Polyphenolic compounds from Korean *Lonicera japonica* Thunb. induces apoptosis via AKT and caspase cascade activation in A549 cells

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Abstract. *Lonicera japonica* Thunb. (*L. japonica* T.) has historically been used in Korean herbal medicine due to its anticancer and protective effects on the respiratory system. In the present study, the polyphenolic compounds in *L. japonica* T. were investigated using high-performance liquid chromatography coupled with tandem mass spectrometry, and its anticancer effects on A549 non-small-cell lung cancer cells were studied. Polyphenolic compounds potentially inhibit A549 cells in a dose-dependent manner. Flow cytometry and western blot analysis demonstrated that polyphenolic compounds induce apoptosis by regulating the protein expression levels of caspases, poly-(ADP-ribose) polymerase and the B-cell lymphoma-2-associated X-protein/B-cell lymphoma-extra large ratio. Furthermore, polyphenolic compounds inhibited mitochondrial membrane potential activity. Caspase-3 activity was increased in a dose-dependent manner and polyphenolic compounds inhibited the activation of protein kinase B by dephosphorylation. These results suggest that polyphenolic compounds in A549 cells indicate the anticancer activity through the induction of apoptosis.

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Introduction

Lung cancer is the most common type of cancer worldwide and it is the leading cause of cancer-associated mortality in numerous countries (1). The majority (75-85%) of lung cancers are non-small-cell lung cancers (NSCLC) (2,3); NSCLC cells have malignant cell properties, including aggressive invasion and metastasis (4,5). The most widely used treatments are chemotherapy, surgery, radiation therapy or a combination of these; however, recuperation and prognosis continue to be problematic (6). Although the survival rate for lung cancer is gradually increasing (7), novel therapeutic agents are required in order to increase the survival rates of patients with adenocarcinoma.

Lonicera japonica Thunb. (*L. japonica* T.; caprifoliaceae) has historically been used in East Asian countries, including

Korea, China and Japan as an agent to treat fever, headache, upper respiratory tract infections, urinary disorders, rheumatoid arthritis and diabetes mellitus (8,9). Previous studies have reported the mechanisms underlying the anti-inflammatory activity of *L. japonica T.* (10). Yip *et al* demonstrated that the aqueous compounds of *L. japonica T.* triggered HepG2 cell death in a c-Jun N-terminal kinase-dependent manner (11). A previous study has indicated that the polyphenolic compounds isolated from Korean *L. japonica T.* induced G_2/M cell cycle arrest and apoptosis in HepG2 cells (12). In addition, luteoin and kaempferol, which are the major active constituents of *L. japonica T.*, were observed to induce lung carcinoma cell apoptosis (13,14). A previous study has also suggested that *L. japonica T.* protects cells from hydrogen peroxide-induced apoptosis through the phosphorylation of mitogen-activated protein kinases and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) (15). However, the molecular mechanisms underlying the anticancer activity of *L. japonica T.* have yet to be elucidated; therefore, the current study used A549 cells to examine the therapeutic effects of the polyphenolic compounds.

Apoptosis has a crucial role in cell homeostasis and differs from cell necrosis (16). During apoptosis, the cells undergo morphological and biological changes, including nuclear fragmentation, cell shrinkage, chromatin condensation and DNA fragmentation (17). Apoptosis is regulated by the B-cell lymphoma-2 (Bcl-2) protein family and also by certain caspases, which are a family of cysteine proteases (18). Apoptosis plays a pivotal role and prevents carcinogenesis by suppressing abnormal cell development or by removing mutated/damaged cells (19). Therefore, apoptosis is essential to the anticancer properties of numerous anticancer agents.

AKT has been observed to phosphorylate >9,000 proteins (20), thus, AKT is a critical regulator of cell growth, proliferation and cell death. AKT controls various signaling pathways, including the PI3K/AKT pathway and the apoptosis-associated proteins Bcl-2-associated X-protein (Bax), certain caspases and tumor protein-21 (p21), through the activation of downstream target proteins (21). The primary aim of the present study was to determine the effects of polyphenolic compounds on A549 cells in order to evaluate the anticancer potential of *L. japonica T.* The cytotoxicity of polyphenolic compounds was evaluated and cell apoptosis was determined using flow cytometry and immunoblotting for proteins specifically involved in apoptosis.

Materials and methods

Chemicals and reagents. RPMI-1640 was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Fetal bovine serum (FBS) and streptomycin/penicillin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT, dimethyl sulfoxide (DMSO), propidium iodide (PI) and RNase A were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Hoechst 33342 was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Anti-poly-(ADP-ribose) polymerase (PARP) (AB16661) and anti-β-actin (MAB1501) primary antibodies were obtained from EMD Millipore (Billerica, MA, USA). Anti-Bax (#2772), anti-B-cell lymphoma-extra large (Bcl-xL) (#2764) and anti-caspase-3 (#9662), -6 (#9762), -8 (#9764) and -9 (#9508) antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Horseradish peroxidase-coupled goat anti-mouse (sc-2005) and rabbit (sc-2030) immunoglobulin (Ig)G and anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). The Fluorescein isothiocyanate (FITC) Annexin-V apoptosis detection kit 1 and carbocyanine dye $DiOC_6$ were purchased from BD Biosciences (San Jose, CA, USA). The materials and chemicals (electrophoresis apparatus and electrophoresis buffer) used for electrophoresis were obtained from Bio-Rad Laboratories, Inc., (Hercules, CA, USA). An enhanced chemiluminescence kit was purchased from GE Healthcare Life Sciences (Chalfont, UK). All other chemicals, including glycine, trizma, sodium chloride and sodium dodecyl sulfate, were of the purest grade available and purchased from Sigma-Aldrich (Merck Millipore).

Purification and isolation of polyphenol components of L. japonica T. Purification and isolation of polyphenol components of *L. japonica T.* was performed according to a previously described method (22). *L. japonica T.* was obtained from the Animal Bio-Resources Bank (Jinju, Korea). The voucher specimen (#00101B) was deposited at the Animal Bio Resources Bank, Gyeongsang National University subsequent to purification in the present study. The lyophilized plant material (100 g) was ground into a powder and added to 500 ml of 70% methanol at 50˚C for 12 h. The material was filtered using a Büchner funnel, and concentrated to ~100 ml at 40˚C using a rotary evaporator. The residue was reconstituted in 0.01 g/ml, methanol and stored at -20˚C until analysis. The mixtures of polyphenolic compounds were reconstituted in methanol (0.01 g/ml), filtered through 0.45-μm cellulose membranes, transferred into silicanized vials and stored at -20˚C prior to high-performance liquid chromatography (HPLC) analysis. HPLC was conducted using an Agilent 1100 series LC system (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was performed on a Zorbax stable bond analytical SB-C_{18} column (4.6x250 mm; 5 μm; Agilent Technologies, Inc.). The flow rate was 0.5 ml/min, with the column temperature maintained at 30˚C and an injection volume of 10 ml in each experiment. PAD spectra were measured over a wavelength range of 240-600 nm in 2 min steps. Tandem mass spectrometry (MS/MS) experiments were conducted on a 3200 Q TRAP LC-MS/MS system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a Turbo V™ source and a turbo ion spray probe operation, at 450˚C.

Cell culture and treatment. A549 human lung carcinoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in an atmosphere containing 5% CO_2 at 37˚C. The cells were grown to 70-80% confluency, and then treated with 0, 200, 400, 800, 1,200, or 1,500 μg/ml polyphenolic compounds (Table I) dissolved in 1% DMSO for 24 h in the complete growth medium (RPMI-1640 medium with 10% FBS and 1% antibiotics; Gibco; Thermo Fisher Scientific, Inc.).

Cytotoxicity assay. An MTT assay was performed to determine the cytotoxicity of the polyphenolic compounds in A549 cells.

Table I. The retention time of identified polyphenolic compounds isolated from Korean *L. japonica* T.

No.	Compound	Rt (min)	MS(M-H)-	MS/MS
1	Caffeoylquinic acid dimer	17.20	707 (708)	353, 191, 179, 161, 127
2	Caffeoylquinic acid	18.09	353 (354)	337, 191, 179, 135
3+4	Caffeoylglycerol + 5-p-coumaroylquinic acid	22.55 22.55	253 (254) 337 (338)	253, 179, 161, 135, 133
5	Feruloylquinic acid	28.78	367 (368)- 369 (368)+	368/367, 179, 161, 135, 369, 163, 145, 135, 117
6	Dicaffeoylquinic acid	34.49	515 (516)	515, 353, 335, 191, 179, 173, 161, 155, 135, 111
7	Dicaffeoylquinic acid	35.37	515 (516)	353, 191, 179, 173, 161, 135
8	Kaempferol 3-O-glucoside	39.43	447 (448) 449 (448)+	447, 285 449, 287
9	Kaempferol- *O*-rutinoside	40.99	595 [M+H]-	449, 287, 269, 241, 153, 135, 105
10	Dicaffeoylquinic acid	41.95	515 (516)	515, 353, 335, 191, 179, 173, 161, 155, 135, 111
11	Apigenin-7-O-glucoside	46.63	431 (432)- 433 (432)+	432/431, 269 433, 271
12+13	Apigenin rutinoside + Feruoyl caffeoylquinic acid	47.47 47.47	579 (578)+ 529 (530)	579, 433, 287, 271, 153
14	Trihydroxymethoxyflavone	49.19	299	299, 284
15+16	Kaempferol + Isorhamnetin glucoside	50.28 50.28	287 (286)+ 477 (478)	287, 269, 153, 137, 119, 477, 357, 314, 299, 285, 271, 257, 243
17	Caffeic acid derivative	53.07	537 (538)	537, 375, 331, 179, 161, 151, 135
18	Feruoyl caffeoylquinic acid	55.71	529 (530)	529, 367, 349, 179, 161, 135

L. japonica T., *Lonicera japonica* Thunb.; Rt, retention time; MS(M-H)-; MS/MS., tandem mass-spectrometry.

Cells were seeded in a 12-well plate at a density of $1x10^5$ cells/ml and incubated for 24 h at 37˚C in a 5% CO_2 atmosphere. The cells were treated with various concentrations of polyphenolic compounds (0, 200, 400, 800, 1,200 or 1,500 μg/ml) for 4 h at 37˚C in a 5% CO_2 atmosphere. Following incubation, 100 μl MTT solution [5 mg/ml in phosphate buffered saline (PBS)] was added to each well and the cells were incubated for 3 h at 37˚C in a 5% CO_2 atmosphere. Subsequently, 500 μl DMSO was added to each well, following the complete removal of the medium, in order to dissolve the formazan crystals. The optical density (OD) of the cells at 540 nm was determined using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Nuclear morphology. Variations in cell morphology were analyzed using light and fluorescence microscopy. A549 cells treated with polyphenolic compounds were centrifuged at 300 x *g* for 5 min at room temperature, fixed for 15 min in PBS containing 4% paraformaldehyde, washed with PBS and then stained with Hoechst 33342 (20 μg/ml) for 10 min. The nuclear morphology was imaged using a Leica DM6000 B fluorescence microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA) with a 350 nm excitation wavelength (blue fluorescence).

Cell cycle analysis. Flow cytometry was performed to analyze the distribution of the cell cycle. The A549 cells ($6.0x10^5$ cells/well; 6 well plate) were treated with polyphenolic compounds (0, 200, 400, 800 and 1,200 μg /ml) and incubated for 24 h at 37˚C in a 5% CO_2 atmosphere. Cells were then trypsinized, washed twice with cold phosphate-buffered saline (PBS) and centrifuged 300 x *g* for 5 min at room temperature. The pellet was fixed with cold 70% (v/v) ethanol for 30 h at 4˚C. The cells were washed once with PBS and resuspended in cold PI (50 μg/ml), containing RNase A (0.1 mg/ml) in PBS (pH 7.4), for 30 min in the dark. The cellular DNA content was analyzed by flow cytometry using a FACS Calibur apparatus (BD Biosciences). Forward light scatter characteristics were used to exclude cell debris from the analysis and $\geq 1x10^4$ cells were used for each analysis. Cell cycle distribution was analyzed using the ModFit LT program (Verity Software House, Topsham, ME, USA) and the relative proportions of cells in the G_0/G_1, S and G_2/M phases were determined for the cell cycle analysis.

Annexin V-FITC/PI double staining assay. A549 cells ($6.0x10^5$ cells/well; 6 well plate) were harvested using trypsin following treatment for 24 h with polyphenolic compounds (0, 200, 400, 800 and 1,200 μg/ml), and the magnitude of apoptosis was determined using the FITC-Annexin-V apoptosis detection kit 1, according to the manufacturer's instructions. Briefly, the cells were washed with ice-cold PBS and resuspended in 100 μl Annexin-V binding buffer containing 10 mM HEPES/NaOH

(pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$. Aliquots of the cells were incubated in 5 μl an Annexin V-FITC solution and 5 μl PI at room temperature for 15 min in the dark. Subsequently, 400 μl binding buffer was added and the apoptotic cells were evaluated using fluorescence-activated cell sorter analysis in a flow cytometer.

Measurement of mitochondrial membrane potential (MMP). Flow cytometry was performed to analyze the MMP in A549 cells using the carbocyanine dye $DiOC_6$. Briefly, A549 cells ($6.0x10^5$ cells/well) were treated with polyphenolic compounds (0, 200, 400, 800 and 1,200 μg/ml) and incubated for 24 h at 37˚C in a 5% CO_2 atmosphere. The cells were trypsinized, washed in PBS and then incubated with $DiOC_6$ (40 nM) at 37˚C for 15 min in the dark. The cells were analyzed immediately with a flow cytometer.

Western blot analysis. Western blot analysis was conducted according to a previously described method (23). A549 cells ($6.0x10^5$ cells/well) were cultured in 6-well plates and incubated for 24 h with various concentrations of polyphenolic compounds (0, 200, 400, 800 and 1,200 μg/ml) or with DMSO as a solvent control. Following washing with ice cold PBS, the cells were lysed by RIPA buffer, and a Bradford assay (Bio-Rad Laboratories, Inc.) was used to determine the protein concentration. An equal amount of protein (20 μg) was loaded into each well and subjected to 12% SDS-PAGE (90 V for 4 h at 4˚C) and the resolved proteins were transferred to a 0.45 mm immunobilon polyvinyldene fluoride membrane (EMD Millipore). The membranes were probed with the aforementioned primary antibodies (dilution, 1:1,000) overnight at 4˚C, and then incubated with the aforementioned secondary antibody (dilution, 1:2,000) for 2 h at room temperature. The membranes were visualized by enhanced chemiluminescence and western blotting detection reagents (GE Healthcare Life Sciences) and exposed to x-ray film (Fuji, Tokyo, Japan). Band expression levels were quantified using ImageJ (version 1.49v for Windows; National Institutes of Health, Bethesda, MD, USA). The densitometry readings of the bands were normalized to β-actin expression.

Caspase-3 activity assay. Caspase-3 activity analysis was measured according to a previously described method (23). Caspase-3 activity was evaluated by the detection of the cleavage of N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, a colorimetric caspase-3 substrate, using a caspase-3 activity assay kit (APT165; EMD Millipore), according to the manufacturer's instructions. Briefly, A549 cells were treated with polyphenolic compounds for 24 h. The floating and adherent cells were collected and lysed in ice-cold RIPA buffer for 30 min in an ice bath. The supernatants were collected and incubated at 37˚C for 2-4 h with the reaction buffer (EMD Millipore), according to the manufacturer's instructions. The OD of the protein samples was evaluated at 405 nm with a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis. The data are presented as the mean ± standard deviation of three independent experiments. The data were analyzed with a Student's *t*-test and one-way analysis of variance with a *post-hoc* Tukey test, using SPSS version 10.0 for Windows (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Characterization of polyphenolic compounds in L. japonica T. The polyphenol components of *L. japonica T.* were isolated using HPLC. The 18 polyphenol components isolated were characterized using HPLC, mass spectra and data from the literature. The HPLC chromatogram and mass data were summarized in Fig. 1 and Table I, respectively (24).

Cell growth inhibition by polyphenolic compounds. To evaluate the effect of cell viability inhibition, the cytotoxicity of various concentrations of polyphenolic compounds (0-1,500 μg/ml) in A549 cells was examined using an MTT assay. As indicated in Fig. 1B, polyphenolic compounds inhibited the viability of A549 cells, as compared with the control cells (vehicle only), in a dose-dependent manner following a 24 h treatment ($P=0.0018$ for 200 μg/ml, $P=0.026$ for 400 μg/ml, $P=0.0011$ for 800 μg/ml, $P<0.0001$ for 1,200 μg/ml and $P<0.0001$ for 1,500 μg/ml). Polyphenolic compounds decreased cell growth by ~38% at 1,200 μg/ml. The 50% inhibitory concentration (IC50) was determined to be ~1,140 μg/ml. Cell viability was not significantly changed by the application of various concentrations of polyphenolic compounds in a normal cell line, a human embryonic fibroblast cell line or the WI-38 lung-derived cell line (data not presented). These results suggest that the extracted polyphenolic compounds had an anti-proliferative effect in A549 cells.

Polyphenolic compounds increase the proportion of cells in the sub-G_1 phase in A549 cells. Flow cytometry was used to investigate the distribution of A549 cell cycle phases following treatment with polyphenolic compounds at various concentrations (0, 200, 400, 800 and 1,200 μg/ml) for 24 h. As presented in Fig. 2A, the polyphenolic compounds significantly increased the proportion of A549 cells in a sub-G_1 phase of 14.9 and 35.7% ($P=0.0414$ and $P<0.0001$) at 800 and 1,200 μg/ml, respectively compared with the control (5.3%), whereas the proportion of cells in the G_1, S, and G_2/M phases were decreased in a dose-dependent manner ($P=0.038$ for 800 μg/ml and $P<0.0001$ for 1,200 μg/ml in G1 phase; $P=0.0059$ for 400 μg/ml, $P=0.0015$ for 800 μg/ml and $P=0.0018$ for 1,200 μg/ml in S phase; $P=0.0044$ for 1,200 μg/ml in G2/M phase). The data presented in Fig. 2B indicate the prevalence of the cell cycle phases at each concentration of the polyphenolic compounds used. These results suggest that polyphenolic compounds are able to induce apoptosis in A549 cells.

Polyphenolic compounds induce apoptosis in A549 cells. PI and Annexin V-FITC/PI double-labeled flow cytometry was used to analyze the rate of apoptosis in A549 cells treated with various concentrations of polyphenolic compounds for 24 h. The treated A549 cells demonstrated that the proportion of cells that underwent early apoptosis (lower right quadrant) was 51.5% at 800 μg/ml polyphenol, whereas the early apoptotic cell proportion was 21.1% at 1,200 μg/ml polyphenol. The late apoptotic cell proportions (upper right quadrant) were 8.3

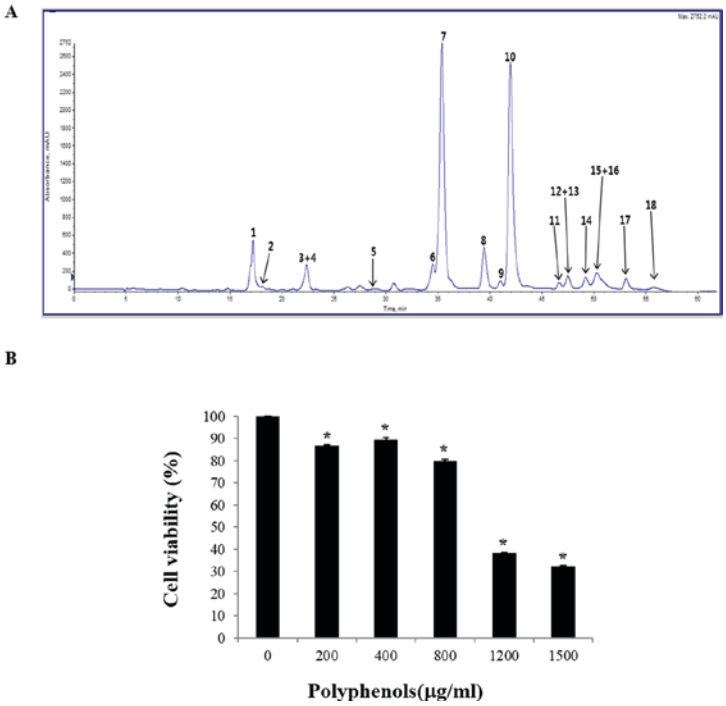

Figure 1. Characterization of polyphenolic compounds identified from Korean *L. japonica* T. in A549 cells. (A) HPLC profiles of Korean *L. japonica* T. at 280 nm: (1) Caffeoylquinic acid dimer; (2) Caffeoylquinic acid; (3) Caffeoylglycerol; (4) 5-p-coumaroylquinic acid; (5) Feruloylquinic acid; (6) Dicaffeoylquinic acid; (7) Dicaffeoylquinic acid; (8) Kaempferol 3-O-glucoside; (9) Kaempferol- *O*-rutinoside; (10) Dicaffeoylquinic acid; (11) Apigenin-7-O-glucoside; (12) Apigenin rutinoside; (13) Feruoyl caffeoylquinic acid; (14) Trihydroxymethoxyflavone; (15) Kaempferol; (16) Isorhamnetin glucoside; (17) Caffeic acid derivative; and (18) Feruoyl caffeoylquinic acid. (B) Growth inhibition of A549 cells following treatment with various concentrations (0-1,500 μg/ml) of polyphenolic compounds for 24 h. *P<0.05 compared with the control. HPLC, high performance liquid chromatography; *L. japonica* T., *Lonicera japonica* Thunb.

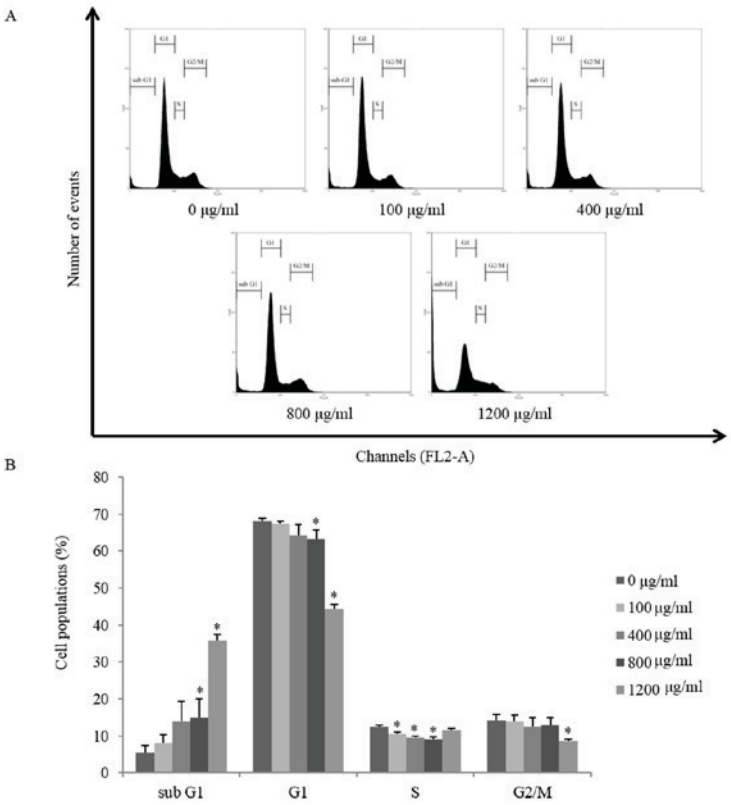

Figure 2. Effect of polyphenolic compounds on the cell cycle distribution in A549 cells. The cells were incubated with various concentrations (0-1,200 μg/ml) of polyphenolic compounds for 24 h and the distribution of the cell cycle was evaluated using FACS analysis. (A) Flow cytometry of cell cycle phase distribution; (B) statistical analysis of cell cycle phase distribution. The data are presented as the mean ± standard deviation of triplicate independent experiments. *P<0.05 compared with the control.

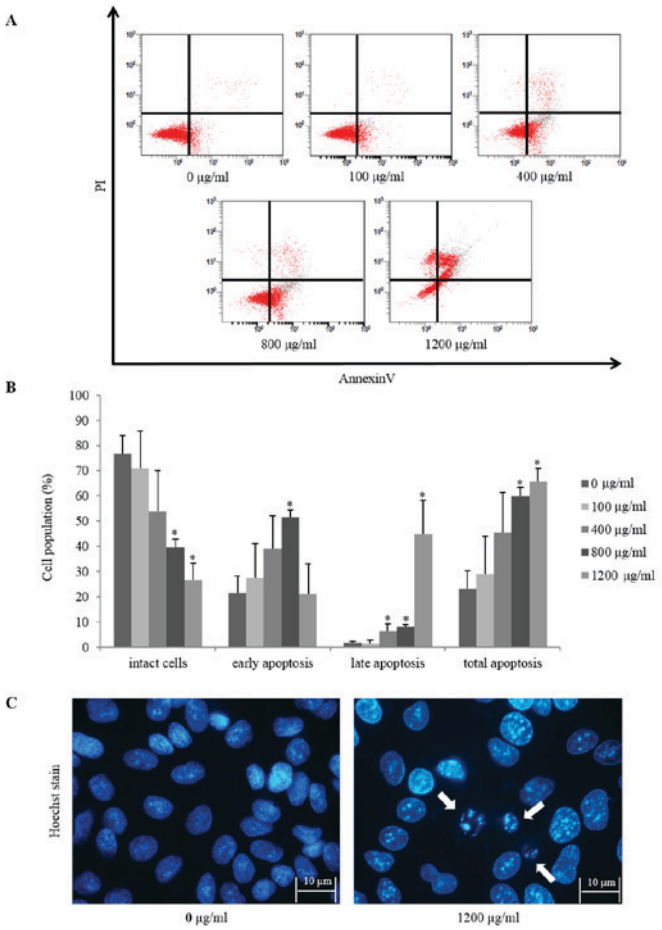

Figure 3. Induction of apoptosis in A549 cells by flavonoids. The apoptosis ratio was detected by Annexin V-FITC/PI double staining. (A) Flow cytometry analysis, (B) statistical analysis for apoptosis detection, (C) the cells were incubated with flavonoids for 24 h, fixed and stained with Hoechst 33342. The cells were imaged using a Leica DM6000 B microscope (magnification, 400x). The white arrows indicate chromatin condensation. The data are presented as the mean ± standard deviation of triplicate independent experiments. *P<0.05 compared with the control. FITC, fluorescein isothiocyanate; PI, propidium iodide.

and 44.8% at 800 and 1,200 μg/ml polyphenol, respectively. The total apoptotic cell proportion was significantly 59.8 and 65.9% at 800 (P=0.0014) and 1,200 μg/ml (P=0.0011), respectively (Fig. 3A and B). Furthermore, the A549 cells treated with polyphenolic compounds at 1,200 μg/ml demonstrated changes in apoptotic features, including nuclear fragmentation and chromatin condensation, which were observed following staining with Hoechest 33342 (Fig. 3C). The results indicate that polyphenolic compounds induce apoptosis in A549 cells.

The effect of polyphenolic compounds on the mitochondrial stability of A549 cells. To further investigate the features of polyphenolic compound-induced apoptosis in A549 cells, the MMP of the A549 cells was detected. Fig. 4A and B demonstrates that 1,200 μg/ml of the polyphenolic compounds induced significant mitochondrial disruption in 18.4% of the cells following a 24 h incubation compared with the control (P<0.0001). The results suggest that the polyphenolic compounds disrupt the MMP, which then triggers the activation and cleavage of certain caspases and the subsequent cell apoptosis.

Polyphenolic compounds induce Bcl-2 family proteins and caspase activation in A549 cells. Following treatment with polyphenolic compounds in A549 cells, Bcl-xL expression levels were decreased 3-fold in a dose-dependent manner (P=0.0232 for 400 μg/ml, P=0.0021 for 800 μg/ml and P=0.0001 for 1,200 μg/ml), whereas Bax expression levels were increased ~5-fold compared with the control (P=0.0243 for 1,200 μg/ml). A densitometric analysis of the western blotting bands revealed that treatment of A549 cells with polyphenolic compounds increased the Bax/Bcl-xL ratio in a dose-dependent manner (P<0.0001 for 1,200 μg/ml) (Fig. 5A). In addition, polyphenolic compounds decreased the expression levels of pro-caspases-3 (P=0.0042 for 400 μg/ml, P=0.0019 for 800 μg/ml and P=0.0006 for 1,200 μg/ml), -6 (P=0.0432 for 400 μg/ml, P=0.0219 for 800 μg/ml and P=0.0012 for 1,200 μg/ml), -8 (P=0.0046 for 400 μg/ml, P=0.0029 for 800 μg/ml and P=0.0097 for 1,200 μg/ml) and -9 (P=0.0003 for 400 μg/ml, P=0.0016 for 800 μg/ml and P=0.0004 for 1,200 μg/ml), decreased the expression levels of PARP (P=0.0016 for 100 μg/ml P=0.0011 for 400 μg/ml, P=0.0026 for 800 μg/ml and P=0.0001 for 1,200 μg/ml) and increased caspase-3 activity in a dose-dependent manner (P=0.035 for 100 μg/ml, P=0.027 for 400 μg/ml, P=0.004 for 800 μg/ml and P=0.001 for 1,200 μg/ml) (Fig. 5B). These results suggest that polyphenolic compounds-induce apoptosis by increasing the Bax/Bcl-xL ratio and activating certain caspases.

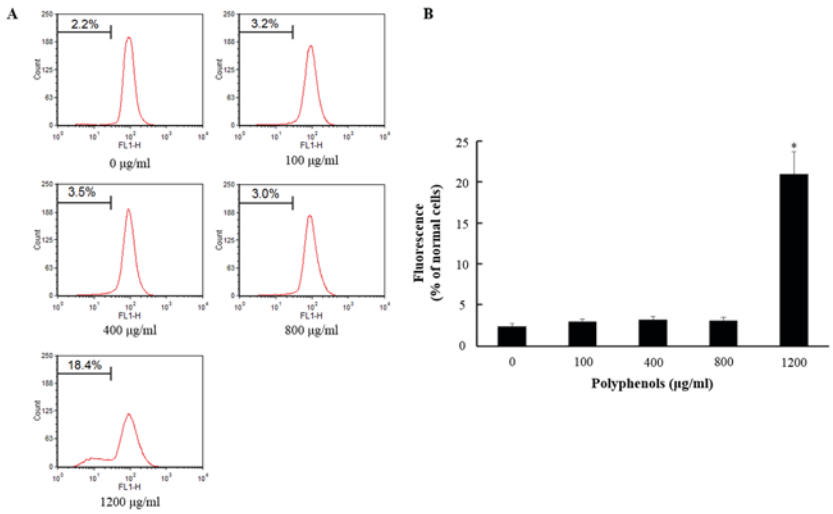

Figure 4. Polyphenolic compounds induced mitochondrial membrane potential stability. Cells were treated with various concentrations of polyphenolic compounds (0-1,200 μg/ml) for 24 h. (A) The cells were stained with the carbocyanine dye $DiOC_6$ and flow cytometry was performed to determine the MMP stability. (B) Fluorescence light was detected by flow cytometry and expressed as a bar graph. The data are presented as the mean ± standard deviation of triplicate independent experiments. *P<0.05 compared with the control. MMP, mitochondrial membrane potential.

Polyphenolic compounds decrease p-AKT in A549 cells. To determine the effect of polyphenolic compounds on the expression levels of phosphorylated (P)-AKT in A549 cells, western blot analysis was performed on those cells treated with polyphenolic compounds for 24 h. As presented in Fig. 5C, the expression levels of p-AKT in treated cells were decreased in a dose-dependent manner (P=0.0211 for 400 μg/ml, P=0.0165 for 800 μg/ml and P=0.0005 for 1,200 μg/ml). However, the total level of AKT expressed did not differ, revealing that polyphenolic compounds dephosphorylate AKT in A549 cells.

Discussion

Plant-derived herbal medicines have historically been used as traditional treatments in certain Asian countries, including Korea and China (25). Over half of the anticancer drugs identified originated from plants and a number of studies have been conducted on cancer prevention through the use of dietary polyphenolic compounds (10). Numerous studies have successfully demonstrated that naturally occurring dietary polyphenolic compounds have antiproliferative properties and, therefore, these compounds are considered to have anticancer effects (26). In the present study, the anticancer properties of the polyphenolic compounds isolated from *L. japonica T.* were investigated in A549 cells.

The regulation of apoptosis is essential for the maintenance of cell homeostasis. Apoptotic signals are regulated primarily by caspases, which perform as inactive zymogens in cells and produce a cascade of catalytic effects when apoptosis is initiated (27). Numerous studies have aimed to elucidate the mechanisms underlying the caspase signaling pathway mediated cell apoptosis (16). Following stimulation by natural killer T-cells, caspase-3 activation triggers multiple signaling cascades, and this cascade reaction acts to stimulate mitochondria to release cytochrome c. An excess of cytochrome *c* recruits caspase-9 for the activation of caspase-3 (28). It was previously determined that kaempferol was able to induce apoptosis in pancreatic cancer cells through DNA damage (29). In the current study the expression levels of pro-caspase -3, -6, -8 and -9 were significantly downregulated. Furthermore, the upregulation of caspase-3 activity indicates that polyphenolic compound-induced apoptosis is dependent on caspase-3. The Bcl family also has an important role in cell apoptosis. The Bcl proteins are apoptotic regulatory proteins that control the mitochondrial apoptotic process (30). Bcl-xL interacts with the mitochondrial plasma membrane to inhibit the other apoptotic factors, including Bax and Bak. This prevents induced cytochrome *c* from interacting with the mitochondrial plasma membrane. Furthermore, kaempferol is able to induce apoptosis in specific ovarian cancer cell lines by the inhibition of Bcl proteins expression (31). The Bax/Bcl-xL ratio may be a key mechanism underlying apoptosis as the results demonstrate that the Bax/Bcl-xL ratio was increased in the A549 cells treated with polyphenolic compounds.

Mitochondria power eukaryotic cells and MMP has become a frequently used tool for monitoring changes in the cells; therefore, MMP is a key indicator of cell health or injury. During cellular stress, MMP may be altered by the dysregulation of intracellular ionic charges, including Ca^{2+} and K^+, thus leading to a failure in adenosine triphosphate production and the collapse of cell homeostasis (32). The current study evaluated MMP in A549 cells using $DiOC_6$ fluorescence dye. Treatment of the cells with polyphenolic compounds at 1,200 μg/ml significantly increased MMP, compared with the control. The results indicate that polyphenol induces apoptosis through a mitochondria-dependent signaling pathway in A549 cells.

AKT is a serine/threonine protein kinase that serves as a pivotal regulator of diverse cellular responses (21). The AKT signaling pathway is one of the most important signaling pathways in various types of human cancer; AKT is typically overexpressed in human cancers (33). Previous studies have identified that the inhibition of AKT induces apoptosis or cell

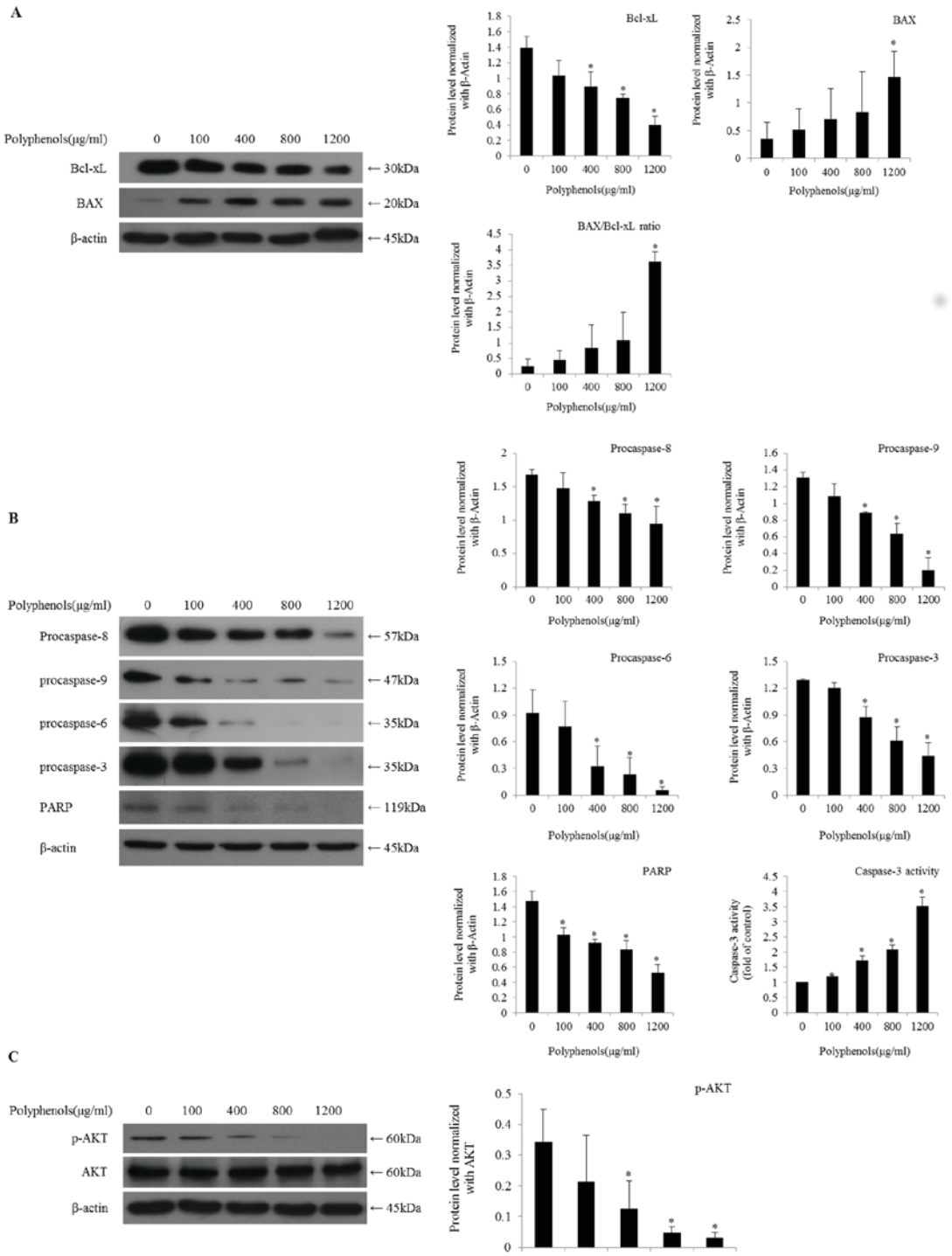

Figure 5. Effect of polyphenolic compounds on apoptosis-associated protein (Bcl-xL, BAX, caspases, PARP and AKT) expression levels and caspase-3 activity in A549 cells. The cells were treated with polyphenolic compounds (0, 100, 400, 800 and 1,200 μg/ml) for 24 h. (A) Whole cell lysates were subjected to SDS-PAGE and analyzed Bcl-xl and Bax by western blotting. (B) Densitometry analysis of the effect of flavonoids on the expression levels of apoptosis-associated proteins and caspase-3 activity is depicted. (C) p-AKT was normalized to the respective total AKT, and is presented relative to the value for the untreated control cells. The data are presented as the mean ± standard deviation of triplicate independent experiments. *P<0.05 compared with the control. Bcl-xL, B-cell lymphoma-extra large; BAX, Bcl-2-associated x-protein; PARP, poly-(ADP-ribose) polymerase; AKT, protein kinase B; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; p-AKT, phosphorylated AKT.

cycle arrest and increases the effects of radiation and certain chemotherapeutic agents in numerous types of cancer (34-36). Cell cycle progression may be affected by AKT through the suppression of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) (37). In addition, the upregulation of p-AKT serves to provide cells with a survival signal that allows them to resist apoptotic stimuli, by inhibiting specific pro-apoptotic proteins (38). Clinical studies have demonstrated that AKT activation is a common feature of numerous types of cancer, and that it correlates with poor prognosis (39). To evaluate

the regulation of AKT by polyphenolic compounds, p-AKT was investigated in A549 cells. The results demonstrated that polyphenolic compounds suppressed the expression levels of p-AKT in a dose-dependent manner (Fig. 5C). These results indicate that the downregulation of p-AKT by polyphenolic compounds triggered apoptosis through the upregulation of pro-apoptotic proteins.

In conclusion, the results of the present study demonstrate the anticancer properties of polyphenolic compounds isolated from *L. japonica T.* in A549 cells. The polyphenolic compounds induced apoptosis in A549 cells by downregulating certain pro-caspases (caspase-3, -6, -8 and -9) and PARP, and increasing the Bax/Bcl-xL ratio, caspase-3 activity, MMP dysfunction and the dephosphorylation of AKT, inducing apoptosis by a mitochondria-dependent signaling pathway. Flow cytometry demonstrated that polyphenolic compounds are able to induce apoptosis by increasing the sub-G_1 cell population, which was also indicated by FITC-Annexin V double staining in A549 cells. The results suggest that the polyphenolic compounds isolated from *L. japonica T.* must be evaluated in further studies as a potential therapeutic agent for the treatment of human lung cancer.

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