

Crude Extracts of *Solanum lyratum* Induced Cytotoxicity and Apoptosis in a Human Colon Adenocarcinoma Cell Line (Colo 205)

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Abstract. The effects of the crude extract of *Solanum lyratum* (SLE) on human colon cancer colo 205 cells were investigated. The cell viability, morphological changes of the cells, cell cycle arrest, apoptosis, reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$) and cell cycle- and apoptosis-associated protein levels and gene expressions were examined in colo 205 cells after exposure to various concentrations of SLE for different time periods. The results indicated that SLE decreased the percentage of viable colo 205 cells accompanied by morphological changes. The most effective concentration of SLE was 300 $\mu\text{g/ml}$ (SLE 300) and this concentration was used for further investigations. SLE induced S-phase arrest and apoptosis (sub-G1) in the colo 205 cells and those effects were dose- and time-dependent. DAPI staining and DNA gel electrophoresis confirmed that SLE induced apoptosis in colo 205 cells. Flow cytometric analysis also showed that SLE 300 promoted ROS production and decreased the $\Delta\Psi_m$. Western blotting analysis indicated that SLE 300 increased Bax levels and decreased Bcl-2 levels, which caused the loss of $\Delta\Psi_m$ followed by cytochrome c release and caspase-9 and -3 activation, finally leading to apoptosis. SLE 300 also promoted p53 and p27, but decreased the levels of cyclin B1 thus causing S-phase arrest. The gene expression associated with those proteins was also confirmed by PCR methods. The findings show that SLE might be used as a colon cancer therapeutic agent in the future.

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In Taiwan, 15.03 per 100,000 people die per year of colon cancer according to the 'People Health Bureau of Taiwan'. Surgery, radiotherapy and chemotherapy are used in the clinical therapy of colon cancer, but the strategies for treatment are not yet satisfactory. Apoptosis has a critical function in the development and homeostasis of multicellular organisms and it is also a well regulated and organized death process which is involved in physiological and pathological conditions (1). The apoptotic morphological changes including chromatin condensation, DNA fragmentation and membrane blebbing (2-7), apoptotic bodies, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet occur after a cascade of cell signaling and caspase-mediated events that regulate pro-apoptotic and anti-apoptotic proteins (4, 8). Two major pathways are involved in apoptosis, one is the death receptor-induced pathway and the other is the mitochondria-mediated pathway. The death receptor-induced apoptotic pathway includes Fas/FasL, tumor necrosis factor (TNF)/TNF receptors and death receptor (DR)3/DR3 receptors, and their down-stream molecules such as caspase-8 (8, 9). The mitochondria-apoptosome-mediated pathway includes apoptotic stimuli induced by radiation and chemotherapy; decreased mitochondrial membrane potential ($\Delta\Psi_m$) could cause cytochrome c release leading to the activation of the caspase-cascade, Apaf-1 and caspase-9, initiated by the formation of apoptosomes (4, 7, 9). Cross-talk between these two apoptotic pathways also exists (7, 9).

Solanum lyratum Thunberg (Solanaceae), well known as "Herba Solani Lyrati", is one of the traditional medicines in China. It has been used for regulating immune function (10), and against allergic diseases (11). *Solanum lyratum* is also commonly used as an anticancer drug to treat cancers

of the liver, lung, esophagus and other. Recent studies have demonstrated that *Solanum lyratum* extract (SLE) significantly inhibited human hepatoma BEL-7402 cell, gastric carcinoma SGC-7901 cell and A375-S2 cell proliferation *in vitro* and *in vivo* (12, 13). In studies on the anticancer mechanisms, SLE induced HeLa cell apoptosis by up-regulating expression of Fas/FasL (14) and also promoted the formation of cyclic adenosine monophosphate (cAMP) (14) and the activity of protein kinase A (PKA) in gastric cancer cells (15, 16).

However, there has been no report as to whether *Solanum lyratum* Thunb. could induce apoptosis in colon cancer cells. Therefore this study focused on the apoptosis-inducing action of extracts of *Solanum lyratum* Thunb. on the human colon cancer cell line colo 205.

Materials and Methods

Plant material and preparation of crude extracts of *Solanum lyratum*. The SLE was collected from Nantou County Sinyi Township Dongpu located in the middle part of Taiwan in September 2002. Voucher specimens (CMU SL 0222) were deposited in the School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan. The *Solanum lyratum* (600 g) was extracted repeatedly with 50% EtOH at room temperature. The combined EtOH extracts were filtered and evaporated under reduced pressure to yield a brownish viscous residue (58.44 g). For the present experiments, the crude extracts were dissolved in dimethyl sulfoxide (DMSO).

Chemicals and reagents. Propidium iodide (PI), potassium phosphates, triton X-100, Tris-HCl, trypan blue and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMSO and TBE buffer were purchased from Merck Co. (Darmstadt, Germany). The 90% RPMI 1640 medium, 2 mM L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Cell culture. The human colon adenocarcinoma colo 205 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The colo 205 cells were placed into 75 cm³ tissue culture flasks and grown at 37°C under a humidified 5% CO₂ atmosphere in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% FBS, 10,000 U/ml penicillin and 10 mg/ml streptomycin.

Viability and morphological changes. The colo 205 cells were plated in 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. Different concentrations of SLE were then added to the cells for a final concentration of 0, 50, 100, 200, 300 or 400 µg/ml, while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO₂ and 95% air for different periods of time. On the other hand, we also compared the SLE with the clinical therapy medicine 5-fluorouracil (5-FU). For determining cell viability, the flow cytometry assay and trypan blue exclusion were used as described previously (17, 18). For determining the morphological changes, the cells were examined and photographed under a phase-contrast microscope as described previously (17, 18).

Table I. PCR primers.

Primer size (bp)		Sequence: F, forward primer R, reverse primer		Product name
		5'	3'	
Cdk1	F	TTTTCAGAGCTTTGGGCACT		142
	R	AAATCCAAGCCATTTTCATCC		
Cdc25b	F	TTGAACCAAGGCCCTAACAG		125
	R	CAAGAGGAAATGTCACAGAAAGC		
Cdc25c	F	GCAGCCTTGAGTTGCATAGAG		131
	R	GACCAACGCTCTTGCATAGCC		
p27	F	AAACGTGCGAGTGTCTAACGGGA		
	R	CGCTTCCTTATTCTGCGCATTG		
p21	F	AGTGGACAGCGAGCAGCTGA		
	R	TAGAAATCTGTCATGCTGGTCTG		
MDM2	F	ATGGCCTGCTTACATGTGC		209
	R	TGAATTGAGGCATTTTCTCAC		
Wee 1	F	ACTGATAGAATCCAGTTTGC		321
	R	ATGCAATGCCTACAAAGTGC		
Bcl-2	F	CGACTTCGCCGAGATGTCCAGCCAG		389
	R	ACTTGTGGCTCAGATAGGCACCCAG		
Apaf-1	F	AGAGTTTCAAAACATACAAGAGAGA		161
	R	TTCATGCAGTCGGTCA		
Topoiso-merase IIα	F	ACACTCAGCCTCTTATGTGC		272
	R	ATCAAATGTTGTCCCCG		
Topoiso-merase IIβ	F	GCCCAAAGAGCACAACATT		204
	R	CAGACAGTAGTGACATTTCAATG		
NF-κB	F	GATGAATATGTGTGTATCCG		658
	R	TTTGTGTGCTTGAGAACC		
I-κB	F	CATGTTTACACACACAATCAGC		240
	R	GGAGTAGGAGACTTTCACAAGTGG		
GAPDH	F	CCATGGAGAAGGCTGGGG		
	R	CAAGAGTTGTCATGGATGACC		

Cell cycle and apoptosis of colo 205 cells. About 2x10⁵ cells/well of colo 205 cells in 12-well plates with 0, 50, 100, 200, 300 or 400 µg/ml of SLE were incubated for 6, 12, 24, 48 or 72 h. The cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) and were fixed in 70% ethanol (in PBS) at 4°C overnight, and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in a dark room for 30 min at 37°C, and analyzed with a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength. Then the cell cycle and sub-G1 group (apoptosis) were determined as described previously (17, 19).

DNA fragmentation. About 1x10⁶ cells/well of colo 205 cells in 12-well plates with 0, 100, 200, 300 or 400 µg/ml of SLE were incubated for 48 h or treated with 300 µg/ml of SLE for 0, 6, 12, 24 h. The cells were harvested by centrifugation, the DNA was isolated and DNA gel electrophoresis was performed. After EtBr staining the cells were photographed under fluorescence light as described previously (19, 20).

Detection of reactive oxygen species (ROS). About 2x10⁵ cells/well of colo 205 cells in 12-well plates were treated with 0 µg (control) or 300 µg/ml of SLE (SLE 300) and were incubated for 4, 8, 12 or 24 h to detect the changes of ROS. The cells were harvested and washed with 1X PBS

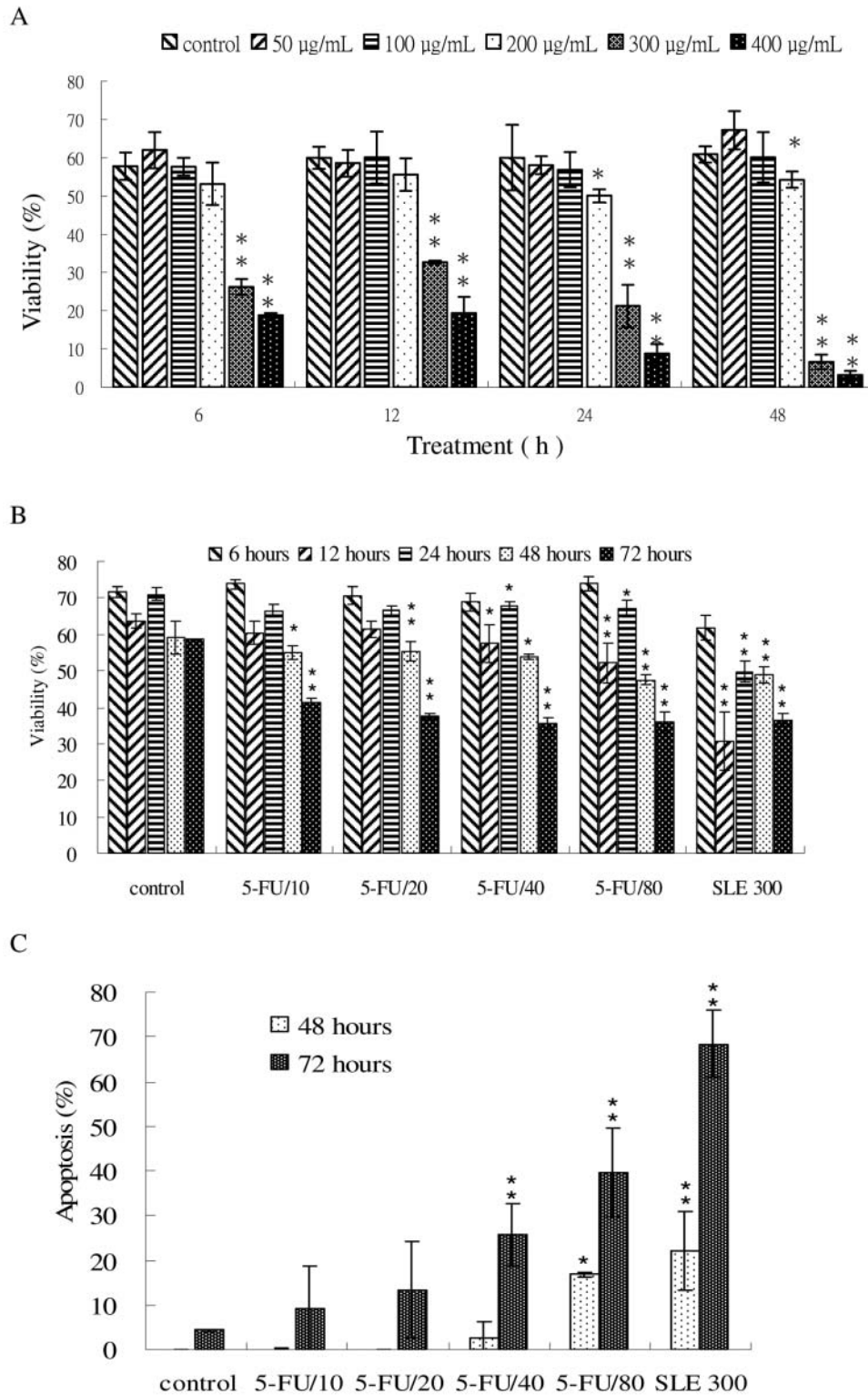


Figure 1. Viability of human colon adenocarcinoma colo 205 cells after exposure to extract of *Solanum lyratum*. The colo 205 cells (2×10^5 cells/well) were incubated with different concentration of SLE for 6, 12, 24, 48 or 72 h. Panel A: viability of colo 205 after different concentration and time of SL treatment; panel B: compared the viability of 5-fluorouracil (5-FU), concentration were 10, 20, 40, 80 μ M and SLE 300 (50% ethanol extract of *Solanum lyratum*, concentration was 300 μ g/ml); panel C: compared the apoptosis cells of various concentration 5-FU and SLE 300. Each point is mean \pm SD of three experiments. * $p < 0.05$.

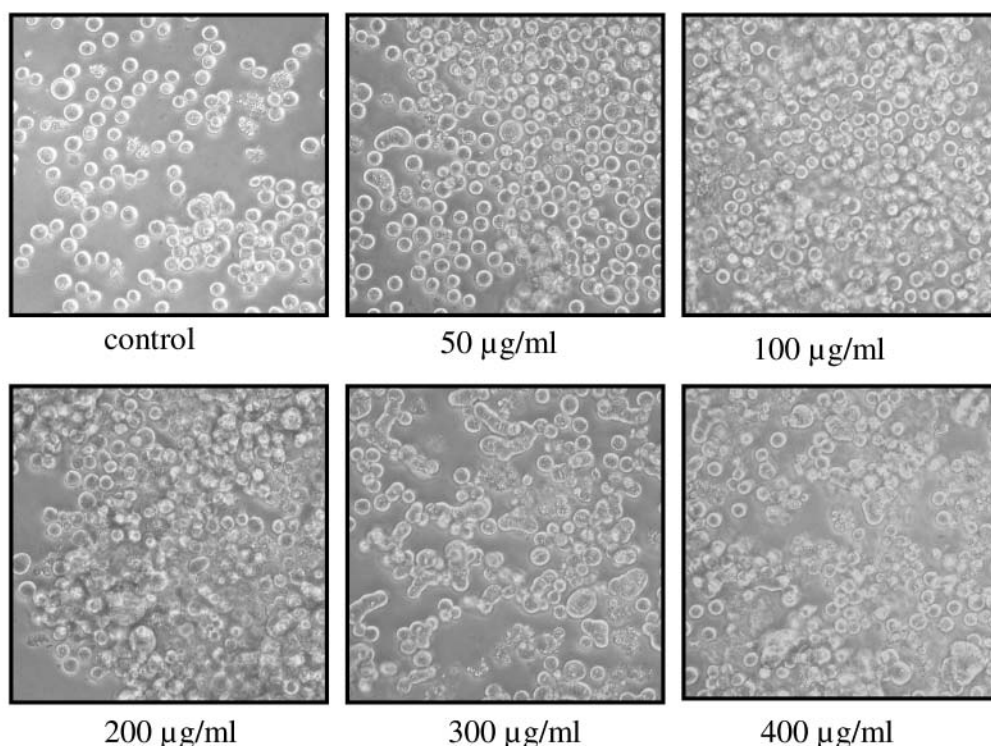


Figure 2. Morphological changes of colo 205 cells after exposure to SLE. The colo 205 cells (2×10^5 cells/well) were incubated with various concentrations of SLE (50, 100, 200, 300 or 400 $\mu\text{g/ml}$) or DMSO (control) for 48 h and examined under a phase-contrast microscope at $\times 200$.

twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM), incubated at 37°C for 30 min and then analyzed by flow cytometry as described previously (17, 19).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$). About 2×10^5 cells/well of colo 205 cells in 12-well plates were treated with 0 μg (control) or 300 $\mu\text{g/ml}$ of SLE and were incubated for 4, 8, 12 or 24 h to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed with 1X PBS twice, re-suspended in 500 μl of 3,3'-dihexyloxacarbocyanine iodide (DiOC_6) (Calbiochem) (4 mol/L), and incubated at 37°C for 30 min and analyzed by flow cytometry as described previously (17, 19).

Western blotting for examination of the levels of proteins associated with cell cycle and apoptosis. About 2×10^5 cells/well of colo 205 cells in 12-well plates were treated with 300 $\mu\text{g/ml}$ of SLE and were incubated for 4, 8, 12 or 24 h. The cells were harvested and were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor (Sigma Chemical Co.) and 1% phosphatase inhibitor mixture II (Sigma Chemical Co.). For protein determination the cell lysates (40 μg of each) were separated by SDS-PAGE on a polyacrylamide gel followed by electrotransfer onto a sequi-blot polyvinylidene difluoride membrane (Bio-rad, Richmond, CA, USA). Phosphorylation-specific rabbit polyclonal antibodies were purchased from New England Biolabs (Beverly, MA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat antirabbit or antimouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) was used as a secondary antibody for

enhanced chemiluminescence (NEN Life Science Products, Inc., Boston, MA, USA) as described previously (17, 21).

RNA preparation. About 2×10^6 cells/well of colo 205 cells in 12-well plates were treated with 0, 100, 200, 300 or 400 $\mu\text{g/ml}$ of SLE and were incubated for 24 h. The total RNA was extracted from the cells using the total RNA miniprep purification kit (TRIzol reagent according to the manufacturer's procedure: Invitrogen), and then the amount of extracted total RNA was determined as described previously (22).

Reverse transcriptase PCR. The extracted total RNA was dissolved in diethylpyrocarbonated (DEPC)-treated water and the RNA was treated with deoxyribonuclease I (Invitrogen, catalog No. 18068-015). Reverse transcription PCR was conducted with Super-Script One-Step RT-PCR for Long Templates (Invitrogen, catalog No. 11922-028) with a PTC programmable thermal controller (MJ Research, Inc.) according to the manufacturer's procedures. The primer sets for the associated genes and GAPDH (control for RNA sample's quality) are shown in Table I. The thermo-cycler parameters were: 5 min at 95°C ; denaturation 1 min at 95°C ; annealing 1 min at 55°C ; 40 cycles of: 15 sec at 94°C , 30 sec at 55°C and 1 min at 72°C ; final extension of 10 min at 72°C , as described previously (22). The reaction products were analyzed by electrophoresis on 1% agarose gel as described previously (22).

Statistical analysis. The Student's *t*-test was used for the statistical analysis between the SLE treated and control groups. The data represent mean \pm SD of three experiments with $p < 0.05$ considered significant.

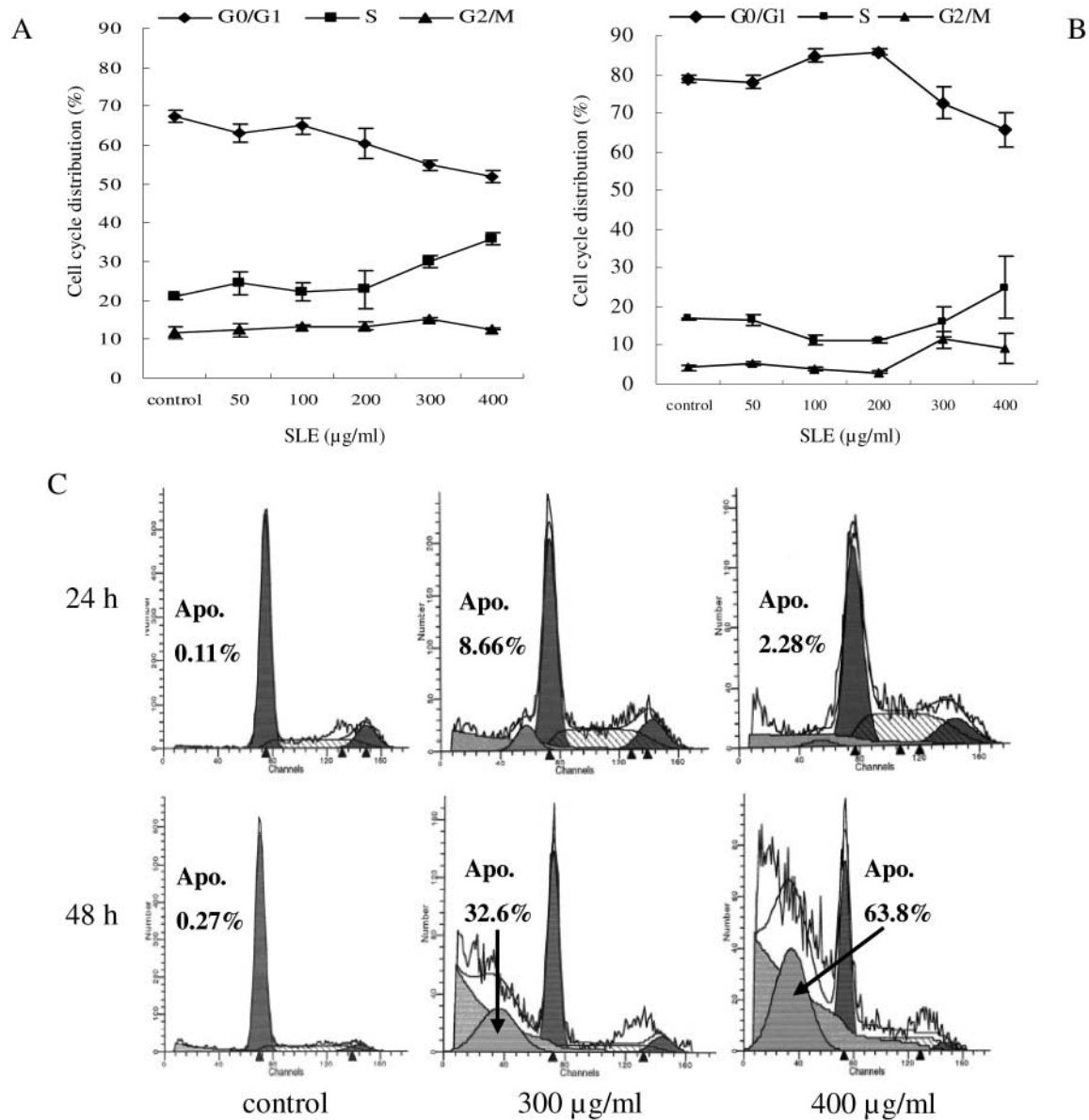


Figure 3. The effects of SLE on cell cycle and apoptosis in colon adenocarcinoma colo 205 cells. The colo 205 cells (2×10^5 cells/well) were incubated with various concentrations of SLE for 24 or 48 h. The cell cycle and apoptosis were determined by flow cytometric analysis. Panel A: representative profile after 24 and 48 h incubation, Apo. was mean apoptosis; panel B, C: 24 and 48 h the percentage of cell cycle phase; data represents mean \pm SD of three experiments. * $p < 0.05$.

Results

Viability after exposure to SLE. The SLE at 300 µg/ml significantly decreased by 40% the viable cells (Figure 1A). The results from Figure 1B also showed that SLE 300 treatment for all time periods decreased the percentages of viable cells more significantly than that of the clinical drug (5-FU: 10, 20, 40, 80 µM) for colon cancer patients. The percentage of apoptosis from SLE 300 and various concentrations of 5-FU (Figure 1C) indicated that SLE 300

induced a higher percentage of apoptosis in colo 205 cells than that of different concentrations of 5-FU after 48 and 72 h of treatment, respectively.

Morphological changes. After the colo 205 cells were incubated with SLE at final concentrations of 50, 100, 200, 300 and 400 µg/ml or DMSO (control) for 48 h they were photographed under a phase-contrast microscope. SLE 300 induced morphological changes (Figure 2) and also decreased the cell viability.

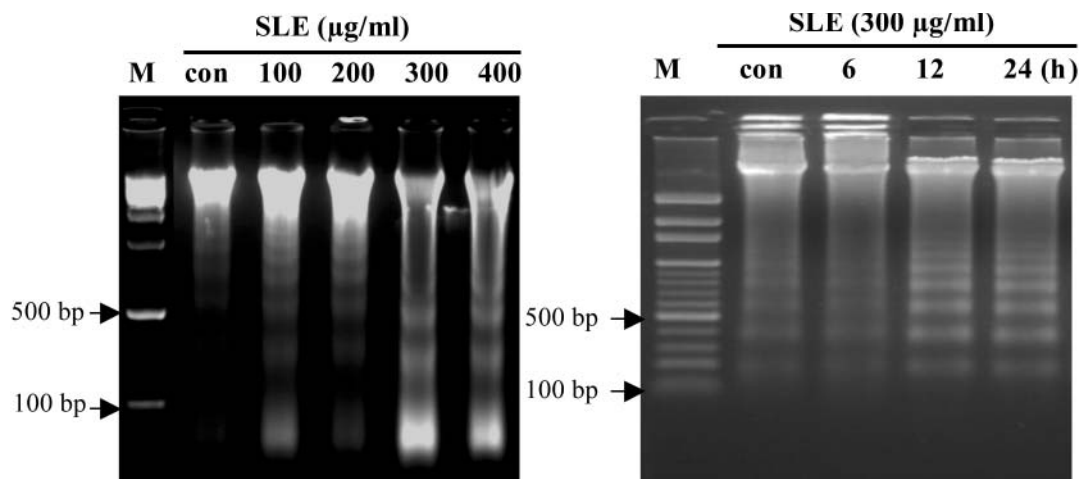


Figure 4. The effects of SLE on DNA fragmentation in colo 205 cells. The colo 205 cells were treated with various concentrations of SLE for 48 h (A) or treated with 300 µg/ml of SLE for various times (B).

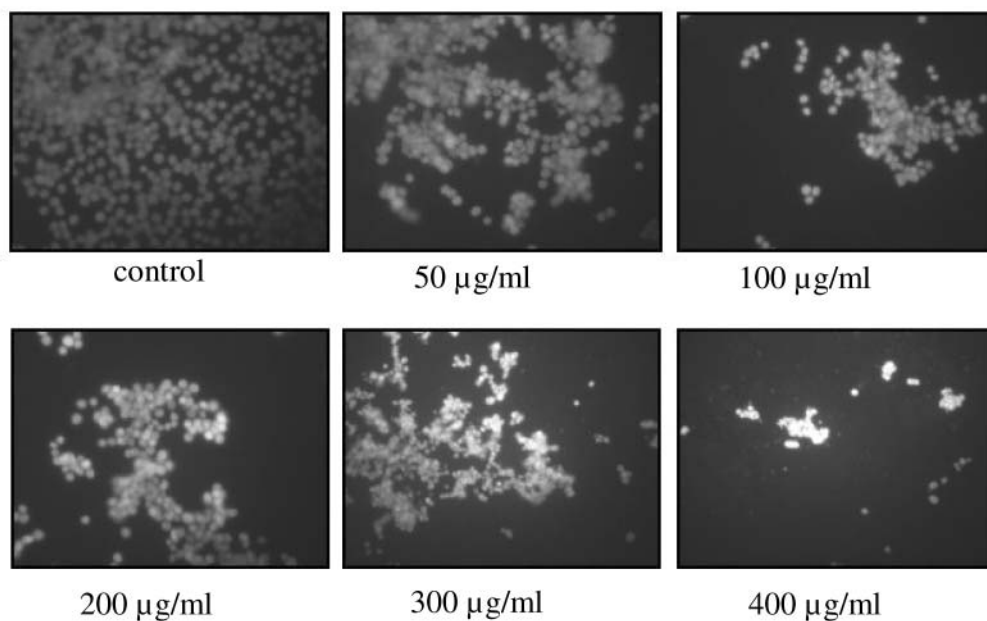


Figure 5. The effects of SLE on DAPI staining in colo 205 cells. The colo 205 cells (2×10^5 cells/well) were treated with various concentrations of SLE for 48 h and apoptosis was determined by DAPI staining and were photographed under a phase-contrast microscope at x200.

The effects of SLE on cell cycle and apoptosis. The results indicated that SLE at 300 µg/ml induced S-phase arrest [Figure 3A (24 h) and B (48 h)] and apoptosis (Figure 3C). The apoptotic effects were time-dependent.

The effects of SLE on DNA fragmentation. After the colo 205 cells were treated with various concentrations of SLE for 48 h or treated with 300 µg/ml of SLE for various times, the results indicated that SLE at 300 µg/ml induced DNA fragments which indicated the occurrence of apoptosis (Figure 4).

The effects of SLE on DAPI staining in colo 205 cells. As shown in Figure 5, SLE induced apoptosis in a concentration-dependent manner. At a higher concentration of SLE, a higher proportion of cells were stained by DAPI.

The effects of SLE on reactive oxygen species (ROS) and $\Delta\Psi_m$. The results indicated that SLE 300 induced ROS production (Figure 6A and B) and decreased the $\Delta\Psi_m$ (Figure 6C and D) in the examined cells.

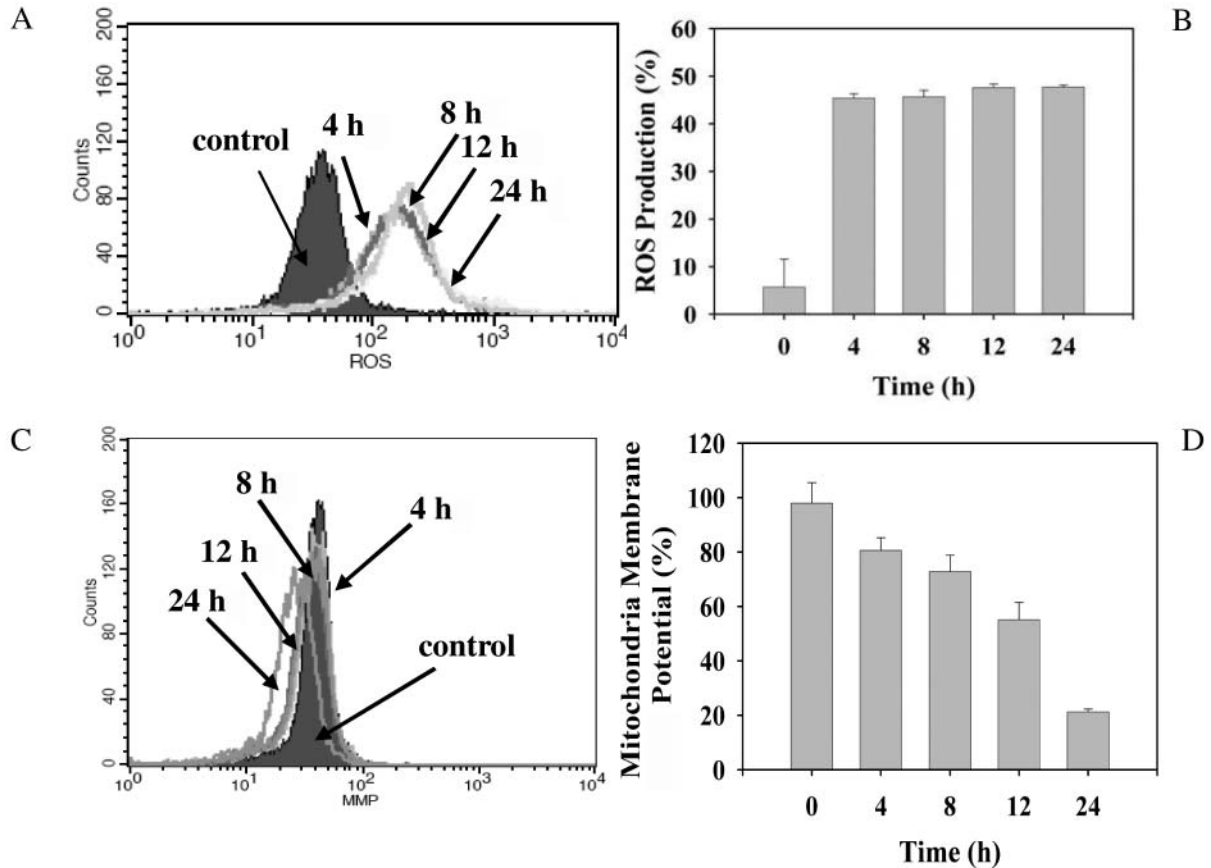


Figure 6. The effects of SLE on reactive oxygen species (ROS) and mitochondria membrane potential ($\Delta\Psi_m$). The colo 205 cells (2×10^5 cells/well) were treated with 300 $\mu\text{g/ml}$ of SLE (control: 0 μg SLE) for various times to detect the changes of ROS and $\Delta\Psi_m$. Panels A and B: the percentage of cells that were stained by DCFH-DA dye for ROS; panels C and D: stained by DiOC₆ dye for $\Delta\Psi_m$. *Differences between SLE and control $p < 0.05$.

Effects of SLE on the levels of Cdk1, p27, p53, cyclin B1, cyclin E, caspase-3 and -8, procaspase-9, Bax, Bcl-2, cytochrome c, and NF- κ B p65 and p50 in colon cancer cells. The results from Western blotting are presented in Figure 7A, B and C. SLE increased the levels of p27, p53, cyclin B1, active-caspase-3 and Bax, but decreased the levels of Cdk1, pro-caspase-9, Bcl-2, and NF- κ B p65 and p50.

Effect of SLE on the gene expression (mRNA) of Cdk1, Cdc25b, Cdc25c, p27, p21, MDM2, Wee 1, Bcl-2, Apaf-1, Topo II α , II β , NF- κ B and I- κ B. The RT-PCR analysis results (Figure 8A, B and C) indicated that SLE increased the gene expression of Cdk1, Cdc25c, Wee1 and p27 but decreased the gene expression of MDM2, Bcl-2, Apaf-1 and topoisomerase II α .

Discussion

In the present study, the SLE induced morphological changes of the colo 205 cells in a dose- and time-dependent manner. The morphological changes were also accompanied

by cell death and a decrease in the percentage of viable cells was shown. The SLE also induced apoptosis in the colo 205 cells in a time-dependent manner. In order to find out the possible signal pathway, we used Western blotting and PCR was used to examine the apoptosis-associated genes and associated proteins expressions.

Further evidence that SLE treatment led to apoptosis was provided by sub-G1 occurrence as analyzed by flow cytometric analysis (Figure 3) and confirmed by DAPI staining (Figure 5), while the DNA gel electrophoresis (Figure 4) indicated that DNA fragments were present. Such observations of apoptotic characteristics have been accepted by many investigators (23-25). DNA damage was also shown to occur by using the Comet assay (data not shown) and ROS production (Figure 6) in the cells treated with SLE. Flow cytometric analysis also showed that SLE decreased the $\Delta\Psi_m$ in the colo 205 cells. It is well known that the $\Delta\Psi_m$ is based on the balance of the ratio of Bax/Bcl-2 (26, 27). Bax is a pro-apoptotic protein which promotes apoptosis while Bcl-2 is an anti-apoptotic protein

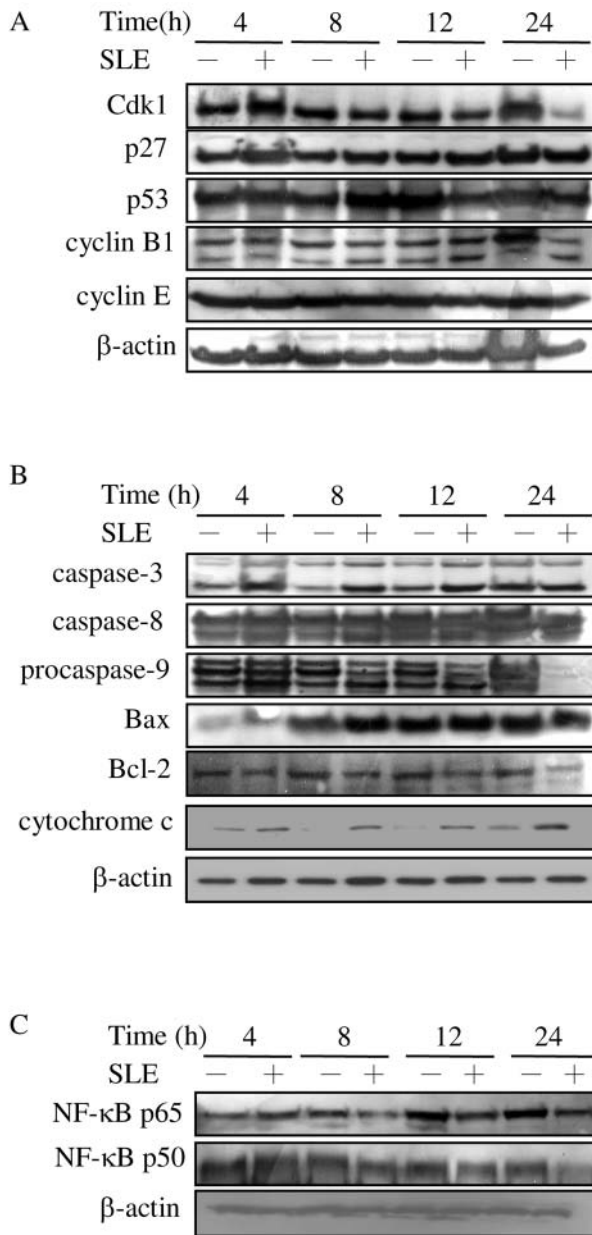


Figure 7. Effects of SLE on the levels of Cdk1, p27, p53, cyclin B1, cyclin E, caspase-3, 8, procaspase-9, Bax, Bcl-2, cytochrome c, NF-κB p65 and p50. The colo 205 cells ($1 \times 10^5/\text{ml}$) were treated with 300 $\mu\text{g}/\text{ml}$ SLE for 4, 8, 12 or 24 h, and then cytosolic total protein was prepared and the individual protein levels were estimated by Western blotting.

which reduces apoptosis (28, 29). It has also been reported that changes of the Bax/Bcl-2 ratio influence apoptosis because they affect the $\Delta\Psi_m$ (27, 30). Evidence that SLE increased the levels of p53 which promoted Bax and Bcl-2 to increase mitochondrial membrane permeability (the loss of $\Delta\Psi_m$) was also provided. Western blotting showed that

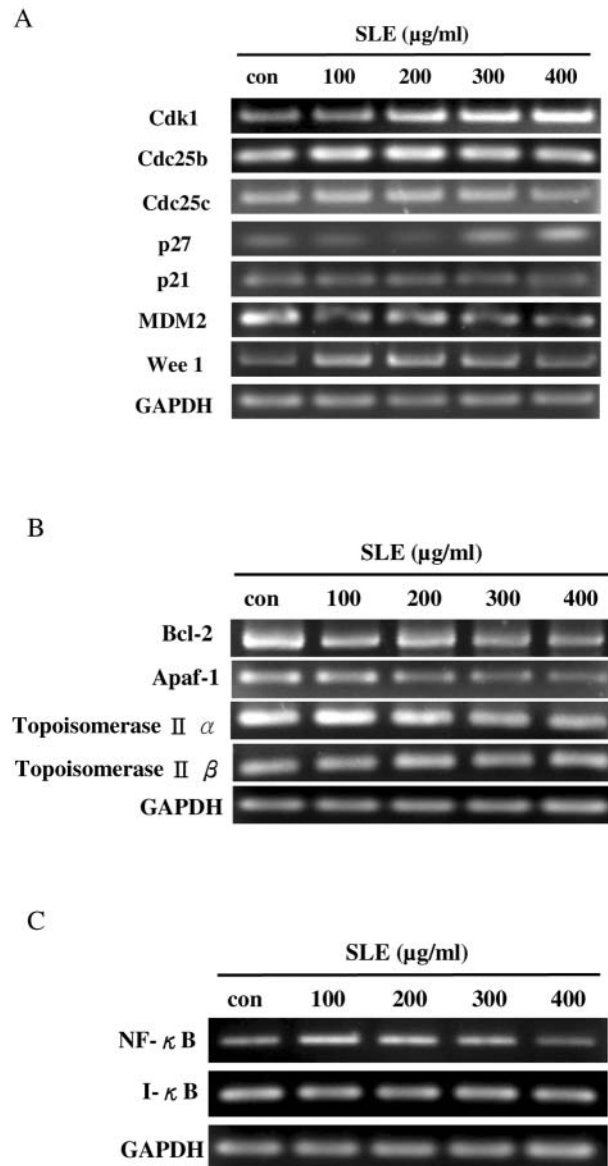


Figure 8. Effect of SLE on the expression of Cdk1, Cdc25b, Cdc25c, p27, p21, MDM2, Wee 1, Bcl-2, Apaf-1, Topo II α , II β , NF- κ B and I- κ B. The colo 205 cells were incubated with different concentrations of 300 $\mu\text{g}/\text{ml}$ SLE for 24 h. The RNA was extracted and subjected to RT-PCR analysis using specific primers for Cdk1, Cdc25b, Cdc25c, p27, p21, MDM2, Wee 1, Bcl-2, Apaf-1, Topo II α , II β , NF- κ B, I- κ B and GAPDH, and then the PCR-amplified cDNA derived from the mRNA was applied to agarose gel-electrophoresis.

SLE 300 decreased the Bcl-2 levels and promoted the Bax levels, and flow cytometric analysis showed that the $\Delta\Psi_m$ was decreased. Our results also showed that SLE induced S-phase arrest (Figure 3). Western blotting was used to show that SLE promoted p53, p27, Cdc25c and Wee1 levels but decreased Cdk1 and cyclin B1 levels (Figure 7A and B).

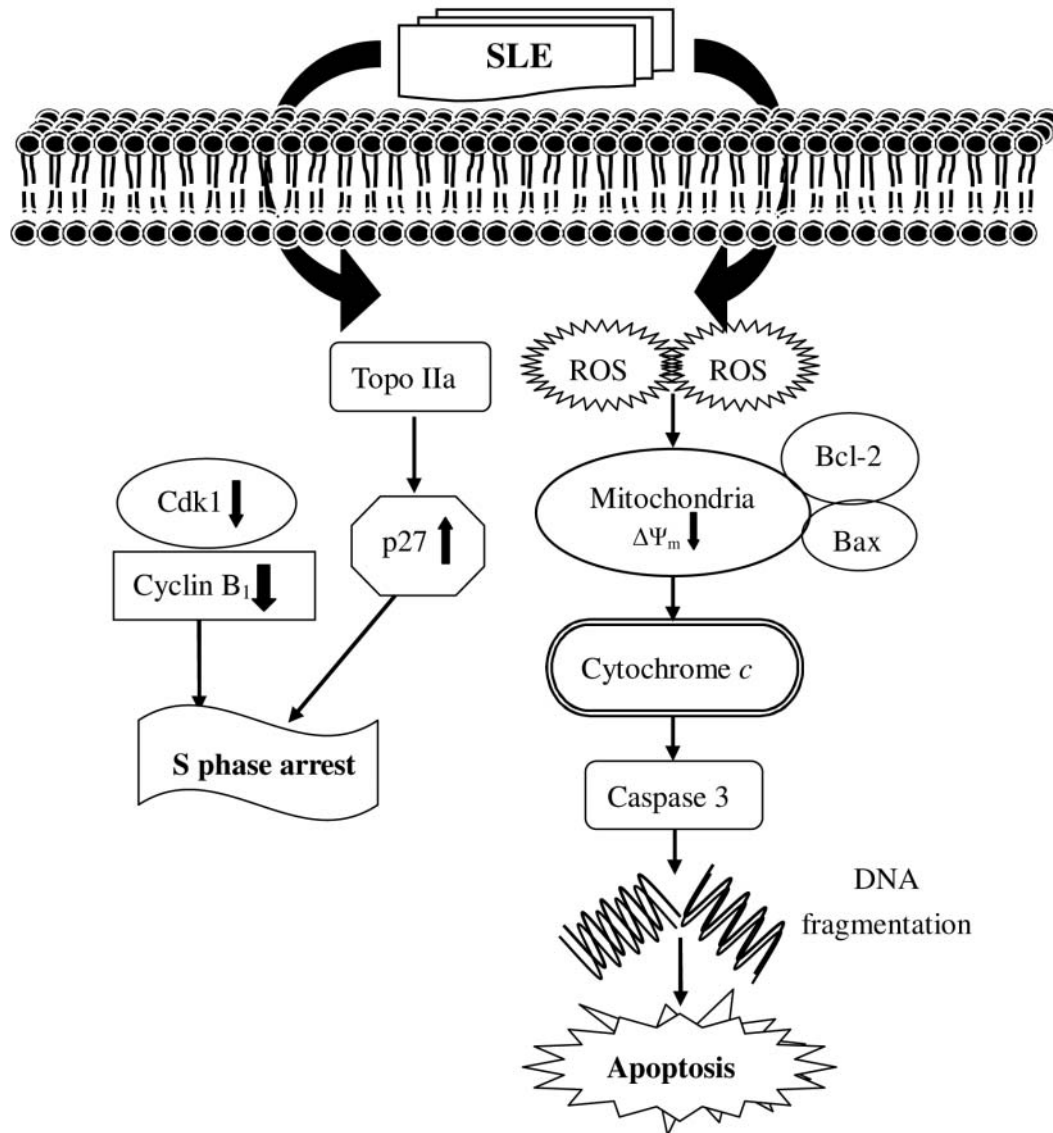


Figure 9. Proposed model of SLE mechanism of action for apoptosis in colo 205 cells.

Those proteins have been demonstrated to be involved in the cell cycle, particularly in the S-phase (31) and cell cycle phase, such as G0/G1, S and G2/M are also associated with check point enzymes (32). The SLE effects on the colo 205 cells that were associated with cell cycle arrest and apoptosis were also confirmed by PCR analysis (Figure 8A, B and C) and indicated that SLE affected gene expression of S-phase and apoptosis-associated genes and in turn affected those protein levels and finally led to apoptosis.

In conclusion, SLE induced ROS production and DNA damage, affected anti- and pro-apoptotic proteins which caused the loss of $\Delta\Psi_m$ then followed by activation of caspase-3 leading to apoptosis (Figure 9). Therefore, SLE may be useful for treating colon cancer in the future.

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