Safrole Induces G_0/G_1 Phase Arrest *via* Inhibition of Cyclin E and Provokes Apoptosis through Endoplasmic Reticulum Stress and Mitochondrion-dependent Pathways in Human Leukemia HL-60 Cells

CHUN-SHU YU[1], AN-CHENG HUANG[2], JAI-SING YANG[3], CHIEN-CHIH YU[1], CHIN-CHUNG LIN[4,5], HSIUNG-KWANG CHUNG[6,7], YI-PING HUANG[8], FU-SHIN CHUEH[9*] and JING-GUNG CHUNG[10,11*]

[1]School of Pharmacy, Departments of [3]Pharmacology, [8]Physiology, [10]Biological Science and Technology, [7]Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan, R.O.C.; [2]Department of Nursing, St. Mary's Medicine Nursing and Management College, Yilan, Taiwan, R.O.C.; [4]Department of Chinese Medicine, Fong-Yuan Hospital, Department of Health, Executive Yuan, Taichung, Taiwan, R.O.C.; [5]Department of Nursing, Hung Kung University, Taichung, Taiwan, R.O.C.; [6]Department of Otorhinolaryngology-Head and Neck Surgery, China Medical University Hospital, Taichung, Taiwan, R.O.C.; Departments of [9]Health and Nutrition Biotechnology, and [11]Biotechnology, Asia University, Taichung, Taiwan, R.O.C.

Abstract. *Safrole, a component of Piper betle inflorescence, is a carcinogen which has been demonstrated to induce apoptosis on human oral cancer HSC-3 cells in vitro and to inhibit HSC-3 cells in xenograft tumor cells in vivo. In our previous study, safrole promoted phagocytosis by macrophages and natural killer cell cytotoxicity in normal BALB/c mice. The cytotoxic effects of safrole on HL-60 cells were investigated by using flow cytometric analysis, comet assay, 4',6-diamidino-2-phenylindole (DAPI) staining, western blotting and confocal laser microscopy. The obtained results indicate that safrole induced a cytotoxic response through reducing the percentage of viable cells and induction of apoptosis in HL-60 cells in a dose-dependent manner. DAPI staining and comet assay also showed that safrole induced apoptosis (chromatin condensation) and DNA damage in HL-60 cells. The flow cytometric assay showed that safrole increased the production of reactive oxygen species (ROS) and Ca^{2+} and reduced the mitochondrial membrane potential in HL-60 cells. Safrole enhanced the levels of the pro-apoptotic protein BAX, inhibited those of the anti-apoptotic protein BCL-2 and promoted the levels of apoptosis-inducing factor (AIF) and endonuclease G (Endo G) in HL-60 cells. Furthermore, safrole promoted the expression of glucose-regulated protein 78 (GRP78), growth arrest- and DNA damage-inducible gene 153 (GADD153) and of activating transcription factor 6α (ATF-6α). Based on these findings, we suggest that safrole-induced apoptosis in HL-60 cells is mediated through the ER stress and intrinsic signaling pathways.*

Chewing betel quid containing *Piper betle* inflorescence reportedly generates a high concentration of safrole (420 μM) in the saliva (1). Safrole can bind to DNA and generate safrole-DNA adducts (2-4), causes marked increase in intracellular Ca^{2+} and reduces the cell viability of human osteosarcoma cells (5). Additionally, safrole increased the levels of Ca^{2+} release from the endoplasmic reticulum (ER) in Madin-Darby canine kidney (MDCK) cells (6). Recently, in our laboratory, we have found that safrole induced apoptosis in human oral cancer HSC-3 cells *in vitro* through a mitochondria-dependent pathway and reduced the HSC-3 cells in a xenograft mouse model *in vivo* (7). Although safrole is a group 2B carcinogen and a documented rodent carcinogen (8), there is no adequate evidence to show that

*These Authors contributed equally to this study.

Correspondence to: Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. Tel: +886 422053366 ext. 2161, Fax: +886 422053764, e-mail: jgchung@mail.cmu.edu.tw and Fu-Shin Chueh, Department of Health and Nutrition Biotechnology, Asia University, No. 500, Lioufeng Road, Taichung 41354, Taiwan, R.O.C. Tel: +886 423323456 ext 1848, Fax: +886 423321126, e-mail: fushin@asia.edu.tw

Key Words: Safrole, human leukemia HL-60 cells, apoptosis, ER stress, mitochondria, HSC-3 cells.

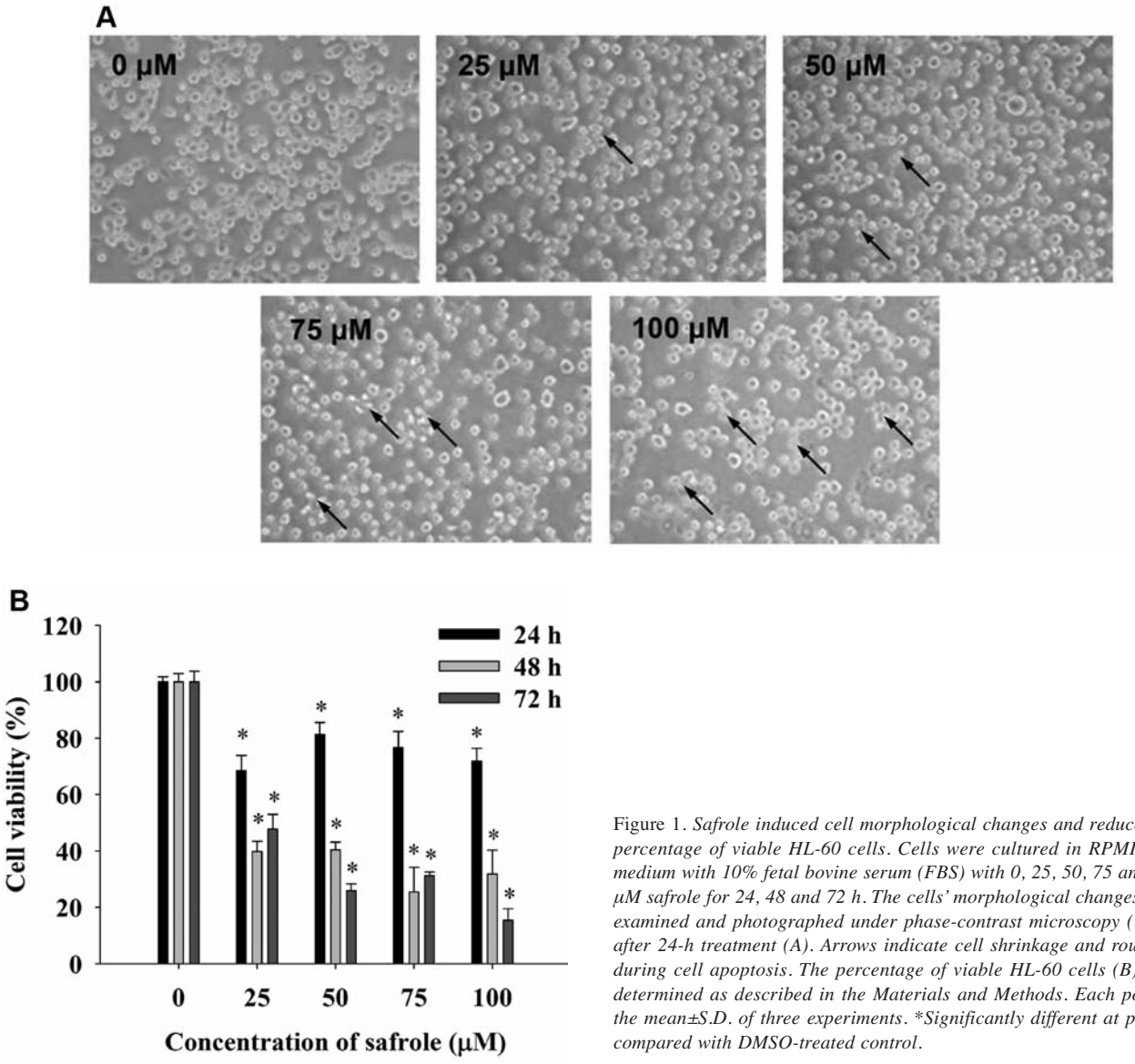

Figure 1. *Safrole induced cell morphological changes and reduced the percentage of viable HL-60 cells. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) with 0, 25, 50, 75 and 100 μM safrole for 24, 48 and 72 h. The cells' morphological changes were examined and photographed under phase-contrast microscopy (×200) after 24-h treatment (A). Arrows indicate cell shrinkage and rounding during cell apoptosis. The percentage of viable HL-60 cells (B) were determined as described in the Materials and Methods. Each point is the mean±S.D. of three experiments. *Significantly different at p<0.05 compared with DMSO-treated control.*

exposure to safrole can induce human cancer cell apoptosis. We investigated whether or not safrole could induce apoptosis in human leukemia HL-60 cells, because there is no available information regarding its cytotoxic effects on human leukemia cells.

Materials and Methods

Materials and chemicals. Safrole, dimethyl sulfoxide (DMSO), propidium iodide (PI), trypan blue and Triton X-100 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Culture medium and supplements were obtained from Gibco Life Technologies (Carlsbad, CA, USA). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The fluorescent probes 4',6-diamidino-2-phenylindole (DAPI), 2',7'-dichlorofluorescin diacetate (H_2DCF-DA), 1H-indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl] amino]-3-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester (Indo-1/AM) and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes Life Technologies.

Cell line. The human leukemia cell line (HL-60) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC), and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in an incubator with a 5% CO_2 and 95% air (9, 10).

Morphological changes and viable cell examinations. Cells were seeded on 24-well plates at the density of 2×10^5 viable cells/well, and were treated with 0, 25, 50, 75 and 100 μM safrole, or with a vehicle (DMSO, 0.1% in culture media), and all cells were incubated for 24, 48 and 72 h. For the examination of morphological changes, cells in the wells were photographed under a phase-contrast microscope. For the determination of the percentage of viable cells, a PI exclusion method and a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) equipped with BD CellQuest Pro software were used, as previously described (11-13).

Determination of cell cycle distribution and apoptosis. Cells (2×10^5/well) seeded on 24-well plates, were treated with or without 0, 25, 50, 75 and 100 μM safrole, or with vehicle (DMSO, 0.1% in culture media) for 24 h. Cells from each treatment were harvested and gently fixed with 70% ethanol at –20˚C overnight. Then cells were washed twice with phosphate buffered saline (PBS) and were then incubated with 20 μg/ml PI, 100 μg/ml RNase and 0.1% Triton X-100 in PBS for 30 min in the dark. Finally, the PI-stained cells were analyzed for cell cycle distribution and sub-G_1 phase population (apoptosis) by using flow cytometry, as described elsewhere (11, 14).

DAPI staining and comet assay for apoptosis and DNA damage. Cells placed in 24-well plates at a density of 2×10^5 viable cells/well were treated with or without 0, 25, 50, 75 and 100 μM safrole, or with vehicle (DMSO, 0.1% in culture media) and all cells were incubated for 24 h. Cells in each treatment were individually fixed with 4% formaldehyde (Sigma-Aldrich Corp.) for 15 min and were stained with DAPI (Molecular Probes Life Technologies) for nucleic acid condensation (apoptosis), as described elsewhere (15, 16). The examined cells were prepared as previously described (16, 17) for the comet assay, and the DNA was stained by PI in order to determine the DNA tail in safrole-treated HL-60 cells. All samples were photographed on a fluorescence microscope (9, 10).

Assays for mitochondrial membrane potential ($\Delta\Psi m$), reactive oxygen species (ROS) and cytosolic Ca^{2+} release. Cells were seeded on 24-well plates at a density of 2×10^5 cells/well and treated with or without 75 μM safrole, or with vehicle (DMSO, 0.1% in culture media) and all cells were incubated for 0, 0.5, 1, 3, 6, 12 or 24 h to determine the level of $\Delta\Psi_m$, as well as the production of ROS and cytosolic Ca^{2+}. All cells in each treatment were harvested, washed twice by PBS, then re-suspended in 500 μl of $DiOC_6$ (1 μmol/l) for $\Delta\Psi_m$, in 500 μl of H_2DCF-DA (10 μM) for ROS and in 500 μl of Indo-1/AM (3 μg/ml) for cytosolic Ca^{2+} production at 37˚C in a dark room for 30 min. Then all samples were analyzed immediately by flow cytometry, as previously described (18-20).

Western blotting analysis for the changes of apoptosis-associated protein levels. Cells at a density of 1×10^6 cells/well were exposed to 75 μM safrole, or vehicle (DMSO, 0.1% in culture media) and all cells were incubated for 0, 6, 12, 24, 48 and 72 h. Then cells were harvested and washed twice with PBS for the determination of protein levels [cyclin D and E, cyclin-dependent kinase 6 (CDK6), p16, p27, p57, checkpoint kinase 2 (CHK2), B-cell lymphoma 2 (BCL-2), BCL-2-associated X protein (BAX), apoptotic protease activating factor-1 (APAF-1), cytochrome *c*, apoptosis-inducing factor (AIF), X-linked inhibitor of apoptosis protein (XIAP), caspase-9, fatty acid synthase (FAS), caspase-3, caspase-8, poly (ADP-ribose) polymerase (PARP), glucose-regulated protein 78 (GRP78), growth arrest- and DNA damage-inducible gene 153 (GADD153) and activating transcription factor-4 (ATF-4) and ATF6-α] associated with G_0/G_1 arrest and of apoptosis, which were determined by western blotting. Cells from each treatment were lysed by using the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea), as described previously (13, 21, 22). Samples containing equal amounts of protein (40 μg) from the lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 8.0) for 1 h at 25˚C, prior to incubation with the specific

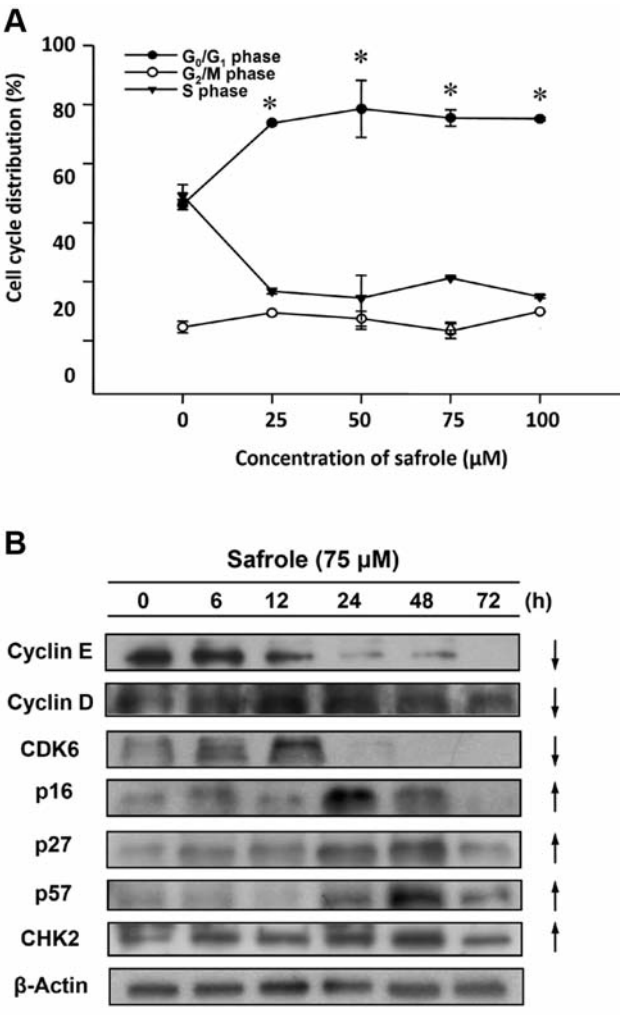

Figure 2. *Safrole affected the cell cycle distribution and the levels of the associated proteins in HL-60 cells. Cells were cultured with 0, 25, 50, 75 and 100 μM safrole for 24 h. The cells were examined and analyzed for cell cycle distribution (A) and the associated protein levels (B) by flow cytometry and western blotting as, described in the Materials and Methods. Each point is the mean±S.D. of three experiments. *Significantly different at p<0.05 compared with DMSO-treated control.*

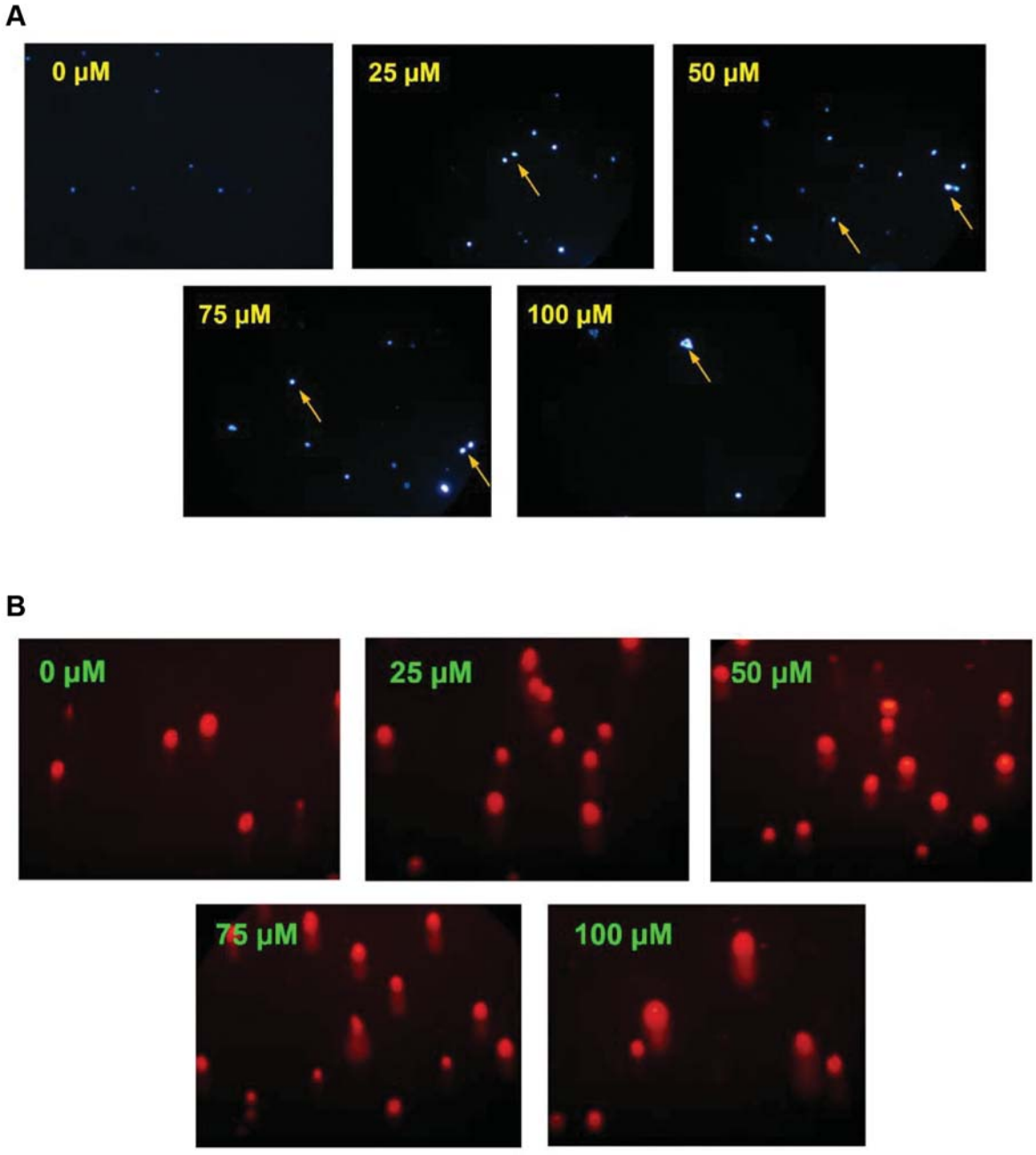

Figure 3. *Safrole induced apoptosis and DNA damage in HL-60 cells. Cells were incubated with 0, 25, 50, 75 and 100 μM safrole for 24 h and then were harvested and were examined for apoptosis by DAPI staining (A) and for DNA damage by the comet assay (B), as described in the Materials and Methods. Results are representative of three independent experiments.*

antibodies overnight at 4˚C. Then they were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies that were detected by the enhanced chemiluminescence (ECL) reagent kit (Immobilon Western HRP substrate, Millipore) and then with autoradiography using X-ray film (GE Healthcare, Piscataway, NJ, USA) (14, 20, 23). For ensuring equal protein amounts, each membrane was re-probed with anti-β-Actin antibody.

Confocal laser scanning microscopy for protein translocation. Cells were seeded in 4-well chamber slides at a density of 2×10^4 cells and then treated or not with 75 μM safrole for 24 h. Cells in each chamber were fixed in 4% formaldehyde in PBS for 15 min, and where then permeabilized with 0.3% Triton-X 100 in PBS for 1 h. The fixed cells were stained with antibodies against AIF and GADD153 (1:100 dilution) overnight before being washed twice

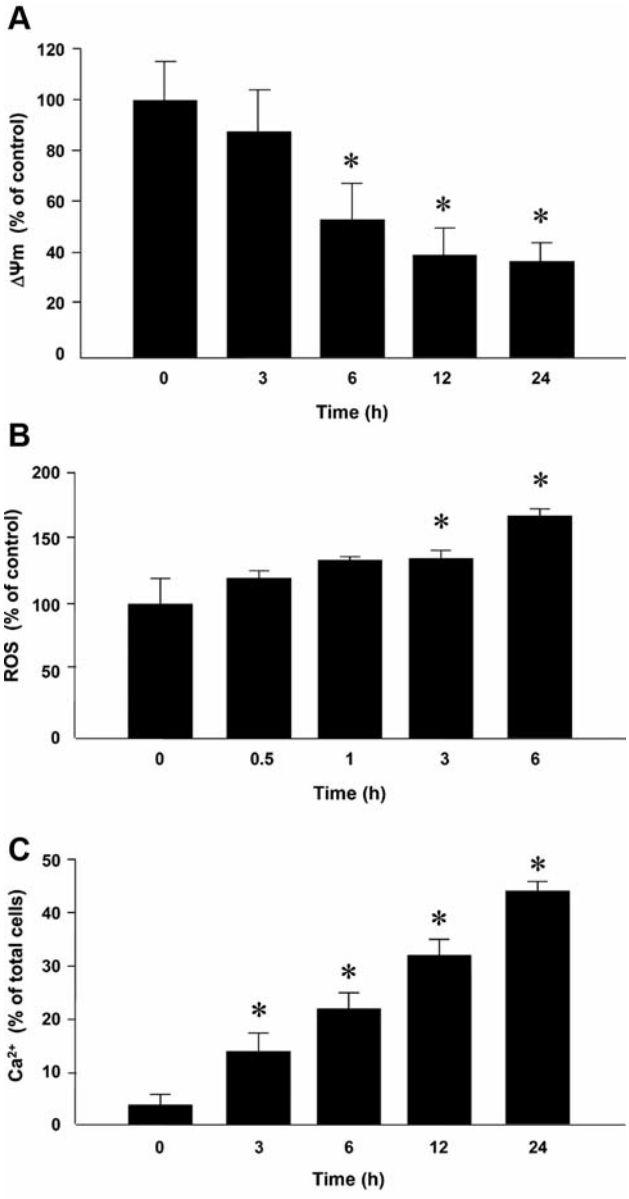

Figure 4. *Safrole altered the mitochondria membrane potential (ΔΨm), and the levels of reactive oxygen species (ROS) and Ca^{2+} in HL-60 cells. Cells were treated with 75 μM safrole for indicated periods of time before being collected, and stained with $DiOC_6$ (1 μmol/l) for ΔΨm (A), H_2DCF-DA (10 μM) for ROS (B) and Indo-1/AM (3 μg/ml) for cytosolic Ca^{2+} (C), as described in the Materials and Methods. Each experiment was carried out with triplicate sets mean±S.D.: *Significantly different at p<0.05 compared with DMSO-treated control.*

with PBS, and were then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (green fluorescence) at 1:100 dilution, followed by DNA staining with PI (red fluorescence), as previously described (14, 16). All samples were examined and photomicrographed by the use of a Leica TCS SP2 Confocal Spectral Microscope (19, 24).

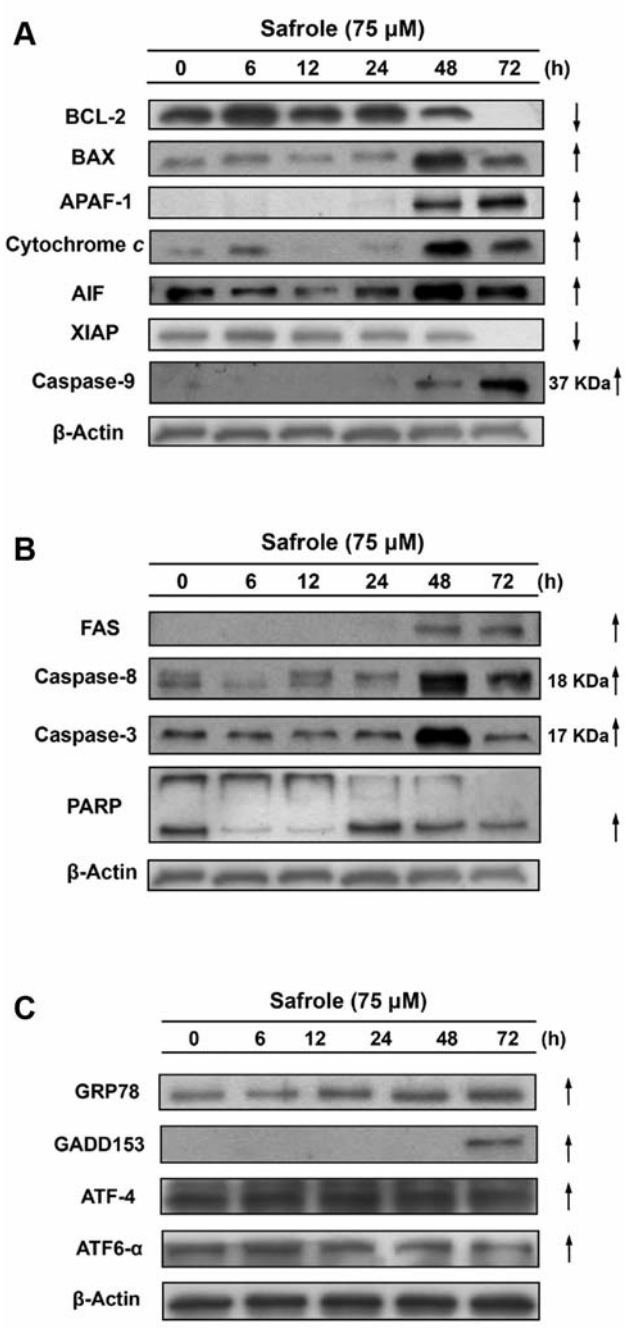

Figure 5. *Representative western blotting showing changes in the levels of apoptosis and endoplasmic reticulum (ER) stress-associated proteins in HL-60 cells after exposure to safrole. Cells were treated with 75 μM safrole for 0, 6, 12, 24, 48 and 72 h before total proteins were prepared and determined. The levels of apoptosis-related protein expressions were estimated by western blotting analysis, as described in the Materials and Methods. BCL-2 (B-cell lymphoma 2), BAX (BCL-2-associated X protein), APAF-1 (apoptotic protease activating factor-1), cytochrome c, AIF (apoptosis-inducing factor), XIAP (X-linked inhibitor of apoptosis protein), caspase-9, FAS (fatty acid synthase), caspase-3, caspase-8, PARP (poly (ADP-ribose) polymerase), GRP78 (glucose-regulated protein 78), GADD153 (growth arrest- and DNA damage-inducible gene 153) and ATF-4 (activating transcription factor-4) and ATF6-α.*

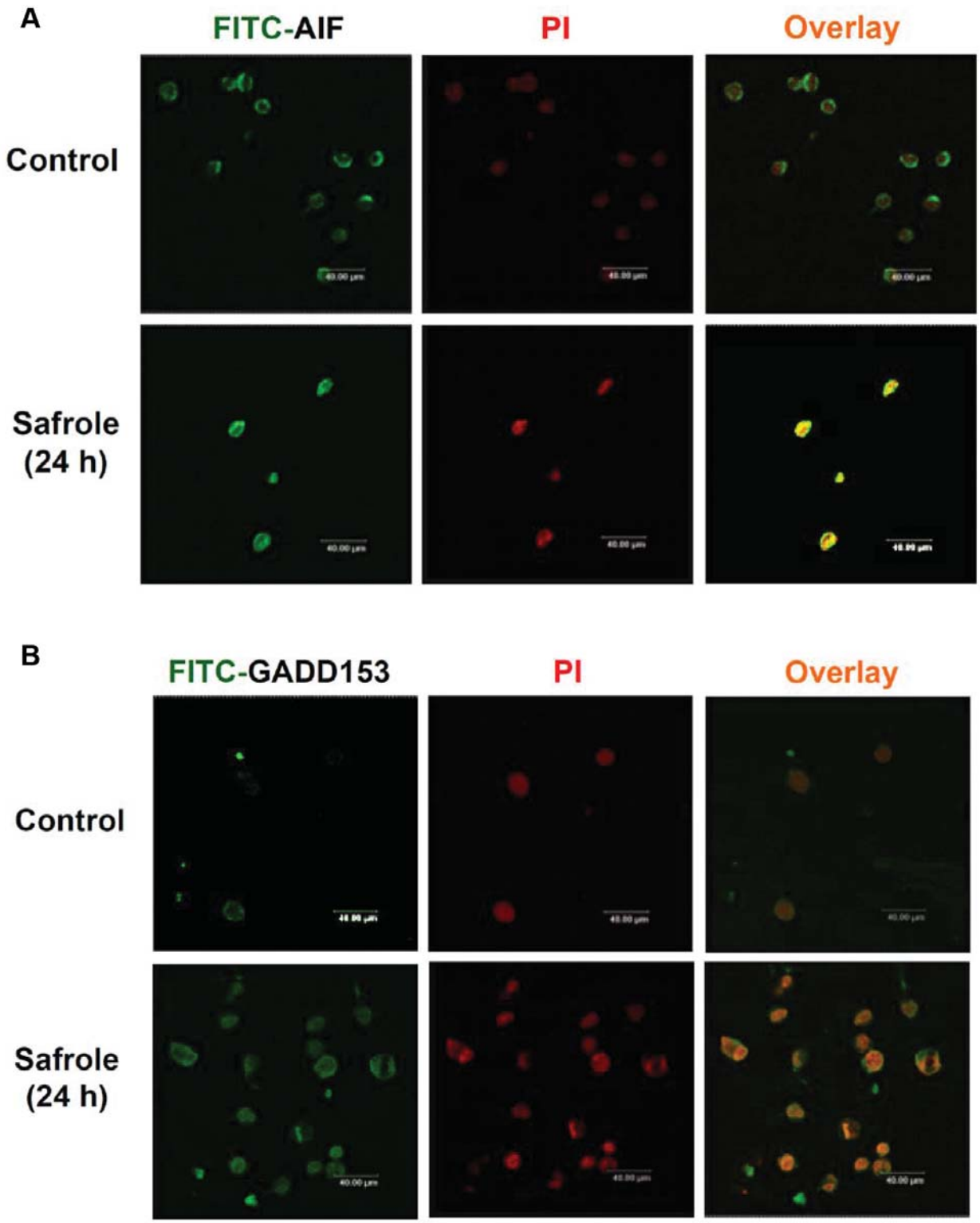

Figure 6. *Safrole treatment resulted in translocation of apoptosis-inducing factor (AIF) and growth arrest- and DNA damage-inducible gene 153 (GADD153) in HL-60 cells. Cells were incubated with or without 75 μM safrole for 24 h, and then were fixed and stained with anti-AIF (A) and GADD153 (B) antibodies before the fluorescein isothiocyanate (FITC)-labeled secondary antibodies were used (green fluorescence) and then they were detected by a confocal laser microscope. The nuclei were stained by PI (red fluorescence). Areas of co-localization of AIF and GADD153 expression, respectively, and nuclei in the merged panels are yellow. Scale bar, 40 μm.*

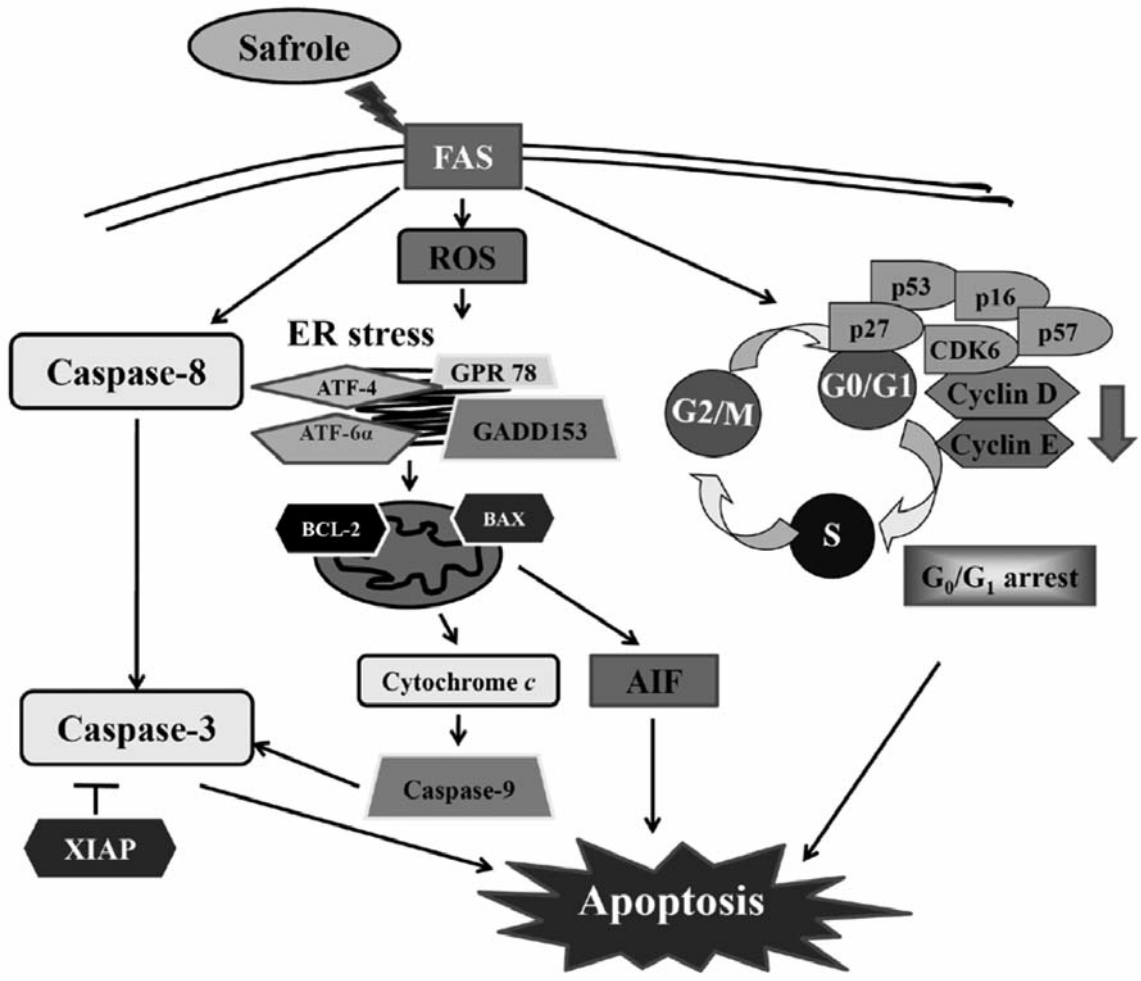

Figure 7. *The proposed model of possible signaling pathways for human leukemia HL-60 cells after exposure to safrole.*

Statistical analysis. The quantitative data are presented as the mean±SD. The statistical differences between the safrole-treated and control samples were calculated by the Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

Results

Safrole induced morphological changes and reduced the percentage of viable HL-60 cells. Cells were treated with or without various doses of safrole for 24, 48 and 72 h, and the induced morphological changes were examined and photographed under a phase-contrast microscopy. The results shown in Figure 1A indicate that safrole induced morphological changes such as membrane blebbing and cell shrinkage in a dose-dependent manner. Cells were harvested for determining the percentage of viable cells from each treatment by the PI exclusion method. Results are presented in Figure 1B, indicating that safrole decreased the percentage of viable HL-60 cells.

Safrole induced cell cycle arrest and apoptosis of HL-60 cells. Cells were treated with different concentrations of safrole for 24 h and were then isolated for determining the cell cycle distribution. The results shown in Figure 2A indicate that the number of cells in each stage of the cell cycle was expressed as a percentage that of the total number of cells. The results indicate that safrole induced G_0/G_1 phase arrest in HL-60 cells (Figure 2A).

Safrole induced chromatin condensation and DNA damage in HL-60 cells. Cells were treated with different doses of safrole for 24 h and were then harvested for determining the apoptosis by DAPI staining and the DNA damage by the comet assay and results are shown in Figure 3A and B, respectively. DAPI staining assay demonstrated that safrole induced chromatin condensation (apoptosis) (Figure 3A). The comet assay demonstrated that the treatment with safrole (25-100 μM) for 24 h induced DNA damage (Figure 3B).

Safrole affected the mitochondria membrane potential ($\Delta\Psi_m$) and levels of ROS and cytosolic Ca^{2+} in HL-60 cells. Cells were treated with 75 μM safrole for different periods of time, and were collected to measure $\Delta\Psi_m$, the levels of ROS and cytosolic Ca^{2+} release. The results in Figure 4A, B and C, respectively, show that there was a significant decrease in $\Delta\Psi_m$ (Figure 4A) and an increase in intracellular ROS (Figure 4B) and cytosolic Ca^{2+} levels (Figure 4C) in the safrole-treated cells, as compared to the untreated cells. Safrole significantly decreased the levels of $\Delta\Psi_m$ in HL-60 cells after 6-h exposure (Figure 4A). After 3-h treatment with safrole, the levels of cytosolic Ca^{2+} significantly increased (Figure 4B). In addition, the 3- and 6-h treatment with safrole led to significantly increased ROS levels (Figure 4C).

Safrole affected the levels of G_0/G_1 phase-, apoptosis- and ER stress-associated proteins levels in HL-60 cells. Cells were treated with 75 μM safrole for 0, 6, 12, 24, 48 and 72 h and were then isolated for determinations for G_0/G_1 phase, apoptosis- and ER-associated protein levels; the results are shown in Figures 2B and 5. Figure 2B shows that safrole inhibited the expression of cyclins D and E, and CDK6, but it promoted the expression of p16, p27, p57 and CHK2, which may lead to G_0/G_1 phase arrest. The results also indicated that safrole inhibited the expression of BCL-2, XIAP, and procaspase-9 but promoted the expression of BAX, APAF-1, cytochrome c (Figure 5A) and FAS, caspase-3 and -8 (Figure 5B), which are involved in apoptosis, in HL-60 cells. Furthermore, safrole also promoted the expression of GRP78, GADD153, ATF4 and ATF-6α (Figure 5C), which are involved in ER stress in HL-60 cells.

Safrole promoted translocation of AIF and GADD153 in HL-60 cells. Cells were treated with 75 μM safrole for 24 h and then the location of AIF and GADD153 was determined. Results shown in Figure 6 indicate that safrole induced the translocation of AIF (Figure 6A) and of GADD153 (Figure 6B) to nuclei, leading to cell apoptosis.

Discussion

Our previous study indicated that safrole could induce apoptosis on human oral cancer cells (7). There is no report to show that safrole induced cell cycle arrest and apoptosis in human leukemia cells. In this study, we showed that safrole triggered apoptosis in HL-60 cells through the ER stress-associated signaling and mitochondria-dependent pathways based on these observations: i) safrole induced cell morphological changes and reduced the percentage of viable HL-60 cells; ii) safrole induced G_0/G_1 phase arrest in HL-60 cells; iii) Safrole induced apoptosis of HL-60 cells dependent on ER stress activation and mitochondria-dependent pathways; iv) safrole promoted the expression of BAX protein and inhibited the expression of BCL-2; v) the biological effects of safrole on HL-60 cells seem to be explained, at least in part, through the ER stress, including promotion of the expression of GRP78, GADD153, ATF-6α and ATF4. Collectively, these findings indicate that the effects of safrole in leukemia may be worthy of further study.

In the present study, our analyses showed that safrole induced a significant increase in BAX (a pro-apoptotic protein) (14) and reduced the levels of BCL-2 (an anti-apoptotic protein) (16) in HL-60 cells, which may have led to the decrease of mitochondrial membrane potential and we suggest that safrole induced apoptosis through the mitochondria-dependent pathway. It is well known that anticancer agents can induce apoptosis through mitochondria-dependent pathways (25). It has also been reported that dysfunction of mitochondria can be induced (reduction of mitochondrial membrane potential) mediated by BAX/BCL2 (14). Our results clearly indicate that safrole increased the BAX/BCL-2 ratio, which may have led to the induction of apoptosis by safrole in HL-60 cells.

Our results also showed that safrole-induced apoptosis may occur through the ER stress pathway based on the production of ROS and the increase of hallmarkers of ER stress such as GRP78 and GADD153 in HL-60 cells (Figure 5C). Therefore, we present the first findings to show that safrole induced apoptosis in human leukemia HL-60 cells through the ER stress signaling pathway.

In conclusion, the present study demonstrated that safrole can induce cytotoxic effects in human leukemia HL-60 cells *in vitro*. Safrole triggered apoptosis *via* ER stress and mitochondria-dependent signaling pathways. These possible signaling pathways are summarized in Figure 7.

Acknowledgements

This study was supported by the grant CMU100-TC-10 from China Medical University, Taichung, Taiwan.

References

1 Wang CK and Hwang LS: Phenolic compounds of betel quid chewing juice. Food Sci *20*: 458-471, 1993.

2 Lee JM, Liu TY, Wu DC, Tang HC, Leh J, Wu MT, Hsu HH, Huang PM, Chen JS, Lee CJ and Lee YC: Safrole-DNA adducts in tissues from esophageal cancer patients: clues to areca-related esophageal carcinogenesis. Mutat Res *565*: 121-128, 2005.

3 Daimon H, Sawada S, Asakura S and Sagami F: *In vivo* genotoxicity and DNA adduct levels in the liver of rats treated with safrole. Carcinogenesis *19*: 141-146, 1998.

4 Daimon H, Sawada S, Asakura S and Sagami F: Analysis of cytogenetic effects and DNA adduct formation induced by safrole in Chinese hamster lung cells. Teratog Carcinog Mutagen *17*: 7-18, 1997.

5 Lin HC, Cheng HH, Huang CJ, Chen WC, Chen IS, Liu SI, Hsu SS, Chang HT, Huang JK, Chen JS, Lu YC and Jan CR: Safrole-induced cellular Ca^{2+} increases and death in human osteosarcoma cells. Pharmacol Res *54*: 103-110, 2006.

6 Chen WC, Cheng HH, Huang CJ, Lu YC, Chen IS, Liu SI, Hsu SS, Chang HT, Huang JK, Chen JS and Jan CR: The carcinogen safrole increases intracellular free Ca^{2+} levels and causes death in MDCK cells. Chin J Physiol *50*: 34-40, 2007.
7 Yu FS, Yang JS, Yu CS, Lu CC, Chiang JH, Lin CW and Chung JG: Safrole induces apoptosis in human oral cancer HSC-3 cells. J Dent Res *90*: 168-174, 2011.
8 IARC monographs on the evaluation of the carcinogenic risk of chemicals to man: some naturally occurring substances. IARC Monogr Eval Carcinog Risk Chem Man *10*: 1-342, 1976.
9 Su CC, Yang JS, Lin SY, Lu HF, Lin SS, Chang YH, Huang WW, Li YC, Chang SJ and Chung JG: Curcumin inhibits WEHI-3 leukemia cells in BALB/c mice *in vivo*. In Vivo *22*: 63-68, 2008.
10 Yu FS, Wu CC, Chen CT, Huang SP, Yang JS, Hsu YM, Wu PP, Ip SW, Lin JP, Lin JG and Chung JG: Diallyl sulfide inhibits murine WEHI-3 leukemia cells in BALB/c mice *in vitro* and *in vivo*. Hum Exp Toxicol *28*: 785-790, 2009.
11 Tan TW, Tsai HR, Lu HF, Lin HL, Tsou MF, Lin YT, Tsai HY, Chen YF and Chung JG: Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells via MMP changes and caspase-3 activation. Anticancer Res *26*: 4361-4371, 2006.
12 Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL, Lu HF, Lee TH, Wood WG, Chung JG: Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res *54*: 967-976, 2010.
13 Kao ST, Yeh CC, Hsieh CC, Yang MD, Lee MR, Liu HS and Lin JG: The Chinese medicine Bu-Zhong-Yi-Qi-Tang inhibited proliferation of hepatoma cell lines by inducing apoptosis via G_0/G_1 arrest. Life Sci *69*: 1485-1496, 2001.
14 Chiu TH, Lai WW, Hsia TC, Yang JS, Lai TY, Wu PP, Ma CY, Yeh CC, Ho CC, Lu HF, Wood WG and Chung JG: Aloe-emodin induces cell death through S-phase arrest and caspase-dependent pathways in human tongue squamous cancer SCC-4 cells. Anticancer Res *29*: 4503-4511, 2009.
15 Yang JS, Hour MJ, Huang WW, Lin KL, Kuo SC and Chung JG: MJ-29 inhibits tubulin polymerization, induces mitotic arrest, and triggers apoptosis via cyclin-dependent kinase 1-mediated Bcl-2 phosphorylation in human leukemia U937 cells. J Pharmacol Exp Ther *334*: 477-488, 2010.
16 Chiang LC, Ng LT, Lin IC, Kuo PL and Lin CC: Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett *237*: 207-214, 2006.
17 Chen YY, Chiang SY, Lin JG, Yang JS, Ma YS, Liao CL, Lai TY, Tang NY and Chung JG: Emodin, aloe-emodin and rhein induced DNA damage and inhibited DNA repair gene expression in SCC-4 human tongue cancer cells. Anticancer Res *30*: 945-951, 2010.
18 Lin ML, Lu YC, Chung JG, Li YC, Wang SG, N GS, Wu CY, Su HL and Chen SS: Aloe-emodin induces apoptosis of human nasopharyngeal carcinoma cells via caspase-8-mediated activation of the mitochondrial death pathway. Cancer Lett *291*: 46-58, 2010.
19 Lo C, Lai TY, Yang JH, Yang JS, Ma YS, Weng SW, Chen YY, Lin JG and Chung JG: Gallic acid induces apoptosis in A375.S2 human melanoma cells through caspase-dependent and -independent pathways. Int J Oncol *37*: 377-385, 2010.
20 Chiang JH, Yang JS, Ma CY, Yang MD, Huang HY, Hsia TC, Kuo HM, Wu PP, Lee TH and Chung JG: Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways. Chem Res Toxicol *24*: 20-29, 2011.
21 Chung JG, Yang JS, Huang LJ, Lee FY, Teng CM, Tsai SC, Lin KL, Wang SF and Kuo SC: Proteomic approach to studying the cytotoxicity of YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice. Proteomics *7*: 3305-3317, 2007.
22 Ip SW, Liao SS, Lin SY, Lin JP, Yang JS, Lin ML, Chen GW, Lu HF, Lin MW, Han SM and Chung JG: The role of mitochondria in bee venom-induced apoptosis in human breast cancer MCF7 cells. In Vivo *22*: 237-245, 2008.
23 Harikumar KB, Kuttan G and Kuttan R: Phyllanthus amarus inhibits cell growth and induces apoptosis in Dalton's lymphoma ascites cells through activation of caspase-3 and downregulation of Bcl-2. Integr Cancer Ther *8*: 190-194, 2009.
24 Wu SH, Hang LW, Yang JS, Chen HY, Lin HY, Chiang JH, Lu CC, Yang JL, Lai TY, Ko YC and Chung JG: Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. Anticancer Res *30*: 2125-2133, 2010.
25 Soto-Cerrato V, Llagostera E, Montaner B, Scheffer GL and Perez-Tomas R: Mitochondria-mediated apoptosis operating irrespective of multidrug resistance in breast cancer cells by the anticancer agent prodigiosin. Biochem Pharmacol *68*: 1345-1352, 2004.

Received February 29, 2012
Revised April 3, 2012
Accepted April 4, 2012