27(Kip1) and p21(Cip1) in the anti-angiogenic mechanism of compounds **1** and **2** remains to be clarified.

In summary, this is the first report to demonstrate that the EA fraction of *B. pilosa* extract and polyacetylene aglycones, namely 1,2-dihydroxytrideca-5,7,9,11-tetrayne and 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne, exhibit significant and potent anti-angiogenic activities. The ability of both compounds to block angiogenesis is possibly in part through induction of p27(Kip1) and regulation of other cell cycle mediators including p21(Cip1) and cyclin E. The relationship of apoptosis and the angiogenic inhibition mediated by both compounds **1** and **2** in HUVEC are under investigated. More studies are warranted to ascertain the detailed mechanism of the action of compounds **1** and **2** on angiogenesis and the potential application of these compounds as anti-angiogenic drugs in cancer therapy.

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