J. Korean Soc. Appl. Biol. Chem. **53**(5), 584-592 (2010) **Article**

Identification of Plant Extracts that Inhibit Cellular Senescence in Human Fibroblasts, Endothelial Cells, and Vascular Smooth Muscle Cells

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Received May 6, 2010; Accepted June 22, 2010

To identify plant extracts capable of inhibiting cellular senescence, the effects of plant extracts on adriamycin-induced cellular senescence in human fibroblasts, human umbilical vein endothelial cells (HUVECs), and vascular smooth muscle cells were investigated. After adriamcyin treatment, the effects of plant extracts on cellular senescence were evaluated by measuring senescence-associated β-galactosidase activity of the cells. Among 799 plant extracts, those from Rhei Rhizoma, Cirsii Radix, and Plantagnis Semen repressed adriamycin-induced cellular senescence in fibroblasts. Extracts of Cinnamomi Cortex and Cinnamomi Cortex Spissus were effective in endothelial cells. Extracts of Euonymi Lignum Suberalatum, Salicis Radicis Cortex, Polygoni aviculari Herba, and Chaenomelis langenariae Radix reduced senescence in vascular smooth muscle cells. These results suggested the effectiveness of plant extracts in reducing cellular senescence of human cells. These plant extracts could be used to develop dietary supplements or cosmetics for modulating tissue aging or aging-associated diseases.

Key words: aging intervention, cellular senescence, human primary cells, plant extracts

Cellular senescence is a stress response in which cell proliferation ceases after a finite number of cell divisions in normal somatic cells [Hayflick and Moorhead, 1961]. Cellular senescence contributes to organismal or tissue aging [Patil *et al.*, 2005] as well as to cancer formation or development [Campisi, 2001]. Diverse factors (i. e., telomere shortening, activation of oncogenes or tumor suppressor genes, oxidative stress, irradiation, cytotoxic chemicals, and inflammatory cytokines) induce cellular senescence [Collado *et al.*, 2007]. Senescent cells show characteristic phenotypes, including flattened and enlarged morphology, increases in senescence-associated heterochromatic foci (SAHFs) in the nucleus and senescence-associated β-galactosidase (SA-β-gal) activity, and up-regulation of p53 and p16INK4 proteins. In addition, senescent cells secrete inflammatory cytokines such as insulin-like growth factor binding proteins (IGFBPs), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), and interferons. [Kuilman and Peeper, 2009]. Although cellular senescence is induced by a variety of factors, p53 and p16/Rb tumor suppressor pathways are crucial components in the regulation of cellular senescence [Campisi, 2005; Ye *et al.*, 2007].

Cellular senescence is also involved -directly or indirectly- in the pathophysiology of aging-associated diseases. In human tissues, especially skin [Dimri *et al.*, 1995] and liver [Paradis *et al.*, 2001], the number of senescent cells increases with age. It is well known that ultraviolet light from the sun is a key factor in inducing skin aging. UV irradiation causes cellular senescence in fibroblasts and keratinocytes, which, in turn, results in age-associated skin damages such as wrinkles and pigmentation [Makrantonaki and Zouboulis, 2007]. Fibroblast senescence in skin ulcers also affects treatment efficacy and healing prognosis [Wall *et al.*, 2008]. Senescent cells are observed in many inflammatory tissues and tumor tissues such as rheumatoid arthritis [Schmid *et al.*, 2004], osteoarthritis [Price *et al.*, 2002; Dai *et al.*, 2006], hepatitis [Paradis *et al.*, 2001], chronic

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doi:10.3839/jksabc.2010.090

skin diseases [Harding *et al.*, 2005], atherosclerosis [Minamino *et al.*, 2003], benign prostate hyperplasia [Choi *et al.*, 2000], and liver cancer [Paradis *et al.*, 2001]. Accumulation of senescent cells accelerates tissue damage due to the secretion of inflammatory cytokines and degrading enzymes and decreases the repair or regeneration capabilities of the tissue, contributing to the pathogenesis of aging-associated diseases [Campisi, 2005].

Small molecules such as *N*-acetylcysteine [Kim *et al.*, 2009a; Kim *et al.*, 2009b], an antioxidant, and resveratrol [Demidenko and Blagosklonny, 2009], an SIRT1 activator, are known to repress cellular senescence. Various plant extracts, including *Persicaria hydropiper*, *Filipendula glaberrima*, *Nymphaea tetragona*, *Camellia japonica* [Kim *et al.*, 2007], horse chestnut [Fujimura *et al.*, 2007], *Malus doumeri* [Leu *et al.*, 2006], *Paeoniae lactiflora* [Lee *et al.*, 2006b], and *Melothria heterophylla* [Cho *et al.*, 2006], have been reported to repress senescence phenotypes in fibroblasts. However, because these plant extracts were assessed by measuring the inhibitory effects on the expression of matrix metalloproteinase in UV-irradiated fibroblasts, no direct evidence was provided as to whether these extracts inhibit cellular senescence in human cells or tissues.

Adriamycin, a chemotherapeutic, induces cellular senescence in numerous cell types at low concentrations, which was confirmed by increases in ROS level, p53 level, and SA-β-gal activity [Song *et al.*, 2005]. In addition, SA-β-gal activity is the most common and reliable method for identifying cellular senescence [Dimri *et al.*, 1995]. Therefore, we screened for plant extracts capable of inhibiting cellular senescence in human primary cells (i.e., fibroblasts, endothelial cells, vascular smooth muscle cells) using the adriamycin-induced cellular senescence model system and the SA-β-gal activity staining method. Among the 799 plant extracts tested, we identified several plant extracts capable of effectively suppressing cellular senescence phenotypes.

Materials and Methods

Materials. Human dermal fibroblasts (HDFs), human umbilical vein endothelial cells (HUVECs), human vascular smooth muscle cells (VSMCs), endothelial cell growth medium-2 (EGM-2) and smooth muscle cell growth medium-2 (SmGM-2) were purchased from Lonza (Basel, Switzerland). Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from WelGENE Inc. (Deagu, Korea). The p53 antibody was purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA). A rabbit polyclonal antibody against glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was kindly donated by Dr. K. S. Kwon (KRIBB, Daejeon, Korea). Adriamycin was purchased from Ildong Pharmaceutical (Seoul, Korea). Fig. 1 shows the general experimental scheme for identifying plant extracts that inhibit adriamycin-induced cellular senescence in human primary cells.

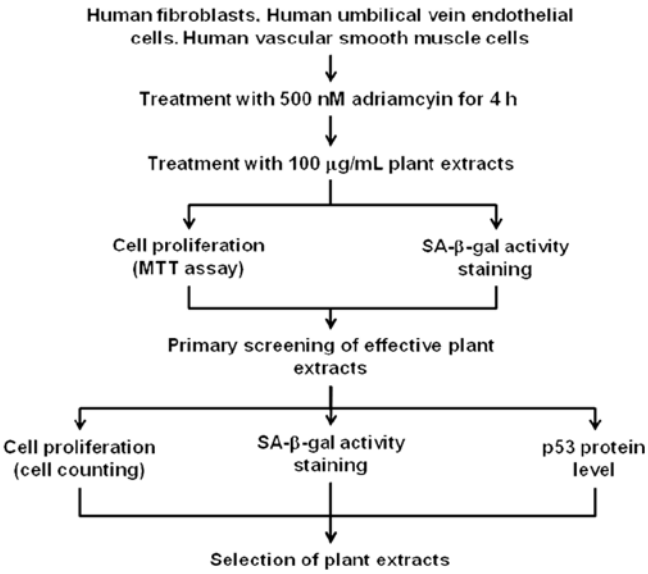

Fig. 1. Brief experimental scheme for determining which plant extracts are capable of inhibiting adriamycin-induced cellular senescence in human primary cells.

Preparation of crude extracts. Crude plant extracts were purchased from Korea Plant Extract Bank, Korea Research Institute of Bioscience & Biotechnology (Daejon, Korea). We used 399 crude ethanol extracts (CA01-001 to CA01-100, CA02-001 to CA02-100, CA03-001 to CA03-100, and CA04-001 to CA04-100) and 400 crude water extracts (CW01-001 to CW01-100, CW02-001 to CW02-100, CW03-001 to CW03-100, and CW04-001 to CW04-100), which were prepared by decocting dried plants in earthen pots. Each extract was dissolved in dimethyl sulfoxide (DMSO) and used for the treatment of cells.

Cell culture. HDFs in DMEM with 10% FBS and 1% antibiotic (penicillin 10,000 unit/mL and streptomycin 10,000 μg/mL) were plated at 2×10^5 cells per 100-mm culture plate and cultured at 37°C in 5% CO_2 humidified air. When subcultures reached 80-90% confluence, serial passaging was performed by trypsinization. HUVECs in EGM-2 and VSMCs in SmGM-2 media were cultured under the same conditions. The number of population doublings (PDs) was monitored for further experiments. PD was calculated using the geometric equation: $PD=\log_2F/\log_2I$ (F, final cell number; I, initial cell number). HDFs in PD<40, HUVECs in PD<30, and VSMCs in

PD<30 were used for the experiments.

Induction of cellular senescence by adriamycin treatment. HDFs in DMEM were seeded at 1×10^5 cells per 100-mm culture plate. HUVECs in EGM-2 and VSMCs in SmGM-2 media were plated at 2×10^5 cells per 100-mm culture plate. After incubation at 37°C in a CO_2 incubator for 3 days, cells were washed two times with DMEM containing 1% antibiotics and treated with 500 nM adriamycin for 4 h. After rinsing three times with DMEM containing 1% antibiotics, HDFs in DMEM containing 10% FBS and 1% antibiotics, HUVECs in EGM-2, and VSMCs in SmGM-2 were incubated in a 5% CO_2 incubator for 4 days. Adriamycin-induced cellular senescence was confirmed by senescence-associated β-galactosidase (SA-β-gal) activity staining.

Treatment with crude plant extracts. To investigate the effects of crude extracts on adriamycin-induced cellular senescence, cells treated with adriamycin for 4 h were detached by trypsinization. HDFs were plated at 500 cells/well in 96-well plates, and HUVECs and VSMCs were plated at 1,000 cells/well in 96-well plates. Cells were incubated at 37°C in 5% CO_2 humidified air for 24 h before treatment with 100 mg/mL of crude plant extracts, 0.5% DMSO or 5 mM NAC for three days. Cell proliferation was measured by the MTT assay, and cellular senescence was determined by SA-β-gal activity staining.

3-(4,5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with 0.1% MTT solution for 3 h. After aspiration of the medium containing MTT solution, the resulting formazan product was solubilized with 100 μL DMSO. Viability was assessed by measuring the absorbance at 550 nm with a microplate reader.

Cell counting. HDFs (5×10^3 cells/well), HUVECs (1×10^4 cells/well), and VSMCs (1×10^4 cells/well) were seeded on 24-well culture plates and treated with 500 nM adriamycin for 4 h. After washing cells twice with DMEM containing antibiotics, cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. Cells were treated with indicated concentrations of plant extracts for 3 days and harvested by trypsinization. Cell number was determined by counting with hemocytometers.

Senescence-associated β-galactosidase (SA-β-gal) activity assay. SA-β-gal activity was measured as described previously [Dimri *et al.*, 1995]. Cells were fixed with 3.7% paraformaldehyde in PBS and incubated in SA-β-gal staining solution (40 mM citric acid/ phosphate, pH 5.85, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/mL X-gal) at 37°C for 16-18 h. After washing the cells twice with PBS, they were stained with a 1% eosin solution for 5 min. A total of 50 cells were counted in three randomized fields, and the percentage of blue cells was calculated.

Protein extractions. Cells were seeded at 1×10^5 in 60-mm culture dishes and incubated for 24 h. Cells were pretreated with the indicated concentrations of crude extracts for 1 h and treated with 500 nM adriamycin for 4 h. Cells were lysed in 50 μL ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate, 5 mM NaF, a protease inhibitor, or 1 mM PMSF). The particulate debris was removed by centrifugation at 12,000×g for 10 min at 4°C. Protein concentration in the supernatant was quantified by the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL), using bovine serum albumin as a standard.

Western blot analysis. Proteins (30 μg) were separated on 10% SDS-polyacrylamide gels and were transferred to nitrocellulose membranes. After blocking the membranes with Tween-20 Tris-buffered saline (TTBS) containing 5% skim milk for 30 min, the membranes were incubated overnight with the p53 antibody at room temperature. After washing three times in TTBS, horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies were applied for 3 h. Antigen-antibody complexes were detected using Western Blotting Luminol Reagent (Santa Cruz Biotech, Santa Cruz, CA). Images of membranes were captured using a LAS-3000 image system (Fujifilm, Stamford, CT). GAPDH was used as a control for protein loading.

Statistical analysis. All data are presented as means ± SD. Statistical significance was determined by Student's *t* test. A p-value of < 0.05 was considered statistically significant.

Results

Screening of plant extracts to inhibit adriamycin-induced cell senescence in human fibroblasts. We evaluated the effects of 799 plant extracts on SA-β-gal activity in human fibroblasts treated with adriamycin. DMSO, a solvent used to dissolve plant extracts, and *N*-acetylcysteine, an antioxidant known to repress cellular senescence, were used as controls. Extracts CW01-047 (Rhei Rhizoma), CW02-007 (Cirsii Radix), and CW03-065 (Plantaginis Semen) effectively inhibited adriamycin-induced cellular senescence (Fig. 2A). Among these three extracts, CW02-007 (Cirsii Radix) treatment also inhibited SA-β-gal activity in old cells, which was induced by replicative senescence (Fig. 2B). In addition, effects of the three extracts on cell proliferation and cytotoxicity were determined by the MTT assay and cell counts,

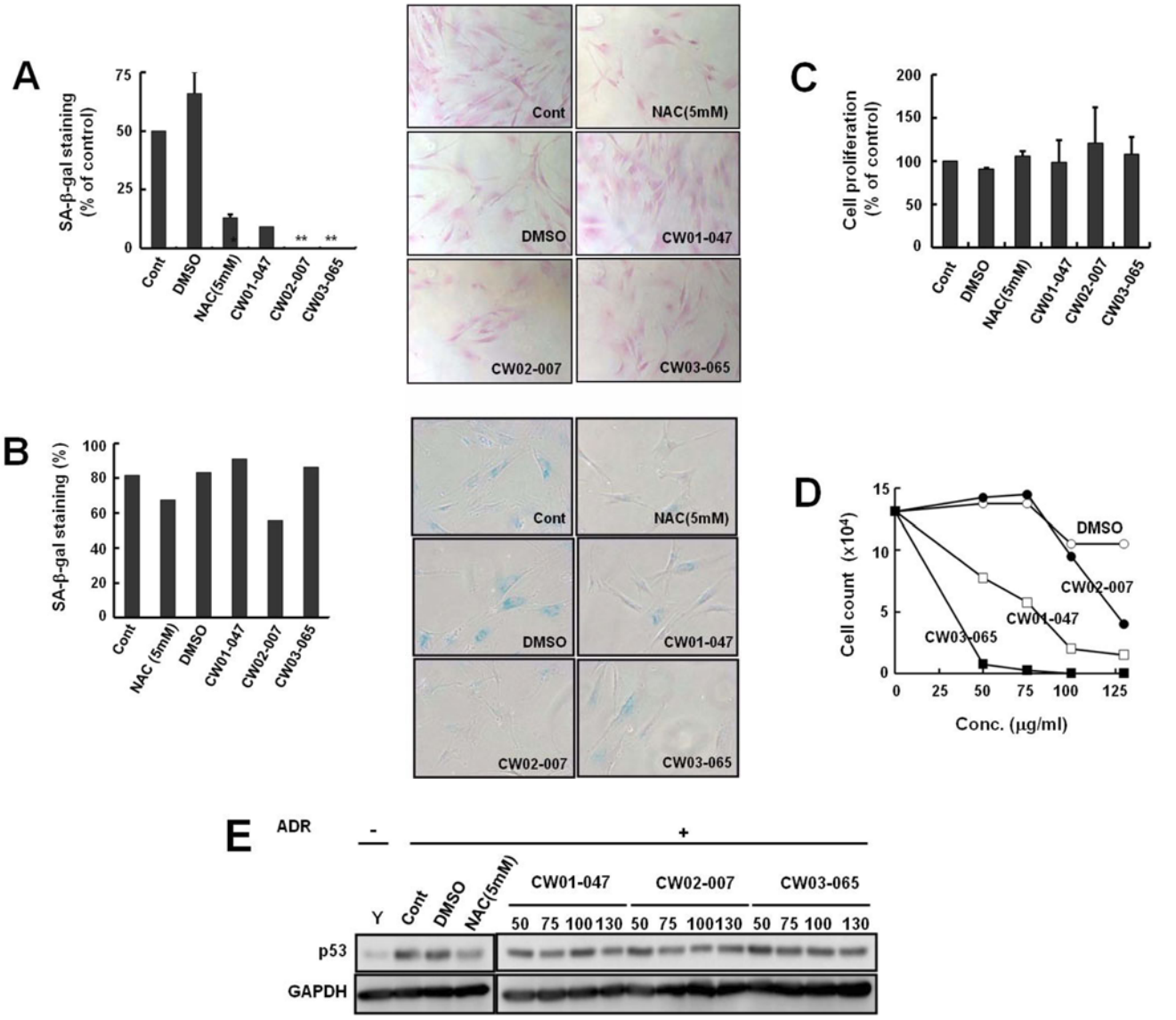

Fig. 2. Identification of plant extracts that inhibit adriamycin-induced cellular senescence in human fibroblasts. A. Effects of plant extracts on SA-β-gal activity in adriamycin-treated cells. Cells treated with 500 nM adriamycin for 4 h were seeded at 500 cells/well in 96-well plates. After treatment with 100 μg/mL of crude plant extract (n=799) for three days, SA-β-gal activity was measured. B. Effects of plant extracts on replicative senescence. Old cells (PD>60) were treated with 100 μg/mL plant extracts for three days, and SA-β-gal activity was measured. C. Effects of plant extracts on cell proliferation. Cells were seeded at 500 cells/well in 96-well plates and treated with 100 μg/mL of plant extract. Cell proliferation was measured by the MTT assay. D. Cytotoxicity of plant extracts. Cells were seeded at 5×10^3 cells/well in 24-well plates and treated with increasing concentrations of plant extract. Cell proliferation was determined by cell counts. E. Effects of plant extracts on the p53 protein level, which increases by adriamycin treatment. Cells (1×10^5) were seeded on 60-mm culture dishes and treated with indicated concentrations of plant extract for 1 h prior to 500 nM adriamycin treatment. After incubation for 4 h, cells were harvested, and proteins were extracted. The level of p53 protein was measured by Western blotting using a p53 antibody. Protein loading of each lane was assessed by a GAPDH antibody. Values are means±SDs of triplicates of three independent experiments. Representative data of three independent experiments are shown. $^{**}p<0.01$, Student's *t*-test; Y, young cells; Cont, control; DMSO, dimethyl sulfoxide; NAC, *N*-acetylcysteine; ADR, adriamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

respectively. None of the three extracts increased cell proliferation (Fig. 2C) at the concentration used (100 μg/mL). Although CW01-047 (Rhei Rhizoma) and CW03-065 (Plantaginis Semen) exhibited cytotoxicity in a dose-dependent manner, CW02-007 (Cirsii Radix) was not cytotoxic up to 100 μg/mL in fibroblasts (Fig. 2D). We further examined the ability of these three extracts to reduce the expression level of p53 protein, which increased

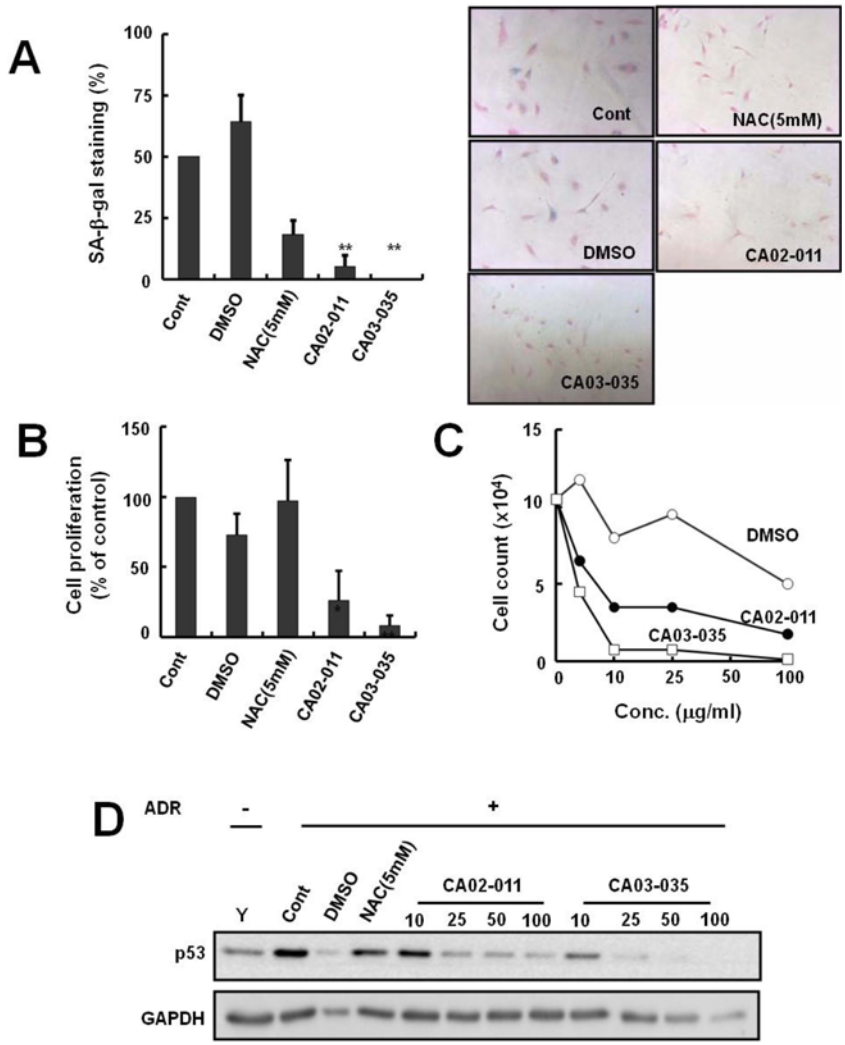

Fig. 3. Identification of plant extracts that inhibit adriamycin-induced cellular senescence in human endothelial cells. A. Effects of plant extracts on SA-β-gal activity in adriamycin-treated cells. Cells treated with 500 nM adriamycin for 4 h were seeded at 1,000 cells/well in 96-well plates. After treatment with 100 μg/mL of crude plant extract (n=799) for 3 days, SA-β-gal activity was measured. B. Effects of plant extracts on cell proliferation. Cells were seeded at 1,000 cells/ well in 96-well plates and treated with 100 μg/mL of plant extract. Cell proliferation was measured by the MTT assay. C. Cytotoxicity of plant extracts. Cells were seeded at 1×10^4 cells/ well in 24-well plates and treated with increasing concentrations of plant extract. Cell proliferation was measured by cell counts. D. Effects of plant extracts on the p53 protein level increased by adriamycin treatment. Cells (1×10^5) were seeded on 60-mm culture dishes and treated with indicated concentrations of plant extract for 1 h prior to 500 nM adriamycin treatment. After incubation for 4 h, cells were harvested, and proteins were extracted. The level of p53 protein was measured by Western blotting using a p53 antibody. Protein loading of each lane was assessed by a GAPDH antibody. Values are means±SDs of triplicates of three independent experiments. Representative data of three independent experiments are shown. $^{**}p<0.01$, Student's *t*-test; Y, young cells; Cont, control; DMSO, dimethyl sulfoxide; NAC, *N*-acetylcysteine; ADR, adriamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

in adriamycin-treated cells. All three extracts decreased the p53 protein level in fibroblasts. Results showed that, among the three extracts, CW02-007 (Cirsii Radix) has the greatest potential for effectively reducing adriamycin-induced cellular senescence in fibroblasts.

Screening of plant extracts to inhibit adriamycin-induced cell senescence in human umbilical vein endothelial cells. Among the 799 plant extracts, two extracts, CA02-011 (Cinnamomi Cortex) and CA03-035 (Cinnamomi Cortex Spissus), effectively suppressed adriamycin-induced cellular senescence (Fig. 3A). Cell proliferation decreased with treatment (100 μg/mL) by CA02-011 (Cinnamomi Cortex) or CA03-035 (Cinnamomi Cortex Spissus) (Fig. 3B). Although CA02-011 (Cinnamomi Cortex) and CA03-035 (Cinnamomi Cortex Spissus) had cytotoxic effects in endothelial cells in a dose-dependent

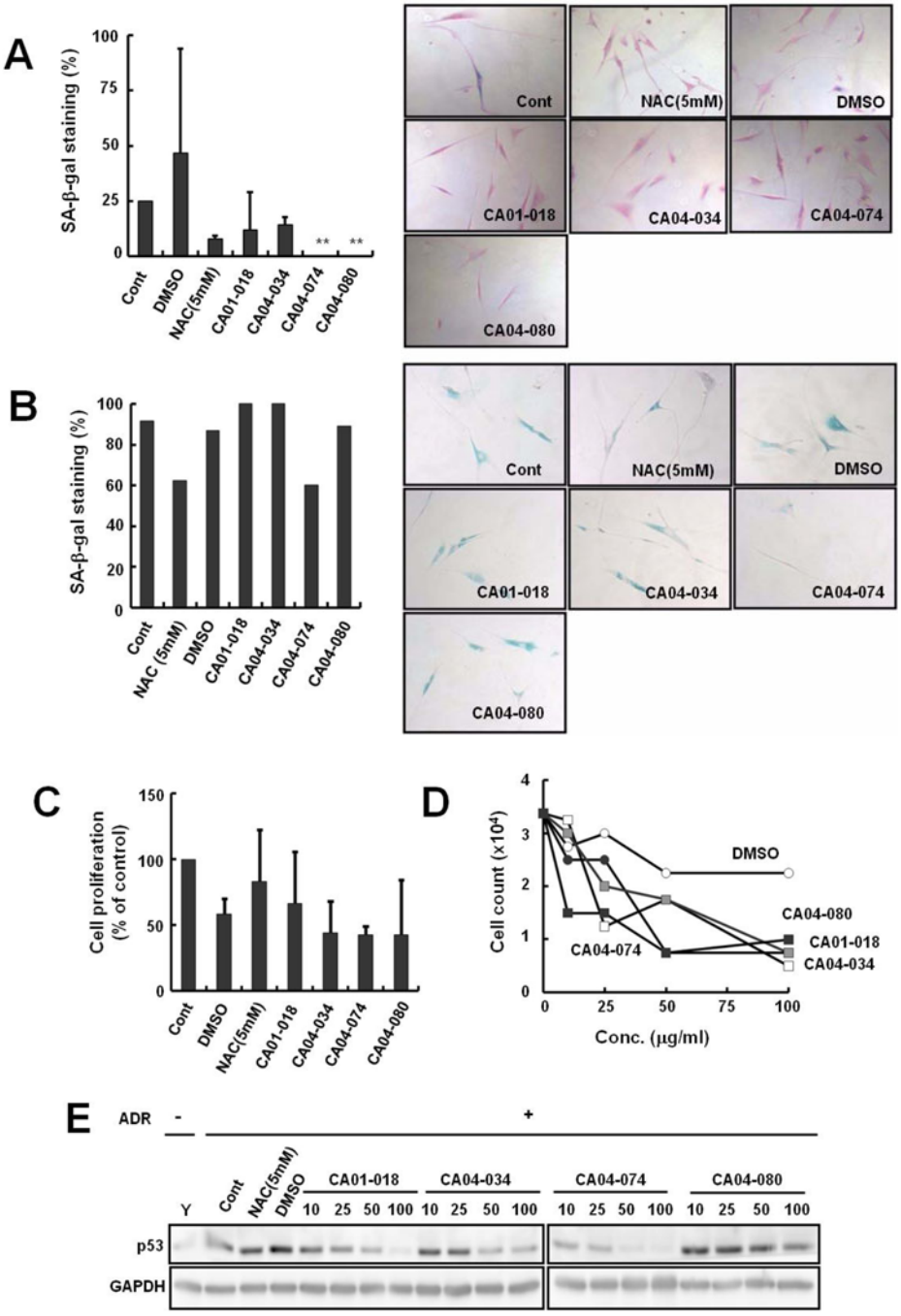

Fig. 4. Identification of plant extracts that inhibit adriamycin-induced cellular senescence in human vascular smooth muscle cells. A. Effects of plant extracts on SA-β-gal activity in adriamycin-treated cells. Cells treated with 500 nM adriamycin for 4 h were seeded at 1,000 cells/well in 96-well plates. After treatment with 100 μg/mL of crude plant extract (n=799) for 3 days, SA-β-gal activity was measured. B. Effects of plant extracts on replicative senescence. Old cells (PD>45) were treated with 100 μg/mL plant extract for 3 days, and SA-β-gal activity was measured. C. Effects of plant extracts on cell proliferation. Cells were seeded at 1,000 cells/well in 96-well plates and treated with 100 μg/mL plant extract. Cell proliferation was measured by the MTT assay. D. Cytotoxicity of plant extracts. Cells were seeded at 1×10^4 cells/well in 24-well plates and treated with increasing concentrations of plant extract. Cell proliferation was measured by cell counts. E. Effects of plant extracts on the p53 protein level, which is increased by adriamycin treatment. Cells (1×10^5) were seeded on 60-mm culture dishes and treated with indicated concentrations of plant extract for 1 h prior to 500 nM adriamycin treatment. After incubation for 4 h, cells were harvested, and proteins were extracted. The level of p53 protein was measured by Western blotting using a p53 antibody. Protein loading of each lane was assessed by a GAPDH antibody. Values are means±SDs from triplicates of three independent experiments. Representative data of three independent experiments are shown. $^{**}p<0.01$, Student's *t*-test; Y, young cells; Cont, control; DMSO, dimethyl sulfoxide; NAC, *N*-acetylcysteine; ADR, adriamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

manner, the cytotoxic effect of CA02-011 was lower than that of CA03-035 (Fig. 3B and 3C). The expression level of p53 protein, which increased in adriamycin-treated cells, was also reduced by treatment with CA02-011 and CA03-035 (Fig. 3D).

Screening of plant extracts for inhibition of adriamycin-induced cell senescence in human vascular smooth muscle cells. Among the 799 plant extracts studied, CA01-018 (Euonymi Lignum Suberalatum), CA04-034 (Salicis Radicis Cortex), CA04-074 (Polygoni avicularis Herba), and CA04-080 (Chaenomelis langenariae Radix) effectively suppressed adriamycin-induced cellular senescence (Fig. 4A). Of these four extracts, CA04-074 treatment also inhibited SA-β-gal activity in old cells, which was induced by replicative senescence (Fig. 4B). All four extracts showed some dose-dependent cytotoxic effects in the vascular smooth muscle cells (Fig. 4C and 4D). Furthermore, the expression level of p53 protein, which increased in adriamycin-treated cells, was reduced by all four extracts (Fig. 4E).

Discussion

The present study shows that among 799 plant extracts, several plant extracts reduced adriamycin-induced cellular senescence in various human primary cells. CW01-047 (Rhei Rhizoma), CW02-007 (Cirsii Radix), and CW03-065 (Plantaginis Semen) were effective in fibroblasts, CA02-011 (Cinnamomi Cortex) and CA03-035 (Cinnamomi Cortex Spissus) were effective in endothelial cells, and CA01-018 (Euonymi Lignum Suberalatum), CA04-034 (Salicis Radicis Cortex), CA04-074 (Polygoni avicularis Herba), and CA04-080 (Chaenomelis langenariae Radix) were effective in vascular smooth muscle cells. To our knowledge, this is the first report on the ability of plant extracts to directly inhibit cellular senescence in human primary cells as found by measuring SA-β-gal activity induced by adriamycin treatment.

Rhei Rhizoma contains a variety of components that have diverse biological activities; anthraquinone derivatives -including rhein and emodin- were reported to have antibacterial, antioxidant and antitumor activity [Huang *et al.*, 2007]. In addition, tannin components -as antioxidants- have antiviral, antibacterial, anticarcinogenic, and anti-inflammatory effects [Koleckar *et al.*, 2008]. Silymarin components in Cirsii Radix has hepatoprotective roles including inhibition of leukotriene production, enhancement of glutathione secretion, anti-inflammatory effects through suppression of NF-κB-regulated gene products, and antitumor activity in a variety of tumor cells [Agarwal *et al.*, 2006]. Cinnamic aldehydes in Cinnamomi Cortex were found to have diverse activities such as vasodilation effects in peripheral vessels and inhibitory effects on platelet aggregation [Yun *et al.*, 2001], antibacterial activity [Chang *et al.*, 2001], stimulation of gastric juice secretion and antitumor activity [Cabello *et al.*, 2009], and anti-inflammatory effects [Huss *et al.*, 2002]. Carvacrols have antitumor and antibacterial activities [Baser, 2008]. Euonymi Lignum Suberalatum showed antidiabetic effects in db/db mice [Park, 2007]. Salicis Radicis Cortex inhibits platelet aggregation [Krivoy *et al.*, 2001] and is used to treat osteoarthritis [Beer and Wegener, 2008]. Flavonoid components in Salicis Radicis Cortex have antioxidant, antitumor, and antimetastatic effects [Bonfili *et al.*, 2008], and they increase NO synthase activity [Tunon *et al.*, 2009]. β-Sitosterol in *Salicis radicis* cortex, as a vitamin D precursor, lowers LDL cholesterol levels [Lugasi, 2009], prevents cancer development and metastasis [Ovesna *et al.*, 2004], and is effective in wound healing, angiogenesis, and cardiovascular disease [Plat and Mensink, 2001]. In Polygoni avicularis Herba, camperols exhibit antitumor activity [Griffiths *et al.*, 1999], and avicularin has hypotensive effects by increasing diuresis [Fukunaga *et al.*, 1989]. Chaenomelis langenariae Radix contains antioxidants [Beekwilder *et al.*, 2005]. Campesterol, β-sitosterol, and quercetin in Chaenomelis langenariae Radix inhibit tumor cell proliferation [Hirpara *et al.*, 2009] and have antibacterial and anti-inflammatory effects [Bischoff, 2008]. Results of various studies suggest that the plant extracts isolated in the present study have some direct or indirect effects on cellular senescence or tissue aging. Inhibitory effects of cellular senescence by the plant extracts could be directly due to the regulation of SA-β-gal activity or indirectly due to antioxidant activity and other effects of the plant extracts. Our finding that different plant extracts reduced adriamycin-induced cellular senescence in each cell type suggests the inhibitory effects of plant extracts on cellular senescence could be cell context-dependent or specific regulatory target-dependent. Since down-regulation of SA-β-gal activity in fibroblasts and HeLa cells was reported to have no effects on cellular senescence [Lee *et al.*, 2006a], further study is necessary to elucidate which components of the plant extracts are effective for reducing cellular senescence and to determine the mechanisms by which the plant extracts inhibit cellular senescence in human cells.

Acknowledgment. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (20090063425).

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