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Anticancer Effects of *Alpinia pricei* Hayata Roots

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The leaves and roots of *Alpinia pricei* Hayata are used as a traditional wrapping for food and as a cooking substitute for fresh ginger. Our previous study showed that ethanol extracts from the roots of *A. pricei* Hayata (EEAP) and its phenolic compounds have anti-inflammatory effects. The aims of this work were to further study the in vitro anticancer activity of EEAP and its active compounds with respect to various cancer cells. The results from an MTT assay demonstrated that EEAP decreased the cell population growth of CH27, HL-60, and A549 cells. Flow cytometric analysis of HL-60 cells exposed to EEAP showed that the number of apoptotic cells increased in a time- and dose-dependent manner. Western blot data revealed that EEAP stimulated an increase in the level of protein expression of Fas, FasL, caspase-8, and tBid. Moreover, the ratio of the expression levels of pro- and anti-apoptotic Bcl-2 family members was changed after treatment with EEAP. EEAP-induced apoptosis involved the release of mitochondrial cytochrome *c* and subsequently induced the activation of caspase-9 and caspase-3, which were followed by the cleavage of poly(ADP-ribose) polymerase (PARP). The results also demonstrated that phenolic compounds (caffeic acid, apigenin, curcumin, and pinocembrin) from EEAP decreased the rate of population growth of HL-60 cells. Treatment of HL-60 cells with these phenolic compounds caused the loss of mitochondrial membrane potential. Our finding could provide critical information regarding the chemopreventive potential of ethanol extracts from *A. pricei* Hayata. These results also demonstrate that the EEAP-induced apoptotic ability in HL-60 cells might be related to the phenolic compounds.

KEYWORDS: ***Alpinia pricei*** **Hayata; phenolic compound; HL-60 cells; apoptosis**

INTRODUCTION

Apoptosis, or programmed cell death, is characterized by various biochemical and morphological changes, including cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing, and the breakdown of the cell into apoptotic bodies (*1*). Apoptosis can be activated through two main pathways, ultimately classified into the mitochondrion-dependent pathway (the intrinsic pathway) and the death receptor-dependent pathway [the extrinsic pathway (*2*)]. Cytochrome *c* appears to be an important regulator of programmed cell death. Cory and Adams (*3*) indicated that the release of cytochrome *c* from mitochondria to the cytosol can be controlled by the Bcl-2 family of proteins. The Bcl-2 family plays a crucial role in apoptosis since it includes both anti-apoptotic members (Bcl-2) and pro-apoptotic members (Bax, Bad, and Bak) (*4*). The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is thought to play a crucial role in determining the survival or death of cells (*5*). Activation of caspase-3 results in the terminal execution phase of apoptosis and can be induced by a variety of stimuli (*6*). Green and Reed (*7*) defined the loss of the mitochondrial membrane potential as an early stage of apoptosis, where the release of cytochrome *c* from the mitochondria to the cytosol is followed by caspase-3/caspase-9 cascade activation.

Alpinia pricei Hayata is cultivated throughout Asia and is endemic to Taiwan. The leaves of this plant are used as a traditional wrapping for foods. Its roots have been used as a traditional cooking substitute for fresh ginger. The phytochemistry and biology of *Alpinia* plants have been investigated for various uses, including treating metabolic syndrome, as well as antioxidant, anti-inflammatory, anticancer, immunostimulating, and hepatoprotective activities. Chou et al. (*8*) indicated that metabolic syndrome induced by sucrose-containing drinking water in C57BL/6J mice was reduced by the use of 70% ethanol extracts of roots from *A. pricei* Hayata. *Alpinia* plants caused a decrease in the rate of population growth of various cancer cells, such as human HT-1080 fibrosarcoma cells, murine colon 26-L5 carcinoma cells, CORL23 lung cancer cells, MCF7 breast cancer cells, and human carcinoma KB cells (*9*−*11*). The reports indicated that *Alpinia* species contain phenolic compounds (e.g., chlorogenic acid, rutin, quercetin, epicatechin, catechin, cardamonin, protocatechuic acid, zingerone, curcumin, apigenin, and pinocembrin) (*12*−*18*). Lin et al. (*19*) indicated that flavokawain B from *A. pricei* suppresses LPS-induced NO and PGE_2 production by inhibition of NF-κB nuclear translocation. In our previous work, a high-performance liquid chromatography

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(HPLC) profile indicated that ethanol extracts of *A. pricei* Hayata (EEAP) contain caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, rutin, apigenin, curcumin, and pinocembrin (*20*). However, literature regarding the anticancer activity of EEAP and its phenolic compounds remains scarce.

The objective of this study was to investigate the anticancer activity of EEAP and its active compounds. In this study, various human cancer cells (including CH27, HL-60, A549, HT-29, HCT 116, COLO 205, and HepG2 cells) were used to investigate anticancer activity in vitro. The anticancer effects of EEAP on intrinsically and extrinsically mediated pathways in human cancer cells were also investigated.

MATERIALS AND METHODS

Materials. *A. pricei* Hayata was collected at Ping-tung, Taiwan, in March 2006. Seventy percent ethanol extracts of roots from *A. pricei* Hayata (EEAP) were provided by S.-Y. Wang (National Chung Hsing University). MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, rutin, apigenin, curcumin, pinocembrin, sulfanilamide, and anti-β-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium, fetal bovine serum, L-glutamine, and the antibiotic mixture (penicillin/streptomycin) were purchased from Invitrogen Co. (Carlsbad, CA). Anti-Bad, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-caspase-8, anti-Fas, anti-FasL, anti-cytochrome *c*, and anti-PARP [poly(ADP-ribose) polymerase] antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-Bid antibody was purchased from Biosource (Camarillo, CA). The anti-caspase-9 antibody was obtained from BioVision. Anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies were purchased from Bethyl Laboratories (Montgomery, TX), and protein molecular mass markers were obtained from Pharmacia Biotech (Saclay, France). Polyvinyl difluoride (PVDF) membranes for Western blotting were obtained from Millipore (Bedford, MA). All other chemicals were reagent grade.

Cell Culture. Human acute promyelocytic leukemia cells (HL-60 cells), human colorectal carcinoma cells (HCT 116 cells), human lung carcinoma cells (A549 cells), and human hepatoblastoma cells (HepG2 cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Human lung squamous carcinoma cells (CH27 cells) were provided by S.-L. Hsu (Taichung Veterans General Hospital, Taichung, Taiwan). Human colorectal carcinoma cells (COLO 205 cells and HT-29 cells) were provided by M.-H. Pan (National Kaohsiung Marine University, Kaohsiung, Taiwan). A549 cells and CH27 cells were grown in a medium consisting of 90% RPMI 1640 with 10% fetal bovine serum supplemented with 0.1 mM nonessential amino acid, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/mL penicillin/streptomycin. COLO 205 cells were grown in a medium consisting of 90% RPMI 1640 medium with 10% fetal bovine serum supplemented with 1.5 g/L sodium bicarbonate and 100 units/mL penicillin/streptomycin. HCT 116 cells were grown in a medium consisting of 90% McCoy's 5a medium with 10% fetal bovine serum supplemented with 1.5 mM L-glutamine and 100 units/mL penicillin/streptomycin. HepG2 cells were grown in a medium consisting of 90% minimum essential medium (Eagle) with 10% fetal bovine serum supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 100 units/mL penicillin/streptomycin. HL-60 cells were grown in a medium consisting of 80% Iscove's modified Dulbecco's medium and 20% fetal bovine serum supplemented with 1.5 g/L sodium bicarbonate and 100 units/mL penicillin/streptomycin. HT-29 cells were grown in a medium consisting of 90% RPMI 1640 medium and 10% fetal bovine serum supplemented with 2 mM L-glutamine and 100 units/mL penicillin/streptomycin. Human peripheral blood lymphocytes were obtained from healthy male donors and were separated by the Ficoll-Paque premium (GE Healthcare, Uppsala, Sweden). The cell culture condition was 37 °C in a humidified incubator with 5% CO_2.

Cell Viability Determined by the MTT Assay. The MTT assay was performed according to the method of Mosmann (*21*). Cancer cells were plated into 96-well microtiter plates at a density of 1×10^4 cells/well. After 24 h, the culture medium was replaced with 200 μL serial dilutions of EEAP (0−500 μg/mL) or phenolic compounds (0−100 μM), and the cells were incubated for 48 h. The final concentration of solvent was $< 0.1\%$ in the cell culture medium. The culture medium was removed and replaced with 90 μL of fresh culture medium. Ten microliters of sterile filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) was added to each well, producing a final concentration of 0.5 mg of MTT/mL. After 5 h, unreacted dye was removed and the insoluble formazan crystals were dissolved in 200 μL of DMSO per well and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies, Offenburg, Germany) at 570 nm. The relative cell viability (percent) related to control wells containing a cell culture medium without samples was calculated as A_{570}[sample]/A_{570}[control] × 100. The IC_{50} value was calculated as the concentration of the compound at which cell growth was inhibited by 50% compared to untreated controls.

Analyses of Cell Apoptosis (PI staining method) by Flow Cytometry. The cells were plated into a 6 cm culture dish at a density of 1×10^6 cells/dish. The cancer cells stimulated with 0−200 μg/mL EEAP for 0−72 h were assayed for cell cycle progression and/or apoptosis by the PI staining method, as previously described (*22*). Briefly, cells were harvested, washed with PBS twice, and fixed in 80% ethanol at 4 °C for 30 min, followed by incubation with 100 μg/mL RNase for 30 min at 37 °C. The cells were then stained with 40 μg/mL PI for 15 min at room temperature and subjected to flow cytometric analysis of the DNA content using FACScan flow cytometry (Becton-Dickinson Immunocytometery Systems, San Jose, CA). Approximately 1×10^4 counts were made for each sample. The percentage of the distribution of the cell cycle phase and apoptosis were calculated with CELL Quest. The nuclear morphology of cells was examined using fluorescence microscopy (Olympus, Tokyo, Japan).

Annexin V-FITC/PI Assay. An annexin V-FITC/PI assay was performed using an annexin V-FITC kit (ANNEX100F, SEROTEC) according to the manufacturer's instructions. Briefly, 1×10^6 cells were plated in a 6 cm dish. Cells were treated with 0−200 μg/mL EEAP for 24 and 48 h. The cells were labeled with annexin V-FITC/PI according to the manufacturer's instructions. The annexin V-FITC−/PI− population was determined to consist of normal healthy cells, while the annexin V-FITC+/PI− cells were taken to represent cells undergoing early apoptosis, Annexin V-FITC+/PI+ and annexin V-FITC−/PI+ cells represented those undergoing late apoptosis and necrosis, respectively. Approximately 1×10^4 events were acquired and analyzed using CELL Quest.

Nuclear Staining with PI and DAPI. Apoptosis was evaluated by staining with PI and DAPI. Cells were stimulated with 0−25 μg/mL EEAP for 48 h. Cells stained with PI were fixed with 80% ethanol for 30 min and incubated with a 40 μg/mL PI solution for 30 min in the dark. Cells stained with DAPI were fixed with 4% paraformaldehyde for 30 min and incubated with a 1 μg/mL DAPI solution for 30 min in the dark. The nuclear morphology of the cells was examined by fluorescence microscopy (Olympus). Typical apoptotic changes included chromatin condensation, chromatin compaction along the periphery of the nucleus, and segmentation of the nucleus.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Analysis. The mitochondrial membrane potential was determined using a MitoPT 100 test kit (Immunochemistry Technologies, LLC, Bloomington, MN). JC-1 is a cationic dye that exhibits potential-dependent accumulation in the mitochondria and indicates a shift in the fluorescence emissions from green to red. Cells were seeded in 12-well plates. After 24 h, the cells were treated with EEAP (0−50 μg/mL) or phenolic compounds (0−25 μM) for 0−24 h. Routine passage consisted of rinsing cells in 12-well plates once with PBS, followed by harvesting with 0.1 mL of a TE solution, addition of 1 mL of fresh culture medium, and thorough dispersion. Aliquots of the resultant cell suspensions were placed in Eppendorf vials with 1×10^6 cells per vial containing 1 mL of culture medium. After being centrifuged, cells were incubated with 10 μg/mL JC-1 at 37 °C for 15 min in a humidified incubator with 5% CO_2. Cells were collected and washed with 1× assay buffer (MitoPT 100 test kit). The cells were resuspended in an adequate amount of the same solution and analyzed using a FLUOstar galaxy

fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 590 nm for red fluorescence. Apoptotic cells generate a lower reading of red fluorescence, and the changes in the mitochondrial membrane potential ($\Delta\Psi_m$) can be most accurately assessed by comparing the red fluorescence of cells that were untreated and the red fluorescence of those that were treated with EEAP. The morphology of the cells was examined by fluorescence microscopy (Olympus).

Western Blot Analysis and Measurement of Caspase-3 and -9 Activity. Cells (1×10^7 cells/10 cm dish) were incubated with 25 μg/mL EEAP for 0−6 h. Cells were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF]. The Fas, FasL, caspase-8, tBid, Bax, Bcl-2, Bad, cytochrome *c*, caspase-9, caspase-3, PARP, and β-actin proteins were assessed in HL-60 cells. The protein concentrations of the extracts were estimated with a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Total proteins (50−60 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane, and the membrane was blocked with 5% skim milk in PBST [0.05% (v/v) Tween 20 in PBS (pH 7.2)] for 1 h. Membranes were incubated with a primary antibody (1:5000) at 4 °C overnight and then with a secondary antibody (1:5000) for 1 h. Membranes were washed three times in PBST for 10 min between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative protein expression was quantified densitometrically using LabWorks version 4.5 and calculated according to the β-actin reference bands. Caspase-3 and caspase-9 activities were assayed using commercial kits as specified by the manufacturers (Upstate Biotechnology, Lake Placid, NY; BioVision). Fluorescence was measured with a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies Ltd.).

Statistical Analysis. Statistical analysis was performed using SAS. Analyses of variance were performed using ANOVAs, while significant differences ($p < 0.05$) between the means were identified using Duncan's multiple-range tests. Each treatment was performed in triplicate.

Table 1. Effect of EEAP on the Cell Growth of Various Human Cancer Cells

cell line	IC_{50} (μg/mL)[a]
CH27	49.0 ± 5.4
HL-60	59.0 ± 5.4
A549	89.9 ± 4.1
HT-29	157 ± 8
HCT 116	205 ± 4
COLO 205	234 ± 6
HepG2	348 ± 7

[a] Cells were treated with 0−500 μg/mL EEAP for 72 h. Values are reported as means ± SD ($n = 5$). EEAP indicates 70% ethanol extracts of *A. pricei* Hayata.

RESULTS

Effects of EEAP and Its Phenolic Compounds on Cell Population Growth. The inhibitory effects of EEAP on the cell population growth of human lung squamous carcinoma cells (CH27 cells), human lung carcinoma cells (A549 cells), human colorectal carcinoma cells (HCT 116 cells, COLO 205 cells, and HT-29 cells), human hepatoblastoma cells (HepG2 cells), and human acute promyelocytic leukemia cells (HL-60 cells) were determined by MTT assays. As shown in **Table 1**, the IC_{50} (inhibits growth by 50%) values of EEAP on CH27 cells, HL-60 cells, A549 cells, HT-29 cells, HCT 116 cells, COLO 205 cells, and HepG2 cells were

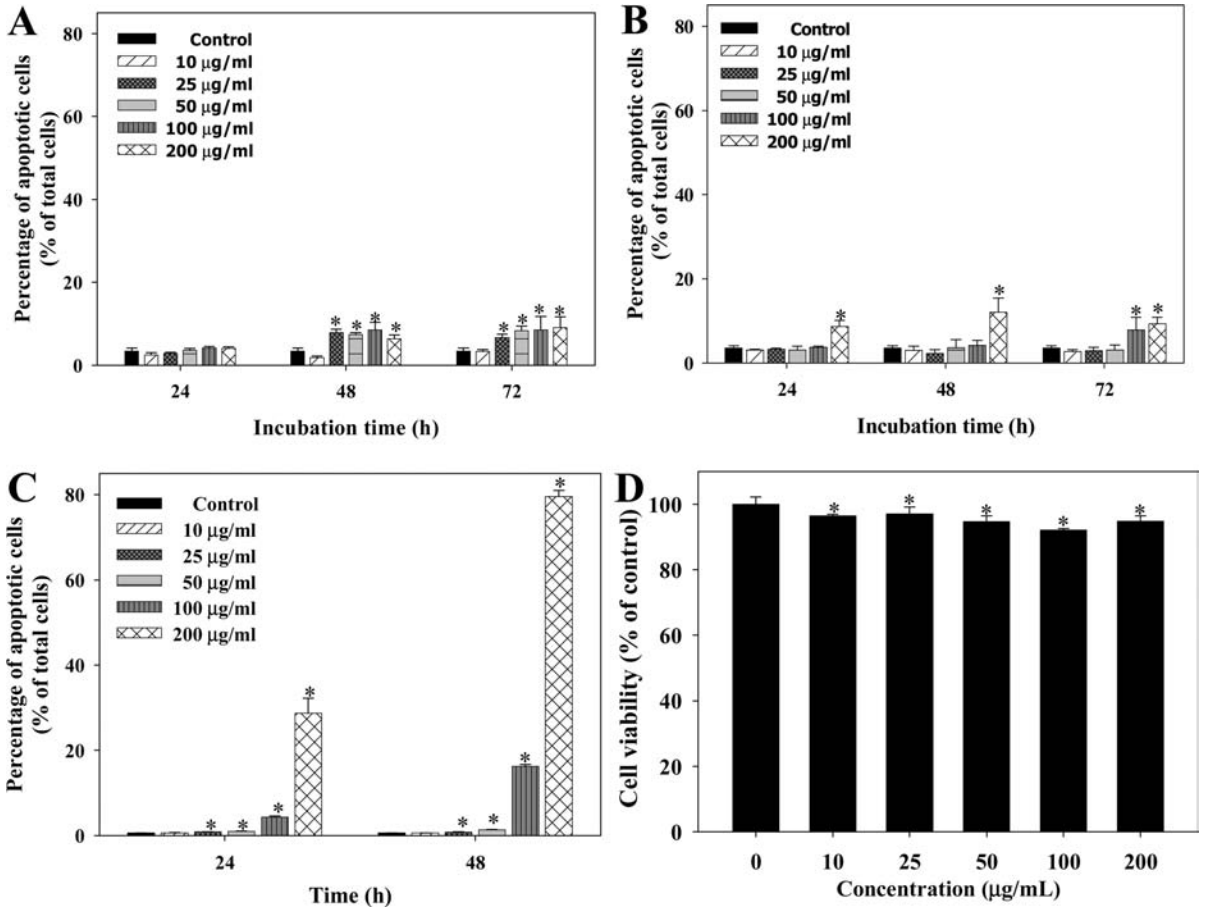

Figure 1. Effect of EEAP on cell apoptosis in A549 cells (**A**), CH27 cells (**B**), and HL-60 cells (**C**) and on the viability of human lymphocyte cells (**D**). Cells were treated with 0−200 μg/mL EEAP for 72 h. Values are reported as means ± SD ($n = 3$). An asterisk indicates a significant difference ($p < 0.05$) from the untreated group.

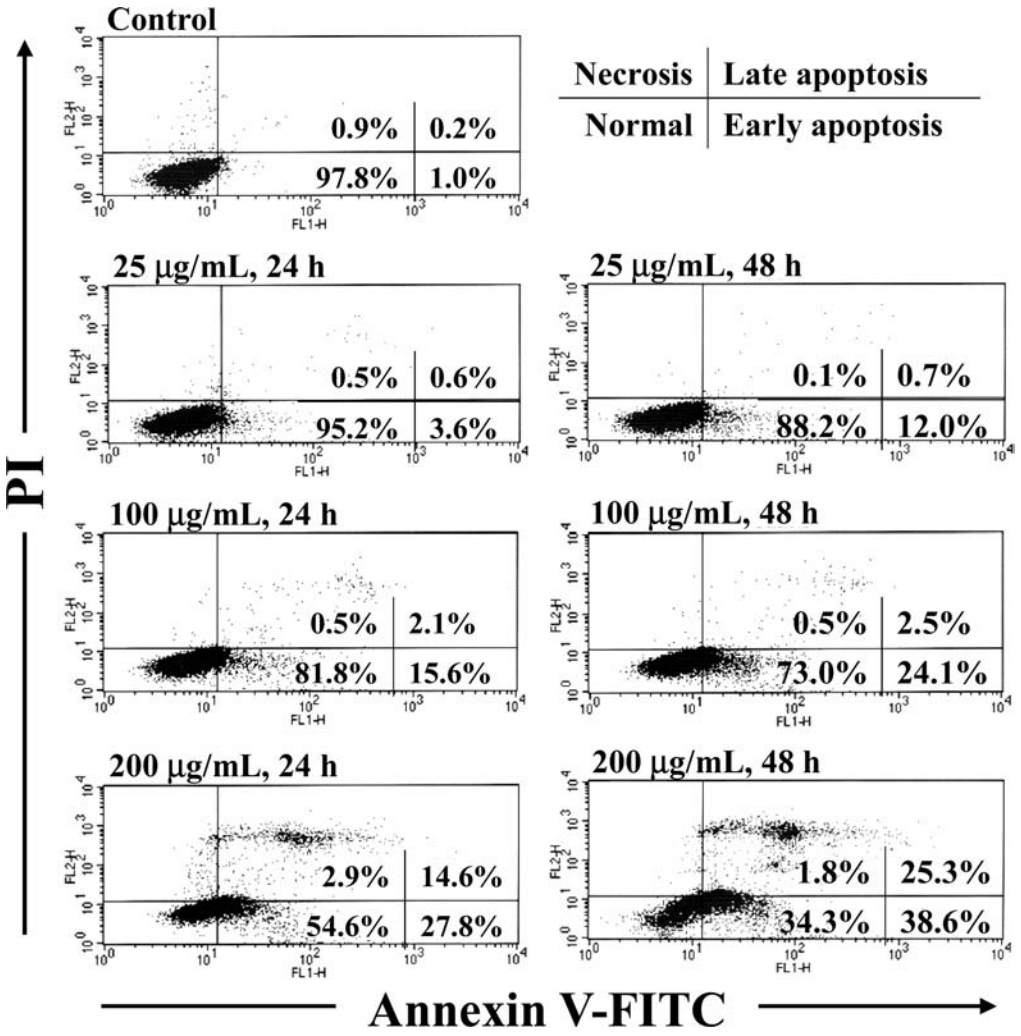

Figure 2. Effect of EEAP on apoptotic and necrotic cells in HL-60 cells. Cells were treated with 0−200 μg/mL EEAP for 24−48 h. Cells doubly stained with annexin V-FITC/PI were analyzed by flow cytometry.

49.0, 59.0, 89.9, 157, 205, 234, and 348 μg/mL, respectively. EEAP had a strong inhibitory effect on cell growth in CH27 cells, HL-60 cells, and A549 cells.

EEAP-Induced Cell Apoptosis. On the basis of the results listed in **Table 1**, CH27 cells, HL-60 cells, and A549 cells were selected for the study of the induction of EEAP on cell apoptosis. The results are shown in **Figure 1**. The addition of EEAP to A549 cells, CH27 cells, and HL-60 cells resulted in a marked increase in the level of accumulation of the sub-G1 phase (apoptotic cells) in a time- and dose-dependent manner (**Figure 1A−C**). The data indicated that EEAP had the highest percentage of apoptotic cells in HL-60 cells. Consequently, HL-60 cells were selected for all subsequent studies. Moreover, EEAP did not affect the survival of normal peripheral blood lymphocytes (**Figure 1D**). **Figure 2** shows the effect of EEAP on the percentage of normal, early apoptotic, late apoptotic, and necrotic cells in the HL-60 cell line. The results indicated that EEAP decreased the number of HL-60 cells in a time- and dose-dependent manner. The number of apoptotic cells, including both early apoptotic (annexin V-FITC+/PI−) and late apoptotic cells (annexin V-FITC+/PI+), increased in a time- and dose-dependent manner. The nuclear morphology of untreated and treated cells is shown in panels **A** and **B** of **Figure 3** using PI and DAPI staining, respectively. PI and DAPI staining showed apoptotic bodies when cells were treated with 25 μg/mL EEAP for 48 h.

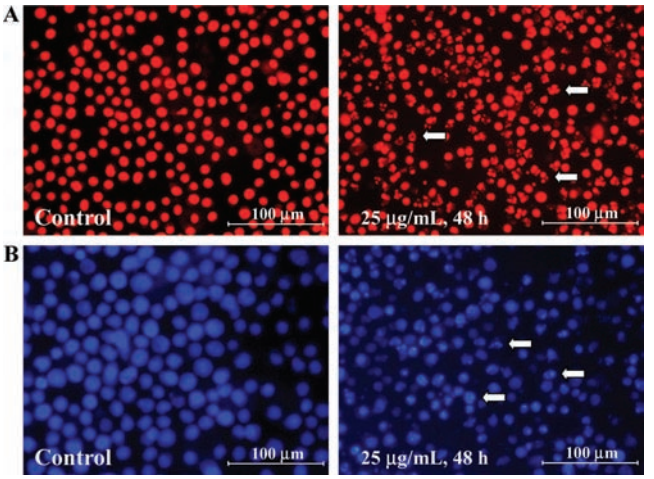

Figure 3. Effect of EEAP on morphological changes to HL-60 cells. Cells were treated with 25 μg/mL EEAP for 48 h and then stained with PI (**A**) or DAPI (**B**). Arrowheads indicate condensed chromatin.

Disruption of Mitochondrial Membrane Potential ($\Delta\Psi_m$) by EEAP. The effect of EEAP on the mitochondrial membrane potential in HL-60 cells was investigated. Alterations in mitochondrial function have been shown to play a crucial role in apoptosis. In this assay, cell morphology indicated that nonapoptotic cells with

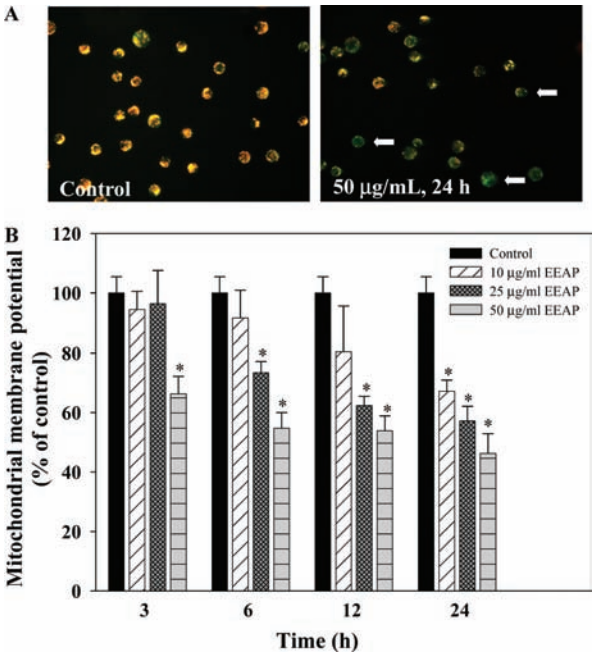

Figure 4. Effect of EEAP on the mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells. Cells were treated with 0−50 μg/mL EEAP for 3−24 h. (**A**) Morphological changes to cells. Arrowheads indicate apoptotic cells. (**B**) Percentages of mitochondrial membrane potential. Results are expressed as percentages of mitochondrial membrane potential in comparison with the untreated control (mean ± SD; n = 3). An asterisk indicates a significant difference ($p < 0.05$) from the untreated group.

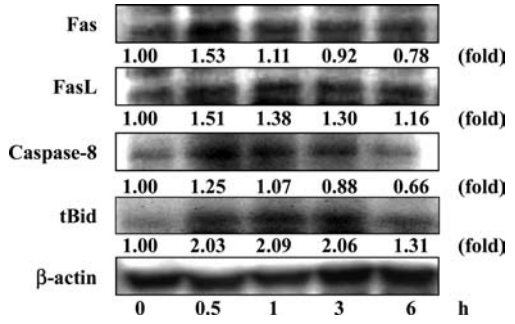

Figure 5. Effect of EEAP on the protein expression of Fas, FasL, caspase-8, and tBid in HL-60 cells. Cells were treated with 25 μg/mL EEAP for 0−6 h. The relative expression of proteins was quantified densitometrically using LabWorks version 4.5 and was calculated according to the β-actin reference bands.

healthy mitochondria appear as red fluorescent cells and apoptotic cells appear as green fluorescent cells. HL-60 cells showed a significant ($p < 0.05$) decrease in the intensity of red fluorescence when treated with 0−50 μg/mL EEAP for 0−24 h (**Figure 4**). This study indicates early damage to the mitochondrial membrane potential that may further activate the intrinsic pathways of apoptosis.

EEAP Induces Apoptosis via Intrinsically and Extrinsically Mediated Pathways. The effects of EEAP on the protein expression of Fas, FasL, caspase-8, and tBid in HL-60 cells are shown in **Figure 5**. Treatment of HL-60 cells with EEAP (25 μg/mL) for 0.5 h resulted in significant 1.53- and 1.51-fold increases in the levels of Fas and FasL expression, respectively. Treatment with 25 μg/mL EEAP for 0.5 h significantly increased the level of caspase-8 expression from 1.00-fold (control) to 1.25-fold. Treatment with 25 μg/mL EEAP for 3 h significantly increased

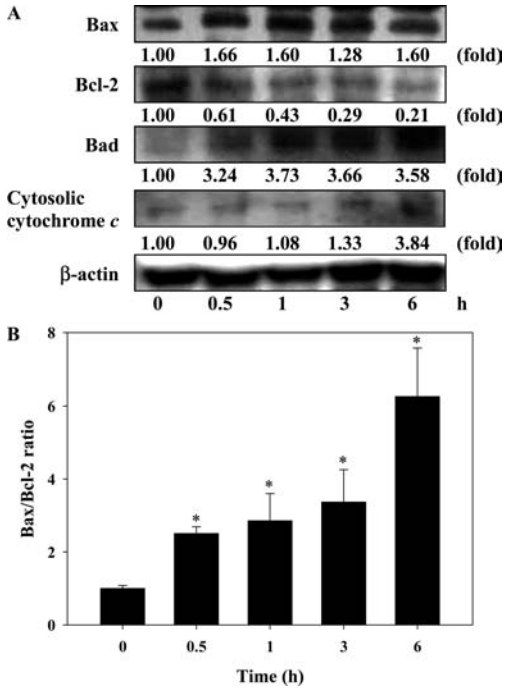

Figure 6. Effects of EEAP on the protein expression of Bax, Bcl-2, Bad, and cytochrome c (**A**) and the Bax:Bcl-2 ratio (**B**) in HL-60 cells. Cells were treated with 25 μg/mL EEAP for 0−6 h. The relative level of expression of proteins was quantified densitometrically using LabWorks version 4.5 and was calculated according to the β-actin reference bands. Values are reported as means ± SD (n = 3). An asterisk indicates a significant difference ($p < 0.05$) from the untreated group.

levels of tBid expression 2.06-fold from those observed with the control. The effects of EEAP on the protein expression of the Bcl-2 family and cytosolic cytochrome c in HL-60 cells are shown in **Figure 6A**. The level of pro-apoptotic protein expression of Bax and Bad was increased by 1.60- and 3.73-fold, respectively, in comparison to the control after treatment with 25 μg/mL EEAP for 1 h. EEAP treatment at 25 μg/mL for 6 h significantly decreased the level of Bcl-2 (antiapoptotic protein) expression to 0.21-fold in comparison to the control. Cytochrome c release in the cytosolic fraction following EEAP treatment was then investigated. Treatment with EEAP (25 μg/mL, 6 h) resulted in a significant increase in the level of cytosolic cytochrome c expression, it increasing 3.84-fold in comparison to the control. A significant time-dependent shift in the ratio of Bax to Bcl-2 was observed after EEAP treatment at 25 μg/mL for 0−6 h (**Figure 6B**). The effects of EEAP on the protein expression of caspase-9, caspase-3, and PARP in HL-60 cells are shown in **Figure 7A**. The results showed that exposure of HL-60 cells to EEAP (25 μg/mL, 0.5 h) caused the degradation of pro-caspase-9 and pro-caspase-3, which generated a fragment of caspase-9 and caspase-3. EEAP treatment at 25 μg/mL for 6 h significantly increased the level of expression of cleaved PARP 2.68-fold in comparison to the control. The results indicated that EEAP treatment caused a significant increase in the activity of caspase-9 and caspase-3 (**Figure 7B**).

Effects of Phenolic Compounds from EEAP on Cell Population Growth. In our previous study, the high-performance liquid chromatography (HPLC) profile indicated that EEAP contains caffeic acid, chlorogenic acid, ferulic acid, p-hydroxybenzoic acid, rutin, apigenin, curcumin, and pinocembrin (*20*). In this study, the effects of chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, ferulic acid, rutin, apigenin, curcumin, and pinocembrin on

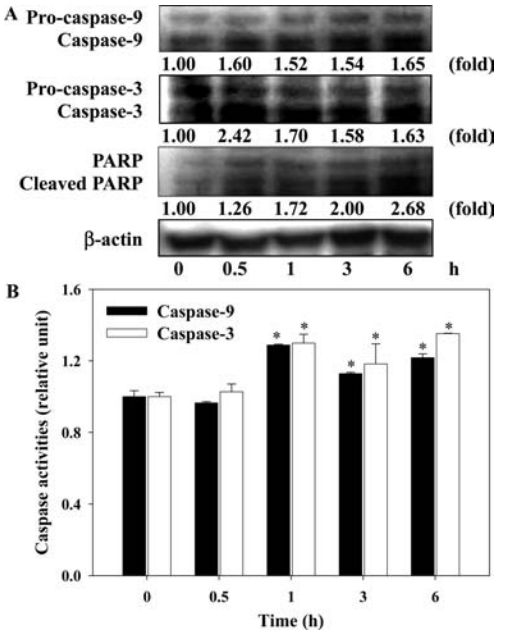

Figure 7. Effect of EEAP on the protein expression of caspase-9, caspase-3, and PARP (**A**) and activities of caspase-9 and caspase-3 (**B**) on HL-60 cells. Cells were treated with 25 μg/mL EEAP for 0−6 h. The relative level of expression of proteins was quantified densitometrically using LabWorks version 4.5 and was calculated according to the β-actin reference bands. Values are reported as means ± SD (n = 3). An asterisk indicates a significant difference (p < 0.05) from the untreated group.

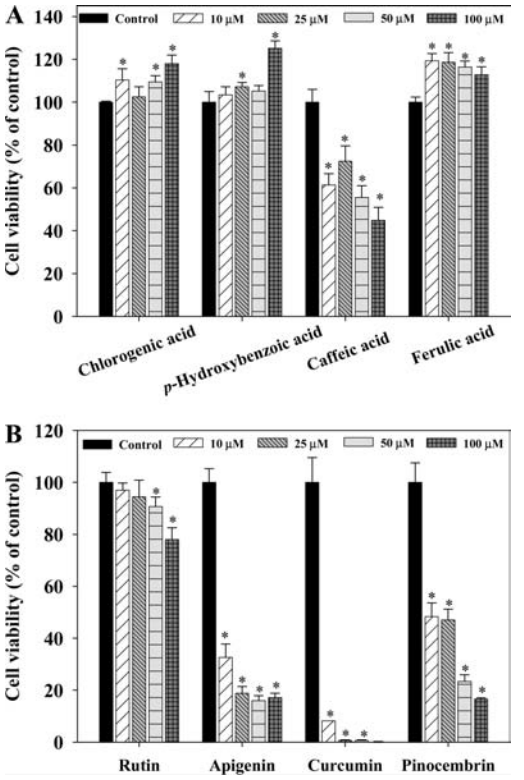

Figure 8. Effects of chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, rutin, apigenin, curcumin, and pinocembrin on the viability of HL-60 cells. Cells were treated with 0−100 μM chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, rutin, apigenin, curcumin, or pinocembrin for 48 h. Values are reported as means ± SD (n = 6). An asterisk indicates a significant difference (p < 0.05) from the untreated group.

the cell viability of HL-60 cells are shown in **Figure 8**. The results showed that the addition of caffeic acid, apigenin, curcumin, and pinocembrin to the growth medium decreased the rate of cell population growth of HL-60 cells.

Disruption of Mitochondrial Membrane Potential ($\Delta\Psi_m$) by Phenolic Compounds from EEAP. Effects of caffeic acid, apigenin, curcumin, and pinocembrin on the mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells are shown in **Figure 9**. HL-60 cells showed a significant (p < 0.05) decrease in the intensity of red fluorescence when treated with 0−25 μM caffeic acid, apigenin, curcumin, or pinocembrin for 12 h. The data indicated that curcumin exhibited the highest disruption of mitochondrial membrane potential in HL-60 cells.

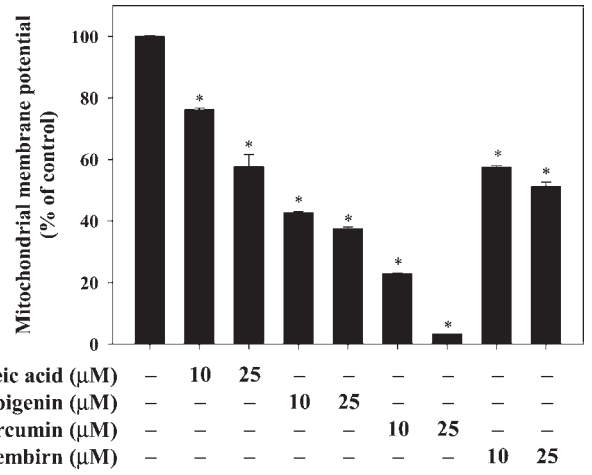

Figure 9. Effects of caffeic acid, apigenin, curcumin, and pinocembrin on the mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells. Cells were treated with 10 and 25 μM caffeic acid, apigenin, curcumin, or pinocembrin for 12 h. Results are expressed as percentages of mitochondrial membrane potential in comparison to the untreated control (mean ± SD; n = 3). An asterisk indicates a significant difference (p < 0.05) from the untreated group.

DISCUSSION

Results from our previous study indicated that EEAP and its phenolic compound, pinocembrin, suppressed LPS-induced NO and PGE_2 production by inhibition of NF-κB nuclear translocation and ROS generation (*20*). However, the literature regarding the effects of EEAP and its phenolic compounds on cell population growth and apoptosis in human cancer cells remains unclear. The effects of EEAP and its phenolic compounds on cell growth inhibition and apoptosis in human cancer cells were further investigated in this study. We found that EEAP caused a significant decrease in the rate of cell population growth of various human cancer cells at 72 h (**Table 1**). Treatment with EEAP had a stronger inhibitory effect on cell growth in A549 cells, CH27 cells, and HL-60 cells. The data also indicated that EEAP had the highest percentage of apoptotic cells in HL-60 cells (**Figure 1C**). However, EEAP did not affect the survival of normal peripheral blood lymphocytes (**Figure 1D**). Our previous study indicated that EEAP contains caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, rutin, apigenin, curcumin, and pinocembrin (*20*). Phenolic compounds of caffeic acid, apigenin, curcumin, and pinocembrin had the strongest inhibitory effects on the cell population growth of HL-60 cells (**Figure 8**). The results indicated that EEAP increased the number of early and late apoptotic cells but not necrotic cells (**Figures 2** and **3**). Changes in the membrane phosphatidylserine (PS)

externalization are generally observed at a later stage than the loss of mitochondrial membrane potential (*23*). In this study, treatment of HL-60 cells with EEAP and its phenolic compounds (caffeic acid, apigenin, curcumin, and pinocembrin) decreased the mitochondrial membrane potential (**Figures 4** and **9**). Many studies have indicated that the phenolic compounds could inhibit the population growth and the induction of apoptosis in HL-60 cells. Chen et al. (*24*) indicated that caffeic acid, quercetin, and rutin exhibit strong cytotoxic effects against HL-60 cells. Pan et al. (*25*) indicated that the curcumin induces apoptosis in HL-60 cells through the release of cytochrome *c* and the activation of caspases. Tan et al. (*26*) indicated that curcumin-induced cell cycle arrest and apoptosis occurred in HL-60 cells through MMP changes and caspase-3 activation. Vargo et al. (*27*) indicated that apigenin induces apoptosis in leukemia cells via activation of PKCδ and caspases.

The Fas (CD95)/Fas Ligand (FasL; CD95L) system is an important extracellular pathway for apoptotic signaling in diverse cell types and tissues (*28*). Our data indicated that EEAP significantly increased levels of Fas, FasL, caspase-8, and tBid expression (**Figure 5**). Cleavage of Bid by caspase-8 has been reported in many cancer cells undergoing apoptosis induced by Fas and FasL (*29*). The Bcl-2 family of proteins has been determined to be involved in apoptosis, including the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2. Moreover, the ratio of Bax to Bcl-2 is a decisive factor that plays an important role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death (*30*). Our data indicated that the ratio of expression levels of pro- and anti-apoptotic Bcl-2 family members was changed by the EEAP treatment (**Figure 6**). EEAP also caused the release of mitochondrial cytochrome *c* into the cytosol (**Figure 6**). Madesh et al. (*31*) indicated that mitochondria undergoing permeability transition cause a loss of mitochondrial membrane potential and cytochrome *c* release in the cytosol. Treatment of HL-60 cells with EEAP revealed the protein expression and activation of caspase-9 and caspase-3 (**Figure 7**). Hseu et al. (*32*) indicated that the ethanol extracts of *A. pricei* rhizome induce cell cycle arrest in KB cells. In an in vivo study, the tumor volume at the end of the experiment showed a significant reduction in the 10 mg/kg *A. pricei* extract treatment group as compared with the control group. Jaszewska et al. (*33*) indicated that induction of apoptotic ability in HTB-140 human skin melanoma cells by defatted seeds of *Oenothera paradoxa* Hudziok might be related to the phenolic compounds.

In conclusion, this study shows that the treatment of HL-60 cells with EEAP leads to death receptor (Fas/FasL) signaling, loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), release of cytochrome *c* from the mitochondria into the cytosol, and the subsequent activation of caspase-8, caspase-9, and caspase-3, followed by the cleavage of PARP. The ratio of expression levels of pro- and anti-apoptotic Bcl-2 family members is also affected by EEAP treatment. We found that EEAP-induced apoptotic ability in HL-60 cells can be related to its phenolic compounds, including caffeic acid, apigenin, curcumin, and pinocembrin. These results suggest a potential molecular mechanism for EEAP and the apoptosis induced by phenolic compounds in HL-60 human acute promyelocytic leukemia cells. *A. pricei* Hayata root may provide beneficial human health effects.

ABBREVIATIONS USED

DAPI, 4,6-diamidine-2-phenylindole; DMSO, dimethyl sulfoxide; EEAP, ethanol extracts of *A. pricei* Hayata; IC_{50}, 50% inhibitory concentration; $\Delta\Psi_m$, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinyl difluoride; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TE, trypsin-EDTA.

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