

Induction of Apoptosis in Human Promyelocytic Leukemia HL-60 Cells by *Ampelopsis cantoniensis* Crude Extract

TZU-WEI TAN¹, HUEI-YANN TSAI^{1,3}, YUH-FUNG CHEN¹ and JING-GUNG CHUNG²

Departments of ¹Pharmacology and ²Microbiology, China Medical University, Taichung 400;

³Department of Pharmacy, China Medical University Hospital, 91 Hsueh-Shih Road, Taichung 400, Taiwan, Republic of China

Abstract. *The crude extract of Ampelopsis cantoniensis induced apoptosis in human promyelocytic leukemia HL-60 cells and this induction was investigated by flow cytometric analysis, DNA gel electrophoresis and poly (ADP-ribose) fluorescence staining. The results demonstrated that this extract induced dose-dependent cytotoxicity and apoptosis. The level of active caspase-3 was increased after treatment with the crude extract for 24 hours.*

Ampelopsis cantoniensis is a member of the *Ampelopsis Vitaceae* plant, used in Taiwan as a part of folk-medicine for deinfection and antibacterial functions. Our previous studies have demonstrated that *Ampelopsis cantoniensis* has analgesic and anti-inflammatory effects (1) and inhibits N-acetyltransferase activity (2). The present study was performed to determine the effects of a crude extract of *Ampelopsis cantoniensis* on human leukemia cells.

Materials and Methods

Chemicals and reagents. Fresh *Ampelopsis cantoniensis* was collected from the mountains (Taichung Hsien, Taiwan, ROC). Trypan blue, Tris-HCl, Triton X-100, propidium iodide and ribonuclease A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Distilled H₂O, potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). All chemicals were reagent grade.

Human leukemia cell line (HL-60). The human promyelocytic leukemia cell line (HL-60) was obtained from the Food Industry

Correspondence to: Dr. Jing-Gung Chung, Department of Microbiology, China Medical University, No 91, Hsueh-Shih Road, Taichung 400, Taiwan, ROC. Tel: (04)2205-3366-8501, Fax: (04)2205-3764, e-mail: jgchung@mail.cmu.edu.tw

Key Words: *Ampelopsis cantoniensis*, leukemia HL-60 cells, apoptosis.

Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were cultured in 75-cm³ tissue culture flasks at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and L-glutamine (2 mM).

Cell viability determination by flow cytometric assays. Cells were cultured in 12-well plates at a density of 5x10⁵ cells/well for 24 hours. Various concentrations of crude extract of *Ampelopsis cantoniensis* were then added while distilled H₂O (solvent) was used as the control. The cells were grown at 37°C in 5% CO₂ and 95% air for 24 hours. For determining cell viability (%), the propidium iodide (PI) stain protocol was used. The cells were harvested, 500 µl of PI were added and the cell suspension was then analyzed by flow cytometry as described in the manual of flow cytometry protocols (3-5).

Cell morphology of HL-60 cells treated with crude extract of Ampelopsis cantoniensis. HL-60 cells were plated in 12-well plates at a density of 1x10⁵ cells/well and grown for 24 hours. Then various concentrations of crude extract of *Ampelopsis cantoniensis* were added and the cells were incubated at 37°C in a humidified 5% CO₂ for different time periods. For the cell morphology experiment, the culture plates were examined under phase contrast microscope and photographed (5).

Flow cytometric analysis of DNA content of HL-60 cells treated with crude extract of Ampelopsis cantoniensis for 24 hours. To estimate the degree of apoptosis of HL-60 cells after treatment with various concentrations of a crude extract of *Ampelopsis cantoniensis*, the cellular DNA content was measured by flow cytometry as described by Ormerod (6). The cells were harvested by centrifugation, fixed gently (drop by drop) in 70% ethanol in ice overnight and finally resuspended in PBS containing 40 µg/mL PI, 0.1 mg/mL Rnase (Sigma) and 5% of Triton X-100. After 30 minutes at 37°C, the cells were analyzed on a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm wavelength. Then the percentage of apoptosis was determined (5).

Poly (ADP ribose) monoclonal antibody assay for apoptosis of HL-60 cells treated with various concentrations of crude Ampelopsis cantoniensis extract. Cells were plated in 12-well plates (34 mm) at a density of 1x10⁶ cells/well and grown at 37°C in humidified-5%

CO₂ atmosphere for 24 hours. They were then centrifuged to remove and discard the medium, and washed in PBS. After centrifuging at 1500 rpm at 4 °C for 5 minutes, the supernatant was removed and the cells resuspended with PBS. This procedure was repeated twice. The cells were then transferred to 96-well dishes, and the dishes centrifuged at 1500 rpm for 4 minutes at 4 °C, the supernatant removed and 100 µl of 3.7% formaldehyde added at 4 °C for 15 minutes. The cells were then washed again with PBS, centrifuged to remove the supernatant and 100 µl of 0.2% NP-40 was added for 15 minutes. After centrifugation to remove the supernatant, PBS with 5% non-fat milk formaldehyde was added for 60 minutes. The cells were washed and then 1% monoclonal antibody (poly ADP-ribose) (Alexis, San Diego, CA, USA) was added diluted in PBS with 5% non-fat milk and incubated at 4 °C overnight. After that, the supernatant was removed and 50 µl 1% FITC-conjugated goat anti-mouse IgG antibody (secondary antibody) was added for 30 minutes in dark conditions. The cells were then washed with PBS, observed under a fluorescent microscope and photographed (5).

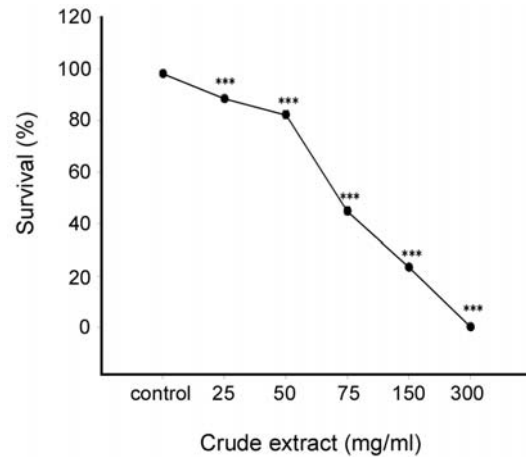


Figure 1. Viability of HL-60 cells after treatment with crude extract of *Ampelopsis cantoniensis* for 24 hours.

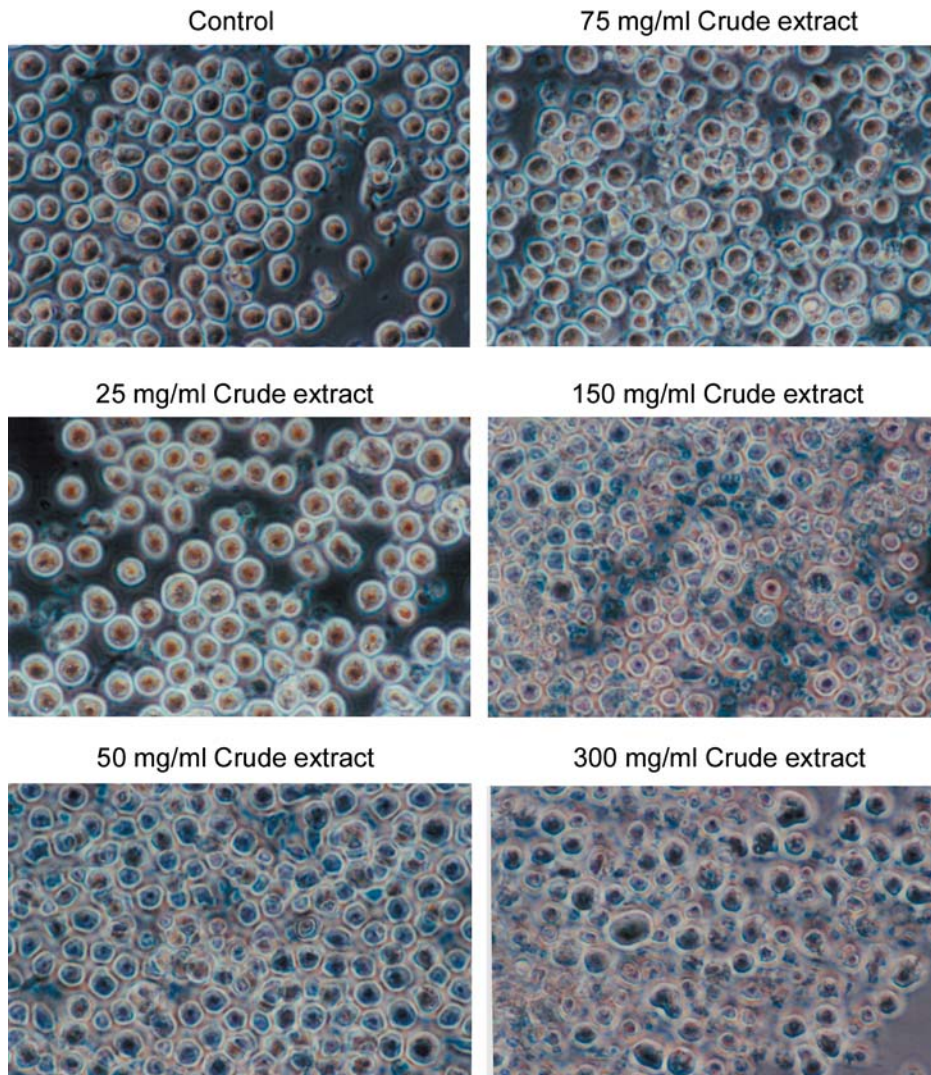


Figure 2. Morphological changes of HL-60 cells after being treated with or without crude extract of *Ampelopsis cantoniensis* for 24 hours.

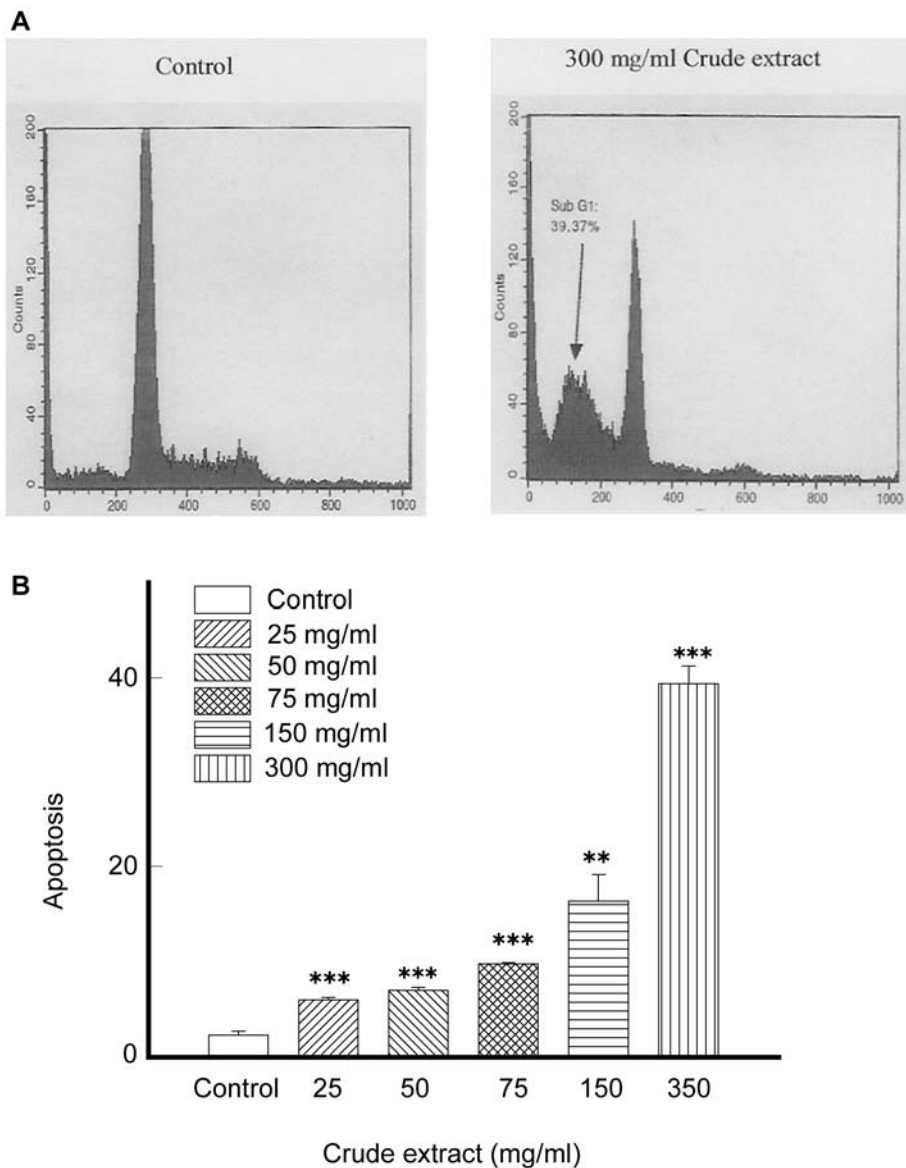


Figure 3. Induction of apoptosis of HL-60 cells by the extract of *Ampelopsis cantoniensis*. The HL-60 cells were incubated with various concentrations of crude extract of *Ampelopsis cantoniensis* for 24 hours and were analyzed by flow cytometry (Panel A). The data was plotted (Panel B) as described in Materials and Methods.

DNA fragmentation electrophoresis analysis. HL-60 cells were cultured in 12-well plates at a density of 5×10^6 cells/well for 24 hours. A 25-300 mg/ml crude extract of *Ampelopsis cantoniensis* was then added while distilled H_2O (solvent) was used as the control. The cells were grown at $37^\circ C$, in a humidified 5% CO_2 atmosphere, for 24 hours. The DNA was prepared using the GNOME DNA isolation kit protocol (BIO 101, La Jolla, CA, USA). The loading buffer [10 mM EDTA, 0.2% (w/v) bromophenol blue and 50% (v/v) glycerol] was individually added to each DNA sample set at a ratio of about 1.5. Approximately 50 μl of DNA was loaded into each well of an 1.6% agarose gel and electrophoresis was carried out at 50 V in TBE buffer for 30

minutes. After electrophoresis, the DNA was visualized by soaking the gel in the TBE buffer containing 0.1 mM ethidium bromide. DNA was observed by UV light and photographed (5).

Reverse transcriptase polymerase chain reaction (RT-PCR). The total RNA was extracted from T24 cells by using the Qiagen RNeasy Mini Kit at 24 hours after being treated or not with *Ampelopsis cantoniensis* extract as described previously (7). Total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a micro-centrifuge tube to a final volume of 12.5 μl . The entire mixture was heated at $70^\circ C$ for 10 minutes and chilled on ice for at least 1 minute. The subsequent procedures for

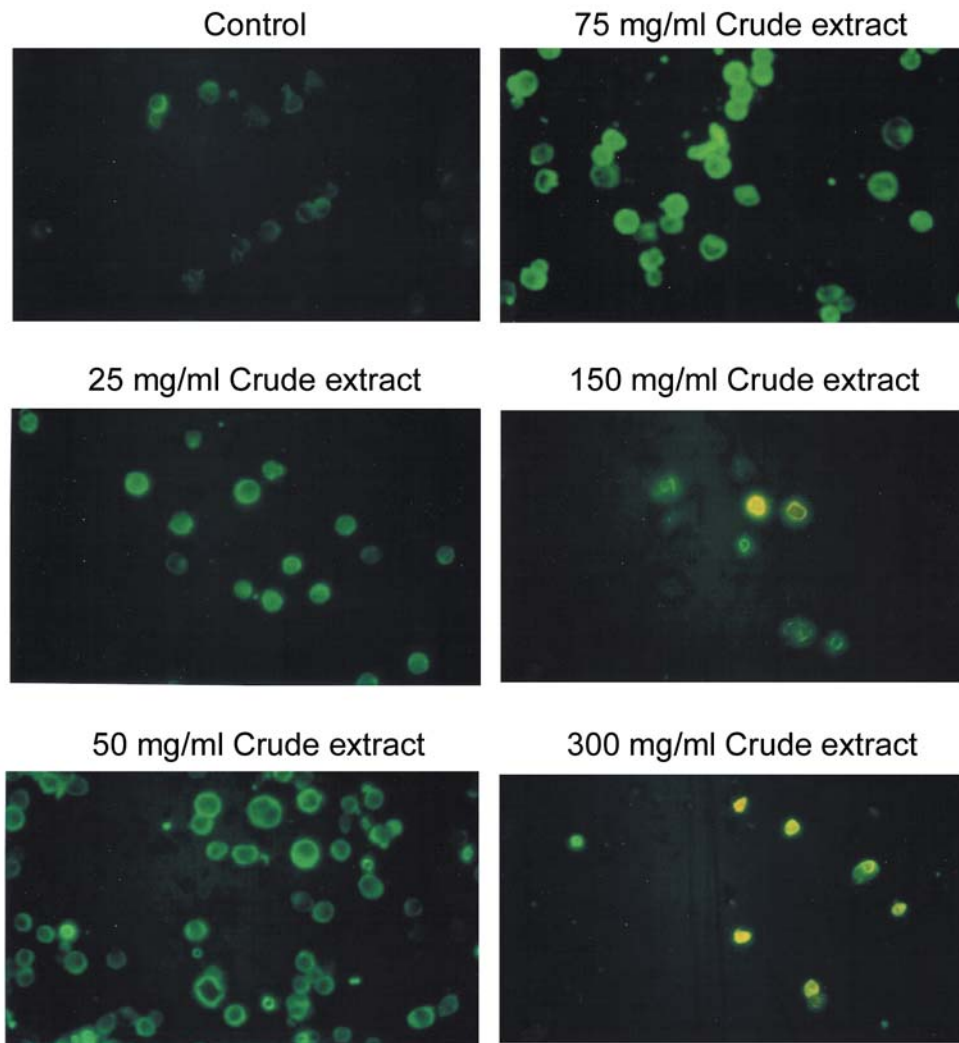


Figure 4. DNA damage of HL-60 cells induced by the crude extract of *Ampelopsis cantoniensis* shown by the PARP monoclonal antibody assay.

conducting reverse transcription were exactly the same as those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. When amplifying target cDNA, components in 50 μ l of solution were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 20 pmoles of each primer, cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers was as follows: Caspase-3: GAATACCCTGGACAACA-3' and ACGCCATGTCATCATCAA. Act b: GCTCGTCGTCGACAACGGCTC and CAAACATGATCTGGGTCATCTTCTC (8-10).

Results

Effects of the crude extract of Ampelopsis cantoniensis on the cell viability of human leukemia HL-60 cells. The results from the PI stain analysis indicated that <2% of HL-60 (human

leukemia cells) were stained when they were incubated in RPMI 1640 medium containing 10% FBS (viability >98%). The crude extract of *Ampelopsis cantoniensis* (25-300 mg/ml) induced a concentration-dependent cytotoxicity in HL-60 (* p <0.05; Figure 1). However, doses lower than 25 mg/ml did not cause any significant effects.

Effects of the crude extract of Ampelopsis cantoniensis on HL-60 cell morphology. Morphological changes of HL-60 cells were substantially observed at 24 hours after the addition of the crude extract of *Ampelopsis cantoniensis* by light-phase microscope and were photographed. Damaged cells characterized by cellular shrinkage were observed while the control HL-60 cells were normal (Figure 2). The cell damage appeared after treatment with 50-300 mg/ml of crude extract of *Ampelopsis cantoniensis*.

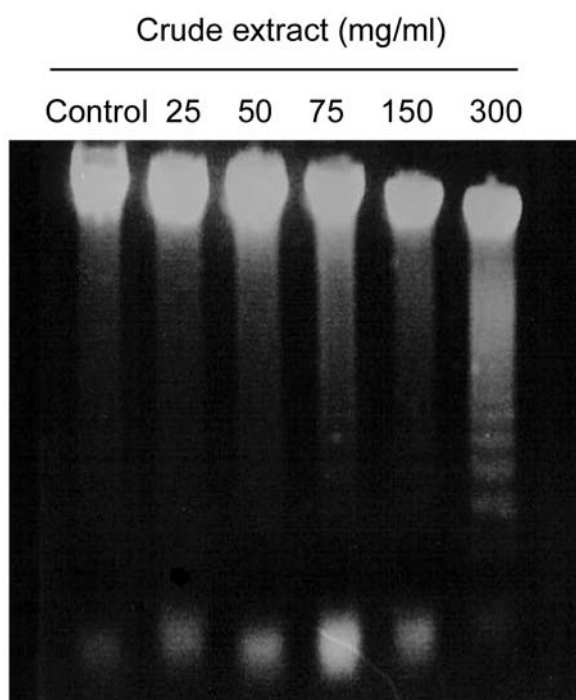


Figure 5. DNA fragmentation of the HL-60 cells induced by the crude extract of *Ampelopsis cantoniensis* shown by gel-electrophoresis as described in Materials and Methods.

Induction of apoptosis in HL-60 cells by the crude extract of Ampelopsis cantoniensis. Flow cytometric analysis was performed to identify the % of apoptotic cells based on their light-scattering properties and DNA content. After fixation and permeabilisation, the cells were incubated with PI and assayed for cell cycle and apoptosis. As shown in Figures 3A and B, the percentage of apoptotic cells was increased with increasing concentrations of the crude extract of *Ampelopsis cantoniensis*. The apoptosis of HL-60 cells was confirmed by using a poly (ADP-ribose) fluorescence stain followed by microscopic examination, some cells with damaged DNA producing poly (ADP-ribose) polymerase (PARP) for DNA repair. Figure 4 shows PARP-positive fluorescence at 50-300 mg/ml of crude extract of *Ampelopsis cantoniensis*.

Since the main characteristic of apoptosis is the cleavage of nuclear DNA into multiple fragments, DNA from treated and non-treated cells was analyzed in 1.6% agarose gel electrophoresis (Figure 5). The low-molecular weight DNA from control cells was undegraded while increased amounts of DNA fragments occurred in cells treated with 75-300 mg/ml crude extract of *Ampelopsis cantoniensis*.

Effects of the crude extract of Ampelopsis cantoniensis on the expression of caspase-3. Activation of caspase-3 was examined

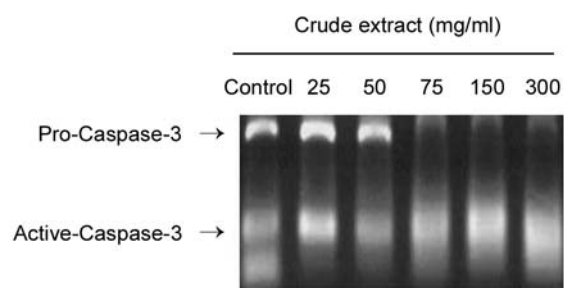


Figure 6. Representative Western blot showing the effects of the crude extract of *Ampelopsis cantoniensis* on the expression of caspase-3 of human HL-60 cells. HL-60 cells were incubated with or without 25-300 mg/ml crude extract of *Ampelopsis cantoniensis* for 24 hours and total RNA was extracted for PCR.

by PCR (Caspase-3: GAATACCCTGGACAACA-3' and ACGCCATGTCATCATCAA). In the control cells, a high level of pro-caspase-3 was observed at 24 hours, but the crude extract of *Ampelopsis cantoniensis* treatment groups showed decrease in the levels of pro-caspase-3 (Figure 6) and increased phosphorylation of caspase-3. This means that the activity of caspase-3 was increased.

Discussion

The hypothesis that was tested in this study was whether a crude extract of *Ampelopsis cantoniensis* could impact cell viability to HL-60 cells via apoptosis. Our data demonstrated that crude extract of *Ampelopsis cantoniensis* treatment of the HL-60 cells resulted in significant cell growth inhibition (cytotoxicity) (Figures 1 and 2), and apoptosis (Figures 3-5) in a dose-dependent manner. Apoptosis induced by crude extract of *Ampelopsis cantoniensis* was confirmed by characteristic DNA ladders in DNA gel electrophoresis and cellular morphological changes. These are important findings because the regulation of apoptotic machinery is important in the development of neoplasms. Currently apoptosis has gained increasing attention as a target against cancer (11-15).

We also analyzed the levels of caspase-3 in HL-60 cells by PCR. The levels of pro-caspase-3 were decreased in the examined cells since the phosphorylation of caspase (active caspase-3) was increased with increased concentrations of the extract (Figure 6). Therefore, it was also indicated that the mechanism of apoptosis induction might involve caspase-3 (Figure 6). Since obviously the extract of *Ampelopsis cantoniensis* is interfering in the apoptosis pathway (16-19) of the HL-60 cells, further investigations on identifying the particular ingredients of the extract should be justified.

Acknowledgements

This work was supported by grants CMC89-M-16 and CMC90-M-23 from the China Medical University, Taichung, Taiwan.

References

- 1 Tan TW, Tsai HY, Chung JG, Hsueh WT and Chiu NY: Analgesic and anti-inflammatory effects of *Ampelopsis cantoniensis*. *China Med Coll J* 6: 129-136, 1997.
- 2 Tan TW, Tsai HY, Chung JG and Hsueh WT: Effects of crude extract from *Ampelopsis cantoniensis* on arylamine N-acetyltransferase in activity in rat blood and liver tissue. *China Med Coll J* 7: 55-62, 1998.
- 3 Jaroszeski MJ *et al*: *Flow Cytometry Protocols*: Humana Press. pp.77-80, 1998.
- 4 Su CC, Chen GW, Yeh CC, Yang MD, Hung CF and Chung JG: Luteolin induces N-acetylation and DNA adduct of 2-aminofluorene accompanying N-acetyltransferase activity and gene expression in human bladder cancer T24 cell line. *Anticancer Res* 23: 355-362, 2003.
- 5 Lee YM, Wu TH, Chen SF and Chung JG: Effect of 5-methoxypsoralen (5-MOP) on cell apoptosis and cell cycle in human hepatocellular carcinoma cell line. *Toxicol In Vitro* 17: 279-287, 2003.
- 6 Ormerod MG *et al*: *Flow Cytometry: A Practical Approach*. Oxford University Press. pp. 69-81, 1990.
- 7 Chiu TH, Chen JC and Chung JG: N-acetyltransferase is involved in gypenosides-induced N-acetylation of 2-aminofluorene and DNA adduct formation in human cervix epidermoid carcinoma cells (Ca Ski). *In Vivo* 17: 281-288, 2003.
- 8 Kuida k, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P and Flavell RA: Reduced apoptosis and cytochrome-c-mediated caspase activation in mice lacking caspase 9. *Cell* 94: 325-337, 1998.
- 9 Uehara T, Kikuchi Y and Nomura Y: Caspase activation accompanying cytochrome c release from mitochondria is possibly involved in nitric oxide-induced neuronal apoptosis in SH-SY5Y cells. *J Neurochem* 72: 196-205, 1999.
- 10 Chen HC, Hsieh WT, Chung WC and Chung JG: Aloe-emodin induced *in vitro* G2/M arrest of cell cycle in human promyelocytic leukemia HL-60 cells. *In Vivo* (in press), 2004.
- 11 Buolamwini JK: Cell cycle molecular targets in novel anticancer drug discovery. *Curr Pharmaceut Des* 6: 379-392, 2000.
- 12 Hajduch M, Havlieek L, Vesely J, Novotny R, Mihal V and Strnad M: Synthetic cyclin dependent kinase inhibitors. New generation of potent anti-cancer drugs. *Adv Exp Med Biol* 457: 341-353, 1999.
- 13 Sherr CJ: Cancer cell cycles. *Science* 274: 1672-1677, 1996.
- 14 Ahmad N, Feyes DK, Agarwal R and Mukhtar H: Photodynamic therapy results in induction of WAF1/CIP1/P21 leading to cell cycle arrest and apoptosis. *Proc Nat Acad Sci USA* 95: 6977-82, 1998.
- 15 Sugikawa E, Hosoi T, Yazaki N, Gamanuma M, Nakanishi N and Ohashi M: Mutant p53 mediated induction of cell cycle arrest and apoptosis at G1 phase by 9-hydroxyellipticine. *Anticancer Res* 19: 3099-3108, 1999.
- 16 Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogasawara S, Momosaki S and Kojiro M: The herbal medicine sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. *Cancer Res* 54: 448-454, 1994.
- 17 Brunet CL, Gunby RH, Benson RS, Hickman JA, Watson AJ and Brady G: Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptotic markers. *Cell Death Diff* 5: 107-115, 1998.
- 18 Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251-306, 1980.
- 19 Salvesen GS and Dixit VM: Caspase activation: the induced-proximity model. *Proc Nat Acad Sci USA* 96: 10964-10967, 1999.

Received November 24, 2004

Revised March 15, 2004

Accepted May 13, 2004