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Alpinia pricei rhizome extracts induce apoptosis of human carcinoma KB cells via a mitochondria-dependent apoptotic pathway

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ABSTRACT

Alpinia pricei Hayata (*A. pricei*) is well known in Taiwan as a traditional Chinese medicine. In this study, the ability of ethanol (70%) extracts of *A. pricei* rhizome (AP extracts) to induce apoptosis in cultured human carcinoma KB cells was investigated. Treatment of KB cells with various concentrations of AP extracts (25–200 μg/mL) resulted in sequences of events marked by apoptosis, such as loss of cell viability, morphology change, and internucleosomal DNA fragmentation. AP extract-induced apoptotic cell-death was associated with loss of mitochondrial membrane potential, cytochrome *c* translocation, caspase-3 and -9 activation, and poly ADP-ribose polymerase (PARP) degradation in KB cells. This increase in AP extract-induced apoptosis was also associated with a reduction in the levels of Bcl-2, a potent cell-death inhibitor, and an increase in levels of the Bax protein, which heterodimerizes with and thereby inhibits Bcl-2. Furthermore, AP extracts induced a dose-dependent elevation of reactive oxygen species (ROS) in KB cells. Our findings suggest that *A. pricei* exerts antiproliferative action and growth inhibition on human carcinoma KB cells through a mitochondria-dependent apoptotic pathway. *A. pricei* may, therefore, have anticancer properties valuable for application in food and drug products.

1. Introduction

Alpinia pricei (*A. pricei*) Hayata is a perennial rhizomatous herb indigenous to Taiwan. The rhizomes of the Zingiberaceae family are a vegetable widely used in many Asian countries, and their medicinal functions have been broadly discussed and accepted in many traditional recipes. It has been reported that *Alpinia* plants (family Zingiberaceae) possess antioxidant (Shi et al., 2006), anti-inflammatory (Israf et al., 2007), anticancer (Ali et al., 2001; Lee and Houghton, 2005), immunostimulating (Bendjeddou et al., 2003), hepatoprotective (Kadota et al., 2003) and antinociceptive (Arambewela et al., 2004) activities. Further, the rhizomes of *Alpinia* species (*galanga*) are used to treat problems associated with the digestive system, and to relieve symptoms of bronchitis, measles, rubella and cholera (Brown, 1995). Traditional medicine in Algeria as for centuries used the roots of *Alpinia* species for the treatment of respiratory infections (Bendjeddou et al., 2003). *A. pricei* is used in traditional Chinese medicine to treat abdominal distension and increase stomach secretion and peristalsis (Chen et al., 2008; Cheng et al., 2008), however, very few biological activity tests are reported.

Chemoprevention, which refers to the administration of naturally occurring agents to prevent initiation and promotion events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasms (Surh, 2003; Sporn and Suh, 2000). Increasing evidence suggests that the related processes of neoplastic transformation, progression and metastasis involve alteration of the normal apoptotic pathways (Bold et al., 1997). Morphological hallmarks of this process includes loss of cell volume, hyperactivity of the plasma membrane, and condensation of peripheral heterochromatin, followed by cleavage of the nucleus and cytoplasm into multiple membrane-enclosed bodies containing chromatin fragments (Wyllie et al., 1980; Wyllie, 1987). Apoptosis, a phenomenon associated with many physiological and pathological processes including cancer, has gained recognition as an ideal way to eliminate precancerous and cancer cells (Kiechle and Zhang, 2002; Nicholson, 2000). Many studies have shown associations between apoptosis and cancer, in as much as the apoptosis-inducing agents are being appreciated as weapons for the management of cancer (Stewart, 2003; Schmitt, 2003).

Cervical cancer, a slow growing squamous cell carcinoma, is a common disease in women. Carcinoma of the uterine cervix is one of the highest causes of mortality in female cancer patients worldwide, and due to non-existent or inadequate screening,

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disease is normally detected at late stage. Chemical as well as biological agents that induce apoptosis have been reported to be promising interventions in the management of malignant cancer. In this study, the anticancer effects of ethanol (70%) extracts of *A. pricei* (AP extracts) (25–200 μg/mL) on cultured human cervical carcinoma KB cells were investigated. Human carcinoma KB cells represent a subclone of human HeLa cells (Masters, 2002). In addition, the biochemical steps linking *A. pricei* to the apoptotic process in these cells are investigated.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), DMEM, penicillin–streptomycin (PS), glutamine (GIBCO Laboratories, Grand Island, NY), rabbit polyclonal antibody against cytochrome *c*, caspase-3, -8, and -9, Bcl-2, and Bax (Santa Cruz Biotechnology Inc., Heidelberg, Germany), PARP rabbit polyclonal antibody (Upstate biotechnology, Lake Placid, NY), and mouse monoclonal antibody against actin (Sigma Chemical Co., St. Louis, MO) were obtained from various suppliers. All other chemicals were of the highest grade commercially available and supplied either by Merck or Sigma.

2.2. Preparation of AP extracts

The roots of *A. pricei* were colleted in March, 2007 in Ping-tung County, an administrative region located in southern Taiwan, and were identified by Dr. Yen-Hsueh Tseng (National Chung-Hsin University). The voucher specimen was deposited in the herbarium of the same university. The air-dried roots (2 kg) of *A. pricei* were extracted with 10 L 70% ethanol (EtOH) at room temperature. The crude extracts (166 g) were concentrated in a vacuum and freeze dried to form a powder. The stock was then stored at −20 °C for subsequent analysis of its anticancer properties. The yield of AP extracts was 8.3%. For preparation of the stock solution, the powder samples were solubilized in 70% EtOH at 25 °C. The stock solution was stored at −20 °C before analysis for apoptotic properties.

2.3. Cell culture and assessment of cell growth and viability

The human carcinoma cell line, KB (HeLa derivative), and the human gingival fibroblast cell line, HGF, were obtained from the American type Culture Collection (Rockville, MD). KB cell line was used by the NCI for some of the earliest in vitro anticancer drug-screening work, and has been thought to be derived from an epidermoid carcinoma of the oral cavity (Shoemarker, 2006). KB cells were once thought to be derived from an oral cancer, but in fact they were derived from a glandular cancer of cervix (Masters, 2002). These cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1% penicillin–streptomycin–neomycin in a humidified incubator (5% CO2 in air at 37 °C). Cells were seeded in 6- or 12-well plates prior to the addition of AP extracts. The KB and HGF cells were incubated with AP extracts (0–200 g/mL for KB cells and HGF cells) for 24, 48 and 72 h. Cultures were harvested and monitored for cell number by counting cell suspensions with a hemocytometer. Cell viability (3.0×10^5 cells/12-well) and growth (1.0×10^5 cells/6-well) were checked before and after treatment with AP extracts using trypan blue exclusion and examined using phase contrast microscopy.

2.4. TUNEL assay for DNA apoptotic fragmentation

DNA fragmentation was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) with the KLENOW FrgEL DNA fragmentation detection kit (Calbiochem, San Diego, Calif) (Gavrieli et al., 1992). Briefly, apoptotic KB cells (5×10^5 cells/6-well) were harvested, fixed with 4% formaldehyde and applied to glass slides. Fixed cells were permeabilised with 20 g/mL protease K in TBS and endogenous peroxidase was inactivated by 3% H_2O_2 in methanol. Apoptosis was detected by labeling the 3′-OH ends of fragmented DNA with biotin-dNTP using klenow at 37 °C for 1.5 h. The slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3,3′-diaminobenzidine and H_2O_2. Apoptotic cells were identified by their dark brown nuclei seen under a light microscope.

2.5. Flow cytometric analysis

Cellular DNA content was determined by flow cytometric analysis of PI-labeled cells (Hseu et al., 2007). KB cells were grown to exponential phase, seeded at a density of 1×10^6 cells/60-mm dish, and treated with the indicated concentrations of AP extracts (25–200 g/mL) for 24, 48, and 72 h. After treatment, the cells were collected by trypsinization, and fixed in ice-cold 70% ethanol at −20 °C overnight. The cells were re-suspended in PBS containing 1% Triton X-100, 0.5 mg/mL RNase, and 4 g/mL PI at 37 °C for 30 min. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm) was used for flow cytometric analysis. Forward and right-angle light scatter, which correlate with the size of the cell and the cytoplasmic complexity, respectively, were used to establish size gates and exclude cellular debris from the analysis. DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system. The cell cycle was determined and analyzed using ModFit software (Verity Software House, Topsham, ME). Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence.

2.6. Preparation of total cell extract and immunoblot analysis

KB cells (3.0×10^6 cells/100 mm dish) were detached, washed once in cold PBS, and then suspended in 100 μL lysis buffer (10 mM Tris–HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfonyl fluoride). The suspension was put on ice for 20 min and then centrifuged at 13,000 rpm for 20 min at 4 °C. Total protein content was determined using Bio-Rad protein assay reagent, with bovine serum albumin as the standard. Protein extracts were reconstituted in sample buffer (0.062 M Tris–HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated on 8–15% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes overnight. Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were reacted with primary antibodies for 2 h. They were then incubated with either a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h before being developed using the SuperSignal ULTRA chemiluminescence substrate (Pierce, Rockford, IL). Band intensities were quantified by densitometry with absorbance of the mixture at 540 nm determined using an ELISA plate

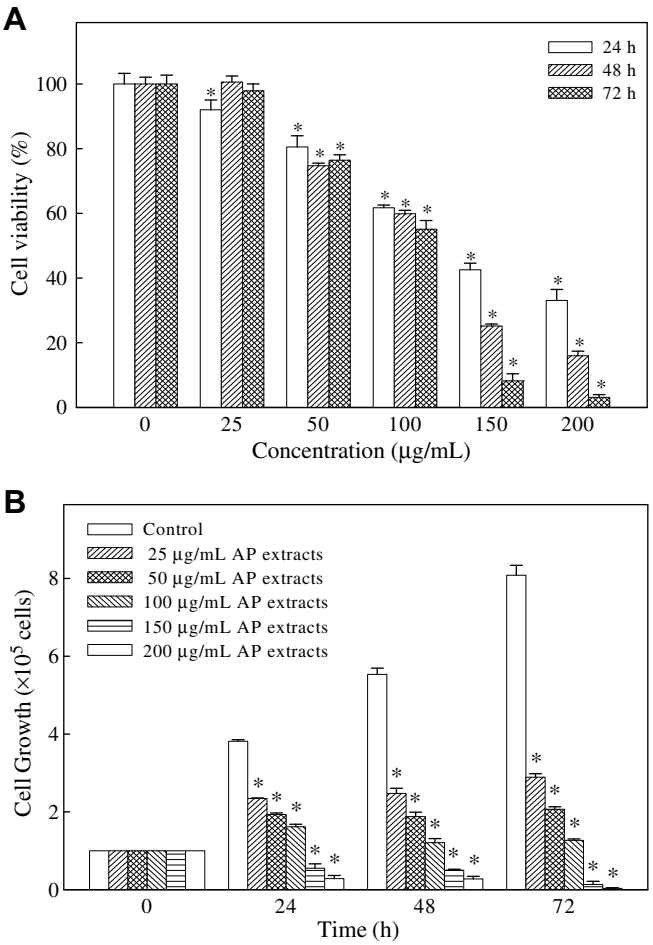

Fig. 1. Effects of AP extracts on KB cell viability (A) and growth (B). Cells were treated with 0, 25, 50, 100, 150, or 200 μg/mL of AP extracts for 24, 48, or 72 h. Control cells were maintained in the vehicle for the indicated time periods. Results are presented as meanSD of three assays. * indicates significant difference in comparison to control group ($p < 0.05$).

reader. Western blot analysis, with antibodies against cytochrome *c*, caspase-3, -8, and -9, PARP, Bcl-2, and Bax was performed as described previously (Yang et al., 2006).

2.7. Analysis of mitochondrial membrane potential

The loss of mitochondrial membrane potential was assessed by flow cytometry. Cells (5×10^5 cells/6-well) were harvested and washed twice, re-suspended in 500 μL of DiOC6 (20 μM) and incubated at 37 °C for 30 min. The excitation wavelength was 488 nm, with monitoring at 530 nm (DiOC6). Cell percentage was calculated using ModFit software.

2.8. Measurement of ROS generation by flow cytometry

Intracellular ROS production was detected by flow cytometry using 2′,7′-dihydrofluorescein-diacetate (DCFH-DA) (Yang et al., 2006). Cells (5×10^5 cells/6-well) were cultured, with renewal of the culture medium when the cells reached 80% confluence. Cells were then incubated with 10 μM DCFH-DA in culture medium at 37 °C for 30 min before the end of each experiment. The intracellular ROS, as indicated by dichlorofluorescin (DCF) fluorescence, was measured with a FACSCalibur flow cytometer.

2.9. Statistics

Mean data values are presented with their standard deviation (mean ± SD). All data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $p < 0.05$ for all tests.

3. Results

In this study, KB, a human carcinoma cell line, was used to investigate the capability of the 70% ethanol extracts of *A. pricei* (AP extracts) (25–200 μg/mL) to induce apoptosis, and to elaborate the molecular mechanism(s) involved.

3.1. Effect of AP extracts on cell viability and growth of KB cells

To investigate the potential effects of AP extracts on proliferation and survival of KB cells, the cells were exposed to 0–200 g/mL of AP extracts for 24, 48 and 72 h. Fig. 1A and B shows that AP extracts induced cell-death in a dose- and time-dependent manner, as determined using trypan blue exclusion. The concentrations of AP extracts required for 50% inhibition of KB cell growth (IC50) were approximately 119, 104, and 95 g/mL for 24, 48 and 72 h, respectively.

3.2. Induction of apoptosis by AP extracts

It was also demonstrated that, after 24 h of incubation, the majority of KB cells treated with AP extracts (at 0, 50, 100, and 200 g/mL) contained condensed nuclei. Fig. 2 is an image of a characteristic population of AP extract-treated KB cells using TUNEL assay for DNA apoptotic fragmentation. Apoptotic cells were identified by their dark nuclei under a light microscope.

3.3. Effect of AP extracts on sub-G1 in KB cells

The profile of the DNA content of the AP extract-treated KB cells was obtained using flow cytometric analysis to measure the fluorescence of PI-DNA binding. Cells with less DNA staining relative to diploid analogs were considered apoptotic. It was noted that there was a remarkable accumulation of subploid cells, the so-called sub-G1 peak, in AP extract-treated KB cells (0–200 μg/mL for 24, 48, or 72 h) when compared with the untreated group (Table 1). Our findings suggest that *A. pricei* promotes cell growth inhibition by inducing apoptosis in cancer cells.

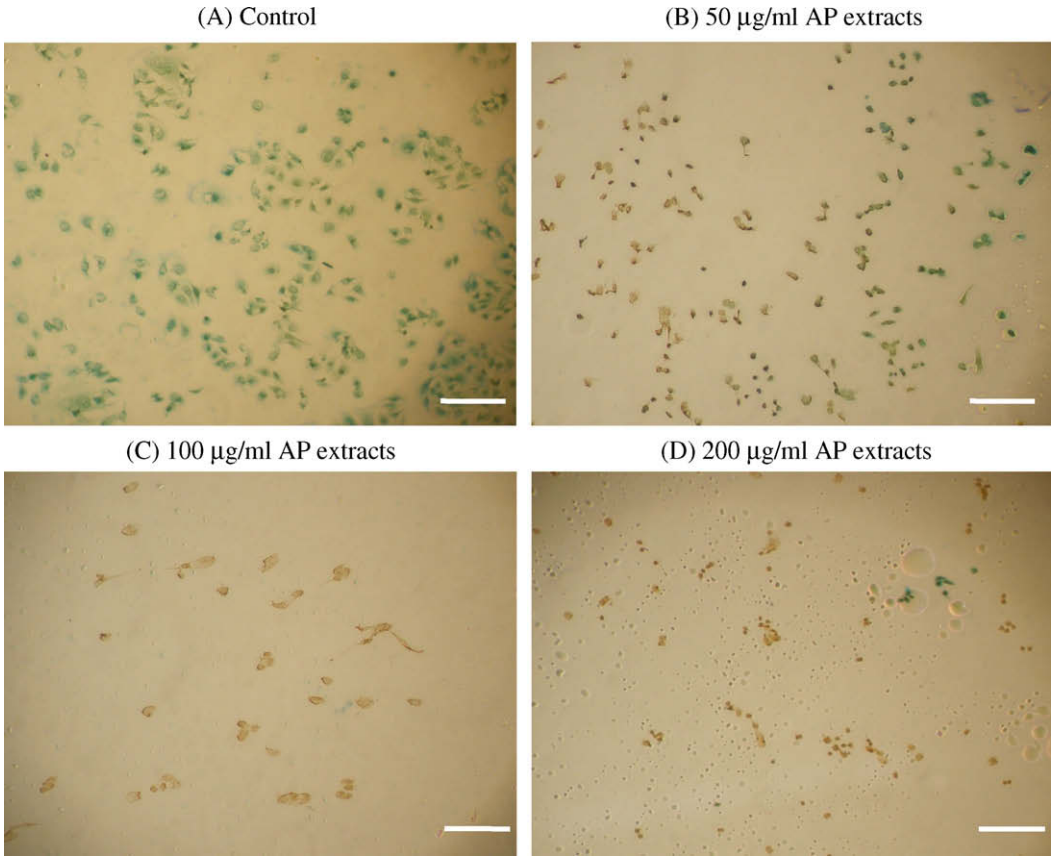

Fig. 2. TUNEL assay of KB cells exposed to AP extracts. KB cells treated with 0, 50, 100, or 200 μg/mL of AP extracts for 24 h were examined under a microscope. A typical result from three independent experiments is shown. Bar represents 25 μm.

Table 1
Effect of AP extracts on sub-G1 in KB cells

μg/mL	Sub-G1 population (%)		
	24 h	48 h	72 h
0	0.3 ± 0.1	0.4 ± 0.1	1.0 ± 0.6
50	0.3 ± 0.1	1.0 ± 0.8	0.7 ± 0.1
100	1.4 ± 0.6*	2.1 ± 0.9*	19.0 ± 1.6*
150	5.0 ± 0.6*	8.5 ± 0.7*	31.8 ± 1.8*
200	5.4 ± 0.2*	14.0 ± 1.5*	41.2 ± 5.4*

Cells were treated with 0, 50, 100, 150, or 200 g/mL of AP extracts for 24, 48, or 72 h, stained with PI, and analyzed for sub-G1 using flow cytometry. Apoptotic nuclei were identified as a subploid DNA peak and distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Cellular distribution (as percentage) in sub-G1 after AP extracts treatment is shown. Results are presented as meanSD of three assays.
* Indicates significant difference in comparison to control group ($p < 0.05$).

3.4. Effect of AP extracts on mitochondrial membrane potential in KB cells

To determine whether early loss of mitochondrial membrane potential occurred during AP extract treatment, the KB cells were grown in the absence (control) or the presence of AP extracts (150 μg/mL) for 0, 2, 4, or 8 h, and stained with DiOC6. Mitochondrial membrane potential was then analyzed by flow cytometry. Fig. 3A and B shows that AP extract treatment resulted in the loss of mitochondrial membrane potential in KB cells, indicating that *A. pricei* can induce mitochondrial dysfunctions in KB cells.

3.5. Effect of AP extracts on cytochrome c release, caspase-3, -8, and -9 activity, and PARP cleavage

Studies indicate that treatment of cells with a variety of chemotherapeutic agents is accompanied by increased cytosolic translocation of cytochrome *c*, activation of caspase-3, and degradation of PARP (Green and Reed, 1998). In the present study, the cytosol levels of cytochrome c were examined using Western blot analysis. Our results revealed that AP extracts induced the release of cytosolic cytochrome *c* in a dose- and time-dependent manner (Fig. 4A and B). As cytochrome *c* is reportedly involved in the activation of the caspases that trigger apoptosis, we investigated the role of caspase-3 and -9 in the cellular response to AP extracts. Immunoblotting analysis revealed that AP extracts induced proteolytic cleavage of pro-caspase-3 and pro-caspase-9 into the active form in treated KB cells (Fig. 4A and B). There was no effect on the caspase-8 protein, however (Fig. 4A and B). Since PARP-specific proteolytic cleavage by caspase-3 is considered to be a biochemical characteristic of apoptosis, a Western blotting experiment was conducted using the antibody against PARP. PARP is a nuclear enzyme involved in DNA repair, and it has been demonstrated that the 115 kDa PARP proteins is cleaved into a 85 kDa fragment (Tewari et al., 1995). Fig. 4A and B shows that PARP was cleaved into a 85 kDa fragment after the addition of AP extracts.

3.6. Effect of AP extracts on Bcl-2 and Bax protein

As shown in Fig. 5A and B, incubation of KB cells with AP extracts dramatically reduced the level of Bcl-2, a potent cell-death inhibitor, and increased the level of Bax protein in a dose and time-dependent manner (Fig. 5A and B). These results indicate that AP extracts induced dysregulation of Bcl-2 and Bax in KB cells.

3.7. ROS generation in AP extracts-treated KB cells

Flow cytometric analysis using DCFH-DA as a fluorescence probe can be used for estimating the generation of ROS (Yang et al., 2006). Basal DCFH-DA fluorescence was demonstrated in the untreated KB cells (control) in our study. Incubation of cells with AP extracts (150 g/mL for 0, 30, 60, 90, or 180 min) caused a significant increase in fluorescence response, with a maximum ROS increase observed 90 min after treatment (Fig. 6A and B). These results indicate that *A. pricei* induces ROS generation in KB cells in a time-dependent manner.

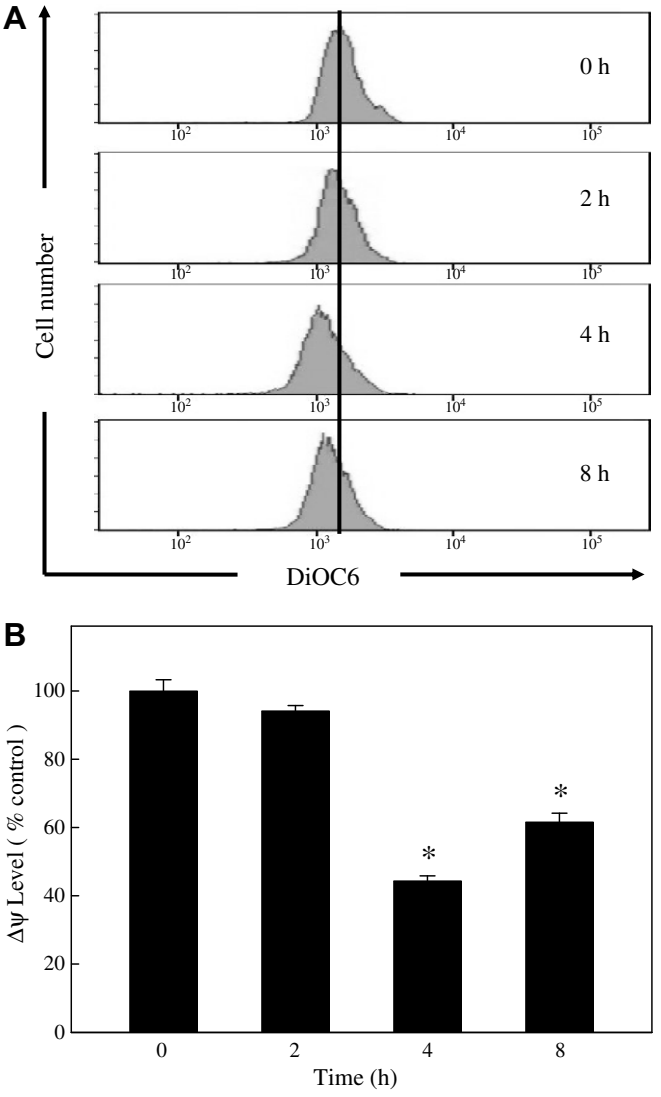

Fig. 3. Effect of AP extracts on mitochondrial membrane potential of KB cells. (A) Cells were grown in the absence (control) or presence of AP extracts (150 g/mL) for 0, 2, 4, or 8 h, stained with DiOC6, and analyzed by flow cytometry as described in Section 2. Representative flow cytometry patterns are shown. (B) The mitochondrial membrane potential (% control), as indicated by DiOC6 fluorescence, after AP extract treatment is shown. Results are the mean ± SD of three assays. * indicates significant difference in comparison to the control group ($p < 0.05$).

3.8. Effects of AP extracts on the viability of HGF cells

To test whether AP extracts affect healthy human HGF cells, the effects of AP extracts on the viability of HGF cells were also examined. At 24 h and 48 h, concentrations of 0, 50, and 100 g/mL did not affect the number of HGF cells; however, 200 g/mL proved to be cytotoxic (Fig. 7). There was a decrease in cell number at 72 h with AP extracts in a dose-dependent manner (Fig. 7). Experiments were conducted to compare the response of KB and HGF cells to treatment with AP extracts. Cell viability dropped in response to

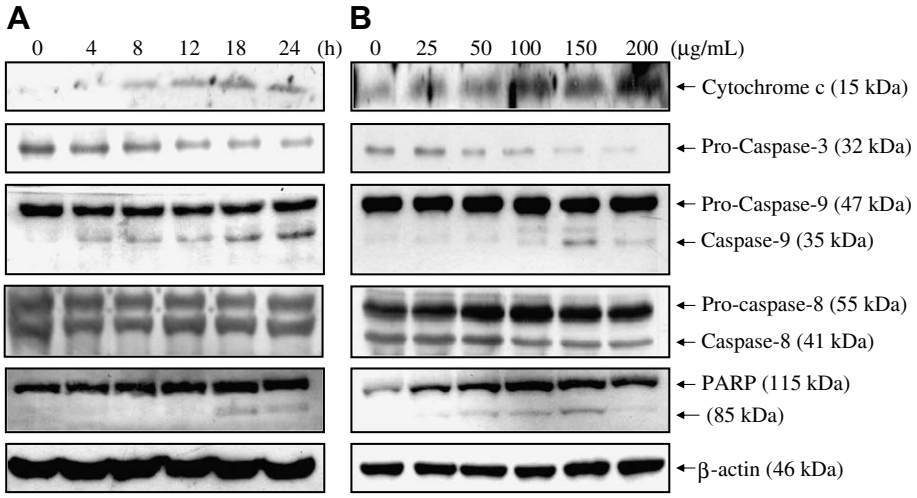

Fig. 4. Western blot analysis of cytochrome *c*, caspase-3, -8 and -9, and PARP protein levels exposed to AP extracts. (A) The KB cells were treated with AP extracts (150 g/mL) for 0, 4, 8, 12, 18, or 24 h. (B) Cells treated with 0, 25, 50, 100, 150, or 200 μg/mL of AP extracts for 24 h. Protein (50 μg) from each sample was resolved on 8–15% SDS–PAGE and Western blot was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. A typical result from three independent experiments is shown.

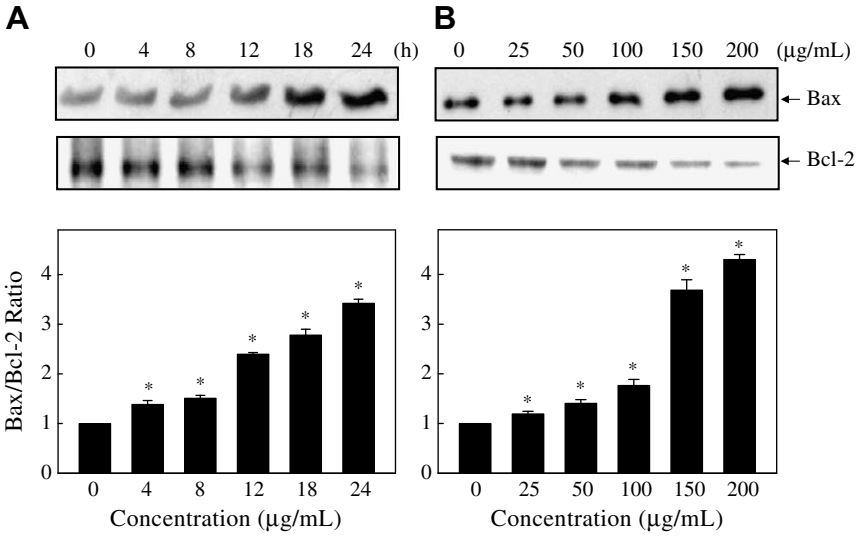

Fig. 5. Western blot analysis of Bcl-2 and Bax protein levels in KB cells after exposure to AP extracts. (A) The KB cells were treated with AP extracts (150 g/mL) for 0, 4, 8, 12, 18, or 24 h. (B) Cells treated with 0, 25, 50, 100, 150, or 200 g/mL of AP extracts for 24 h. Protein (50 μg) from each sample was resolved on 15% SDS–PAGE and Western blot was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. A typical result from three independent experiments is shown. * indicates significant difference in comparison to the control group ($p < 0.05$).

treatment in both cell lines, but the reduction in cell viability was more pronounced in KB cells than in HGF cells.

4. Discussion

Several studies have demonstrated that a number of herbal medicines or mixtures have anticancer potential in vitro and in vivo (Hu et al., 2002). Typically, herbal medicines comprise whole extracts from a plant mix or from complex formulations (Sporn and Suh, 2002). The present research documents a parallel study showing the effect of ethanol (70%) extracts of *A. pricei* (AP extracts) treatment in cell culture models involving human cervical carcinoma KB cells. Based on our findings, we propose that AP extracts directly inhibit cell viability and growth of KB cells by inducing apoptosis of KB cells through a mitochondria-dependent apoptotic pathway. Studies in the past decade have shown that concurrent chemotherapy programs have the potential to improve the overall survival of patients with squamous cell carcinoma. In the current work, *A. pricei* markedly suppressed the proliferation of human cervical carcinoma (KB) cells. Interestingly, *A. pricei* has been found to have lesser cytotoxicity towards the normal human fibroblast (HGF) cells. These results suggest that *A. pricei* may be useful for the prevention and/or treatment of patients with cervical cancer. However, further *in vivo* studies using animal models and human patients are necessary to elaborate and exploit this nascent promise.

Disturbances of apoptosis in cancer cells have been studied in detail, and induction of apoptosis in these cells is one of the strategies for anticancer drug development (Carnero, 2002). In this study, we present evidence demonstrating that treatment of KB cells with a variety of concentrations of AP extracts resulted in dose- and time-dependent sequences of events marked by

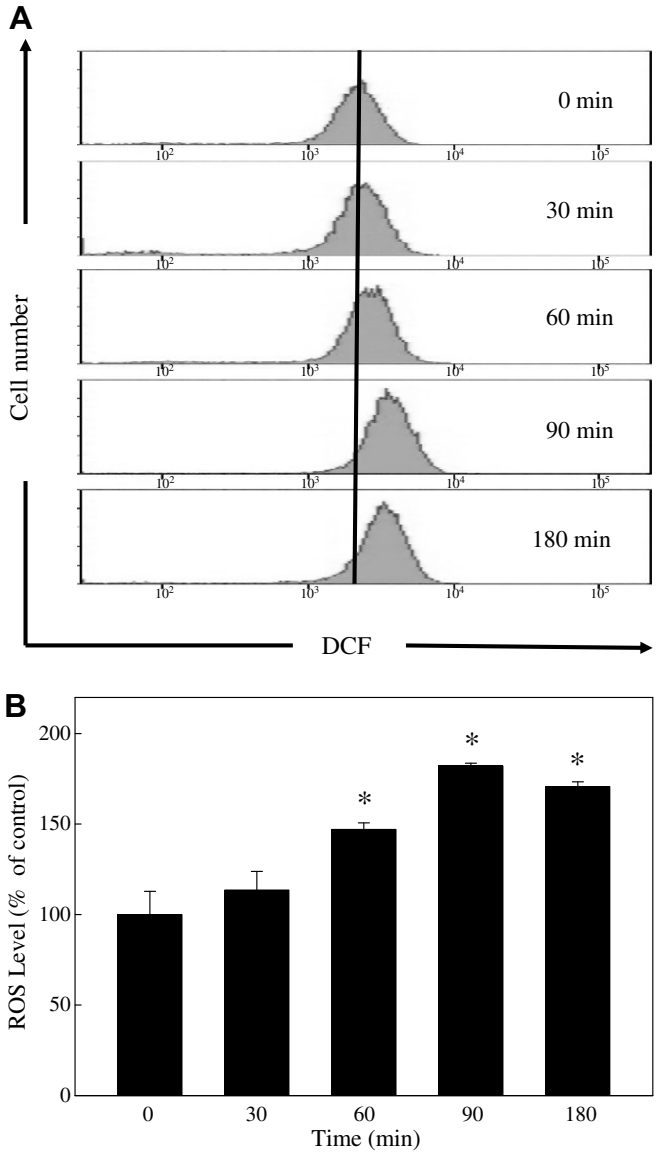

Fig. 6. Effects of AP extracts on intracellular ROS Level in KB cells. (A) Cells were treated with 150 μg/mL of AP extracts for 0, 30, 60, 90, or 180 min. The non-fluorescent cell-membrane permeable probe DCFH-DA was added to the culture medium at a final concentration of 10 μM 30 min before the end of each experiment. The DCFH-DA was used to penetrate the cells, react with cellular esterases and ROS, and then metabolize into fluorescent DCF. Representative flow cytometry patterns are shown. A typical result from three independent experiments is shown. (B) The intracellular ROS level (% control), as indicated by DCF fluorescence, was measured with a flow cytometer. Results are mean ± SD of three assays. * indicates significant difference in comparison to the control group ($p < 0.05$).

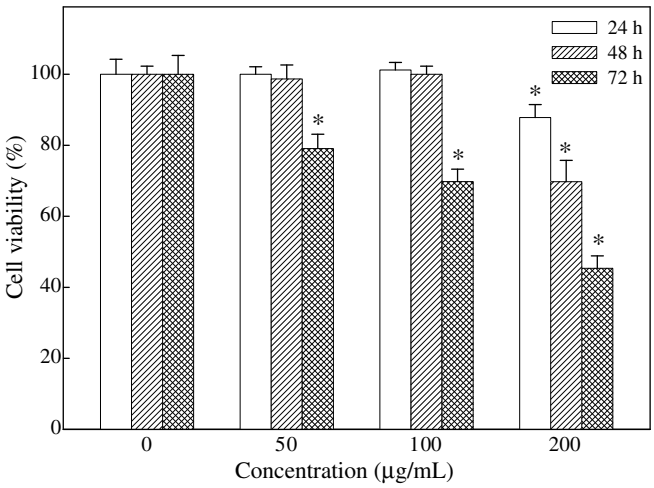

Fig. 7. Effects of AP extracts on cell viability of HGF cells. Cells were treated with 0, 50, 100, 150, and 200 μg/mL of AP extracts for 24, 48, or 72 h. Cultures were then harvested, and cell numbers were obtained by counting cell suspensions with a hemocytometer. Results are presented as mean ± SD of three assays. * indicates a significant difference in comparison to the control group ($p < 0.05$).

apoptosis, such as loss of cell viability, internucleosomal DNA fragmentation, and sub-G1 phase accumulation. This study also defines those events, most of which are used as biomarkers of apoptosis, that were associated with AP extracts induced KB apoptotic cell-death. Apoptosis is controlled by two major pathways, a mitochondrial pathway (Green and Reed, 1998) and a membrane death receptor (DR) pathway (Ashkenazi and Dixit, 1999). Mitochondrial dysfunctions, including the loss of mitochondrial membrane potential and release of cytochrome *c* from the mitochondria into the cytosol are associated with apoptosis (Green and Reed, 1998). Cell-free systems have been used to demonstrate that mitochondrial release of cytochrome *c* into the cytosol is rate limiting in terms of the activation of caspases and endonucleases (Green and Reed, 1998). Cytosolic cytochrome *c* activates pro-caspase-9 by binding to Apaf1 in the presence of dATP, leading to activation of caspase-9 and, subsequently, to the activation of downstream effector caspases (including caspase-3), triggering apoptosis (Li et al., 1997). Activated caspase-3 is responsible for the proteolytic degradation of poly ADP-ribose polymerase, which occurs at the onset of apoptosis. In this study, we provide evidence demonstrating that AP extract-induced apoptosis of KB cells is mediated by loss of mitochondrial membrane potential, increased cytosolic translocation of cytochrome *c*, activation of caspase-3, and degradation of PARP. However, there were no significant differences in the expression of caspase-8 after AP extracts treatment. Caspase-8 is a proximal caspase that plays a critical role in the DR-mediated apoptotic pathway (Ashkenazi and Dixit, 1999). Our data indicate that *A. pricei* induces apoptosis of KB cells through a mitochondria-dependent pathway.

Recently, it has been shown that the Bcl-2 family plays an important regulatory role in apoptosis, either as an activator (Bax) or as an inhibitor (Bcl-2) (Voehringer, 1999; Adams and Cory, 1998). Of the Bcl-2 family members, the Bcl-2 and Bax protein ratio has been recognized as a key factor in regulation of the apoptotic process (Jacobson and Raff, 1995). In the present study, the increase in AP extracts induced apoptosis was associated with an increase in levels of Bax protein, which heterodimerizes with, and thereby inhibits, Bcl-2. Negligible Bcl-2 reduction was observed, however. Our data indicate that *A. pricei* may lead to apoptosis of KB cells by disturbing the Bcl-2/Bax ratio.

Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity (Buttke and Sandstrom, 1994). Indeed, factors that induce oxidative stress, such as ROS production, lipid peroxidation, downregulation of the antioxidant defenses characterized by reduced glutathione levels, and reduced transcription of superoxide dismutase, catalase, and thioredoxin, have been observed in some apoptotic processes (Hockenbery et al., 1993; Briehl and Baker, 1996; Marchetti et al., 1996). Moreover, ROS can also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell-death pathway (Hockenbery et al., 1993; Briehl and Baker, 1996; Marchetti et al., 1996). All of these factors point to a significant role for intracellular oxidative metabolites in the regulation of apoptosis. It was also reported that many stimuli, such as anticancer

drugs, could induce cells to produce reactive oxygen species (ROS), which mediate mitochondrion-initiated apoptosis by inducing the loss of mitochondrial membrane potential (Zhuge and Cederbaum, 2006; Hu et al., 2006). Growth inhibition and ROS generation induced by *A. pricei* in KB cells indicate that ROS production probably causes apoptotic cell-death via the mitochondrial pathway.

Natural products, including plants, provide rich resources for anticancer drug discovery (Schwartsmann et al., 2002). As the different components in a given herb may have synergistic activities or buffer toxic effects, mixtures or extracts of these herbs may offer greater therapeutic or preventive activity when used in combination (Vickers, 2002; Li et al., 2000). In our previous study, a number of compounds, including desmethoxyyangonin, cardamonin, and flavokawain B were isolated from EtOH extracts of the *A. pricei* root (Cheng et al., 2008). These results imply that desmethoxyyangonin, cardamonin, and flavokawain B, the fractions of AP extracts, may act as potential chemopreventive agents with respect to inhibition of the proliferation (growth) of human carcinoma cancer through the induction of apoptosis *in vitro*. Further investigation is required, however, to identify the main active constituents of *A. pricei*.

In conclusion, *A. pricei* exhibits an antiproliferative effect by inducing loss of cell viability, morphology change, internucleosomal DNA fragmentation, sub-G1 phase accumulation, loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome *c* translocation, caspase-3 and -9, activation, poly ADP-ribose polymerase (PARP) degradation, dysregulation of Bcl-2 and Bax, and ROS generation in KB cells. Our data confirm the potential of *A. pricei* as an agent of chemotherapeutic and cytostatic activity in human carcinoma cancer. These data suggest that *A. pricei* may possess anticancer properties and, therefore, may be potentially valuable for application in food and drug products.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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