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Induction of Apoptosis by the *Lactuca indica* L. in Human Leukemia Cell Line and Its Active Components

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Lactuca indica L. (Compositae family) is used as a folk medicine in anti-inflammatory, antibacterial, antidiabetic, and other medications in Asia. The objectives of this study were to evaluate the antiproliferative effect of ethanol extracts of *Lactuca indica* L. (EEL) on human leukemic HL-60 cell lines and its active components. The results showed that EEL exhibited strong cytotoxic effects against HL-60 cells; the IC_{50} value was 313 μg/mL. Flow cytometric analysis of the externalization of phosphatidylserine (PS) using the annexin V/PI method on EEL-treated HL-60 cells showed a concentration-dependent increase of apoptosis. Moreover, EEL could induce typical DNA fragmentation in a concentration- and time-dependent manner as determined by electrophoresis and TUNEL assays. The treatment of HL-60 cells with EEL induced significant accumulation of cells in the G_0/G_1 phase, indicating that EEL is a cell-cycle-dependent anticancer agent. Our results also indicate that EEL-induced apoptosis in HL-60 cells is associated with the loss of mitochondrial membrane potential ($\Delta\Psi_m$). EEL contains 5% phenolic compounds, such as quercetin, caffeic acid, rutin, and chlorogenic acid. Among the four active phenolic compounds, quercetin was found to be the most effective in inhibition against cell viability and in alteration of mitochondrial function. Our results suggest that the induction of apoptosis by EEL might offer a pivotal mechanism for its chemopreventive action.

KEYWORDS: ***Lactuca indica* L.; HL-60 cell; apoptosis; quercetin**

INTRODUCTION

Recent evidence from epidemiological studies has shown that diets rich in fruits and vegetables might reduce the risk of many chronic diseases, including cancer (*1*). Natural phytochemicals such as polyphenolic compounds might contribute to this effect. The chemopreventive effects of polyphenolics have been linked not only to their antioxidant activity but also to their ability to mediate other physiological functions related to cancer suppression, such as antiproliferation and induction of apoptosis (*2*).

In general, most antitumor agents act by inducing apoptosis in tumor cells. Many therapeutic agents for cancer, such as cisplatin (*3*), isothiocyanate (*4*), and adriamycin (*5*), have been reported to eliminate tumor cells by inducing apoptotic cell death. In addition, some flavonoids, such as quercetin, apigenin, and phloretin, also inhibit cancer cell growth through the induction of apoptosis (*6*). In recent years, rapid advances have been made in delineating the molecular mechanisms underlying apoptosis or programmed cell death. Apoptosis is a highly regulated process that involves activation of a series of molecular events leading to cell death, which is characterized by cellular morphological change, chromatin condensation, and apoptotic bodies that are associated with DNA cleavage into ladders (*7*). Apoptosis is initiated either by activation of the tumor necrosis factor (TNF) receptor superfamily at the cell membrane or through other intrinsic signaling pathways such as DNA damage in the nucleus (*8*). Activation of a family of cysteine proteases named caspases, which cleave a variety of cellular substrates that contribute to detrimental biochemical and morphological changes and eventual cell destruction, has been shown to play a vital role in the initiation and execution phases of many models of apoptosis (*9*). Mitochondria also play a pivotal role in activating the caspase cascade in most such pathways, for example, the intrinsic signaling pathway. A central role for mitochondria in the regulation of chemical-induced apoptosis has been demonstrated (*10*). Consequently, understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases.

Researchers have been looking for antitumor agents in natural products to develop novel therapeutic agents for cancer. Compounds or extracts from herbal medicines (*11*), fruits (*2*), and teas (*12*) have been demonstrated to have cytotoxic effects or apoptosis-inducing activity in tumor cell lines. Herbal medicine has been used in China for many years. Furthermore, herbal medicine has attracted a great deal of recent attention as an alternative cancer therapy because of its low toxicity and

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low cost. *Lactuca indica* L. (Compositae family) is an edible wild lettuce widely distributed in Asia. Extracts of *L. indica*, prepared using boiling water or an ethanolic solution, have been popularly used as a folk medicine in anti-inflammatory and antibacterial applications and in the treatment of intestinal disorders (*13*). Recent results have suggested that water extracts of *L. indica* effectively scavenge free radicals and reduce oxidative stress in human promyelocytic leukemia HL-60 and macrophage cell lines (*14*). Methanol extracts of *L. indica* effectively decrease serum levels of total cholesterol and LDL cholesterol (*15*). Hou et al. (*16*) also reported that an aqueous acetone extract of fresh *L. indica* showed significant antidiabetic activity. However, limited scientifically confirmed information is available on other biological functions and clinical efficacies of this plant. In the present work, we report an examination of the antiproliferative effects and molecular mechanisms of the apoptotic effects induced by *L. indica* in human leukemia cells, as well as an investigation into the active components of *L. indica*.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO), agarose, ethidium bromide (EtBr), trypan blue, ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibiotic mixture (penicillin/streptomycin), trypsin/EDTA (T/E), and fetal bovine serum (FBS) were purchased from the Invitrogen Co. (Carlsbad, CA). DNA Zol was obtained from Molecular Research Center (Cincinnati, OH). APO-DIRECT Kit was purchased from Pharmingen (San Diego, CA). RPMI-1640 was purchased from HyClone (Logan, UT). Annexin V/FITC assay kit was obtained from Serotec (Oxford, U.K.). All other chemicals used were of the highest pure grade available.

Sample Extraction. The herbal plant *Lactuca indica* L. was purchased in Chang-Hua, Taiwan. Cold-water extracts (CEL) were prepared by homogenizing fresh plant (10 g) with 500 mL of distilled water. The mixture was then centrifuged at 4 °C and 7780*g* for 30 min. The supernatants of the plant extracts were freeze-dried and then stored at −20 °C. Hot-water extracts (HEL) were prepared similarly to CEL except that the homogenized mixture was boiled at 100 °C for 30 min before centrifugation.

The fresh plant was freeze-dried and then ground into a fine powder with a mill (RT-08, Rong Tsong, Taichung, Taiwan). The powders were passed through an 80-mesh sieve, collected and sealed in a plastic bag, and then stored at −20 °C until use. The herb powders (10 g) were extracted twice with 95% ethanol (500 mL) for 2 h. The ethanol extracts of *Lactuca indica* L. (EEL) were filtered through Whatman No. 2 filter paper and combined; the filtrate was dried under reduced pressure and stored at −20 °C until use.

Cell Cultures. Human leukemia cell line (HL-60) was purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) and was cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin/streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin), and L-glutamine (2 mM) at 37 °C under a humidified 5% CO_2 atmosphere. Human peripheral blood lymphocytes from healthy donors were separated and grown in RPMI 1640 medium supplemented with 20% fetal bovine serum at 37 °C under a humidified 5% CO_2 atmosphere.

Cell Viability Analysis. Cells were plated in 12-well plates at a density of 1×10^5 cells/mL and grown for 24 h. Cells were treated with extracts or the control with ethanol for various periods. The number of cells was determined with a hemocytometer. Cell viability was measured by trypan blue dye exclusion.

Phosphatidylserine Externalization Assay. Apoptosis was assessed by annexin V binding to phosphatidyl serine (PS) externalized early in the process of apoptosis. Annexin V binding to cells was determined using a commercially available annexin V apoptosis detection kit (ANNEX100F, Serotec, Oxford, U.K.) and flow cytometry. Briefly, after treatment of HL-60 cells (1×10^6 cells/mL) with EEL for 48 h, cells were recovered and washed twice with 2 mL of ice-cold phosphate-buffered saline (PBS) each time. Cells were incubated with 100 μL of HEPES buffer containing 2 μL of flourescein isothiocyanate (FITC)-conjugated annexin V and 2 μL of propidium iodide (PI) for 15 min. Following the incubation, without washing of the cells for excess reagents, 400 μL binding buffer was added. The stained cells were immediately analyzed with a FACScan flow cytometer (Becton-Dickinson Immunocytometery Systems USA, San Jose, CA). Approximately 10000 counts were made for each sample. The percentage distributions of apoptotic cells were calculated by CELL Quest software.

DNA Fragmentation Assay. HL-60 cells (1.5×10^5/mL) were treated with or without EEL for 48 h, washed with PBS twice, and then lysed in 1 mL of lysis reagent (DNA-Zol, Cincinnati, OH) for 1 h at 50 °C. DNA was precipitated with 100% ethanol, collected by centrifugation, and then treated with DNA hydration solution (80 μL of Tris-HCl/EDTA buffer and 4 μL of 10 mg/mL RNase) for 1 h at 55 °C. DNA samples were analyzed by 1.8% agarose gel electrophoresis. The agarose gels were run at 100 V for 1.5 h in Tris-borate/EDTA electrophoresis buffer.

TUNEL Assay for DNA Apoptotic Fragmentation. DNA strand breaks during apoptosis were also detected, using terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL), with a DNA fragmentation detection kit (Calbiochem, San Diego, CA) that identifies DNA strand breaks by labeling free 3′-OH termini with modified nucleotides. Following EEL treatment, apoptotic HL-60 cells (1×10^6 cells/mL) were harvested, fixed with 80% ethanol, and applied to a test tube. Fixed cells were permeabilized with 20 μg/mL protease K in Tris-buffered saline (TBS). Cells were labeled with FITC-conjugated dUTP and terminal deoxynucleotidyltransferase enzyme. Nicked DNA was labeled with FITC/dUTP according to the manufacturer's instructions and was detected with a FACScan flow cytometer. Approximately 10000 counts were made for each sample. The percentage of apoptosis was calculated by CELL Quest software.

Cell Cycle Distribution Assay. HL-60 cells (5×10^5 cells/well) after treatment with or without EEL for various periods were harvested by centrifugation. Cells were then washed, pelleted, and fixed with iced 70% ethanol for at least 30 min. After being left to stand overnight at 4 °C, the cell pellets were collected by centrifugation; resuspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase, and 5% Triton X-100 (Sigma, St. Louis, MO); and incubated at 37 °C for 30 min. Cells were analyzed on a flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle was then determined by argon convergent laser at 488 nm. Approximately 10000 counts were made for each sample. The percentage distribution of cell-cycle phases was calculated by CELL Quest software.

Mitochondrial Transmembrane Potential ($\Delta\Psi_m$) Assay. $\Delta\Psi_m$ was determined with a mitochondrial permeability transition detection kit (MitoPT, Immunochemistry Technologies, LLC, Bloomington, MN). The MitoPM dye reagent (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanin iodide, JC-1 dye) is a mitochondrion-selective dye and can be used to detect cells with a loss of $\Delta\Psi_m$. After treatment of HL-60 cells (1×10^5 cell/mL) with or without EEL for 24−48 h, the cells were recovered and loaded into a CO_2 incubator with 100 μL of MitoPT dye reagent at 37 °C for 10−15 min. The stained cells were collected by centrifugation and washed with assay buffer. The change of fluorescence intensity was immediately analyzed by excitation at 485 nm and emission at 590 nm with a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany).

Determination of Total Phenolic Compounds. Total phenolic compounds in EEL were determined with Folin-Ciocalteu reagent and calculated using gallic acid as the standard. Extracts (100 μL) were added to 50% Folin-Ciocalteau reagent (100 μL). After 3 min, 2 mL of 2% Na_2CO_3 solution was added to the mixture, which was then left to stand for 30 min. Absorbance was measured at 750 nm on a spectrophotometer and compared to gallic acid calibration curves. The content of total phenolics is expressed as gallic acid equivalents (GAEs). All analyses were run in three replicates and averaged.

Determination of Flavonoids. The spectrophotometer assay for the quantitative determination of flavonoid content was carried out as described by Jia et al. (*17*). Briefly, the extract (0.5 mL, 1 mg/mL)

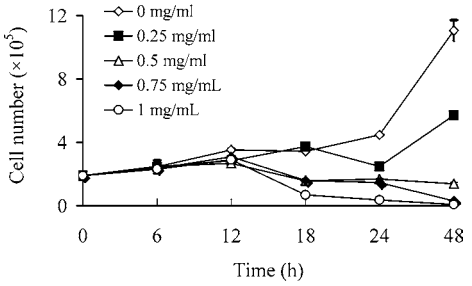

Figure 1. Effect of EEL on cell viability of HL-60 cells. HL-60 cells were plated at a density of 2×10^5 cells/mL and were treated with EEL at various concentrations. The reported values are the means ± SD ($n = 3$).

was mixed with 1.5 mL of distilled water, 100 μL of 10% $Al(NO_3)_3$, and 100 μL of 1 M CH_3COOK. After 40 min, the absorbance of the mixture was determined at 415 nm on a spectrophotometer. The content of total flavonoids in EEL is expressed herein as quercetin equivalents.

Characterization of Phenolic Compounds. Dried EEL (0.1 g) was dissolved in 10 mL of 6 N NaOH and then hydrolyzed under nitrogen at room temperature for 16 h. After alkali hydrolysis, the solution was adjusted with acid to pH 2.0 and was re-extracted three times with 15 mL of ethyl acetate/ethyl ether (1/1, v/v) in each case. The combined ethyl acetate/ethyl ether layer was evaporated to dryness under reduced pressure, and the residue was redissolved in methanol, filtered through a 0.45-μm filter, and analyzed by HPLC. HPLC was performed with a Hitachi liquid chromatograph (Hitachi, Ltd., Tokyo, Japan) consisting of a model L-6200 pump and a model L-3000 photodiode array detector set at 280 nm. A reverse-phase LiChrosphere RP-18 column (Merck, 250 mm × 4.6 mm, 5 μm) was used for HPLC analysis. Elution was carried out at room temperature and utilized 2% (v/v) acetic acid in water as solvent A and 0.5% acetic acid in water and acetonitrile (50:50, v/v) as solvent B. The elution gradient program was as follows: 5% B to 10% B (1−10 min), 10% B to 20% B (10−20 min), 20% B to 30% B (20−30 min), 30% B to 40% B (30−40 min), 40% B to 55% B (40−55 min), 55% B to 80% B (55−60 min), 80% B to 100% B (60−65 min), 100% B to 50% B (65−70 min), 50% B to 30% B (70−75 min), 30% B to 10% B (75−80 min) at a flow rate of 1 mL/min. Phenolic compounds were identified by comparison of their retention time (Rt) values and UV−vis spectra with those of known standards and were quantified by peak areas from the chromatograms.

Statistical Analysis. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($p < 0.05$) between the means were determined using Duncan's multiple range test.

RESULTS

Effect of EEL on Cell Viability. For preliminary screening of the cytotoxic effects of *Lactuca indica* L., HL-60 leukemia cells were treated with cold-water extracts (CEL), hot-water extracts (HEL), and ethanol extracts (EEL) from *Lactuca indica* L. for 48 h. The values of IC_{50} (concentration causing 50% cell death) for HEL and EEL against HL-60 cells were 680 and 313 μg/mL, respectively. CEL exhibited the weakest inhibition on cell viability, having an IC_{50} value of over 1000 μg/mL (data not shown). To further confirm the cytotoxic effect of EEL, HL-60 and lymphocyte cells were incubated with various concentrations of EEL for various periods, and then the viability of the cells was assessed. As shown in **Figure 1**, EEL exhibited cytotoxic effects on HL-60 cells in a dose- and time-dependent manner. Furthermore, EEL appears to preferentially inhibit leukemia cells, as no cytotoxic effect (at 1 mg/mL) was observed against normal peripheral blood lymphocytes (data not shown).

PS Exposure Induced by EEL. The induction of apoptosis by EEL was confirmed by assaying the extent of PS exposure during apoptosis as measured by the annexin V−FITC/PI method. As shown in **Figure 2**, EEL caused a concentration-dependent increase in apoptosis as determined by phosphatidylserine externalization. The amount of annexin V−FITC-positive cells was over 99.5% when HL-60 cells were incubated with EEL at a concentration of 1 mg/mL for 48 h.

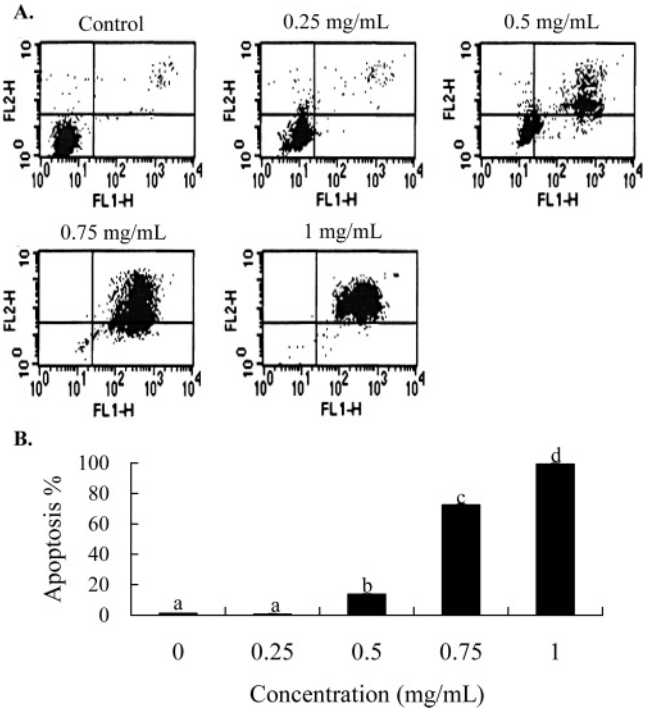

Figure 2. Cell apoptosis, percentage of HL-60 cells after treatment with EEL by flow cytometric analysis. Cells were treated with EEL at various concentrations (0.25, 0.5, 0.75, and 1 mg/mL) for 48 h. After treatment, cells were stained with annexin V/PI and analyzed by flow cytometry. The reported values are the means ± SD ($n = 3$). Bars topped by different letters are significantly different at $p < 0.05$.

DNA Fragmentation Induced by EEL. As a biochemical hallmark of apoptotic cell death, DNA fragmentation was determined. The cell line exhibited apoptosis for treatment with EEL over a concentration of 0.5 mg/mL, as fragmented DNA was seen at around 180−200 bp, and these DNA fragmentation responses were dose-dependent (**Figure 3**). The induction of apoptosis by EEL was also confirmed by in situ TUNEL assay (**Figure 4**). About 61.8% of the cells treated with 1 mg/mL EEL stained positively for TUNEL assay versus 4.3% in the control cell samples. Taken together, these results indicate that EEL induced typical apoptosis in HL-60 cells.

The effects of EEL on the cell cycles were also analyzed using flow cytometry. As shown in **Figure 5**, it was found that a significant increase in the G_0/G_1 phase occurred between the control and the treated sample. Moreover, the S- and G_2/M-phase cell populations gradually decreased with increasing EEL concentration and treatment time. Therefore, the induction of apoptosis by EEL was cell-cycle-dependent.

Effect of EEL on Mitochondrial Membrane Potential ($\Delta\Psi_m$). A decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) is associated with mitochondrial dysfunction. To assess the contribution of mitochondria to EEL-induced apoptosis, we examined changes in $\Delta\Psi_m$ in HL-60 cells. HL-60 cells were treated with various concentrations of EEL for 24 and 48 h and then exposed to lipophilic JC-1 dye, which is taken up by mitochondria and undergoes an orange-red shift in emission

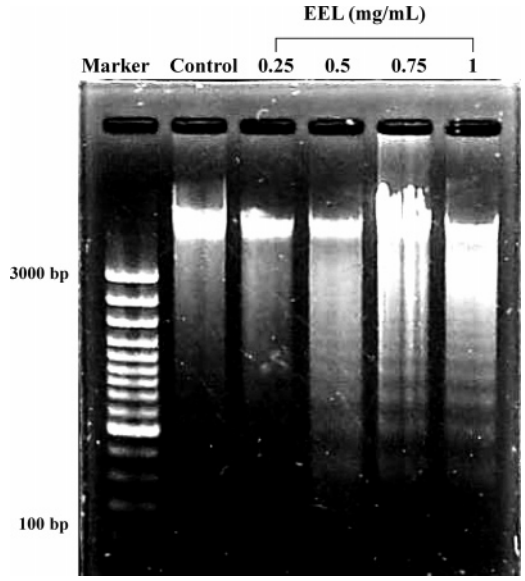

Figure 3. Induction of DNA fragmentation by EEL in HL-60 cells. Cells were incubated with 0–1 mg/mL of EEL for 48 h.

spectrum during changes in $\Delta\Psi_m$. **Figure 6** shows that treatment of HL-60 cells with EEL caused a marked drop in $\Delta\Psi_m$.

Determination of Active Components in EEL. The contents of active components in EEL are listed in **Table 1**. Flavonoids are one of the most diverse and widespread groups of natural phenolics. The data in **Table 1** show that more than 60% of the extracted phenolic substances in EEL are of flavonoidic origin. However, the results from the chromatogram indicate that EEL contains caffeic acid, chlorogenic acid, rutin, and quercetin, identified by comparison of their retention time values and UV spectra with those of known standards (data not shown). Among the four identified phenolic compounds in EEL, the content of caffeic acid is the highest (**Table 1**). These results indicate that other complex phenolic compounds might also be present in EEL.

Apoptosis Induced by Phenolic Compounds in EEL. To determine the roles of the active phenolic compounds in apoptosis induced by EEL, the effects of individual phenolic compounds on the growth of HL-60 cells were compared. As shown in **Figure 7**, the inhibition of phenolic compounds on cell viability decreased in the order quercetin > caffeic acid > rutin > chlorogenic acid. The IC_{50} values for quercetin, caffeic acid, and rutin against HL-60 cells were found to be 50, 91, and 187 μM, respectively. With the exception of chlorogenic acid, the other phenolic compounds caused a marked alteration in $\Delta\Psi_m$. Treatment with quercetin at a concentration of 200 μM for 48 h resulted in mitochondrial damage such that 94% of the HL-60 cells lost their mitochondrial membrane potential (**Figure 8**).

DISCUSSION

Apoptosis is a type of physiological cell death accompanied by a specialized cellular mechanism and some distinctive morphological changes. Apoptosis has been suggested as an underlying mechanism of various anticancer and chemopreventive agents (*18*). In the present study, we investigated the apoptosis of leukemia HL-60 cells induced by ethanol extracts of *Lactuca indica* L. (EEL). Our data revealed that EEL exhibits a cytotoxic capability against HL-60 cells (**Figure 1**). This apoptosis-inducing effect of EEL appears to be leukemia-cell-specific, because EEL did not affect the survival of normal

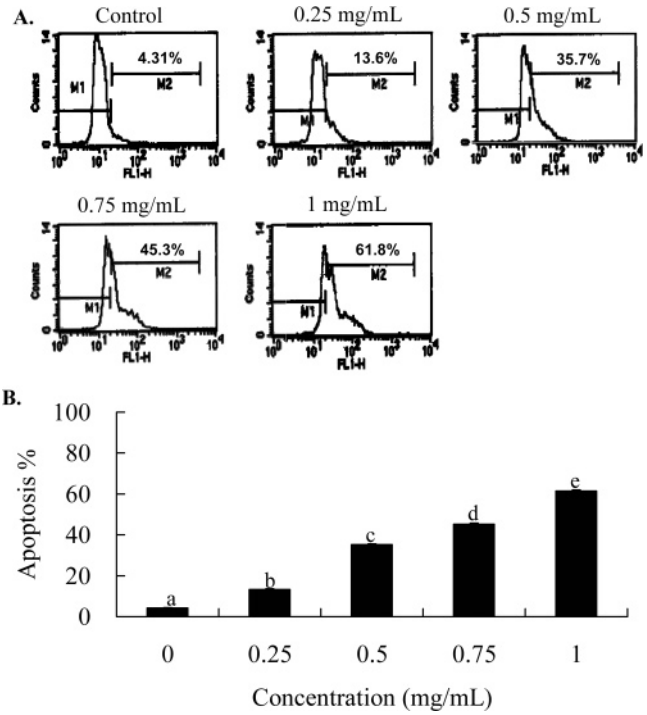

Figure 4. TUNEL assay of HL-60 cells after treatment with EEL for 48 h. Cells were incubated without or with the various concentrations of EEL. The percentage of apoptosis was obtained using flow cytometry based on 1×10^4 cells from each group. The M1 region represents normal viable cells, and the M2 region represents apoptotic cells that stained positive for TUNEL. The reported values are the means ± SD ($n = 3$). Bars topped by different letters are significantly different at $p < 0.05$.

peripheral blood lymphocytes. Zhao et al. (*19*) reported that the cytotoxic activity of tea polyphenols in HL-60 cells relates to their apoptosis-inducing activity. The IC_{50} value was found to be 732.2 μg/mL for 48 h of treatment of HL-60 cells with tea polyphenols. Judging from the IC_{50} value obtained from cytotoxicity data, Kundu et al. (*20*) reported that black tea is as efficient as green tea in the suppression of HL-60 cell proliferation. The IC_{50} value of EEL is lower than that of tea polyphenols, indicating that EEL might be a potential anticancer agent.

One of the hallmark events that occur early in apoptosis is the flipping of phosphatidyl serine from the inner leaflet to the outer leaflet of the cytoplasmic membrane. Annexin V conjugated to fluorescein (annexin V−FITC) can identify apoptotic cells by binding to phosphatidyl serine (PS), and red-fluorescent propidium iodide (PI) binds nucleic acids of necrotic cells, which makes it ideal for detecting membrane changes associated with apoptosis using flow cytometry (*21*). In the present study, EEL was found to induce a significant and dose-dependent increase of annexin V^+/PI^+ apoptotic cells (**Figure 2**).

Apoptosis was further confirmed by DNA fragmentation analysis using agarose gel electrophoresis (**Figure 3**) and TUNEL staining (**Figure 4**). The induction of apoptosis stimulates endonuclease, which involves double-stranded DNA breaking into oligonucleosome-length fragments, resulting in a typical ladder in DNA electrophoresis that is one of the markers of apoptotic cell death (*22*). As evidenced by DNA fragmentation, it appears that apoptosis is the main mechanism for cell killing in the presence of EEL. The apoptosis-inducing effect of EEL in HL-60 cells appeared in a concentration-dependent manner. This efficacy of EEL was found to be similar to its cytotoxic activity in HL-60 cells.

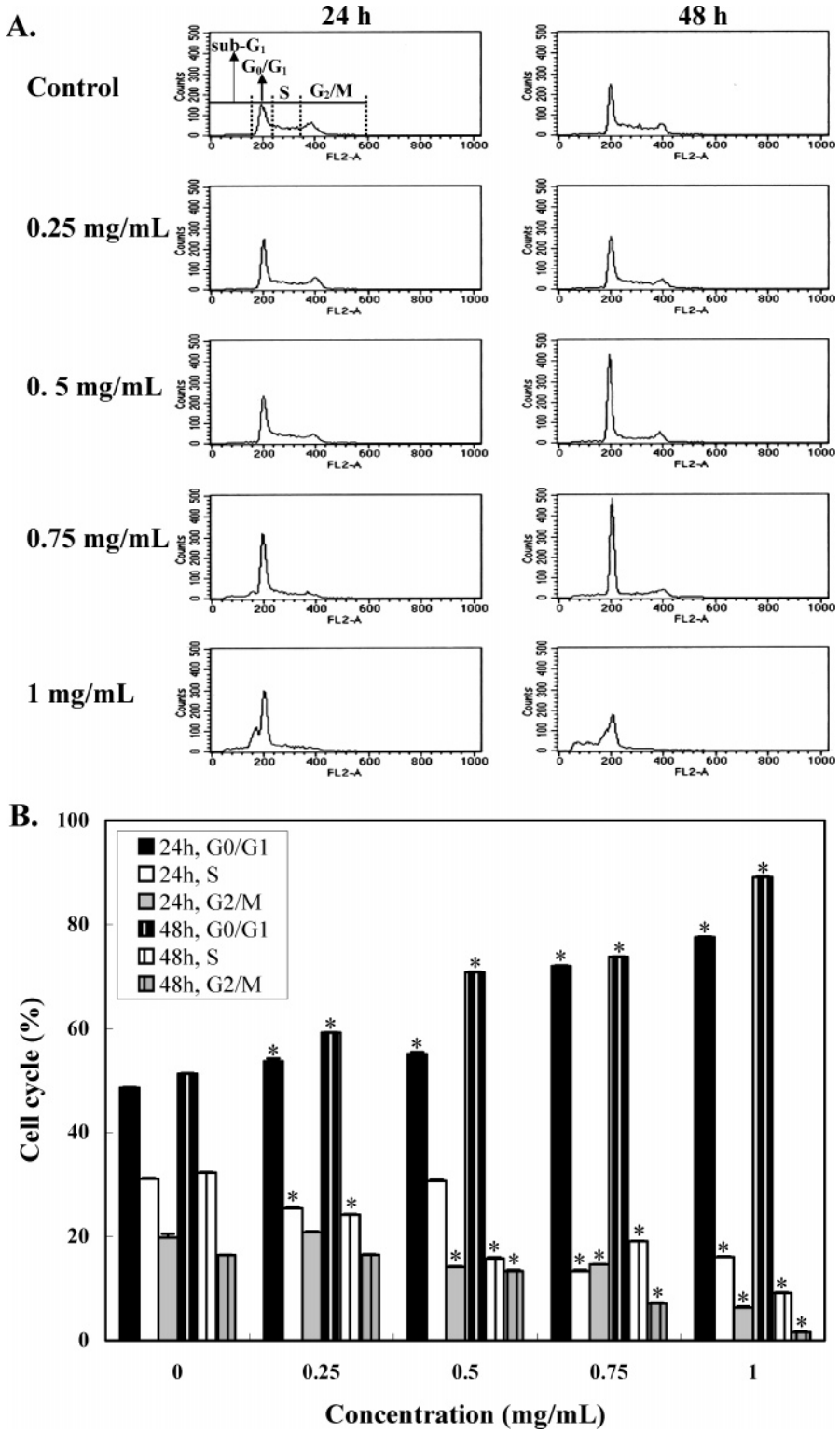

Figure 5. Effect of EEL on the cell cycle of HL-60 cells. Cells were incubated with 0–1 mg/mL EEL for 24 and 48 h. The reported values are the means ± SD ($n = 3$). *$p < 0.05$ significantly different from control.

A flow cytometry analysis of propidium iodide-labeled cells shows that the treatment of HL-60 cells with EEL (0.25−1.0 mg/mL) induces significant accumulation of cells in the G_0/G_1 phase (**Figure 5**). The ratio of G_0/G_1 to S to G_2/M phase in HL-60 cells changed from 49:31:20 in the control to 78:16:6 in the presence of 1 mg/mL EEL for 24 h. The G_0/G_1 cell population increased to 89% in HL-60 cells treated with 1 mg/mL EEL for 48 h. Moreover, a characteristic hypodiploid DNA content peak (sub-G_1) was easily detected after treatment of 1 mg/mL EEL for 48 h (data not shown). A significant increase in the sub-G_1 phase is indicative of induction of apoptosis. Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired mutations in genes that directly regulate their cell cycle (*23*). The study of cell-cycle progression for EEL shows that EEL is a cell-cycle-dependent anticancer agent. Flavonoids have been found to arrest cell-cycle progression either at the G_1/S or G_2/M boundary. Quercetin has been shown to reduce cell proliferation, causing

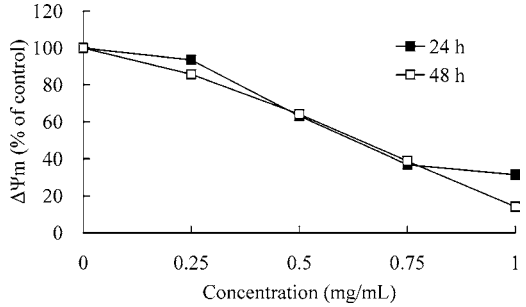

Figure 6. Effect of EEL on the mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells. Cells were incubated with 0–1 mg/mL EEL for 24 and 48 h. The reported values are the means ± SD ($n = 3$).

Table 1. Contents of Total Polyphenols, Flavonoids, and Individual Phenolic Compounds in EEL

compound	content (mg/g of extract)[a]
total polyphenol	48.7 ± 1.7
flavonoids	31.5 ± 0.9
caffeic acid	10.5 ± 0.3
chlorogenic acid	4.9 ± 0.1
rutin	2.9 ± 0.1
quercetin	4.1 ± 0.3

[a] Reported values are the means ± SD ($n = 3$).

cell-cycle arrest in the G_0/G_1 phase (*24*), the S phase (*25*), and the G_2/M phase (*26*) in in vitro experiments with various cell lines. Rusak et al. (*27*) suggested that the different effects of flavonoids on the cycle and proliferation of cells is cell-type-specific. Our data indicate that EEL induces a G_0/G_1 block in HL-60 cells.

Apoptosis of tumor cells can be triggered by a diversity of extracellular and intracellular factors. Recent studies have suggested that mitochondria play a pivotal role in the intrinsic pathway of apoptosis (*10*). Many models of apoptosis show a loss of the mitochondrial transmembrane potential (MTP) mediated by the opening of the megachannel (permeability transition pore), which precedes caspase activation (*28*). In the present study, we demonstrated that EEL can interact with mitochondria and disrupt their function (**Figure 6**), leading to several functional alterations in HL-60 cells, some or all of which might be key factors in the induction of apoptosis. However, the mechanisms responsible for this action remain to be determined.

According to the results of constituent analysis, EEL contains 5% phenolic compounds (**Table 1**). Four major phenolic constituents in EEL, namely, caffeic acid, chlorogenic acid, rutin, and quercetin, were characterized. Wang et al. (*14*) reported that the six phenolic compounds protocatechulic acid, methyl *p*-hydroxybenzoate, caffeic acid, 3,5-dicaffeoylquinic acid, luteolin 7-*O*-β-glucopyranoside, and quercetin 3-*O*-β-glucopyranoside are the major antioxidative constituents in the hot-water extracts from *L. indica*. Hou et al. (*16*) also found that aqueous acetone extracts of fresh *L. indica* show significant antidiabetic activity. Four new compounds (three guaiane-type sesquiterpenes and a furofuran lignan glycoside), two known guaiane-type sesquiterpene lactones (11β,13-dihydrolactucin and cichoriosides B), six known flavonoids (quercetin, quercetin 3-*O*-glucoside, rutin, apigenin, luteolin, and luteolin 7-*O*-glucuronide), and one phenolic compound (chlorogenic acid) were isolated from aqueous acetone extracts of fresh *L. indica*.

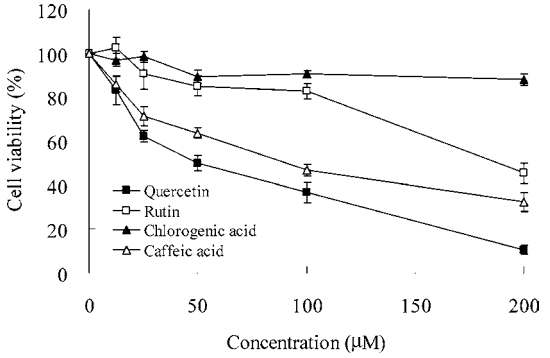

Figure 7. Effects of quercetin, caffeic acid, rutin, and chlorogenic acid on the cell viability of HL-60 cells. Cells were incubated with 0–200 μM samples for 48 h. The reported values are the means ± SD ($n = 3$).

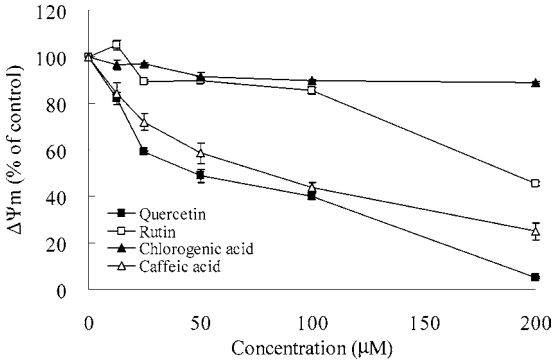

Figure 8. Effects of quercetin, caffeic acid, rutin, and chlorlgenic acid on the mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells. Cells were incubated with 0–200 μM samples for 48 h. The reported values are the means ± SD ($n = 3$).

Among these compounds, latucain C and lactucaside showed significant antidiabetic activities.

Our results further show that the active phenolic compounds in EEL, including quercetin, caffeic aicd, rutin, and chlorogenic acid, decrease HL-60 cell viability (**Figure 7**). The phenolic compounds, especially quercetin, also caused a marked alteration in $\Delta\Psi_m$ (**Figure 8**). Quercetin is a dietary flavonoid commonly found in plants and has been reported to exhibit biological, pharmacological, and medicinal activities. Moreover, quercetin-mediated apoptosis might result from the induction of stress protein, the disruption of microtubules and mitochondria, the release of cytochrome c, the activation of caspases, and a decrease in the expression of receptor tyrosine kinases (*26*, *29*). In our previous study, quercetin-induced apoptosis in SW 872 human liposarcoma cells was found to be associated with the loss of mitochondrial membrane potential ($\Delta\Psi_m$). The apoptosis in SW 872 human liposarcoma cells induced by quercetin is mediated through the activation of caspase-3, Bax, and Bak, followed by cleavage of PARP and down-regulation of Bcl-2 (*30*). Dorta et al. (*31*) determined the energetics of isolated rat liver mitochondria exposed to flavonoids and reported that quercetin is very effective in interacting with the mitochondrial membrane, decreasing the fluidity of the mitochondrial membrane, inhibiting the respiratory chain of mitochondria, and decreasing mitochondrial ATP levels. Kellner and Zunino (*32*) investigated that loss of mitochondrial membrane potential ($\Delta\Psi_m$) leading to apoptosis in acute lymphocytic leukemia cells after exposure to curcumin, carnosol, and quercetin. Treatment of the leukemia-derived cells with these phenolic compounds also increased the levels of NO in the leukemia-derived cells. Overproduction of NO has been linked to prolonged inhibition

of electron transport, resulting in increased electron leakage and generation of reactive oxygen and nitrogen species, which can lead to disruption of $\Delta\Psi_m$ (*33*). Therefore, apoptosis and loss of mitochondrial function induced by quercetin can be correlated to several mechanisms. However, the roles of the synergistic activities of quercetin and other phenolic and non-phenolic compounds in the apoptosis activity of EEL remain as a matter to be studied further.

In conclusion, our study shows clearly that EEL induces apoptosis in HL-60 cells. Furthermore, the results indicate that the cytotoxic properties of EEL are mainly due to the induction of apoptosis as evidenced by collapse of the mitochondrial membrane potential. In addition, these results also suggest that the EEL-induced apoptotic ability can be related to its phenolic compounds, especially quercetin. Further epidemiological studies and clinical efficacies on this plant, for nutraceutical or pharmaceutical applications, are required.

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