

Azorella compacta methanolic extract induces apoptosis via activation of mitogen-activated protein kinase

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Received October 15, 2014; Accepted June 30, 2015

DOI: 10.3892/mmr.2015.4317

Abstract. *Azorella compacta* Phil. (AC) is an alpine medicinal plant used traditionally for antibacterial treatment. Recent studies have revealed that this plant also has anti-diabetic effects, but that it is toxic. The present study investigated the underlying mechanisms of action of AC extract against human leukemia HL60 cells. Apoptosis induction was measured by MTT assay, fluorescence microscopy, DNA fragmentation assay, flow cytometric analysis, reverse transcription quantitative polymerase chain reaction and western blot analyses. It was found that AC extract inhibited the growth of HL60 and other cancer cell lines in a dose-dependent manner. The cytotoxic effects of AC extract on HL60 cells were associated with apoptosis characterized by DNA fragmentation and dose-dependent increases in Annexin V-positive cells, as determined by flow cytometric analysis. AC-extract-induced apoptosis was accompanied by activated/cleaved caspase-3, caspase-9 and poly(adenosine diphosphate-ribose) polymerase (PARP). The increases in apoptosis were also associated with decreases of the apoptosis-inhibitor B-cell lymphoma 2 (Bcl-2), upregulation of pro-apoptotic Bcl-2-associated X (Bax) protein and downregulation of anti-apoptotic Bcl extra large protein. Furthermore, western blot analysis of mitogen-activated protein kinase (MAPK)-associated proteins indicated that treatment with AC extract increased the levels of c-Jun N-terminal kinase, extracellular signal-regulated kinase and p38. In addition, the expression of Bax and cleaved PARP

was blocked when AC treatment was performed in the presence of MAPK inhibitors. It was therefore concluded that AC induced apoptosis in human leukemia HL60 cells via an intrinsic pathway controlled through MAPK-associated signaling.

Introduction

Apoptosis, an active, morphologically distinct form of programmed cell death, has a fundamental role in the normal development and differentiation of multicellular organogenesis, in the control of cell proliferation, in development, and in the pathogenesis of various diseases (1,2). Apoptosis is also characterized by cell shrinkage, blebbing of membranes, nuclear condensation and DNA fragmentation (3-5). In addition, induction of apoptotic cell death is an important mechanism of numerous anti-cancer drugs (6). The intrinsic apoptotic pathway is triggered by a range of physical and chemical stimuli causing mitochondrial dysfunction (7,8). The initiation of the apoptotic cascades leads to the activation of caspase-9 and subsequent activation of effector caspases, such as caspase-3, which in turn cleaves several specific substrates, including poly(ADP-ribose) polymerase (PARP), as well as architectural components of the cell, which eventually leads to apoptosis (9). Proteins of the B-cell lymphoma 2 (Bcl-2) family serve as critical regulators of mitochondrial apoptosis, functioning as either inhibitors or promoters of cell death. Bcl-2 and Bcl extra large protein (Bcl-X_L) inhibit apoptosis by blocking the release of cytochrome C from mitochondria through prevention of channel formation, which is mediated by Bcl-2-associated X protein (Bax) (10,11). Mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK) and p38/MAPK, are activated in response to various stimuli. Subsequent to their activation, they participate in a variety of signaling pathways regulating diverse cellular processes, including cell growth, differentiation and stress responses. Activation of MAPKs is therefore closely associated with stress stimuli-induced apoptosis (12-14).

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Key words: *Azorella compacta*, apoptosis, intrinsic pathway, mitogen-activated protein kinase, HL60

It was previously reported that *Azorella compacta* Phil. (AC), a green, compact, resinous cushion shrub of the Apiaceae family growing in the high Andes of southern Peru and Bolivia, northeastern Chile and northwestern Argentina, contained mulinane and azorellane diterpenoids (15,16). In Chilean folk medicine, AC, together with other *Azorella* and *Laretia* species (collectively known as 'llareta') is used for the treatment of a variety of ailments (15). Historical records indicated that AC has been used to treat the common cold and pain, to reduce blood sugar, and also as an ointment to treat dermatological disorders. AC presents a valuable source of mulinane and azorellane diterpenoids (15,17). AC has also been traditionally used to treat colds, asthma and bronchitis, as well as conditions whose main symptoms include inflammation and pain. Studies on AC have demonstrated anti-bacterial (18) and anti-plasmodial properties (19). However, to the best of our knowledge, no scientific studies are available on their cytotoxicity-based anti-cancer effects.

The present study was the first, to the best of our knowledge, explore the potency of AC extract to induce apoptosis of human leukemia HL60 cells and to elucidate its underlying mechanisms of action. The apoptotic effects of AC extract were assessed by examining the caspase-dependent pathway involving the loss of mitochondrial membrane permeability, the release of cytochrome *c* and the activation of the Bcl-2 family of proteins. In addition, the involvement of MAPK-dependent signaling was assessed using MAPK inhibitors.

Materials and methods

Chemicals. The following reagents and kits were used in the present study: Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA, USA), Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, phosphate-buffered saline (PBS) (Hyclone, Logan, UT, USA), MTT (Amresco, Solon, OH, USA), trypan blue, Hoechst 33342, caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) (Invitrogen Life Technologies, Carlsbad, CA, USA), dimethyl sulfoxide (DMSO), adriamycin, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), agarose, 5X TBE buffer (Bioneer Corp., Daejeon, Korea), apoptotic DNA ladder kit (BioVision, San Diego, CA, USA), Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD Biosciences, Franklin Lakes, NJ, USA), Omniscript RT kit (Qiagen, Hilden, Germany), polymerase chain reaction (PCR) premix (Promega Corp., Madison, WI, USA), TRIzol reagent, enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA) and polyvinylidene difluoride (PVDF) membranes (Merck-Millipore, Billerica, MA, USA), PD98059, SP600125 and SB203580 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Cell line and cell culture. The cell lines HepG2, MCF7, HT1080, A549, SNU-1 and HL60 were procured from the American Type Culture Collection (Manassas, VA, USA). HepG2, MCF7 and HT1080 cells were routinely cultured in DMEM supplemented with 10% FBS. SNU-1 cells were routinely cultured in RPMI-1640 supplemented with 10% FBS.

HL60 cells were routinely cultured in IMDM supplemented with 20% FBS in a humidified incubator maintaining 5% CO₂ at 37°C. For experimental purposes, all cells were harvested by centrifugation for 5 min at 250 x g.

Preparation of AC extract. A methanolic extract of AC was obtained from the International Biological Material Research Center (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). To produce the extracted solid (20.05 g), ground AC seeds (147 g) were treated with methanol and sonicated several times for three days. The dried extract was dissolved in 20 mg/ml DMSO to prepare a stock solution, which was then diluted with PBS.

MTT assay. In the MTT assay, MTT is metabolized into a colored formazan precipitate by mitochondrial dehydrogenases present only in viable cells, which is utilized to quantify the number of viable cells. Cells were seeded into 96-well plates (2.5x10⁴ cells/well in 200 µl medium). AC extract was added to the cells in serial concentrations (0-100 µg/ml) in quadruplets and incubated for 20 h. Adriamycin (2 µg/ml) was used as a positive reference drug. Subsequent to incubation with the drugs, 10 µl MTT solution (5 mg/ml in PBS) was added to each well, followed by incubation for 4 h. The plates were then centrifuged at 250 xg for 10 min and the medium was removed by aspiration. Finally, the formazan crystals were dissolved in 100 µl DMSO and the absorbance at 570 and 630 nm was measured using a 96-well-plate reader (VersaMax, Molecular Devices, Sunnyvale CA, USA). The inhibitory effect of the AC extract on cell growth was expressed as the percentage of viable cells, with the vehicle-treated cells considered 100% viable.

Hoechst 33342 staining. The morphology of the HL60 cells exposed to AC extract was first observed under an inverted microscope. HL60 cells were seeded into six-well plates (2.5x10⁵ cells/well in 1 ml medium). After treatment with AC extract for 24 h at 0, 5, 10 and 20 µg/ml, the cells were harvested, washed in ice-cold PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. The fixed cells were washed with PBS and stained with Hoechst 33342 solution (10 µg/ml) for 20 min at room temperature in the dark. The cells were then washed two times with PBS. Finally, they were observed under a fluorescence microscope and images were captured (Eclipse Ti-U; Nikon, Tokyo, Japan).

DNA fragmentation assay. Apoptosis was assessed by means of electrophoresis of genomic DNA extracted from HL60 cells treated with AC as described previously (20), with certain modifications. Briefly, HL60 cells (1x10⁶ cells/well in 1 ml medium) were treated with various concentrations of AC extract (0, 5, 10 and 20 µg/ml) for 24 h. Subsequently, the cells were harvested and washed in ice-cold PBS. The DNA was harvested using an Apoptotic DNA ladder kit (BioVision, San Diego, CA, USA) following the manufacturer's instructions, according to which the total DNA was analyzed using 1.5% agarose gel electrophoresis.

Annexin V/PI staining and flow cytometric analysis. Phosphatidylserine (PS) exposed on the outer mitochondrial

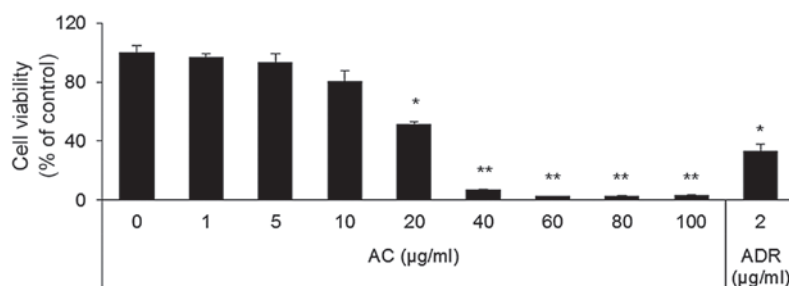


Figure 1. MTT assay showing the effect of AC extract on HL60-cell viability. HL60 cells were cultured in the absence and presence of AC extract (1-100 $\mu\text{g/ml}$) for 24 h, and the cell growth inhibition effect were determined by MTT assay. Values are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$; ** $P < 0.01$ vs. untreated cells. ADR, adriamycin; AC, *Azorella compacta*.

membrane of the apoptotic cells was determined by an Annexin V-FITC apoptosis detection kit (BD Biosciences), as per the manufacturer's instructions. Briefly, following treatment with AC extract for 24 h, cells were harvested by centrifugation (250 \times g, 5 min), washed twice with ice-cold PBS and re-suspended in binding buffer at a density of 1×10^6 cells/ml. Next, 5 μl Annexin V-FITC and 5 μl PI were added to 100 μl cell suspension, which was then incubated in the dark for 15 min. Finally, 400 μl binding buffer was added to the cell suspension. Cells were then analyzed using a flow cytometer (BD Biosciences, San Diego, CA, USA). The data were analyzed with CellQuest software (BD Biosciences).

Measurement of intracellular reactive oxygen species (ROS). First, samples of HL60 cells were treated with AC extract as described above. The production of reactive oxygen species was then measured using the membrane-permeable dye DCFH-DA. The dye was added to cells cultured in six-well plates (2.5×10^4 cells/well in 200 μl medium) at a final concentration of 5 μM , and the plates were incubated at 37°C for 1 h. The fluorescence intensity was measured using a fluorescence plate reader (Victor X3; Perkin-Elmer, Waltham, MA, USA) with excitation at 485 nm and emission at 530 nm.

Caspase-3 activity assay. The activity of caspase-3 was determined using a fluorometric method using the synthetic caspase-3 substrate Ac-DEVD-AFC. Briefly, AC extract-treated or -untreated cells were incubated for 24 h and then harvested by centrifugation. Cell pellets were re-suspended in cold lysis buffer (0.5% Triton X-100, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5) and placed on ice for 15 min. The cell lysates were then collected and incubated with caspase-3 assay buffer (10% glycerol, 2 mM dithiothreitol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5) and caspase-3 substrate DEVD-AFC for 1 h at 37°C. Active caspase-3 was measured by changes in the fluorescence at 485 and 535 nm using a microplate reader (Victor X3).

RNA isolation and reverse transcription quantitative (RT-q) PCR. Total RNA was extracted using TRIzol after the HL60 cells had been treated with AC extract (0, 1, 5, 10 or 20 $\mu\text{g/ml}$) for 24 h. Next, 2 μg RNA from each sample was used to generate cDNA using the Omniscript RT kit (Qiagen) according to the manufacturer's instructions. The cycling conditions included a

denaturation step at 95°C for 5 min, followed by 30-35 cycles of 95°C for 30 sec, 55-60°C for 30 sec and 72°C for 1 min, and a final extension step at 72°C for 10 min. The resulting total cDNA was then used to determine the expression levels of caspase-3, caspase-9, Bax, Bcl-X_L, Bcl-2 and cytochrome C. Equal amounts of PCR products were electrophoresed on 1.2% agarose gels (Bioneer Corp.) and visualized by RedSafe (iNtRON Biotechnology, Seoul, Korea) staining. Images of the gels were captured under ultraviolet light. Expression levels of GAPDH were used as internal control for the integrity of the mRNA. The primer sequences were as follows: Caspase-3 forward, 5'-ACATGGCGTGTGCATAAAATACC-3' and reverse, 5'-CACAAAGCGACTGGATGAAC-3'; caspase-9 forward, 5'-ATGGACGAAGCGGATCGGCGGCTCC-3' and reverse, 5'-GCACCACTGGGGGTAAGGTTTTCTAG-3'; Bax forward, 5'-GTGCACCAAGGTGCCGGAAC-3' and reverse, 5'-TCAGCCCATCTTCTTCCAGA-3'; Bcl-X_L forward, 5'-CCCAGAAAGGATACAGCTGG-3' and reverse, 5'-GCGATCCGACTCACCAATAC-3'; Bcl-2 forward, 5'-GTGAACTGGGGGAGGATTGT-3' and reverse, 5'-GGAGAAATCAAACAGAGGCC-3'; cytochrome C forward, 5'-CTACGGACACCTCAGGCAGT-3' and reverse, 5'-GGTGTGGTCCAAGGAAGAGA-3'; and GAPDH forward, 5'-CAAAAGGGTCATCATCTCTG-3' and reverse, 5'-CCTGCTTACCACCTTCTTG-3'.

Protein extraction and western blot analysis. Following treatment with AC extract for 24 h, HL60 cells were harvested, washed with ice-cold PBS and lysed in lysis buffer containing a protease-inhibitor-cocktail tablet (Roche Diagnostics, Basel, Switzerland). The supernatant was obtained by centrifuging at 2,000 \times g for 15 min. Total protein was extracted and protein concentration was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific). For immunoblotting, 30 μg protein from each sample was subjected to 10% SDS-PAGE and separated proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk at room temperature for 1 h and then incubated with the primary antibodies against caspase-3 (cat. no. 9661), caspase-9 (cat. no. 9505), Bcl-X_L (cat. no. 2762) (1:1,000; Cell Signaling Technology, Danvers, MA, USA), PARP (cat. no. ab194217) (1:1,000; Abcam, Cambridge, MA, USA), phosphorylated (p)-JNK (cat. no. sc-6254), p-ERK (cat. no. sc-7383), p-p38 (cat. no. sc-7973) (1:1,000; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-32233)

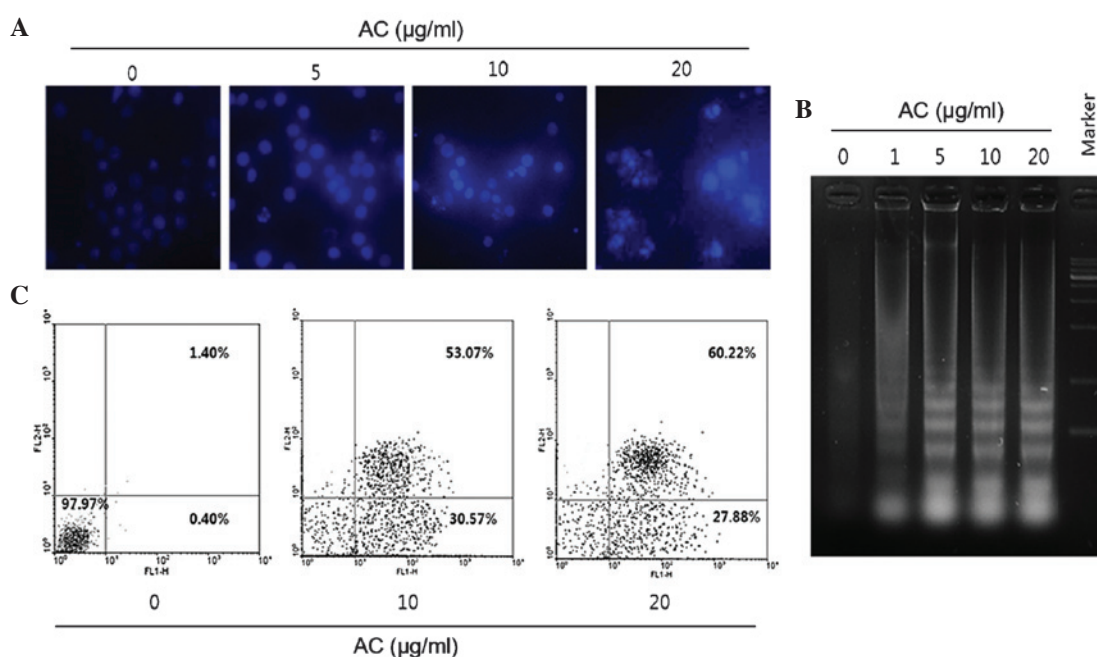


Figure 2. Induction of apoptosis by AC extract in human leukemia HL60 cells. (A) Images of Hoechst 33342-stained nuclei were captured with a fluorescent microscope using a blue filter (magnification, x400). (B) HL60 cells exposed to AC extract at various concentration were analyzed for DNA fragmentation. The genomic DNA subjected to 1.5% agarose gel electrophoresis and visualized under ultraviolet light after staining. (C) Flow cytometric analysis of AC-induced apoptosis and post-apoptotic necrosis in HL60 cells using Annexin V-fluorescein isothiocyanate/propidium iodide double staining. AC, *Azorella compacta*.

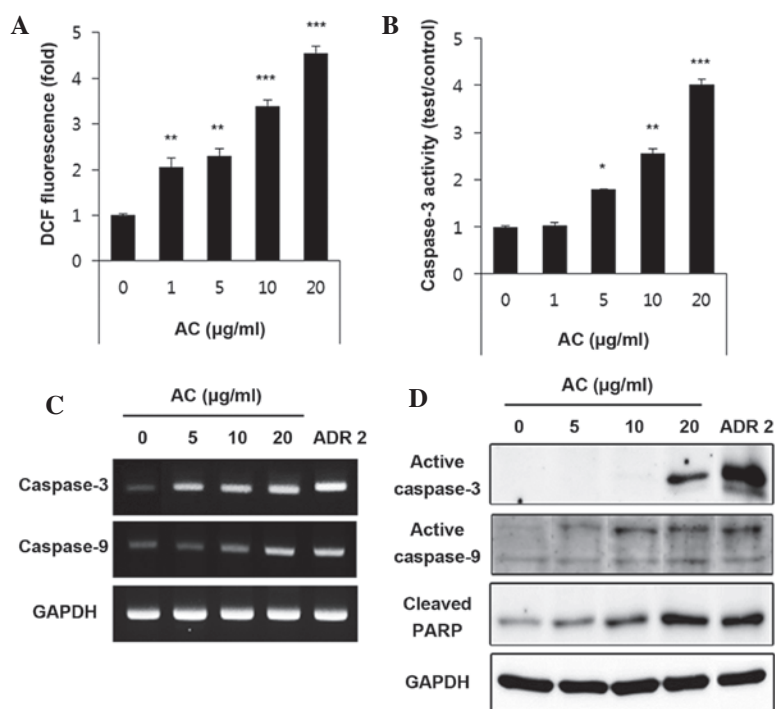


Figure 3. AC extract increases gene expression of caspase-3 and -9 in HL60 cells in a dose-dependent manner. (A) Production of ROS in HL60 cells treated with AC extract. The production of ROS was determined fluorometrically by the DCFH-DA assay. (B) Caspase-3 activity were determined using caspase-3 substrate Ac-DEVD-AFC. Values are expressed as a percentage of the vehicle-treated control \pm standard deviation of three separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control. (C) The expression of mRNA was assessed using reverse transcription quantitative polymerase chain reaction analysis. (D) Western blot analysis of active caspase-3 and -9 as well as cleaved PARP. GAPDH was used as an internal control. PARP, poly(adenosine diphosphate ribose) polymerase; ROS, reactive oxygen species; ADR, adriamycin; AC, *Azorella compacta*.

(1:2,000; Santa Cruz Biotechnology, Inc.), respectively, at 4°C overnight. After washing, the membrane was incubated with anti-rabbit (cat. no. sc-2030) or anti-mouse (cat. no. sc-2005) secondary antibody (1:2,000; Santa Cruz Biotechnology,

Inc.). GAPDH was used as an internal control to monitor equal protein loading and transfer of proteins from the gel to the membranes; for this, blots were stripped with GAPDH antibody. Signals were detected using an enhanced ECL

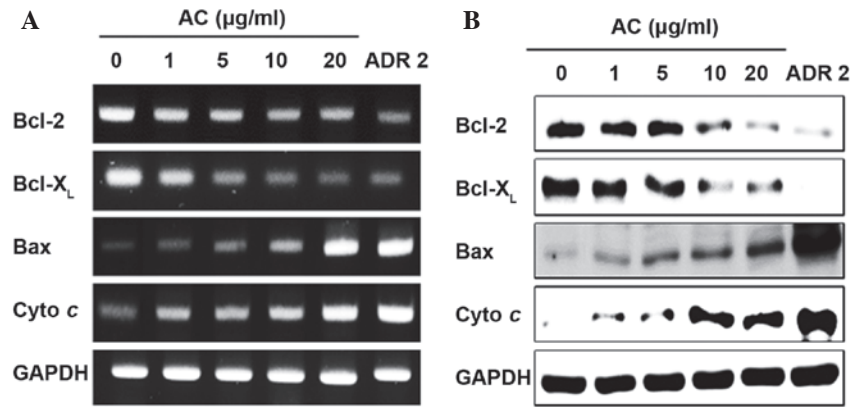


Figure 4. Expression levels of apoptosis-associated genes following AC extract treatment in human leukemia HL60 cells. Cells were seeded at 3×10^5 cells/ml and treated with the indicated concentrations of AC extract for 24 h. (A) The expression of mRNA was analyzed by reverse transcription quantitative polymerase chain reaction analysis. (B) Western blot analysis of Bcl-2, Bcl-XL, Bax and cytochrome c. GAPDH was used as an internal control. AC, *Azorella compacta*; Bcl-2, B-cell lymphoma 2; Bcl-XL, Bcl extra large protein; Bax, Bcl-2-associated X protein; Cyto c, cytochrome; ADR, adriamycin.

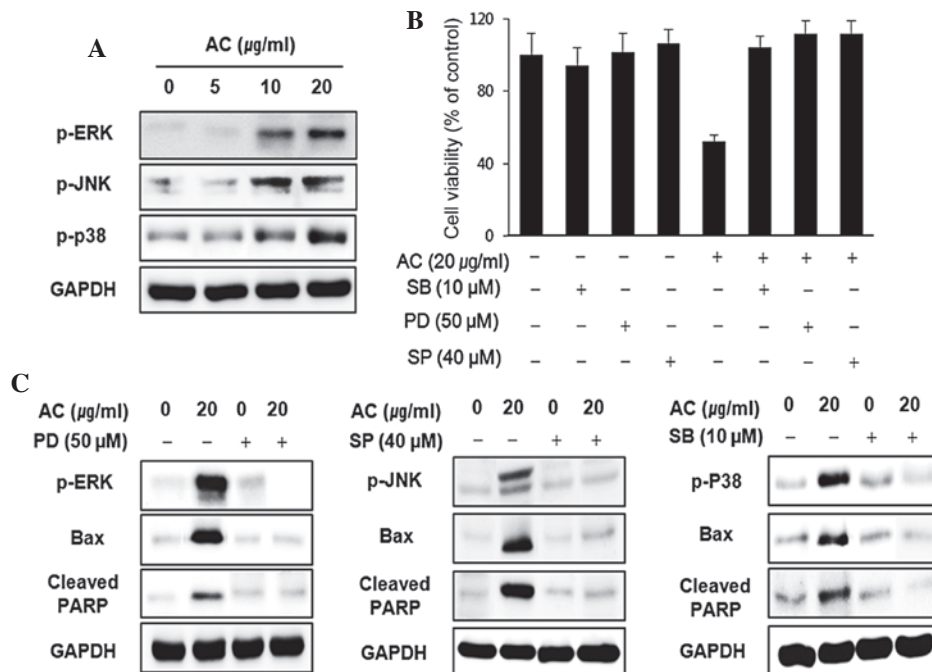


Figure 5. MAPKs were involved in AC extract-induced apoptosis of HL60 cells. (A) HL60 cells were treated with AC extract at indicated concentrations for 24 h, and the activation of MAPKs family proteins was determined by western blotting. (B) Cells pre-treated with SP600125 (40 µM), SB203580 (10 µM) or PD98059 (50 µM) for 1 h were incubated with AC extract for 24 h and cell viability was assessed using an MTT assay. (C) The effects of AC and/or SP600125, SB203580 and PD98059 on the activation of MAPK family proteins PARP and Bax were determined by western blot analysis. MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; Bax, B-cell lymphoma 2-associated X protein; PARP, poly(adenosine diphosphate ribose) polymerase; PD, ERK inhibitor PD98059; SP, JNK inhibitor SP600125; SB, p38 inhibitor SB203580; AC, *Azorella compacta*.

reagent, and an LAS 4000 imaging system (Fujifilm, Tokyo, Japan). The results shown are representative of three independent experiments.

Statistical analysis. All *in vitro* experiments were performed in triplicate, and each data point represents the average of at least three independent experiments. Values are expressed as the mean \pm standard deviation. The comparisons were made between controls and treated cultures using an unpaired Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

AC extract inhibits cell growth and induces apoptosis in human leukemic HL60 cells. To determine the effects of AC extract on the growth of cancer cells, HepG2, SNU-1, MCF7, HT1080, A549 and HL60 cells were treated with AC extract at concentrations of 1–100 µg/ml for 24 h, after which cell proliferation was assessed by MTT assay. AC extract inhibited the growth of HL60 cells in a dose-dependent manner (Fig. 1). At concentrations of 40 µg/ml and higher, AC extract almost completely inhibited the growth of HL60 cells. The IC_{50} of AC

extract was determined to be $34 \pm 4.06 \mu\text{g/ml}$ on HL60 cells. Next, the present study investigated whether the observed inhibitory effects of AC extract on cell viability resulted from apoptotic cell death. Apoptosis is a process of programmed cell death, which is characterized by various biochemical and morphological changes. To evaluate the effects of AC extract on nuclear morphology, Hoechst 33342 staining was performed. The nuclei of cells treated with AC extract at 5, 10 and $20 \mu\text{g/ml}$ were deeply stained and exhibited bright fluorescence, which indicated the condensation of chromatin (Fig. 2A). In addition, as shown in Fig. 2B, DNA ladder formation was confirmed by agarose gel electrophoresis. Efficient DNA laddering was observed in HL60 cells treated with $>10 \mu\text{g/ml}$ AC extract for 24 h. With increasing concentration, a more intense pattern of DNA laddering was detected (Fig. 2B). The apoptosis-inducing effects of the AC extract were also evaluated using Annexin V/PI staining. As shown in Fig. 2C, the apoptotic rate increased in an AC concentration-dependent manner. The early and late apoptotic fractions in control cells were 0.40 and 1.40%, respectively, but increased to 23.80 and 58.82% after treatment with $20 \mu\text{g/ml}$ AC extract (Fig. 2C). PI-positive cells also increased marginally at higher AC concentrations.

AC extract induces ROS production and activation of caspases in human leukemic HL60 cells. ROS levels in AC-extract-treated HL60 cells were quantified 1 h after the addition of DCFH-DA. As displayed in Fig. 3A, intracellular ROS levels significantly increased in an AC concentration-dependent manner. These results suggested that ROS production may be the cause of AC extract-induced apoptosis in HL60 cells. As AC-extract treatment led to enhanced ROS generation, it is possible that alterations in the cell may have a role in AC extract-induced apoptosis. Caspase-3 and -9, known to serve as important mediators of intrinsic apoptotic pathways, also contribute to general apoptotic morphology through the cleavage of various cellular substrates, including PARP (21). As indicated in Fig. 3B, western blot analysis showed that AC extract induced the activation of caspase-3 in a concentration-dependent manner. RT-PCR was used to detect the mRNA expression of caspase-3 and caspase-9 24 h after AC-extract treatment. The change in mRNA expression was normalized against GAPDH expression. Fig. 3C shows that the mRNA expression of caspase-3 and caspase-9 increased in a manner that was AC-extract dose-dependent. Furthermore, the western blots in Fig. 3D showed that AC-extract treatment induced the activation of caspase-3 and -9 in a concentration-dependent manner. In addition, western blot analysis revealed that progressive proteolytic cleavage products of PARP protein, a downstream target of activated caspase-3, occurred in HL60 cells treated with AC extract.

AC extract activates apoptosis-associated signaling in human leukemia HL60 cells. The underlying mechanism by which AC extract induced apoptosis of HL60 cells was delineated by RT-PCR and western blot analyses. As shown Fig. 4A and B, examination of Bax, Bcl-2, Bcl-X_L and cytochrome C expression during apoptosis indicated that AC treatment at 1, 5, 10, or $20 \mu\text{g/ml}$ dose-dependently increased the expression of cytochrome C and pro-apoptotic Bax, whereas the expression

of anti-apoptotic Bcl-2 and Bcl-X_L was downregulated with increasing concentrations of AC extract.

Activation of MAPK is involved in AC-extract-induced apoptosis in human leukemia HL60 cells. Next, the effect of AC-extract treatment on the expression and activities of MAPKs was investigated in order to determine whether these signaling pathways have a role in mediating the observed apoptotic response. To confirm an association between the activation of MAPKs and the induction of apoptosis by the AC extract, the cells were pre-treated with MAPK inhibitors and their viability was assessed using an MTT assay. As shown in Fig. 5A, the levels of p-p38, p-ERK and p-JNK proteins increased in an AC concentration-dependent manner. As Fig. 5B demonstrates, pre-treatment with SB203580 (inhibitor of p38), PD98059 (inhibitor of ERK) and SP600125 (inhibitor of JNK) increased the viability of cells treated with AC extract. To further determine the mechanism of MAPKs activation in HL60 cells by AC extract, the effects of these MAPK inhibitors on the release of Bax and cleaved PARP proteins were also investigated (Fig. 5C). The results showed that MAPK inhibitor blocked pro-apoptotic protein and cleaved PARP protein. These results indicated that AC extract induced apoptosis via these two proteins of the intrinsic apoptotic pathway.

Discussion

The ability to induce tumor-cell apoptosis is an important property of candidate anti-cancer drugs and serves to discriminate between anti-cancer drugs and compounds with toxicity. Much effort has been directed toward identifying compounds that influence apoptosis and toward understanding their mechanisms of action. Extracts prepared from a large variety of plants have been demonstrated to possess the ability to trigger the activation of apoptotic pathways (22,23). The mechanisms of apoptosis induction are complex and not fully known, but certain key events have been identified, which appear essential for the cell to enter apoptosis (24). The notion that apoptosis represents a critical element in control of cell-number in physiological and pathological situations has been well reviewed and its role in oncogenesis is now well established (25). Apoptosis has an important function in the normal development and differentiation of multicellular organisms and is characterized by morphological and biological changes, including chromatin condensation, cytoplasmic shrinkage and DNA degradation (26). Apoptosis also serves as a critical protective mechanism against carcinogenesis caused by mutations of the genetic material in normal cells and against various other forms of carcinogenesis. A variety of stimuli can trigger apoptosis, including death receptor-mediated signaling (extrinsic pathway) or intracellular stresses (intrinsic pathway) (27). The present study aimed to determine the capacity of AC extract to induce apoptosis and to identify the associated biochemical mechanisms in human leukemic cells. The results demonstrated that AC extract inhibits leukemic cell growth by induction of apoptotic cell death, which appeared to account for its anti-proliferative action. Depending on the cell line, AC extract exerted growth-inhibitory effects on cancer cells with IC₅₀-values of 20-100 $\mu\text{g/ml}$ after 24 h treatment. In HL60 cells, a promising level of cytotoxicity was observed;

therefore, the present study pursued the effect of AC extract on human leukemia HL60 cells. It was demonstrated that AC extract exerted a significant anti-proliferative effect against HL60 cells in a dose-dependent manner. Further cellular and biochemical analysis indicated that the proliferation of inhibitory activity of AC extract was associated with the induction of apoptosis. Several sensitive methods for detecting apoptosis have been developed. Staining of apoptotic cells with the fluorescent dye Hoechst 33342 is considered to be a suitable method for evaluating changed nuclear morphology (28). One of the earliest events indicating apoptosis is the loss of plasma membrane polarity, accompanied by translocation of PS from the inner to outer membrane leaflets, thereby exposing PS to the external environment. The phospholipid-binding protein Annexin V has a high affinity for PS and can bind to apoptotic cells with an inverted mitochondrial membrane; the quantity of fluorescently-labeled Annexin-V correlates with the loss of membrane polarity during apoptosis. The perforation and inversion of the mitochondrial membrane precedes the complete loss of membrane integrity that accompanies later stages of cell death, resulting from either apoptosis or necrosis. By contrast, PI only enters cells after loss of membrane integrity. Thus, dual staining with Annexin V and PI allows for a clear discrimination between affected cells, early apoptotic cells and late apoptotic or necrotic cells (29). HL60 cells treated with AC extract at lower concentrations contained a population of Annexin V-positive cells indicating early apoptosis, while Annexin V- and PI-positive cells were present at higher AC concentrations, which revealed the occurrence of post-apoptotic necrosis. The impairment of mitochondrial function has been considered to be a key event in the ROS-mediated apoptotic pathway (30). In the present study ROS generation was indicated to be involved in AC extract-induced cell death. ROS levels were determined in HL60 cells after AC-extract treatment using the peroxide-sensitive fluorescent probe, DCFH-DA, and a four-fold increase was evidenced after 24 h. In general, the mitochondria-mediated intrinsic pathway and the death-receptor-triggered extrinsic pathway can lead to caspase-3 activation (31). In the system of the present study, caspase-9 was significantly activated, which implicated mitochondrial involvement, since caspase-9 is the initiator caspase for the intrinsic apoptotic pathway (32). In the intrinsic pathway, the ratio of the expression of pro-apoptotic proteins such as Bax and anti-apoptotic proteins, including Bcl-2 and Bcl-X_L, ultimately determines cell death or survival through regulation of mitochondrial-permeability transition. This leads to activation of caspase-3 for induction of apoptosis via release of cytochrome C to the cytosol (11). The results of the present study indicated that AC extract induced Bax translocation from the cytosol to the mitochondria, leading to the release of cytochrome C, apoptosome formation, and finally, induction of apoptosis in HL60 cells.

MAPKs, including ERK, JNK and p38, have critical roles in cell survival and apoptosis in various types of cancer cell. It is known that activation of ERK, JNK and p38 leads to induction of apoptosis (33,34). Activation of ERK, JNK and p38 can induce mitochondrial dysfunction with subsequent release of apoptotic proteins, such as cytochrome C, from the mitochondria into the cytosol, and finally activate caspase-9 and caspase-3. In the present study, treatment with

AC extract resulted in upregulation of ERK, JNK and p38 phosphorylation. Therefore, the involvement of ERK, JNK, and p38 activation in the MAPK-signaling pathway during AC-extract-induced apoptosis in HL60 cells was further investigated. As the results indicated, PD98059 (inhibitor of ERK), SP600125 (inhibitor of JNK) and SB203580 (inhibitor of p38) blocked AC-extract-induced apoptosis of HL60 cells by inhibiting the interaction between Bax and activated PARP. The results therefore suggested an association of AC-extract-induced apoptosis with activation of ERK, JNK and p38/MAPK.

In conclusion, the present study demonstrated that AC extract significantly induced apoptosis in leukemia cells by increasing the generation of ROS, by causing translocation of Bax to the mitochondria from the cytosol, and by initiating the release of cytochrome C followed by activation of caspase-9 and caspase-3. AC extract was found to exert its anti-cancer effects via the ERK, JNK and p38/MAPK-mediated intrinsic apoptotic pathway in human leukemia HL60 cells. Future studies will examine the effects of AC on upstream signaling pathways of MAPKs and evaluate its anti-cancer efficacy *in vivo* using nude mouse models. As the present study did not observe any side effects, AC is expected to be a promising anti-cancer drug. The potential use of AC in combination with other drugs may also be investigated.

Acknowledgements

This study was supported by the Ministry of Science, ICT and Future Planning (no. FGC1011433) and the KRIBB Initiative Program (no. KGM1221521) of the Republic of Korea. The authors would like to thank the International Biological Material Research Center (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) for providing the AC extract.

References

1. Danial NN and Korsmeyer SJ: Cell death: critical control points. *Cell* 116: 205-219, 2004.
2. Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251-306, 1980.
3. Earnshaw WC: Nuclear changes in apoptosis. *Curr Opin Cell Biol* 7: 337-343, 1995.
4. Nagata S: Apoptosis by death factor. *Cell* 88: 355-365, 1997.
5. Green DR and Reed JC: Mitochondria and apoptosis. *Science* 281: 1309-1312, 1998.
6. Kaufmann SH and Earnshaw WC: Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 256: 42-49, 2000.
7. Jin Z and El-Deiry WS: Overview of cell death signaling pathways. *Cancer Biol Ther* 4: 139-163, 2005.
8. Kroemer G and Reed JC: Mitochondrial control of cell death. *Nat Med* 6: 513-519, 2000.
9. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC: Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346-347, 1994.
10. Adams JM and Cory S: The Bcl-2 protein family: arbiters of cell survival. *Science* 281: 1322-1326, 1998.
11. Li H, Zhu H, Xu CJ and Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491-501, 1998.
12. Kyosseva SV: Mitogen-activated protein kinase signaling. *Int Rev Neurobiol* 59: 201-220, 2004.
13. Keshet Y and Seger R: The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol Biol* 661: 3-38, 2010.

14. Sumbayev VV and Yasinska IM: Regulation of MAP kinase-dependent apoptotic pathway: implication of reactive oxygen and nitrogen species. *Arch Biochem Biophys* 436: 406-412, 2005.
15. Loyola LA, Bórquez J, Morales G, San-Martín A, Darias J, Flores N and Giménez A: Mulinane-type diterpenoids from *Azorella compacta* display antiparasmodial activity. *Phytochemistry* 65: 1931-1935, 2004.
16. Wachter GA, Franzblau SG, Montenegro G, Suarez E, Fortunato RH, Saavedra E and Timmermann BN: A new antitubercular mulinane diterpenoid from *Azorella madreporica* Clos. *J Nat Prod* 61: 965-968, 1998.
17. Areche C, Rojas-Alvarez F, Campos-Briones C, Lima C, Pérez EG and Sepúlveda B: Further mulinane diterpenoids from *Azorella compacta*. *J Pharm Pharmacol* 65: 1231-1238, 2013.
18. Wachter GA, Matooq G, Hoffmann JJ, Maiese WM, Singh MP, Montenegro G and Timmermann BN: Antibacterial diterpenoid acids from *Azorella compacta*. *J Nat Prod* 62: 1319-1321, 1999.
19. Fuentes NL, Sagua H, Morales G, Bórquez J, San Martín A, Soto J and Loyola LA: Experimental antihyperglycemic effect of diterpenoids of llaleta *Azorella compacta* (Umbelliferae) Phil in rats. *Phytother Res* 19: 713-716, 2005.
20. Lee K, Kwon OK, Xia Y and Ahn KS: Effect of AC-264, a novel indole derivative, on apoptosis in HL-60 cells. *Bull Korean Chem Soc* 31: 3777-3781, 2010.
21. Pink JJ, Wuerzberger-Davies S, Tagliarino C, Planchon SM, Yang X, Froelich CJ and Boothman DA: Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during beta-lapachone-mediated apoptosis. *Exp Cell Res* 255: 144-155, 2000.
22. Ren G, Zhao YP, Yang L and Fu CX: Anti-proliferative effect of clitocine from the mushroom *Leucopaxillus giganteus* on human cervical cancer HeLa cells by inducing apoptosis. *Cancer Lett* 262: 190-200, 2008.
23. Wu SJ, Ng LT, Chen CH, Lin DL, Wang SS and Lin CC: Antihepatoma activity of *Physalis angulata* and *P. peruviana* extracts and their effects on apoptosis in human Hep G2 cells. *Life Sci* 74: 2061-2073, 2004.
24. Andersson B, Janson V, Behnam-Motlagh P, Henriksson R and Grankvist K: Induction of apoptosis by intracellular potassium ion depletion: Using the fluorescent dye PBFI in a 96-well plate method in cultured lung cancer cells. *Toxicol In vitro* 20: 986-994, 2006.
25. Hall PA: Assessing apoptosis: A critical survey. *Endocr Relat Cancer* 6: 3-8, 1999.
26. Hengartner MO: The biochemistry of apoptosis. *Nature* 407: 770-776, 2000.
27. Galluzzi L, Larochette N, Zamzami N and Kroemer G: Mitochondria as therapeutic targets for cancer chemotherapy. *Oncogene* 25: 4812-4830, 2006.
28. Belloc F, Dumain P, Boisseau MR, Jalloustre C, *et al*: A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry* 17: 59-65, 1994.
29. Yang L, Wu S, Zhang Q, Liu F and Wu P: 23,24-Dihydrocucurbitacin B induces G2/M cell-cycle arrest and mitochondria-dependent apoptosis in human breast cancer cells (Bcap37). *Cancer Lett* 256: 267-278, 2007.
30. Wang R, Li C, Song D, Zhao G, Zhao L and Jing Y: Ethacrynic acid butyl-ester induces apoptosis in leukemia cells through a hydrogen peroxide mediated pathway independent of glutathione S-transferase P1-1 inhibition. *Cancer Res* 67: 7856-7864, 2007.
31. Cullen SP and Martin SJ: Caspase activation pathways: Some recent progress. *Cell Death Differ* 16: 935-938, 2009.
32. Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson DW, Lazebnik Y and Flavell RA: Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. *Nat Med* 6: 1241-1247, 2000.
33. Moon DO, Kim MO, Choi YH, Kim ND, Chang JH and Kim GY: Bcl-2 overexpression attenuates SP600125-induced apoptosis in human leukemia U937 cells. *Cancer Lett* 264: 316-325, 2008.
34. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M and Lord JM: Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 256: 34-41, 2000.