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RESEARCH PAPER

Anti-angiogenesis and Anti-adipogenesis Effects of *Anthrisci radix* Extract

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Abstract Natural products in Chonnam, the Republic of Korea were screened *via* anti-angiogenesis experiments, and one candidate product of *A. radix* extract (ARE) was identified. *A. radix* extract (ARE) exerted dose-dependent inhibitory effects against angiogenesis, adipogenesis, and cell adhesion. ARE exhibits an angiogenesis inhibitory effect superior to that of the EGCG from the green tea leaves. The expression level of angiogenesis and adipogenesis-related signal molecules in the Western blotting was reduced by increasing the amount of added ARE. Moreover, a diet supplemented with ARE was deemed more effective in inducing weight loss in LB mice than a representative synthetic diet drug, orlistat, which incidentally caused the side effects of denuding the mice of their hair. These results indicate that ARE may prove to be a useful anti-adipogenic compound, and the *in vitro* results may be reflected at a later time under *in vivo* conditions.

Keywords: *anthrisci radix*, anti-angiogenesis, anti-adipogenesis, cell adhesion, orlistat

1. Introduction

Overweight and obesity are both conditions characterized by abnormal or excessive fat accumulation sufficient to present a risk to health; these conditions are known to be major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases, and cancer [1]. According to WHO statistics on overweight and obesity in 2008, more than 1.4 billion adults were overweight. Of these, over 200 million men and nearly 300 million women were obese [2]. The WHO further projects that by 2015, approximately 2.3 billion adults will be overweight, and more than 700 million will be obese. The fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories expended.

Angiogenesis is the formation of new blood vessels. Angiogenesis is, thus, a necessary requirement for a variety of disease conditions, such as proliferation and metastasis in cancer cells, rheumatism arthritis, and diabetic blindness [3]. A substantial body of evidence shows that neoplastic and non-neoplastic tissue growth is dependent on angiogenesis [4]. Neovascularization and adipogenesis are temporally and spatially coupled processes occurring during prenatal life, and they continue to interact reciprocally throughout adult life, *via* paracrine signaling systems [5]. Activated adipocytes generate multiple angiogenic factors, including leptin, angiopoietins, HGF, GM-CSF, VEGF, FGF-2, and TGF-beta, which either alone or collectively stimulate neovascularization during fat mass expansion. Moreover, neoadipogenesis is preceded or accompanied by an angiogenic response (endothelial cell proliferation, and vessel sprouting) [5-10]. Thus, the antiangiogenic agents constitute a novel therapeutic option for the prevention and treatment of human obesity and its associated disorders [5].

Although alimentotherapy, exercise, and habit amendment should all be part of the routine method to suppress obesity, there have also been advances in the development of chemically synthesized drugs to treat obesity [11]. These drugs can be largely divided into two categories. One class

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of drugs reduces food intake by suppressing appetite; and the other stimulates fat metabolism, suppresses lipogenesis, and increases metabolic activity [12]. However, those anti-obesity agents generally damage not only the adipocytes but also other normal cells, thus inducing some severe side effects on immunological functions *in vivo* [13].

Recently, a great many natural resources have been assessed for their possible utility for anti-obesity treatment [14,15], as natural chemicals frequently pose significantly less risks of deleterious side effects than synthetic chemicals [16]. Among these natural resources, certain herbs have been traditionally used in Asia for anti-obesity treatments. *Anthrisci radix* is one such natural herb, and has long been in use as traditional medicine in Asia [17]. According to a compendium of traditional Korean medicine literature, the Donguibogam, *A. radix* has been associated with a variety of pharmacological effects, including antithrombotic, anti-inflammatory, expectorant, and antioxidant effects. This study describes a new natural anti-obesity candidate - an *A. radix* extract (ARE) - which appears to exert profound inhibitory effects on angiogenesis, adipogenesis, cell adhesion, and *in vivo* diet in LB mice, without any detected side effects.

2. Materials and Methods

2.1. Ethanol extraction and freeze-drying of natural products

Eighteen natural products were purchased from a Korean herbal medicine dealer in Yosu, Chonnam, Korea. The products were homogenized with ethanol at a ratio of 4:6 (w/v). The homogenates were extracted with a heating mantle (Global Labware, Haryana, India) for 5 h at 60°C. The suspensions were filtered with No. 2 paper filters (0.2 μm, Whatman, NJ, USA) and membrane filters (Whatman, NJ, USA). The filtered extracts were then concentrated in a digital water bath (Dynalab Corp., NY, USA), and lyophilized. The powdered extracts were solubilized in DMSO and diluted for this experiment.

2.2. Cell culture of HUVEC, 3T3-L1, and U937

Human umbilical vein endothelial cells (HUVEC) were purchased from Young Science (Seoul, Korea) and cultured in 2% gelatin (Sigma, MO, USA)-coated T75 flasks (Angiotech, Vancouver, Canada). EBM-2 medium (Gibco, NY, USA) was utilized for the HUVEC cultures. Additional supplements added to the culture medium were as follows: hydrocortisone, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin like growth factor-1 (IGF-1), ascorbic acid, epidermal growth factor (EGF), GA-1000, heparin, and 2% fetal bovine serum (FBS). Cell cultures were conducted continuously in a 5% CO_2 incubator at 37°C until more than 80% confluence was achieved.

Mouse embryonic fibroblast adipose like cells (3T3-L1) were obtained from the Department of Food Nutrition, Pukyong University, Korea. DMEM medium (Gibco, NY, USA) was utilized for the 3T3-L1 culture. The following supplements were added to the culture medium: $NaHCO_3$ (3.7 g/L), penicillin G (63 mg/L), streptomycin (100 mg/L), and 10% FBS. Differentiated adipocytes also required the use of the differentiation inductor, MDI (0.5 mM 3-isobuty-1-methyl-zanthine, 1 μM dexamethasone, and 10 μg/mL insulin).

U937 human leukemic monocyte lymphoma cells (American Type Culture Collection, MD, USA) were used for the cell adhesion experiment. The cells were cultured in RPMI-1640 medium (Life Technologies, NY, USA) including 2 mM L-glutamine, penicillin (1 units/mL), streptomycin (100 mg/L), and 10% FBS.

2.3. Inhibitory effect of angiogenesis by natural extracts

HUVEC (2.5 × 104) was plated onto the Matrigel-coated wells of 24-well plates (BD Biosciences, MA, USA). Steady doses of natural extracts were added to the wells using EGCG (Sigma, MO, USA) as a positive control, and cultured for 4 h in a 5% CO_2 incubator at 37°C. The tube formation of HUVEC was photographed using a digital camera (Nikon, Tokyo, Japan), and the tube length was analyzed using Scion Image software (NIH, ML, USA). The tube lengths were compared to those of EGCG, and a candidate from the *A. radix* extracts (ARE) was selected for this study, among the eighteen natural extracts.

2.4. Cell toxic test against ARE

MTT assays were conducted to assess cellular toxicity against ARE. The 1 × 104 HUVECs (C2517A) in one well of a 96-well plate were incubated for 24 h. The ARE was treated at various concentrations (0.1 ~ 25 ppm) and incubated under constant conditions (37°C, 5% CO_2). After 48 h, 0.5% MTT solution (Sigma, MO, USA) was applied and incubated for 3 h. The absorbance of the well plate was scanned at 540 nm using a microplate reader (Biochrom Ltd., Cambridge, UK). The most appropriate concentration (0.2 ppm) was determined and used for this study.

2.5. HPLC fractionation of ARE and its angiogenesis inhibitory effect

Ethanol extract of ARE was subjected to a preparative size exclusion column (500 mm × 21.5 mm; Showa Denko, Tokyo, Japan). The exclusion HPLC apparatus consists of a LC-6AD pump (Shimadzu, Tokyo, Japan), a photodiode array detector (Shimadzu, Tokyo, Japan), an online degasser

(Shimadzu, Tokyo, Japan), an autosampler (Shimadzu, Tokyo, Japan), a fraction collector (Shimadzu, Tokyo, Japan), a system controller (Shimadzu, Tokyo, Japan), and LCsolution (Shimadzu, Tokyo, Japan). The extracts were chromatographed on an Asahipak GS-310 column eluted with methanol at a flow rate of 5.0 mL/min and monitored at 307 nm. The ARE was separated into five fractions. Each fraction was evaluated for its anti-angiogenesis effects. The most superior fraction, fraction 5, was applied at various concentrations (0.05 ~ 0.25 ppm) to HUVEC, and the morphological features were observed *via* microscopy.

2.6. Cell adhesion of U937 on HUVEC

HUVEC cells were incubated in 24-well plates until confluence, and subsequently treated for 6 h with 10 ng/mL of IL-1β (Gibco, NY, USA). The cells were treated overnight with different doses of ARE. U937 (2.5 × 105) was added to the HUVEC cells, and incubated for 30 min at 37°C, washed 3 times with 1% FBS/PBS. The average cell numbers were calculated on 5 randomly photographed areas.

U937 was incubated in 24-well plates until confluence, then treated with 100 mg/L of PMA (Sigma, MO, USA) for 2 h. The cells were treated overnight with different doses of ARE. The adhesion of U937 cells to HUVEC was assessed as described above.

HUVEC and U937 were incubated in 24-well plates until confluence, and the HUVEC cells were treated for 6 h with 10 ng/mL of IL-1β. Both cells were treated overnight with different doses of ARE. The cell adhesion of ARE-treated U937 cells on IL-1β treated HUVEC cells was evaluated as described above.

2.7. Oil red O staining and its inhibitory effect on angiogenesis

3T3-L1 preadipocytes (9.6 × 104) were plated onto 6-well plates (SPL, Gyeonggi, Korea) and cultured for 2 days. MDI and ARE were added to the wells and cultured for an additional 2 days. The medium was exchanged with DMEM/10% FBS, insulin, and ARE. The medium was then exchanged one additional time without insulin. The cells were fixed for 1 h with 10% formalin, washed with 60% 2-propanol, and completely dried. Oil red O working solution was added to the well, and washed 4 times with distilled water [18]. Completely dried cells were washed with 100% 2-propanol, and the eluted Oil red O solution was transferred to 96-well plates, followed by absorbance measurements at a wavelength of 520 nm.

2.8. Western blot analysis

In order to confirm the inhibitory effects of ARE on angiogenesis, Western blot analysis was conducted using the angiogenesis-related signal molecules, VE-cadherin and Akt. HUVEC (1 × 105) was seeded on 100-mm Petri dishes, then incubated in EBM-2 medium with 2% FBS until confluence. ARE was treated with different doses for 24 h, and the cells were washed in serum-free EBM-2 medium. Cells were rinsed with ice-cold PBS including 1 mM vandate, and then solubilized in ice-cold lysis buffer (1% NP, 100 mM MgCl2, 1 M Tris-HCl, pH 8.0, 100 mM sodium fluoric, 100 mM DTT, 2 mM sodium orthovanadate, 2 μg/μL aprotinin, 10 μg/μL pepstatin, 50 mM HEPES), using a gentle side-to-side rocking motion. The cells were scraped, and the lysates were centrifuged (230 × *g*, 10 min, 4°C). The supernatant (cytosol fraction) was collected and applied in a protein assay using BCATM Protein Assay Kit (Pierce, IL, USA). In brief, BCA reagent was added to the 96-well plate at different doses, and standard curves were prepared by measuring the absorbance at 562 nm. Immunoprecipitation was conducted in order to increase sensitivity and specificity, and identical volumes of the protein were employed. Polyclonal anti-VE-cadherin antibody was added to the protein with IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM NaF, 1 mM NaVO4, 5 μg/μl aprotin, 5 μg/μl pepstatin, 1% Triton X-100), and immunoprecipitated with Protein G-Sepharose (GE Healthcare, NJ, USA) at 4°C. After centrifugation (230 × *g*, 5 min, 4°C), the precipitate was washed 3 times in IP washing buffer, and subsequently boiled in Laemmli's sample buffer (Bio-Rad, CA, USA). The samples were then separated on 10% SDS-PAGE gel and transferred to 0.2 mm nitrocellulose membrane (Bio-Rad, CA, USA). The membranes were then blocked with 5% NFDM (Sigma, MO, USA) in PBS containing 0.1% Tween-20, washed with 1× TBST. It was then probed for 12 h at 4°C with either of the following primary antibodies: VEGFR2/flk-1, VE-cadherin, β-catenin, PI3-kinase, and Akt as a dilution ratio of 1:1,000 in 1% NFDM. The membranes were washed with 1× TBST, and conjugated with secondary antibodies (donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP, donkey anti-mouse IgG-HRP) as a dilution ratio of 1:1,000 in 1% NFDM. Immunoreactive bands in the membranes were visualized using SuperSignal West Pico Chemiluminescent Substrate reagent (Pierce, IL, USA), followed by the developer and fixer reagents (Kodak, Tokyo, JAPAN).

In an effort to confirm the inhibitory effects of ARE against adipogenesis, Western blot analysis was conducted on the differentiation substance-treated, ARE-treated group (Group 1), and ARE-added group after differentiation (Group 2) using the adipogenesis-associated signal molecules, C/EBPα, SREBP-1, and PPARγ. The 3T3-L1 cells (1 × 105) were seeded on 100-mm Petri dishes, and incubated to confluence in DMEM medium with 10% FBS. As for the Group 1, the MDI-treated and ARE-treated cells were incubated with DMEM/10% FBS, insulin, and ARE. After

2 days, the medium was exchanged with DMEM/10% FBS and ARE. With regard to the Group 2, 3T3-L1 preadipocytes were wholly differentiated into adipocytes, and ARE was applied at different doses. The cells were rinsed in ice-cold PBS with 1 mM vandate, then solubilized with ice-cold RIPA lysis buffer (Santa Cruz, CA, USA), with gentle side-to-side rocking motion. The cells were scraped and the lysates were centrifuged (230 × *g*, 20 min, 4°C). The supernatant (cytosol fraction) was collected and stored at -80°C before use. Proteins were quantified using BCA reagent. The protein was electrophoresed and transferred, and the membranes were blocked, washed, and probed with one of the following primary antibodies: C/EBPα, SREBP-1, and PPARγ; then visualized as described above.

2.9. Animal experiments

The 4-week-old male C57BL/6J-ob/ob mice were obtained from SLC, Inc. (Shizuoka, Japan) and housed under standard conditions (12-h light/dark cycle, 22°C). The animals were divided into 3 groups (10 individuals/group): a control group, an ARE group, and a positive control group, using the popular agent, orlistat. All 3 mouse groups were fed on high fat diet (45% kcal from fat, D12451 Research Diets, NJ, USA) for the initial 2 weeks of the experiment upon arrival. After that, saline solution, 25 ppm of ARE, and 10 μg of orlistat were subcutaneously injected into the tails of the mice once every 2 days for 4 weeks, respectively. Four weeks later, identical volumes were injected on a daily basis. The body weights were measured using an electronic scale at 2.5-day time intervals. Unconsumed pelleted high fat food was discarded each day and replaced with fresh high fat diet, to ensure consistent food quality was provided to the mice throughout the study. The high fat food was stored at 4°C. The current study protocol was approved by the Ethics Committee at the Chonnam National University for the animal studies. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.10. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). When differences were found among the dietary treatments, Duncan's multiple range test was used to compare the mean difference by using SPSS software (SPSS, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Selection of angiogenesis inhibitory candidate from 18 natural products

We conducted an examination of angiogenesis inhibitory effects to find a candidate among the 18 natural products selected from a compendium of traditional Korean medical literature, the Donguibogam. According to the results of the angiogenesis inhibitor analysis (Fig. 1), several natural products (*S. radix*, *C. furctus*, *A. folium*, *P. semen*, *C. flos*, *S. flos*, *V. rotundifolia*, *A. radix*, and *S. herba*) evidenced inhibitory effects superior to that of the 25 M EGCG in the green tea leaf. Among them, a natural product, *Anthrisci radix*, was selected for an assessment of its anti-angiogenic and anti-adipogenic mechanisms, and was also evaluated as a possible natural anti-obesity suppressor.

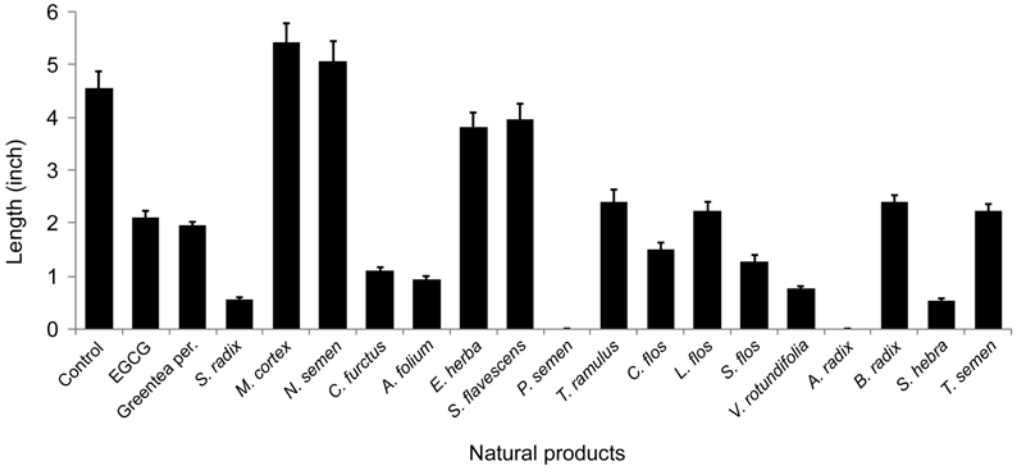


Fig. 1. Anti-angiogenesis experiment of 18 natural products with the positive control, EGCG. HUVEC was cultured on Matrigel-coated wells, and the tube formation of HUVEC cells was randomly photographed using a digital camera, and analyzed by the Scion Image software. Bar indicates standard deviation (n = 3).

3.2. Toxic test of ARE on HUVEC

According to the results of the MTT assay against ARE on HUVEC cells (Fig. 2), no significant changes were noted as compared to those of other ARE concentrations up to 0.2 ppm. More than 95% of HUVEC cells survived after the addition of 0.2 ppm ARE, but the cell viability was reduced dramatically with the addition of ARE at a concentration of 0.5 ppm. The toxicity of a compound occurs *via* the uptake of the compound into the cell or *via* interaction with the cell membrane and associated molecules. Endothelial cell toxicity induced by high levels of ARE may be attributable, at least in part, to an accumulation of intracellular toxicity. The results of the tests of ARE cytotoxicity in this *in vitro* cell culture system have demonstrated that ARE added at a concentration of at least 0.2 ppm is not harmful to cell cultures.

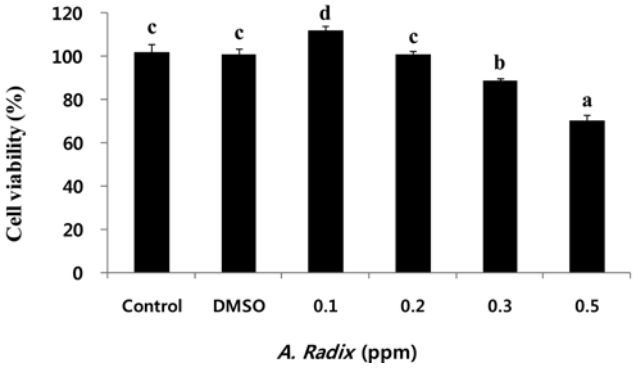


Fig. 2. Cytotoxicity in HUVEC cells with different concentrations of *A. radix* extract (ARE). HUVEC was seeded onto 96-well plates and treated with various doses (0.1, 0.2, 0.3, and 0.5 ppm) of ARE for 48 h. MTT solution was added to the wells, and the 96-well plates were incubated. The absorbances of the 96-well plates were measured with a microplate reader at 540 nm. The value was converted to cell viability based on the control. Bar indicates standard deviation (n = 3). Bars with different letters differ significantly ($P < 0.05$).

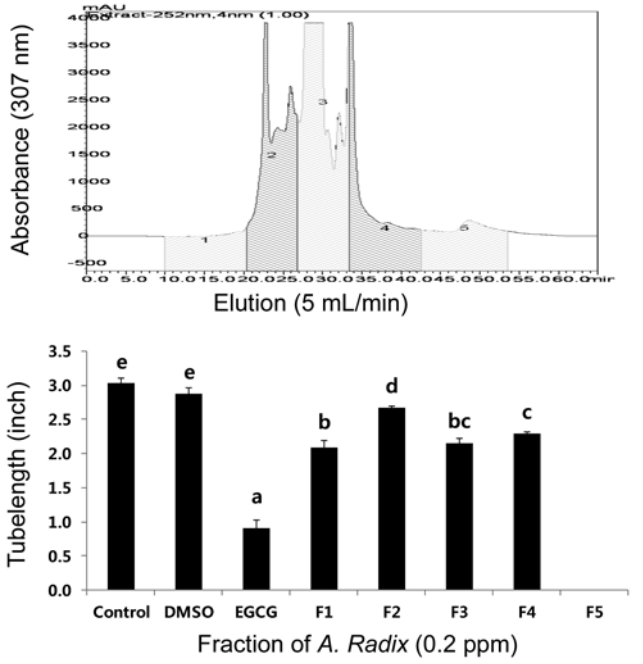
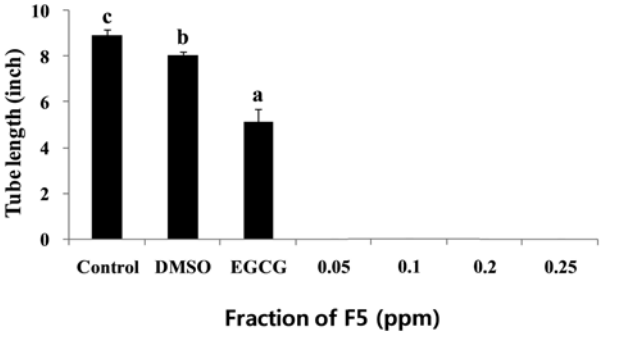


Fig. 3. ARE was subjected to preparative size exclusion column of Asahipak GS-310. ARE was chromatographed on an Asahipak GS-310 column eluted with methanol at a flow rate of 5 mL/min, and monitored at 307 nm. ARE was separated into five fractions. Each fraction was evaluated *via* anti-angiogenesis experiments. HUVEC was cultured on Matrigel-coated wells, and the tube formation of HUVEC cells was randomly photographed using a digital camera, and analyzed by the Scion Image software. Bar indicates standard deviation (n = 3). Bars with different letters differ significantly ($P < 0.05$).

3.3. Angiogenesis inhibitory effect of ARE fractions on HUVEC

ARE was fractionated into 5 fractions *via* HPLC and applied in the angiogenesis inhibitory experiment (Fig. 3). Five ARE fractions (F1, F2, F3, F4, and F5) and a positive control (EGCG) were added to HUVEC cells at identical concentrations (0.2 ppm). Among the fractions, F5 was identified as the optimal angiogenesis suppressor. F5 was further examined in angiogenesis inhibitory experiments at different concentrations (0.05, 0.1, 0.2, and 0.25 ppm). The results demonstrated dose-dependent increases in the observed



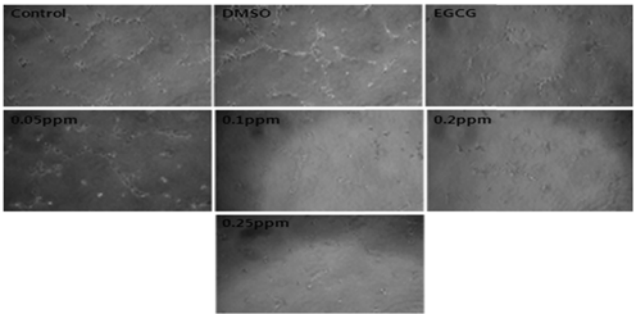


Fig. 4. Anti-angiogenesis effect of ARE fraction 5. Anti-angiogenesis experiments were conducted with different concentrations (0.05, 0.1, 0.2, and 0.25 ppm) of fraction 5. Bar indicates standard deviation (n = 3). Bars with different letters differ significantly ($P < 0.05$). The pictures show the cellular morphology of HUVEC cells treated with different concentrations of fraction 5.

angiogenesis inhibitory effects (Fig. 4). Moreover, 0.25 ppm of fraction 5 proved sufficient to block angiogenesis of the HUVEC cells morphologically. Because the cell culture medium contained low levels of FBS, which harbors residual quantities of growth factors, low levels of tubular formation were also noted in the control cells.

3.4. Inhibitory effect of cell adhesion

The inhibitory effects of cell adhesion were evaluated in *in vitro* experiments conducted under several conditions (Fig. 5). U937 adhered to IL-1β and ARE stimulated HUVEC cells (Fig. 5A). The ARE at concentrations of 0.05, 0.1, 0.15, and 0.2 ppm suppressed U937 cell adhesion by 204, 239, 252, and 276%, respectively. The PMA (phorbol myristyl atetate) and different concentrations (0.05, 0.1, 0.15, and 0.2 ppm) of ARE-stimulated U937 adhered to the HUVEC cells (Fig. 5B). The ARE at 0.05, 0.1, 0.15, and 0.2 ppm suppressed U937 cell adhesion by 12, 79, 79, and 139%, respectively. Different concentrations (0.05, 0.1, 0.15, and 0.2 ppm) of ARE-stimulated U937 adhered to IL-1β and different concentrations (0.05, 0.1, 0.15, and 0.2 ppm) of ARE stimulated HUVEC cells (Fig. 5C). ARE suppressed U937 cell adhesion in a dose-dependent manner, by percentages of 115, 131, 239, and 286%, respectively. As shown in Fig. 5, U937 and HUVEC cells were activated by PMA and IL-1β, individually (Figs. 5A and 5B) or together (Fig. 5C). The best cell adhesion inhibitory effect was noted in HUVEC-adhering PMA and ARE-stimulated U937 cells (Fig. 5B), followed by IL-1β- and ARE-stimulated HUVEC-adhering ARE-stimulated U937 cells (Fig. 5C), and IL-1β- and ARE-stimulated HUVEC-adhering U937 cells (Fig. 5A). The U937 adhesion rate increased significantly when HUVEC cells were stimulated with IL-1β, because IL-1β acts preferentially on HUVEC cells, whereas PMA activates U937 [19,20]. The stimulated adhesion was inhibited clearly by the addition of ARE, and this effect was also observed to occur in a dose-dependent manner. This result demonstrated that ARE exerts an inhibitory effect on cell adhesion, exhibiting an angiogenesis-inhibitory effect.

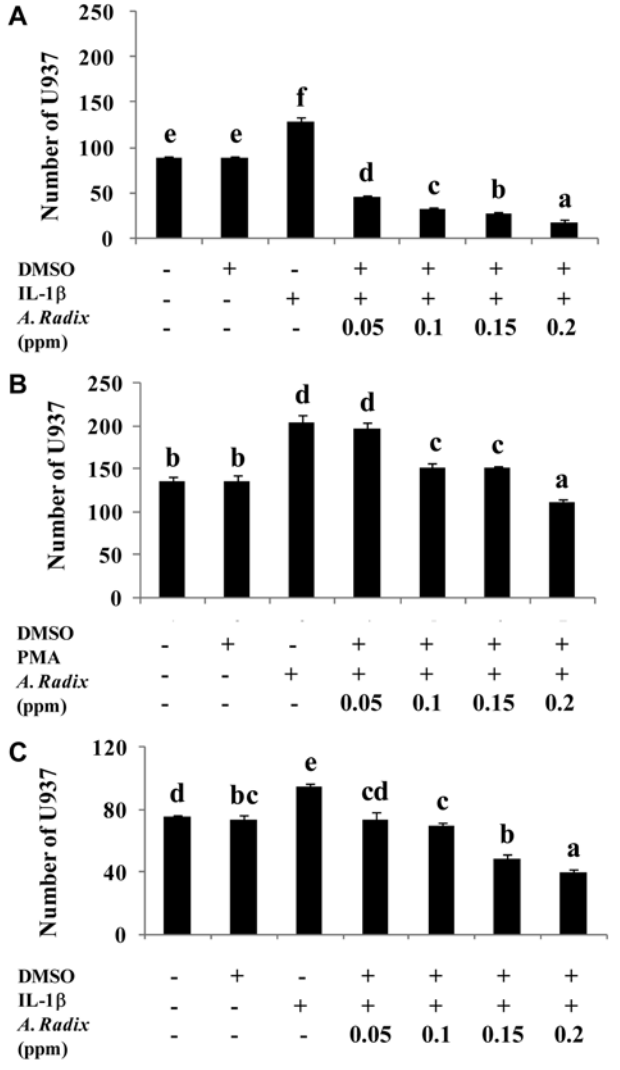


Fig. 5. Various doses (0.05, 0.1, 0.15, and 0.2 ppm) of ARE addition on U937 cell adhesion. (A) Cell adhesion of U937 on IL-1β and ARE stimulated HUVEC, (B) cell adhesion of PMA- and ARE-stimulated U937 on HUVEC, (C) cell adhesion of ARE-stimulated U937 on IL-1β- and ARE-stimulated HUVEC cells. Each treatment in the cell adhesion experiments was conducted as described in the MATERIALS and METHODS section. Bar indicates standard deviation (n = 3). Bars with different letters differ significantly ($P < 0.05$).

3.5. Western blot of angiogenesis-related signal molecules on HUVEC Cells

Because VE-cadherin, β-catenin, and Akt have been identified as important mediators of angiogenesis [21,22], we assessed the role of VE-cadherin, β-catenin, and Akt in angiogenesis under conditions of cell stimulation by VEGF

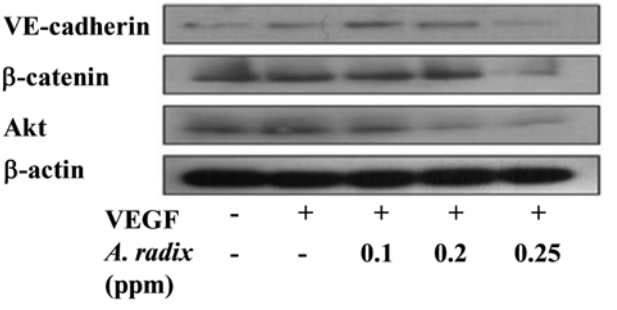


Fig. 6. ARE inhibits the interaction of PI3-kinase with VE-cadherin, β-catenin, and Akt upon cell activation with VEGF. Proteins from HUVEC cells were immunoprecipitated with PI3-kinase antibody and immunoblotted with antibodies to VE-cadherin, β-catenin, and Akt. β-actin was employed as a positive control.

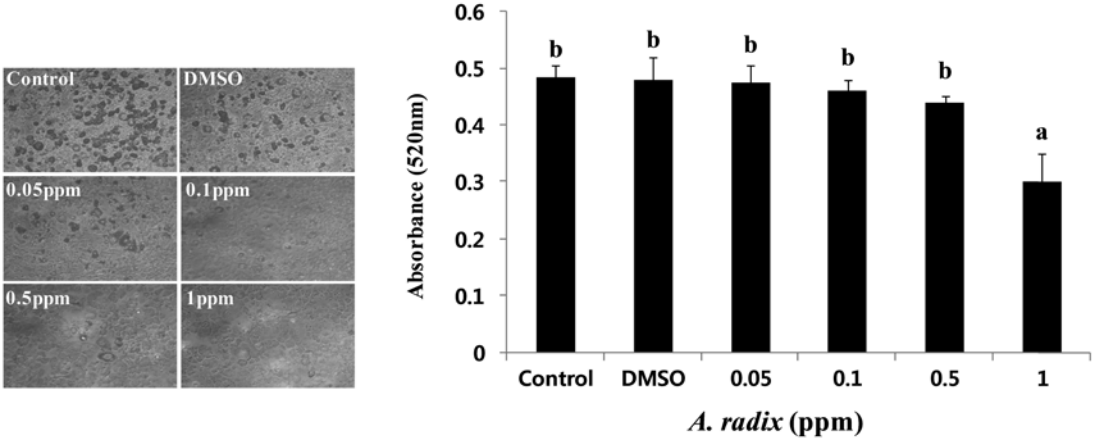


Fig. 7. The effect of ARE addition in the course of 3T3-L1 adipocyte differentiation. 3T3-L1 was treated with ARE at various concentrations (0.05, 0.1, 0.5, and 1 ppm). After differentiation, lipid accumulation was stained with Oil red O solution, and the morphological changes were observed *via* microscopy. The Oil red O stained lipid was quantified with a microplate reader at 520 nm. Bar indicates standard deviation (n = 3). Bars with different letters differ significantly ($P < 0.05$).

and with different concentrations (0.1, 0.2, and 0.25 ppm) of ARE. ARE-induced inhibition of tube formation in HUVEC cells was determined *via* the suppression of the signal pathways of VE-cadherin, β-catenin, and Akt. The expression of signal molecules was evaluated *via* immunoprecipitation and Western blotting analysis. As shown in Fig. 6, VEGF affected a significant increase in the number of signal molecules, and with pre-supplementation of HUVEC with dose-dependent dosages of 0.2 and 0.25 ppm, ARE inhibited the expression of signal molecules - VE-cadherin, β-catenin, and Akt. As the result of the ARE-induced suppression of the pathways from two kinds of signal molecules to NF-κB, angiogenesis was reduced in a dose-dependent manner. Therefore, blocking the function of VE-cadherin, β-catenin, and Akt by the addition of ARE directed against the molecules inhibits its function during the maturation of cells for tube formation.

3.6. Adipogenesis inhibitory effect of 3T3-L1 adipocyte by oil red O staining

Differentiated 3T3-L1 adipocytes were treated with ARE at various concentrations (0.05, 0.1, 0.5, and 1 ppm) for 2 days. The effect of ARE on lipid accumulation was measured by Oil red O staining (Fig. 7). Relative to the control cells, ARE treatment reduced intracellular lipid content in a dose-dependent manner, and the most effective dosage for inhibition was found to be 1 ppm of ARE. The results indicated that ARE could inhibit lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner.

3.7. Western blot of adipogenesis-related signal molecules on 3T3-L1 adipocyte

In order to evaluate the differentiation responses of translational factors before and after differentiation, 3T3-L1 preadipocytes were treated with MDI and different concentrations (0.05, 0.1, and 0.25 ppm) of ARE. Proteins were isolated from 3T3-L1 preadipocytes, and Western blot analysis was conducted. The protein expression level of the adipogenesis-mediated signal molecule, PPARγ, was reduced with the addition of 0.25 ppm ARE (Fig. 8A).

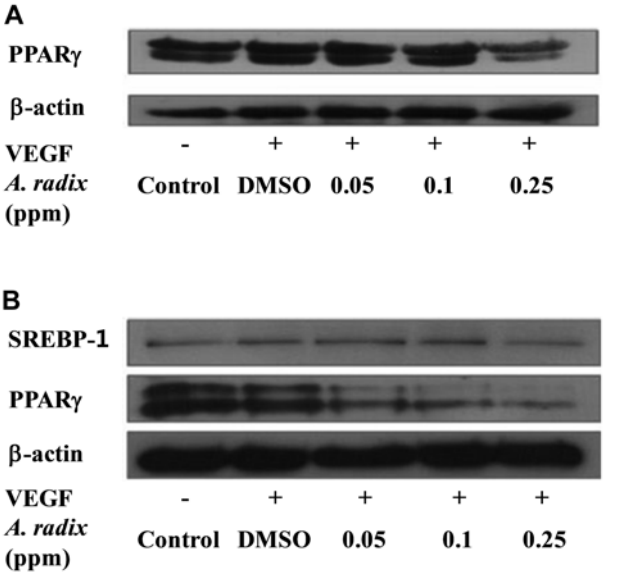


Fig. 8. ARE inhibits the interaction of PI3-kinase with VE-cadherin and Akt upon cell activation with VEGF. Proteins from HUVEC cells were immunoprecipitated with PI3-kinase antibody and immunoblotted with antibodies to VE-cadherin and Akt. β--actin was employed as a positive control. Expression of PPARγ on 3T3-L1 preadipocyte with ARE (A), and expression of SREBP-1 and PPARγ on 3T3-L1 adipocytes with ARE (B). Protein from 3T3-L1 was treated with different concentrations (0.05, 0.1, and 0.25 ppm) of ARE, and extracted with RIPA buffer for Western blotting.

3T3-L1 was completely differentiated and treated with different concentrations (0.05, 0.1, and 0.25 ppm) of ARE. The protein isolated from the 3T3-L1 was subjected to Western blotting using the adipogenesis-mediated signal molecules, SREBP-1 and PPARγ. In the adipogenic signal pathway, the protein expression levels of SREBP-1 and PPARγ began to decrease upon the addition of 0.1 ppm ARE (Fig. 8B). Additionally, the protein levels of SREBP-1 and PPARγ were reduced in a dose-dependent manner after ARE treatment, regardless of differentiation. Therefore, the results demonstrated that ARE was more effective in suppressing adipogenesis in the adipocytes than the preadipocytes *via* the down-regulation of SREBP-1 and PPARγ expression.

3.8. Animal experiments

Significant weight reductions were not observed in any of the groups for the initial four weeks, from the unsteady ARE concentration in the body from supplying ARE once at 2-day intervals. However, the significant weight change was initially observed after 4-weeks of daily ARE supply. No positive effects were noted in the group treated with orlistat, a potent competitive inhibitor of gastric and pancreatic lipase [23,24], but this had a side effect; it denuded the mice of their hair (Fig. 9, inset). The chemically synthesized anti-obesity agent, orlistat, has been reported to exert a variety of side effects, including fecal incontinence, flatulence, and steatorrhea [25,26]. The results demonstrate that the provision of extra nutrients to the mice was blocked by the addition of ARE without any deleterious side effects.

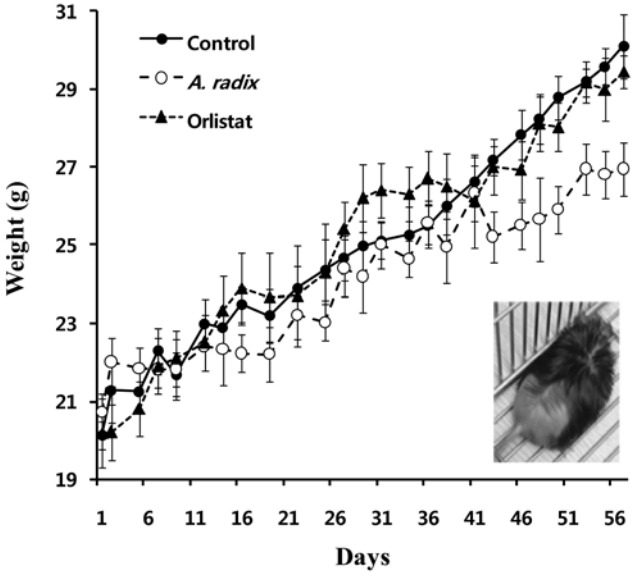


Fig. 9. Weight change of LB mice fed for 8 weeks on a ARE-supplemented diet. The inset shows the side effects of the synthetic obesity agent, orlistat.

4. Discussion

In this study, we have evaluated the effects of ARE on biological functions *in vitro*: anti-angiogenesis, anti-adipogenesis, immune-endothelial cell adhesion, and *in vivo* anti-obesity functions in an animal model. According to the results of the experiments, the addition of ARE suppresses angiogenesis in a dose-dependent manner. At the protein level, the results of Western blot analysis were confirmed using anti-VE-cadherin, anti-β-catenin, and anti-Akt; the protein expression level was reduced in a dose-dependent manner. The results demonstrate that ARE has an angiogenesis inhibitory effect induced by the blockage of intracellular signal molecules and the suppression of the activation of nuclear transcriptional factor, NF-κB.

Angiogenesis is a crucial factor in determining the growth and metastasis of tumor cells. A correlation has been noted to exist between the angiogenesis level and the process and metastasis of tumors in many varieties of malignant tumor [27]. The obesity process also develops through a mechanism similar to that responsible for angiogenesis [28]. VEGF is the most powerful physiological accelerator related with angiogenesis. The VEGF signaling facilitates VE-cadherin, β-catenin, VEGFR-2, PI3-kinase, and Akt complexes, shifts the transcriptional factor NF-κB to the nucleus, and then activates Bcl-2 [29-31]. Those procedures induce the expression of the IL-8 gene, and activate cell proliferation and blood vessel facilitation [32]. Adipocytes in adipose tissue also secrete VEGF, monoburitin, ob-protein, and leptin, and facilitate endithelial cell proliferation and adipocyte differentiation [33]. Moreover, the results of Oil red O staining showed that ARE suppressed the differentiation of 3T3-L1 adipocytes in a dose-dependent manner. The protein expression level was confirmed *via* Western blotting using the diabetes, fat, and tumor growth-mediated signal molecules, PPARγ and SREBP-1. Protein levels were reduced in a dose-dependent manner. The observed reduction in expression demonstrates that ARE suppresses important adipogenesis-related signal molecules, and then inhibits adipogenesis of differentiated adipocytes. As for the cell adhesion inhibitory experiment, ARE suppresses the cell adhesion activators, PMA and IL-1β, as well as angiogenesis. The data generated in this study indicate that ARE has a potential role as a suppressor of angiogenesis and adipogenesis. Further studies are in progress to determine the chemical composition of F5 with the best inhibitory effects among the 5 fractions in the fractionated ARE. In conclusion, ARE evidenced profound adipogenesis inhibitory activity under both *in vitro* and *in vivo* conditions. These results may lead to the development of positive therapies in people, aiding in the common current conditions of energy-dense foods, low physical activity, and urbanization.

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