Food Sci. Biotechnol. Vol. 16, No. 4, pp.537 ~ 542 (2007)

Food Science and Biotechnology

Induction of Apoptosis in Human Oral Epidermoid Carcinoma Cells by Sophoraflavanone G from *Sophora flavescens*

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Abstract *Sophora flavescens* AITON (Leguminosae) is a typical traditional Korean medical herb considered to exhibit antibacterial, anti-inflammatory, and antipyretic effects, and is also used for the treatment of skin and mucosal ulcers, sores, diarrhea, gastrointestinal hemorrhage, arrhythmia, and eczema. In this study, the compound sophoraflavanone G was isolated from the dried roots of *S. flavescens* by bioassay-guided fractionation. We then investigated the effects of various concentrations of sophoraflavanone G on cell viability and the induction of apoptosis in KB cells after an incubation of 24 hr. The results were determined by the following methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT) assay, Hoechst-33258 dye staining, flow cytometry (cell cycle), and Western blotting for caspase-3 and poly (ADP-ribose) polymerase (PARP). We found sophoraflavanone G induced the apoptosis of KB cells in a dose-dependent manner that was verified by DNA fragmentation, apoptotic bodies, the sub-G1 ratio, caspase-3 activity, and cleavage of PARP. These results suggest that sophoraflavanone G has potent anti-proliferative effects on human oral epidermoid carcinoma cells, with the induction of apoptosis.

Keywords: *Sophora flavescens*, sophoraflavanone G, apoptosis, cell cycle, caspase

Introduction

An apoptotic program can be activated by a number of physiological and pathological stimuli, including lack of nutrients, activation of cell surface death receptors, chemicals, ionizing radiation, and direct physical injury (1-4). Apoptosis, distinct from necrosis, is an active process of cell destruction with specific defining biochemical and morphological features characterized by the activation of endogenous proteases (caspase) and endonucleases, nuclear/chromatin condensation, internucleosomal cleavage of DNA (DNA ladder), cell shrinkage, dilated endoplasmic reticulum, membrane blebbing, and the formation of apoptotic bodies (4-7). Caspases can be divided into initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7) (8, 9). Moreover, the effector caspases cleave intracellular substrates such as poly-ADP-ribose polymerase (PARP), and the cleavage of this protein ultimately results in cellular morphological and biochemical alterations that are characteristic of apoptosis (10, 11).

Sophorae radix, the dried root of *Sophora flavescens* AITON (Leguminosae), is a typical traditional Korean medical herb considered to exhibit antibacterial, anti-inflammatory, antioxidant, antimalarial, and antipyretic effects, and is also used for the treatment of skin and mucosal ulcers, sores, diarrhea, gastrointestinal hemorrhage, arrhythmia, and eczema (12-17). The dried roots of *S. flavescens* contain approximately 10 kinds of alkaloids. Among them are matrine, sophoridine, and oxymatrine, and numerous flavonoids such as formononetin, kushenol E, kushenol B, sophoraflavanone G, kushenol L, kushenol M, kuraridin, kurarinone, kushenol N, and kushenol F, which are the main effective constituents used for disease treatment (18-22). Recently, Dai *et al.* (17) reported that these alkaloids are effective against tumors, arrhythmia, and immunodeficiency (21), which generated much attention and interest. Shen *et al.* (22) reported that the lavandulyl side chain found in sophoraflavanone G and kurarinone, has a structural characteristic of possessing a lavandulyl group at C-8 or C-6, together with a hydroxyl group at C-2' (23-25). Recently Lee *et al.* (26) reported that the lavandulyl side chain is essential for the antitumor, antioxidant, and antibacterial activity, as well as phospholipase-Cγ1-inhibition activity, of the flavonoids isolated from this plant (27). Kim *et al.* (28) reported that sophoraflavanone G inhibits prostaglandin E_2 (PGE_2) production from lipopolysaccharide (LPS)-treated RAW cells by cyclooxygenase-2 (COX-2) down-regulation, and has *in vivo* anti-inflammatory activity against mouse croton oil-induced ear edema and rat carrageenan paw edema via oral or topical administration (29). Kim *et al.* (12) has reported that sophoraflavanone G is a rich source of flavonoids with wide ranges of biological activities such as anticancer, anti-inflammatory, antibacterial, antioxidant, antimalarial, and tyrosinase inhibitory properties (27, 28, 30, 31).

In this study, we demonstrated that sophoraflavanone G of *S. flavescens* induced apoptosis in KB cells via the effects of morphology, DNA fragmentation, the cell cycle, caspase-3 activity, and cleavage of PARP.

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Received December 3, 2006; accepted March 12, 2007

Matherials and Methods

Plant material The roots of *S. flavescens* were collected in October 2001, at Jinan in Jeonbuk Province, Korea. A voucher specimen (JS01-3) has been deposited at the Herbarium of the Department of Biomedicinal Chemistry and Cosmetics, College of Technology and Natural Science, Mokwon University.

General 1D-NMR (^{1}H, ^{13}C, DEPT-135, DEPT-90, and DEPT-45) and 2D-NMR spectra, including ^{1}H-^{1}H DQF-COSY, HMQC, and HMBC, were recorded in CD_3OD on a Bruker DMX-600 instrument (Karlsruhe, Germany) operating at 600 MHz for ^{1}H. Chemical shift values (δ) were reported in parts per million (ppm) relative to an appropriate internal solvent standard (CD_3OD; δ_H=3.31/4.83, δ_C=49.15), and coupling constants (J values) were given in Hz. The electrospray ionization (ESI) mass spectra were obtained on a Macro Mass Quatro LC with an electro spray ionization method. Thin layer chromatography (TLC) was performed using Kieselgel $60F_{254}$ (Merck, Rahway, NJ, USA) pre-coated plates, and spots were visualized by spraying with vanillin-sulfuric acid followed by heating.

Extraction and isolation of sophoraflavanone G Dried and coarsely ground roots of *S. flavescens* (5 kg) were extracted by repeated refluxing with methanol (MeOH) for 4 hr at 60°C. The combined MeOH extract was clarified by filtration, and evaporated to obtain a dark brown syrup (210 g). The MeOH extract was suspended in H_2O and partitioned with chloroform ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (BuOH) successively. The organic solvent extracts were dried *in vacuum* at 45°C to yield the $CHCl_3$ soluble fraction (0.23 g), EtOAc soluble fraction (8.94 g), and BuOH soluble fraction (22.47 g). The EtOAc soluble fraction (6.3 g) was subjected to silica gel (Kieselgel 60; 0.063-0.04 mm particle size, 3×50 cm, Merck, Damstadt. Germany) column chromatography. The column was eluted with a gradient elution, using mixtures of MeOH in CH_2Cl_2 (10% with 700, 20% with 500, 30% with 300, and 40% with 200 mL), followed by 300 mL of MeOH. The fractions were combined based on their TLC pattern to yield fractions designated as S1-S6. Fraction S3 (1.4 g) retained activity, thus it was fractionated further by Sepadex LH-20 column chromatography [3×50 cm; stepwise gradient of 10, 20, and 50%(v/v) $CHCl_3$ in MeOH, followed by 300 mL of MeOH; collecting 50 mL fractions]. A portion (275 mg) of the active fraction (SC2) was subjected to recycling preparative HPLC (eluent: MeOH) to yield compound 1 [purity: 96%, t_r = 58 min. 129 mg, 0.03%, w/w, m.p. 173-175°C; $[\alpha]_D^{25}$ -49° (*c* 1.0 in MeOH)].

^{1}H-NMR (600 MHz, CD_3OD) δ 1.48 (3H, *s*, H-7″), 1.57 (3H, *s*, H-6), 1.63 (3H, *s*, H-10″), 2.04 (2H, *m*, H-3″), 2.47 (1H, *m*, H-2″), 2.58 (1H, *m*, H-1″), 2.76 (1H, *dd*, *J*= 17.1, 2.6 Hz, H-3$_\alpha$), 2.99 (1H, *J*=17.1, 13.2Hz, H-3$_\beta$), 4.56 (1H, *brs*, H-9″), 4.97 (1H, *t*, *J*=5.9Hz, H-4″), 5.65 (1H, *d*, *J*=13.2, 2.6 Hz, H-2), 6.00 (1H, *s*, H-6), 6.33 - 6.35 (2H, *m*, H-3′, 5′), 7.30 (1H, *d*, *J*=8.1 Hz, H-6′), and 12.18 (C_5-OH). ^{13}C-NMR (150 MHz, CD_3OD) δ 75.9 (C-2), 43.4 (C-3), 199.2 (C-4), 163.3 (C-5), 96.4 (C-6), 166.6 (C-7), 108.7 (C-8), 162.8 (C-9), 103.4 (C-10), 118.5 (C-1′), 156.8 (C-2′), 103.5 (C-3′), 159.7 (C-4′), 107.8 (C-5′), 128.8 (C-6), 28.1 (C-1″), 48.4 (C-2″), 32.5 (C-3″), 124.9 (C-4″), 132.2 (C-5″), 26.0 (C-6″), 18.0 (C-7″), 149.9 (C-8″), 111.3 (C-9″), and 19.3 (C-10″).

Cell culture Human KB cells of the oral epidermoid carcinoma cell line (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, 10 mg/mL streptomycin, and 0.25 mg/mL fungizone. The KB cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) staining as described by the method of Lee *et al.* (32). Briefly, the KB cells were plated at a density of 10^5 cells/well into 24-well plates, and treated with different concentrations of sophoraflavanone G for 24 hr. The purple crystals produced from the reduction of MTT by the metabolically active cells, were solubilized by dimethyl sulfoxide (DMSO, Sigma). After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 540 nm.

Cell cycle analysis Flow cytometric analysis was performed as previously described by the method of Lim *et al.* (33). The KB cells were plated at a density of 10^5 cells/well into 24-well plates and treated with different concentrations of sophoraflavanone G for 24 hr. Then the cells were collected and fixed with 70% ethanol at 4°C for 1 hr. After washing with phosphate buffered saline (PBS), the cells were treated with RNase A (1 μg/mL, Sigma) and stained with propidium iodide (50 μg/mL, Sigma) for 30 min at 4°C in the dark. The stained cells were quantitatively analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NH, USA).

Nuclear morphology analysis The morphological changes of apoptotic cells were determined by fluorescence microscopy as previously described by the method of Cha *et al.* (34). The KB cells treated with different concentrations of sophoraflavanone G for 24 hr were collected and fixed in 100% ethanol, stained with Hoechst-33258 (Sigma, 0.5 μg/mL) for 15 min at 37°C, and then visualized under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) with UV excitation at 300-500 nm. The cells with nuclei containing condensed chromatin, or with fragmented nuclei, were defined as apoptotic cells.

DNA extraction and DNA gel electrophoresis The characteristic ladder pattern of DNA breakage was analyzed by agarose gel electrophoresis. The KB cells treated with different concentrations of sophoraflavanone G for 24 hr were collected and washed twice with PBS, and then the DNA of the KB cells was isolated by a

Wizard Genomic DNA purification kit (Promega Co., Madison, WI, USA). The isolated genomic DNA was subjected to 2.0% agarose gel electrophoresis at 100 V for 1 hr. The DNA was visualized by staining with ethidium bromide under UV light.

Western blotting The sophoraflavanone G-treated KB cells were collected by centrifugation and washed once with PBS. The washed cell pellets were resuspended in extraction lysis buffer [50 mM HEPES, pH 7.0; 250 mM NaCl; 5 mM ethylenediaminetetraacetate (EDTA, Sigma); 0.1% Nonidet P-40 (NP-40); 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma); 0.5 mM dithiothreitol (DTT, Sigma); 5 mM sodium fluoride (NaF); and 0.5 mM Na orthovanadate) containing 5 mg/mL each of leupeptin and aprotinin, and incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the bicinchoninic acid assay with BSA as standard. The proteins (30 μg) in the cell lysates were separated on 8-12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to screen for proteins involved in apoptosis, such as PARP, caspsase-3, and β-actin, and then transferred to immobilon polyvinylidenedifluoride membranes (PVDF, Millipore Co., Bedford, MA, USA). The membrane was blocked with 1% BSA in PBS-tween 20 for 1 hr at room temperature, and then incubated with anti-PARP, caspase-3, monoclonal antibodies (1:500, Oncogene, Darmstadt, Germany), and β-actin polyclonal antibody (1:1,000, Sigma). After washing in PBS-tween 20 three times, the blot was incubated with secondary antibodies, and bound antibodies were detected by an ECL kit (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England) with chemiluminescence by exposing blots to Hyperfilm (Amersham Pharmacia Biotech).

Statistical analysis Data are presented as the mean and standard error for the indicated number of separate experiments. Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) followed by a *t*-test. *p*-Values less than 0.05 were considered significant.

Results and Discussion

Analysis of sophoraflavanone G Compound 1 was obtained as pale yellow needles and was positive to $FeCl_3$, indicating it had a phenolic group(s) in its structure. In the negative ESI mass spectrometry, a molecular ion peak [M-H^+] was observed at *m/z* 423. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound 1 showed the signals of one lavandulyl group [δ 1.48, 1.57, and 1.63 (each 3H, each *s*, CH_3 ×3); δ 2.04-2.58 (5H, *m*, $=CH_2$×2, CH); δ 4.56 (2H, *brs*, $=CH_2$); and δ 4.97 (1H, *brt*, J= 5.9Hz, $=CH-CH_2$-)] and an ABX-type grouping due to the C-2 (δ 5.65) and C-3 protons (δ 2.76 and 2.99). A two-proton multiplet in 6.33-6.35, assignable to H-3' and H-5', and a doublet at 7.30 (*J*=8.1 Hz), indicated that compound 1 had 2',4'-dioxygenation in the B ring moiety. Also, three typical one-proton double doublets at 2.76 (*dd*, *J*=17.1, 2.6 Hz, H-3$_\alpha$); 2.99 (*J*=17.1, 13.2Hz, H-3$_\beta$); and 5.65 (1H, *d*, *J*=13.2, 2.6 Hz, H-2) showed the A ring was a flavanone. In the ^{13}C-NMR spectrum, four oxygenated aromatic carbons (δ 166.6, 163.3, 159.7, and 156.8) and eight aromatic carbons (96.4, 108.7, 103.4, 118.5, 103.5, 107.8, 128.8, and 146.5) were shown. Two olefinic carbon signals were detected between δ 124.9 (C-4″) -132.2 (C-5″). The position of the lavandulyl group was determined to be at C-8 because the one-proton singlet at δ 12.18 was assigned to a chelated hydroxyl group at C-5, causing a cross peak δ 96.4 in 1H-13C of the long range COSY, and hence, must be at C-6. From these data, compound 1 was postulated as a 5,7,2',4'-tetrahydroxy-8-lavandulylflavanone, named sophoraflavanone G (Fig. 1), and was finally confirmed by the comparison of the NMR data with the reference data (18, 35).

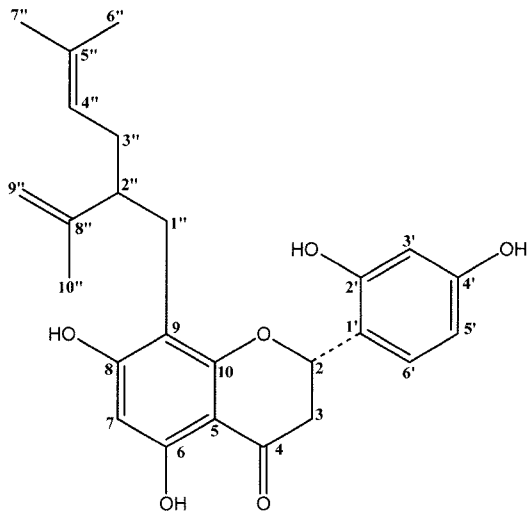

Fig. 1. Structure of 5,7,2',4'-tetrahydroxy-8-lavandulylflavanone (sophoraflavanone G) isolated from *Sophora flavescens*.

Effect of sophoraflavanone G on KB cells proliferation measured by MTT assay The KB cells were treated with different concentrations of sophoraflavanone G for 24 hr, and cell viability was determined as described above by MTT assay. As shown in Fig. 2, sophoraflavanone G inhibited the growth of KB cells in a dose-dependent manner ranging from 0.8 to 100 μg/mL (p<0.01). The *in vitro* antitumor activities of the lavandulyl flavanones of *S. flavescens* have been demonstrated in various cell lines including A549 (non-small cell lung), SK-OV-3 (ovary), SKMEL-2 (skin), XF498 (central nerve system), HCT-15 (colon), HL-60 (human myeloid leukemia), and human MCF-7/6 breast cancer cells (36, 37).

Effect of sophoraflavanone G on cell cycle phase distribution, determination of morphological changes, and DNA fragmentation in KB cells In this study, cell cycle phase redistribution was analyzed after treatments with different concentrations of sophoraflavanone G for 24 hr. In the sophoraflavanone G-treated cells, the proportion of cells in the G0/G1- and S-phases increased, and the proportion in the G2/M-phase decreased, when compared with the control (Fig. 3A). Cells with a sub-G0/G1 DNA

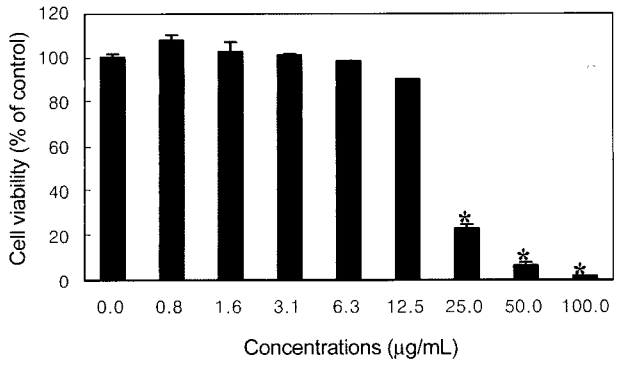

Fig. 2. Effect of sophoraflavanone G isolated from *Sophora flavescens* on cell proliferation in KB cells. The results are expressed as the mean±SE from 3 different experiments with triplicate cultures. *$p<0.01$ compared with control.

content, a hallmark of apoptosis, were seen in the sophoraflavanone G-treated group of KB cells following 24 hr of exposure at concentrations of 25 and 50 μg/mL. Nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in cells treated with different concentrations of sophoraflavanone G for 24 hr (Fig. 3B). The morphological changes and cell deaths of KB cells increased significantly at 25 μg/mL sophoraflavanone G; most cells were detached from the dishes, and cell rounding and shrinking occurred at sophoraflavanone G. Apoptosis-associated nuclear condensation is usually accompanied by oligonucleosomal DNA fragmentation into oligomers of 180 base pairs (10, 38). To determine whether sophoraflavanone G induced apoptosis in KB cells, we investigated DNA fragmentation, which is a biochemical hallmark for apoptosis. The results demonstrated that sophoraflavanone G induced endonucleolytic DNA cleavage in a dose-dependent manner (Fig. 3C).

Effect of sophoraflavanone G on activation of caspase-3 and cleavage of PARP After caspase-3 activation, some specific substrates for caspase-3 such as PARP are cleaved, which is important for the occurrence of apoptosis (9, 11). We investigated whether sophoraflavanone G

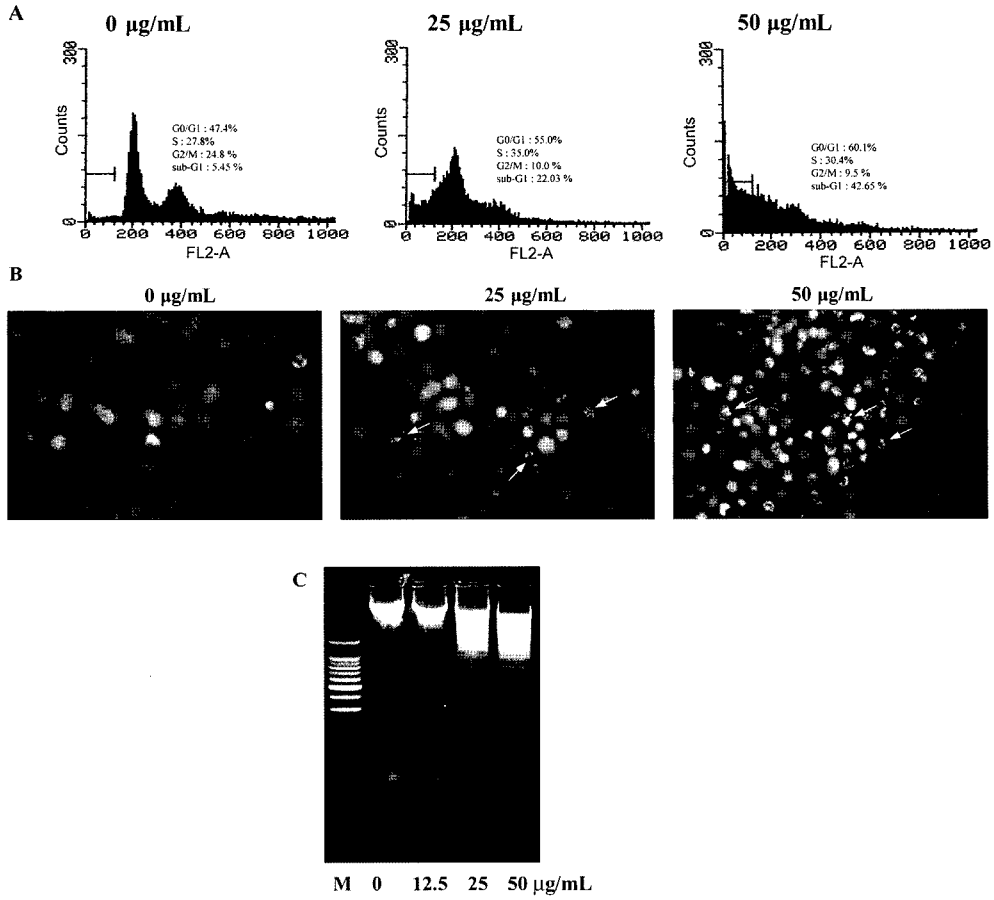

Fig. 3. Sophoraflavanone G isolated from *Sophora flavescens*-induced apoptosis in KB cells. KB cells were treated with sophoraflavanone G (25 and 50 μg/mL) for 24 hr. Non-treated cells were used as a negative control. Following the treatment, cells were collected for 3 kinds of experiments for apoptosis induction. (A) Flow cytometric analysis. The percentages of apoptotic cells were determined by propidium iodide staining followed by flow cytometric analysis. (B) The morphologic change analysis under fluorescence microscopy after staining with Hoechst 33258. The apoptotic cells are indicated with arrows. Normal nuclear morphology is observed in untreated cells; in contrast, small, fragmented, and condensed nuclei with typical apoptotic morphology are observed in treated cells. (C) DNA fragmentation analysis. Intracellular DNAs were isolated and analyzed by agarose gel (2.0%) electrophoresis.

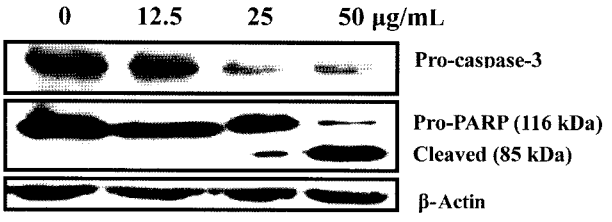

Fig. 4. Western blot analysis of caspase-3 and poly-ADP ribose polymerase (PARP) cleavage in KB cells treated for 24 hr with sophoraflavanone G isolated from *Sophora flavescens*. Proteins in whole cellular lysates and the untreated control were electrophoresed in SDS-PAGE gels and transferred to nitrocellulose membranes. Caspase-3 and PARP were identified using specific antibodies. Pro-caspase-3 was decreased in a dose-dependent manner, and the uncleaved 116 and 85 kDa cleavage products of PARP were detected. This immunoblot is representative of 3 independent assays performed at different times.

induced the activation of these caspases. KB cells were treated with different concentrations of sophoraflavanone G for 24 hr, and the processing of pro-caspases-3 and PARP was monitored by Western blotting (Fig. 4). At concentrations more than 12.5 μg/mL, sophoraflavanone G induced the processing of pro-caspase-3 into the active form within 24 hr, as well as the cleavage of PARP, with accumulation of the characteristic 85 kDa fragments and a concomitant disappearance of the full-length 116 kDa protein (Fig. 4).

In conclusion, sophoraflavanone G from *S. flavescens* induced apoptosis in KB cells. The induction of cancer cell apoptosis or death, without affecting healthy cells or producing side-effects, is a major goal in the development of new therapeutic agents. Our results suggest that sophoraflavanone G could be a candidate as an anticancer agent.

Acknowledgments

We thank Dr. SI Jeong at Institute of Jeonju Biomaterials, Jeonju, Korea.

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