Korean J Physiol Pharmacol
Vol 15: 43-51, February, 2011
DOI: 10.4196/kjpp.2011.15.1.43

Inhibitory Effects of *Corni Fructus* Extract on Angiogenesis and Adipogenesis

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Natural products in Chonnam, Korea were screened via anti-angiogenesis experiments, and 1 candidate product was identified, *Corni fructus*, which exerted dose-dependent inhibitory effects against angiogenesis, adipogenesis, and cell adhesion. *C. fructus* extract (CFE) exhibits an angiogenesis inhibitory effect superior to that of the EGCG from 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 CFE. Moreover, a diet supplemented with CFE was deemed more effective in inducing weight loss in LB mice than a representative synthetic diet drug, orlistat, which incidently caused the side effect of denuding the mice of their hair. These results indicate that *C. fructus* may prove to be a useful anti-adipogenic compound, and these *in vitro* results may be reflected later under *in vivo* conditions.

Key Words: Natural extract, Anti-angiogenesis, Anti-adipogenesis, Orlistat, Animal experiment

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 [1-3]. According to WHO statistics on overweight and obesity in 2005, approximately 1.6 billion adults were overweight; at least 400 million were obese [4]. 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 [5]. A substantial body of evidence now shows that neoplastic and non-neoplastic tissue growth is dependent on angiogenesis [6]. 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 [7]. 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, vessel sprouting) [7-12]. Thus, the antiangiogenic agents constitute a novel therapeutic option for the prevention and treatment of human obesity and its associated disorders [7].

Although alimentotherapy, exercise, and habit amendment should all be part of the routine method to suppress obesity, there have also been some real advances in the development of chemically synthesized drugs to treat obesity [13]. These drugs can be largely divided into two categories; one class of drugs reduces food intake by suppressing appetite, and the other stimulates fat metabolism, suppresses lipogenesis, and increases metabolic activity [14]. However, those anti-obesity agents generally damage not only adipocytes but also other normal cells, thus inducing some severe side effects on immunological functions *in vivo* [15].

Recently, a great many natural resources have been assessed for their possible utility as a substance useful for anti-obesity treatment [16], because natural chemicals frequently pose significantly less risk of deleterious side effects than synthetic chemicals [17]. Among these natural resources, certain herbs have already been traditionally used in Asia for anti-obesity treatments. Although *Corni fructus* is one of these natural herbs and has traditionally

Received February 1, 2011, Revised February 18, 2011, Accepted February 19, 2011

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ABBREVIATIONS: EGCG, epigallocatechin-3-gallate; CFE, *Corni fructus*; HGF, hepatocyte growth factor; GM-CSF, granulocyte-macrophage coloney stimulating factor; TGF, transforming growth factor; DMSO, dimethyl sulfoxide; EBM, endothelial basal medium; DMEM, Dulbecco modified eagle medium; RPMI, Roswell park memorial institute; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole; IL, interleukin; PBS, phosphate buffered saline; VE, vascular endothelial.

been utilized for a long time as a folk medicine in Asia, only a few studies have thus far been conducted on *Corni fructus*, which has been associated with a variety of pharmacological effects, including tuberculosis, asthma, hepatitis, chronic nephritis, anti-inflammation, and diuretic action [18-21]. In this work, we describe a new natural anti-obesity candidate - an *C. fructus* extract - which appears to exert profound inhibitory effects on angiogenesis, adipogenesis, cell adhesion, and *in vivo* diet in LB mice, without any detected side effects.

METHODS

Ethanol extraction and freeze-drying of natural products

18 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 hours at 60°C. The suspensions were filtered with No2 paper filters (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.

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-methylzanthine, 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 (Life Technologies, NY, USA), penicillin (1 units/ml), streptomycin (100 mg/l), and 10% FBS.

Inhibitory effect of angiogenesis by natural extracts

HUVEC (2.5×10^4) 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 hours 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 *C. fructus* extracts (CFE) was selected for this study, among 18 natural extracts.

Cell toxic test against CFE

MTT assays were conducted to assess cellular toxicity against CFE. 1×10^4 cells in one well of a 96-well plate were incubated for 24 hours. CFE was treated at various concentrations (0.1 ~ 100 mg/l) and incubated under constant conditions (37°C, 5% CO_2). After 48 hours, 0.5% MTT solution (Sigma, MO, USA) was applied and incubated for 3 hours. The absorbance of the well plate was scanned at 540 nm using a microplate reader (Biochrom Ltd., Cambridge, UK). The most appropriate concentration (10 mg/l) was determined and used for this study.

HPLC fractionation of CFE and its angiogenesis inhibitory effect

Ethanol extract of CFE was subjected to a preparative size exclusion column (500×21.5 mm; Showa Denko, Tokyo, Japan). The exclusion HPLC apparatus consists of a LC-6AD pump, a SPDM20A photodiode array detector, a DUG-20A3 online degasser, a SIL-20A autosampler, a FRC-10A fraction collector, a CBM-20A system controller, and ver. 1.22sp 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 CFE was separated into six fractions. Each fraction was evaluated for its anti-angiogenesis effects. The superior fractions, fractions 1, 5, and 6, were applied at various concentrations (1~25 mg/l) to HUVEC, and the morphological features were observed via microscopy.

Cell adhesion of U937 on HUVEC

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

U937 was incubated in 24-well plates until confluence, then treated with 100 μg/l of PMA (Sigma, MO, USA) for 2 hours. The cells were treated overnight with different doses of CFE. 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 hours with 10 ng/ml of IL-1β. Both cells were treated overnight with different doses of CFE. The cell adhesion of CFE-treated U937 cells on IL-1β treated HUVEC cells was evaluated as described above.

Adipogenesis inhibitory effect of 3T3-L1 adipocyte by Oil red O staining

3T3-L1 preadipocytes (9.6×10^4) were plated onto 6-well plates (SPL, Gyeonggi, Korea) and cultured for 2 days. MDI

and CFE were added to the wells and cultured for an additional 2 days. The medium was exchanged with DMEM/10% FBS, insulin, and CFE. The medium was then exchanged one additional time without insulin. The cells were fixed for 1 hour 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 [22]. 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.

Western blot analysis

In order to confirm the inhibitory effects of CFE on angiogenesis, western blot analysis was conducted using the angiogenesis-related signal molecules, VE-cadherin and Akt. HUVEC (1×10^5) was seeded on 100-mm Petri dishes, then incubated in EBM-2 medium with 2% FBS until confluence. CFE was treated with different doses for 24 hours, 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 $MgCl_2$, 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 BCA reagent (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 $NaVO_4$, 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 μm 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, and probed for 12 hours 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 CFE against adipogenesis, western blot analysis was conducted on the differentiation substance-treated, CFE-treated group (group 1), and CFE-added group after differentiation (group 2) using the adipogenesis-associated signal molecules, SREBP-1 and PPARγ. 3T3-L1 cells (1×10^5) were seeded on 100-mm Petri dishes, and incubated to confluence in DMEM medium with 10% FBS. As for group 1, the MDI-treated and CFE-treated cells were incubated with DMEM/10% FBS, insulin, and CFE. After 2 days, the medium was exchanged with DMEM/10% FBS and CFE. With regard to group 2, 3T3-L1 preadipocytes were wholly differentiated into adipocytes, and CFE was applied at different doses. The cells were rinsed in ice-cold PBS including 1 mM vandate, then solubilized with ice-cold RIPA lysis buffer (Santa cruz, CA, USA) via a 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: SREBP-1 and PPARγ; then visualized as described above.

Animal experiments

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). Animals were divided into 3 groups (10 individuals/group) - a control group, a CFE group, and a positive control group using the popular agent, orlistat (Roche, SF, USA). 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, 200 μl of CFE (25 mg/l) and orlistat (10 μg) were subcutaneously injected into the tails of the mice once each 2 days for 4 weeks, respectively. The injection concentrations of CFE and orlistat were decided by toxic test in cell level and manufacture's instruction based on animal weight, respectively. During the initial 4 weeks, any negative effect was not observed, and then identical volumes were injected on a daily basis from 4 weeks. 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 fresh high fat diet provided 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 Chonnam National University for 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.

RESULTS

Selection of angiogenesis inhibitory candidate from 18 natural products

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

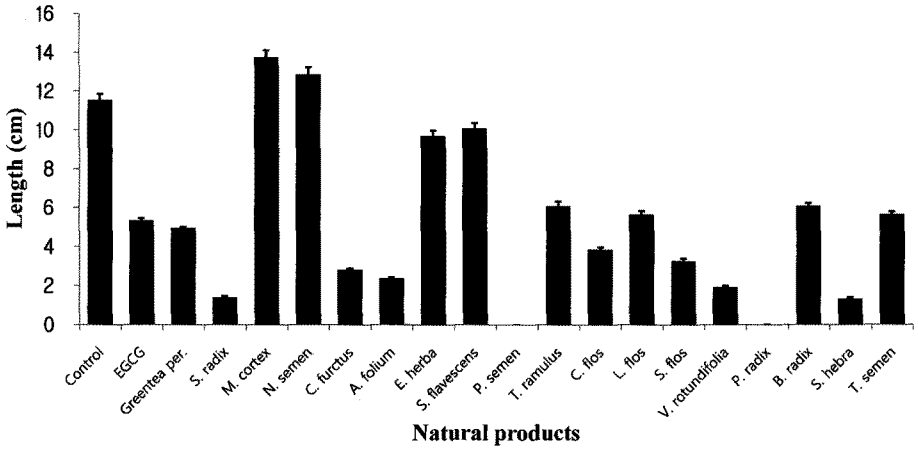

Fig. 1. Anti-angiogenesis experiment of 18 natural products with the positive control, EGCG. HUVEC was cultured on Matrigel-coated wells, and the tube formation of HUVEC cells was randomly photographed using a digital camera, and analyzed by the Scion Image software.

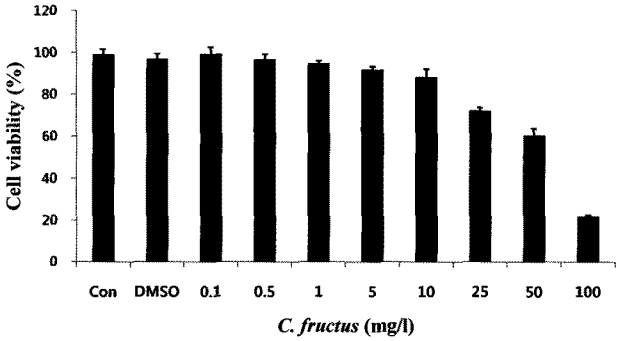

Fig. 2. Cytotoxicity in HUVEC cells with different concentrations of *C. fructus* extract (CFE). HUVEC was seeded onto 96-well plates and treated with various doses (0.1, 0.5, 1, 5, 10, 25, 50, and 100 mg/l) of CFE for 48 hrs. MTT solution was added to the wells, and the 96-well plates were incubated. 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.

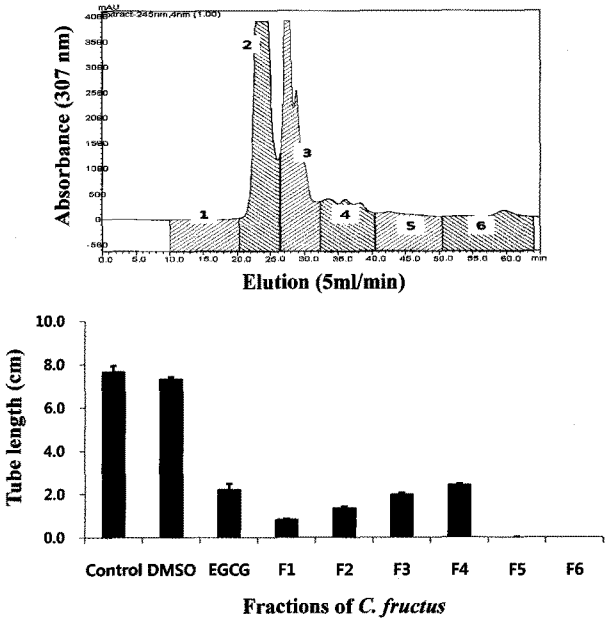

Fig. 3. CFE was subjected to preparative size exclusion column of Asahipak GS-310. CFE was chromatographed on an Asahipak GS-310 column eluted with methanol at a flow rate of 5 ml/min, and monitored at 307 nm. CFE was separated into six fractions. Each fraction was evaluated via anti-angiogenesis experiments. 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.

sible natural anti-obesity suppressor.

Toxic test of CFE on HUVEC

According to the results of the MTT assay toxicity test against CFE on HUVEC cells (Fig. 2), no significant changes were noted as compared to those of other CFE concentrations up to 10 mg/l. More than 90% of HUVEC cells survived after the addition of 10 mg/l CFE, but the cell viability was reduced dramatically with the addition of CFE at a concentration of 100 mg/l. 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 CFE may be attributable, at least in part, to an accumulation of intracellular toxicity. The results of our tests of CFE cytotoxicity in this *in vitro* cell culture system demonstrated that CFE added at a concentration of at least 10 mg/l is not harmful to cell cultures.

Angiogenesis inhibitory effect of CFE fractions on HUVEC

CFE was fractionated into 6 fractions via HPLC and applied in the angiogenesis inhibitory experiment (Fig. 3). 6 CFE fractions (F1, F2, F3, F4, F5, and F6) and a positive control (EGCG) were added to HUVEC cells at identical concentrations (50 mg/l). Among the fractions, F1, F5, and F6 were identified as the optimal angiogenesis suppressor. F1, F5, and F6 were further examined in angiogenesis inhibitory experiments at different concentrations (1, 5, 10, and 25 mg/l). The results demonstrated similar inhibitory effect of F1 with EGCG, and dose-dependent inhibitory effects of F5 and F6 in the observed angiogenesis inhibitory effects over 5 mg/l (Fig. 4). Moreover, the result was coincided with morphological observation of the HUVEC cells proving sufficient to block angiogenesis (data not shown).

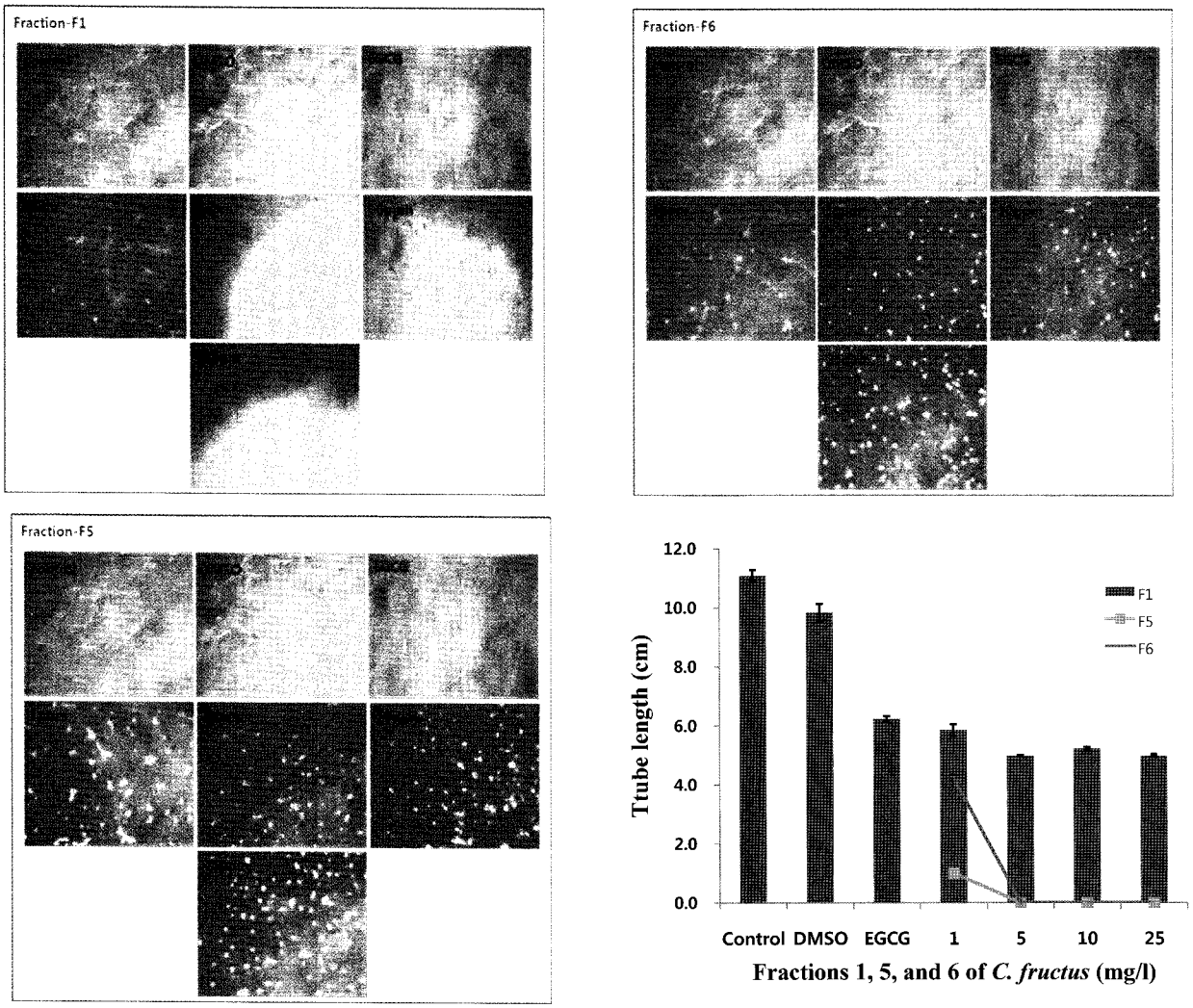

Fig. 4. Anti-angiogenesis effect of SHE fractions 1, 5, and 6. Anti-angiogenesis experiments were conducted with different concentrations (1, 5, 10, and 25 ppm) of fractions 1, 5, and 6. The pictures show the cellular morphology of HUVEC cells treated with different concentrations of fractions 1, 5, and 6.

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 control cells.

Inhibitory effect of cell adhesion

The inhibitory effects of cell adhesion were evaluated in *in vitro* experiments conducted under several conditions (Fig. 5). U937 was adhered to IL-1β and CFE stimulated HUVEC cells (Fig. 5A). Different concentrations of CFE (1, 5, 10, and 20 mg/l) were suppressed the U937 cell adhesion by 12%, 57%, 115%, and 132%, respectively. PMA (phorbol myristyl atetate) and different concentrations (1, 5, 10, and 20 mg/l) of CFE were stimulated to U937, and then the U937 was adhered to the HUVEC cells (Fig. 5B). Different concentrations of CFE (1, 5, 10, and 20 mg/l) were suppressed the U937 cell adhesion by 379%, 504%, 567%, and 664%, respectively. Different concentrations (1, 5, 10, and 20 mg/l) of CFE were stimulated to U937, and adhered to IL-1β and different concentrations (1, 5, 10, and 20 mg/l) of CFE stimulated HUVEC cells (Fig. 5C). CFE was suppressed the U937 cell adhesion in a dose-dependent manner, by percentages of 50%, 112%, 231%, and 297%, respectively. As shown in Fig. 5, U937 and HUVEC cells were activated by IL-1β and PMA, individually (Fig. 5A, B) or together (Fig. 5C). The best cell adhesion inhibitory effect was noted in HUVEC-adhering PMA and CFE-stimulated U937 cells (Fig. 5B) followed by IL-1β- and CFE-stimulated HUVEC-adhering CFE-stimulated U937 cells (Fig. 5C), and IL-1β- and CFE-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 [23,24]. The stimulated adhesion was inhibited clearly by the addition of CFE, and this effect was also observed to occur in a dose-dependent manner. This result demonstrated that CFE exerts an in-

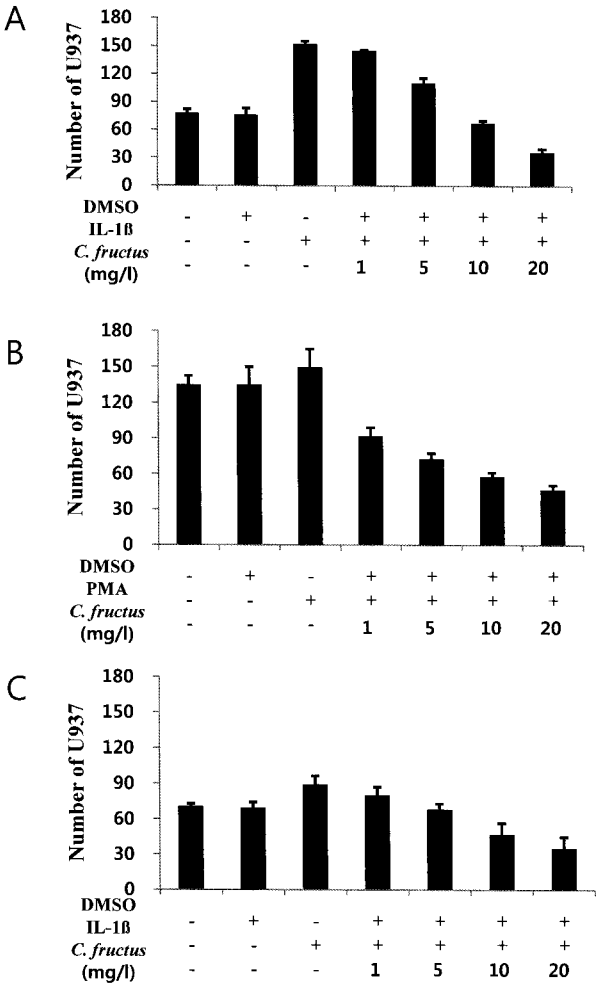

Fig. 5. Various doses (1, 5, 10, and 20 mg/l) of CFE addition on U937 cell adhesion. (A) Cell adhesion of U937 on IL-1β and CFE stimulated HUVEC, (B) Cell adhesion of PMA- and CFE-stimulated U937 on HUVEC, (C) Cell adhesion of CFE-stimulated U937 on IL-1β- and CFE-stimulated HUVEC cells. Each treatment in the cell adhesion experiments was conducted as described in the METHODS section.

hibitory effect on cell adhesion, exhibiting an angiogenesis-inhibitory effect.

Western blot of angiogenesis-related signal molecules on HUVEC cells

Because VE-cadherin and Akt have been identified as important mediators of angiogenesis [25,26], we assessed the role of VE-cadherin and Akt in angiogenesis under conditions of cell stimulation by VEGF and with different concentrations (0.1, 0.5, and 1 mg/l) of CFE. CFE-induced inhibition of tube formation in HUVEC cells was determined via the suppression of the signal pathways of VE-cadherin 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 0.5 and 1

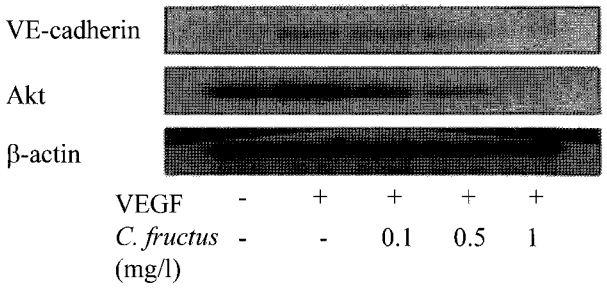

Fig. 6. CFE inhibits the interaction of PI3-kinase with VE-cadherin and Akt upon cell activation with VEGF. Proteins from HUVEC cells were immunoprecipitated with PI3-kinase antibody and immunoblotted with antibodies to VE-cadherin and Akt. β-actin was employed as a positive control.

mg/l, CFE inhibited the expression of both types of signal molecules - VE-cadherin and Akt. As the result of the CFE-induced suppression of the pathways from two kinds of signal molecules to NF-kB, angiogenesis was reduced in a dose-dependent manner. Therefore, blocking the function of VE-cadherin and Akt by the addition of CFE directed against the molecules inhibits its function during the maturation of cells for tube formation.

Adipogenesis inhibitory effect of 3T3-L1 adipocyte by Oil red O staining

Differentiated 3T3-L1 adipocytes were treated with CFE at various concentrations (1, 5, 10, and 25 mg/l) for 2 days. The effect of CFE on lipid accumulation was measured by Oil red O staining (Fig. 7). Relative to the control cells, CFE treatment reduced intracellular lipid content in a dose-dependent manner, and the most effective dosage for inhibition was found to be 25 mg/l of CFE. The results indicated that CFE could inhibit lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner.

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 (1, 5, and 10 mg/l) of CFE. Proteins were isolated from 3T3-L1 preadipocytes and western blot analysis was conducted. The protein expression level of the adipogenesis-mediated signal molecule, PPARβ, was reduced with the addition of 10 mg/l CFE (Fig. 8A). 3T3-L1 was completely differentiated and treated with different concentrations (1, 5, and 10 mg/l) of CFE. 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 10 mg/l CFE (Fig. 8B). Additionally, the protein levels of SREBP-1 and PPARγ were reduced in a dose-dependent manner after CFE treatment, regardless of differentiation. Therefore, the results demonstrated that CFE was more effective in suppressing adipogenesis in the adipocytes rather than the preadipocytes via the down-regulation of SREBP-1 and PPARγ expression.

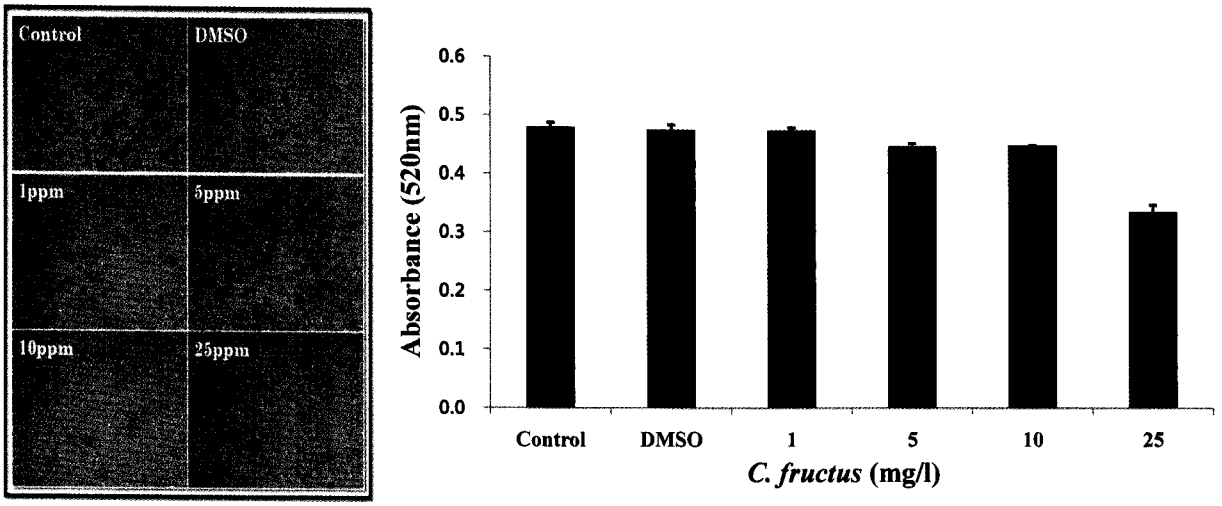

Fig. 7. The effect of CFE addition in the course of 3T3-L1 adipocyte differentiation. 3T3-L1 was treated with CFE at various concentrations (1, 5, 10, and 25 mg/l). After differentiation, lipid accumulation was stained with Oil red O solution and the morphological changes were observed via microscopy. The Oil red O stained lipid was quantified with a microplate reader at 520 nm.

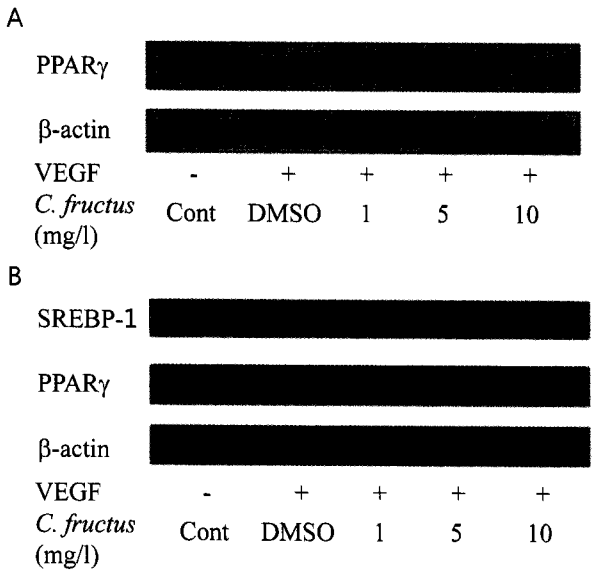

Fig. 8. (A) CFE inhibits the interaction of PI3-kinase with VE-cadherin and Akt upon cell activation with VEGF. Proteins from HUVEC cells were immunoprecipitated with PI3-kinase antibody and immunoblotted with antibodies to VE-cadherin and Akt. β-actin was employed as a positive control. Expression of PPARγ on 3T3-L1 preadipocyte with CFE (A) and expression of SREBP-1 and PPARγ on 3T3-L1 adipocytes with CFE (B). Protein from 3T3-L1 was treated with different concentrations (1, 5, and 10 mg/l) of CFE, and extracted with RIPA buffer for western blotting.

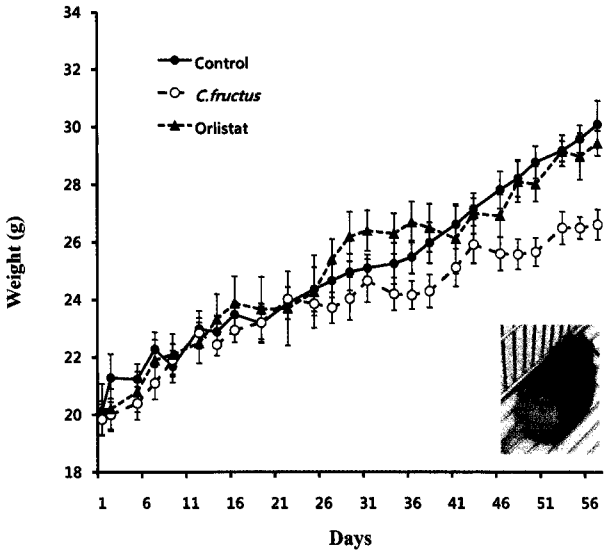

Fig. 9. Weight change of LB mice fed for 8 weeks on a CFE-supplemented diet. The inset shows the side effects of the synthetic obesity agent, orlistat.

Animal experiments

Significant weight reduction and feed intake amount were not observed in all groups for the initial four weeks because of unsteady CFE concentration in the body by supplying CFE once at 2-day intervals. However, the significant weight change was initially observed after 4-weeks of daily CFE supply. No positive effects were noted in the group treated with orlistat, a potent competitive inhibitor of gastric and pancreatic lipase [27,28], 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 [29,30]. The results demonstrate that the provision of extra nutrients to the mice was blocked by the addition of CFE without any deleterious side effects.

DISCUSSION

Based on the previous reports of which activated adipo-

cytes generate multiple angiogenic factors, and stimulate neovascularization during fat mass expansion. Furthermore, neoadipogenesis is preceded or accompanied by an angiogenic response [7-12]. We designed experimental hypothesis by using correlation between angiogenesis and adipogenesis. Then, we have evaluated the effects of CFE 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 our experiments, the addition of CFE suppresses angiogenesis in a dose dependent manner. At the protein level, the results of western blot analysis were confirmed using anti-VE-cadherin and anti-Akt: the protein expression level was reduced in a dose-dependent manner. The results demonstrate that CFE 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. The obesity process also develops through a mechanism similar to that responsible for angiogenesis. 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 [31,32]. Those procedures induce the expression of the IL-8 gene, and activate cell proliferation and blood vessel facilitation [33]. Adipocytes in adipose tissue also secrete VEGF, monoburitin, ob-protein, and leptin, and facilitate endithelial cell proliferation and adipocyte differentiation. Moreover, the results of Oil red O staining showed that CFE 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, SREBP-1 and PPARγ. Protein levels were reduced in a dose-depent manner. The observed reduction in expression demonstrates that CFE suppresses important adipogenesis-related signal molecules, and then inhibits adipogenesis of differentiated adipocytes. As for the cell adhesion inhibitory experiment, CFE suppresses the cell adhesion activators, PMA and IL-1β, as well as angiogenesis. The data generated in this study indicate that CFE has potential power as a suppressor of angiogenesis and adipogenesis. Further studies are in progress to determine the chemical composition of F5 and F6 which showed best inhibitory effects among 6 fractions in the fractionated CFE. In conclusion, CFE evidenced profound adipogenesis inhibitory activity under both *in vitro* and *in vivo* conditions. These results may lead to the development of positive therapies for humans, helping with the adaptation to modern conditions of energy-dense foods, low physical activity, and urbanization.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Education, Science Technology (MEST) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Regional Innovation.

REFERENCES

1. **Han DH, Kim SK, Kang SW, Choe BK, Kim KS, Chung JH.** Matrix metallopeptidase 2 gene polymorphism is associated with obesity in Korean population. *Korean J Physiol Pharmacol.* 2008;12:125-129.
2. **Jung MY, Kim BS, Kim YJ, Koh IS, Chung JH.** Assessment of relationship between fyn-related kinase gene polymorphisms and overweight/obesity in Korean population. *Korean J Physiol Pharmacol.* 2008;12:83-87.
3. **Pataky Z, Bobbioni-Harsch E, Golay A.** Obesity: a complex growing challenge. *Exp Clin Endocrinol Diabetes.* 2010;118: 427-433.
4. **Frezza EE, Wachtel MS, Ewing BT.** The impact of morbid obesity on the state economy: an initial evaluation. *Surg Obes Relat Dis.* 2006;2:504-508.
5. **Folkman J, Shing Y.** Angiogenesis. *J Biol Chem.* 1992;267: 10931-10934.
6. **Doldi N, Bassan M, Gulisano M, Broccoli V, Boncinelli E, Ferrari A.** Vascular endothelial growth factor messenger ribonucleic acid expression in human ovarian and endometrial cancer. *Gynecol Endocrinol.* 1996;10:375-382.
7. **Cao Y.** Angiogenesis modulates adipogenesis and obesity. *J Clin Invest.* 2007;117:2362-2368.
8. **Crandall DL, Hausman GJ, Kral JG.** A review of the microcirculation of adipose tissue: anatomic, metabolic, and angiogenic perspectives. *Microcirculation.* 1997;4:211-232.
9. **Hausman GJ, Richardson RL.** Adipose tissue angiogenesis. *J Anim Sci.* 2004;82:925-934.
10. **Neels JG, Thinnes T, Loskutoff DJ.** Angiogenesis in an in vivo model of adipose tissue development. *FASEB J.* 2004;18:983-985.
11. **Voros G, Maquoi E, Demeulemeester D, Clerx N, Collen D, Lijnen HR.** Modulation of angiogenesis during adipose tissue development in murine models of obesity. *Endocrinology.* 2005; 146:4545-4554.
12. **Christiaens V, Lijnen HR.** Angiogenesis and development of adipose tissue. *Mol Cell Endocrinol.* 2010;318:2-9.
13. **Rudkowska I, Roynette CE, Demonty I, Vanstone CA, Jew S, Jones PJ.** Diacylglycerol: efficacy and mechanism of action of an anti-obesity agent. *Obes Res.* 2005;13:1864-1876.
14. **Obeid OA, Bittar ST, Hwalla N, Emery PW.** Effect of diet supplementation with glutamine, dihydroxyacetone, and leucine on food intake, weight gain, and postprandial glycogen metabolism of rats. *Nutrition.* 2005;21:224-229.
15. **Prestwich TC, Macdougald OA.** Wnt/beta-catenin signaling in adipogenesis and metabolism. *Curr Opin Cell Biol.* 2007;19: 612-617.
16. **Yun JW.** Possible anti-obesity therapeutics from nature--a review. *Phytochemistry.* 2010;71:1625-1641.
17. **Oh S, Kim KS, Chung YS, Shong M, Park SB.** Anti-obesity agents: a focused review on the structural classification of therapeutic entities. *Curr Top Med Chem.* 2009;9:466-481.
18. **Lee SO, Kim SY, Han SM, Kim HM, Ham SS, Kang IJ.** Corni fructus scavenges hydroxy radicals and decreases oxidative stress in endothelial cells. *J Med Food.* 2006;9:594-598.
19. **Choi WH, Chu JP, Jiang MH, Baek SH, Park HD.** Effects of fraction obtained from Korean Corni Fructus extracts causing anti-proliferation and p53-dependent apoptosis in A549 lung cancer cells. *Nutr Cancer.* 2011;63:121-129.
20. **Park CH, Noh JS, Tanaka T, Yokozawa T.** Effects of morroniside isolated from Corni Fructus on renal lipids and inflammation in type 2 diabetic mice. *J Pharm Pharmacol.* 2010;62:374-380.
21. **Yamabe N, Noh JS, Park CH, Kang KS, Shibahara N, Tanaka T, Yokozawa T.** Evaluation of loganin, iridoid glycoside from Corni Fructus, on hepatic and renal glucolipotoxicity and inflammation in type 2 diabetic db/db mice. *Eur J Pharmacol.* 2010;648:179-187.
22. **Ramírez-Zacarías JL, Castro-Muñozledo F, Kuri-Harcuch W.** Quantitation of adipose conversion and triglycerides by staining

intracytoplasmic lipids with Oil red O. *Histochemistry.* 1992;97: 493-497.
23. **Lee DH, MacIntyre JP, Wang E, Hudson DJ, Ishaque A, Conant JA, Pope BL, Lau CY.** A leukocyte lipid up-regulates the avidity of lymphocyte function-associated antigen-1. *Biochem Biophys Res Commun.* 1994;199:319-326.
24. **Vandermeeren M, Janssens S, Borgers M, Geysen J.** Dimethylfumarate is an inhibitor of cytokine-induced E-selectin, VCAM-1, and ICAM-1 expression in human endothelial cells. *Biochem Biophys Res Commun.* 1997;234:19-23.
25. **Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oosthuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E.** Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell.* 1999;98:147-157.
26. **Zhang D, Bar-Eli M, Meloche S, Brodt P.** Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals. *J Biol Chem.* 2004;279: 19683-19690.
27. **Ballinger A, Peikin SR.** Orlistat: its current status as an anti-obesity drug. *Eur J Pharmacol.* 2002;440:109-117.
28. **Jandacek RJ, Woods SC.** Pharmaceutical approaches to the treatment of obesity. *Drug Discov Today.* 2004;9:874-880.
29. **Birari RB, Bhutani KK.** Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discov Today.* 2007;12:879-889.
30. **Weigle DS.** Pharmacological therapy of obesity: past, present, and future. *J Clin Endocrinol Metab.* 2003;88:2462-2469.
31. **Choi YS, Park H, Jeong S.** Distinct role of PI3-kinase/Akt pathway in the activation of etoposide-induced NF- B transcription factor. *J Microbiol Biotechnol.* 2006;16:391-398.
32. **Tang FY, Nguyen N, Meydani M.** Green tea catechins inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule. *Int J Cancer.* 2003;106:871-878.
33. **Tang FY, Meydani M.** Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells through suppression of IL-8 production. *Nutr Cancer.* 2001;41: 119-125.