ORIGINAL RESEARCH

Liriodenine, an aporphine alkaloid from *Enicosanthellum pulchrum*, inhibits proliferation of human ovarian cancer cells through induction of apoptosis via the mitochondrial signaling pathway and blocking cell cycle progression

Noraziah Nordin[1]
Nazia Abdul Majid[2]
Najihah Mohd Hashim[1]
Mashitoh Abd Rahman[1]
Zalila Hassan[3]
Hapipah Mohd Ali[3]

[1]Department of Pharmacy, Faculty of Medicine, [2]Institute of Biological Sciences, [3]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

Abstract: *Enicosanthellum pulchrum* is a tropical plant from Malaysia and belongs to the Annonaceae family. This plant is rich in isoquinoline alkaloids. In the present study, liriodenine, an isoquinoline alkaloid, was examined as a potential anticancer agent, particularly in ovarian cancer. Liriodenine was isolated by preparative high-performance liquid chromatography. Cell viability was performed to determine the cytotoxicity, whilst the detection of morphological changes was carried out by acridine orange/propidium iodide assay. Initial and late apoptosis was examined by Annexin V-fluorescein isothiocyanate and DNA laddering assays, respectively. The involvement of pathways was detected via caspase-3, caspase-8, and caspase-9 analyses. Confirmation of pathways was further performed in mitochondria using a cytotoxicity 3 assay. Apoptosis was confirmed at the protein level, including Bax, Bcl-2, and survivin, while interruption of the cell cycle was used for final validation of apoptosis. The result showed that liriodenine inhibits proliferation of CAOV-3 cells at 37.3 µM after 24 hours of exposure. Changes in cell morphology were detected by the presence of cell membrane blebbing, chromatin condensation, and formation of apoptotic bodies. Early apoptosis was observed by Annexin V-fluorescein isothiocyanate bound to the cell membrane as early as 24 hours. Liriodenine activated the intrinsic pathway by induction of caspase-3 and caspase-9. Involvement of the intrinsic pathway in the mitochondria could be seen, with a significant increase in mitochondrial permeability and cytochrome c release, whereas the mitochondrial membrane potential was decreased. DNA fragmentation occurred at 72 hours upon exposure to liriodenine. The presence of DNA fragmentation indicates the CAOV-3 cells undergo late apoptosis or final stage of apoptosis. Confirmation of apoptosis at the protein level showed overexpression of Bax and suppression of Bcl-2 and survivin. Liriodenine inhibits progression of the CAOV-3 cell cycle in S phase. These findings indicate that liriodenine could be considered as a promising anticancer agent.

Keywords: *Enicosanthellum pulchrum*, liriodenine, ovarian cancer, CAOV-3, apoptosis

Introduction

Enicosanthellum pulchrum (King) Heusden belongs to the family Annonaceae, which is also known as a family of "mempisang" in Malaysia.[1] This species is often found in the middle of the highlands, and the distribution is mostly in the Cameron Highland, Malaysia, as reported by Chua et al.[2] The species is a medium-sized tree that can reach up to 3–5 m in height.[3] Phytochemical analysis of this plant has reported some known alkaloids in the bark and roots, including (−)-asimilobine, liriodenine, (−)-anonaine,

Correspondence: Nazia Abdul Majid
Institute of Biological Sciences,
Faculty of Science, University of Malaya,
50603 Kuala Lumpur, Malaysia
Tel +60 3 7967 5833
Fax +60 3 7967 5833
Email nazia@um.edu.my

(−)-norliridinine (−)-scoulerine,[4] and cleistopholine.[5] Biological activity has also been reported for the species and compounds, including anti-platelet activating factor, antibacterial, and antiulcer activity.[5–7]

Liriodenine (8H-[1,3]benzodioxolo[6,5,4-de]benzo[g] quinolin-8-one), is an isoquinoline alkaloid. This compound is widely distributed and acts as a chemotaxonomic marker in the Annonaceae family.[8] Biological studies in vivo indicate that liriodenine has antiarrhythmic activity,[9] and its potential as antimicrobial, antibacterial, antifungal,[10–12] mutagenic, and antiplatelet agents[13,14] has been demonstrated in in vitro studies. Previous studies have also reported that liriodenine has prominent cytotoxic effects in several cancer cell lines, inducing G1 cell cycle arrest and repressing DNA synthesis in HepG2 and SK-Hep-1 cells.[15] A report by Chen et al showed liriodenine to have potent activity in colon cancer, and that this compound could inhibit the SW480 cell cycle through the nitric oxide-dependent and p53-dependent G1/S phase arrest pathway.[16] In addition, liriodenine suppressed proliferation of A549 human lung adenocarcinoma cells in a time-dependent fashion.[17] These early findings indicate the strong potential of liriodenine as a therapeutic agent for various types of cancers. The present study assessed liriodenine as an anticancer agent, particularly for human ovarian cancer which is the first conducted in-depth study for the mechanism of apoptosis in vitro.

Materials and methods
Plant materials
The plant *E. pulchrum* was from the Cameron Highlands Mountain Forest, Pahang, Malaysia. The specimen was identified by the late Kamaruddin Mat Salleh from the Faculty of Science and Technology, University Kebangsaan Malaysia. A voucher specimen (SM769) was lodged with the Botany Department Herbarium, University Kebangsaan Malaysia. The air-dried roots were ground to 40–60 mesh.

Root extraction
A total of 100 g of roots were extracted successively by the maceration technique using *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH) (Merck, Whitehouse Station, NJ, USA) solvents. The EtOAc extract (1.96 g) was taken and separated by vacuum liquid chromatography using silica gel type H (Sigma-Aldrich, St Louis, MO, USA) on a gradient of *n*-hexane-CHCl$_3$ (100:0, 70:30, 50:50, 30:70, and 0:100) and CHCl$_3$-MeOH (100:0, 50:50, and 0:100). In total, five fractions were obtained after 10 fractions were combined based on thin layer chromatography analysis.

Preparative HPLC separation
Preparative high-performance liquid chromatography (HPLC) was performed by injecting 2 mL of filtered MeOH fraction 4 onto a Prep Nova-Pak (10 mm ×20 mm ×30 mm) HR C-18 reversed-phase HPLC column (Waters, Milford, MA, USA). The fractions were eluted using a 322 Gilson pump (Gilson, Fairfield, NJ, USA) at a permanent flow rate of 12 mL/min. The solvent system consisted of 10% acetonitrile in water to 100% acetonitrile (v/v) and flow gradiently for 90 minutes. Liriodenine (8.0 mg, 0.4% of yield) was detected at 254 nm absorbance using a Gilson 156 ultraviolet-visible absorbance detector. The compound was collected at 26–28 minutes.

Cell viability assay
Human ovarian cancer (CAOV-3 and SKOV-3) and human hepatic (WRL-68) cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA), and an immortalized human ovarian epithelial cell line (SV-40) was purchased from ABM Inc (Richmond, BC, Canada). Briefly, confluence cells were harvested and centrifuged at 1,800 rpm for 5 minutes. The concentration of cells was adjusted to 1×10^6 cells/mL. The cells were then treated with liriodenine at various concentrations in a 96-well plate and incubated for 24, 48, and 72 hours. A total of 20 µL of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Invitrogen, Carlsbad, USA) was added to each well and incubated for 3 hours. The plate was recorded at an absorbance of 570 nm to determine the IC$_{50}$ value. Paclitaxel and cisplatin were used as the positive control in this assay.

Acridine orange/propidium iodide double staining
Apoptosis were counted by propidium iodide (PI) and acridine orange (AO) double staining according to the standard procedure and were examined under a fluorescent microscope (Leica, Wetzlar, Germany) attached to Q-Floro software. In brief, the assay was carried out in a 25 mL culture flask (Nunc®, Sigma-Aldrich). CAOV-3 cells were seeded at density of 1×10^6 cells/mL and treated with an IC$_{50}$ concentration of liriodenine. The flasks were then incubated at 37°C for 24, 48, and 72 hours. The harvested cells were washed and stained with fluorescent dyes (AO/PI) comprising 10 µL of AO (10 µg/mL) and 10 µL of PI (10 µg/mL). The morphology of the cells was observed under an ultraviolet-fluorescent BX60 microscope (Olympus, Tokyo, Japan) within 30 minutes, before the intensity of fluorescent started to disappear.

Annexin-V-FITC assay

CAOV-3 cells (5×10^4 cells/mL) were seeded into a six-well plate. After exposure to liriodenine for 24, 48, and 72 hours, the cells were harvested and washed with phosphate-buffered saline. The assay was performed using an FITC (fluorescein isothiocyanate) Annexin V Apoptosis Detection Kit I (BD Pharmingen™, San Diego, CA, USA). The treated cells were centrifuged at 1,600 rpm for 5 minutes. Next, 100 µL of each sample were taken and placed into a tube containing 5 µL of FITC Annexin V and 10 µL of PI stain. The suspension was mixed, and 100 µL of 1× Assay buffer was added per tube. All samples were then analyzed using a flow cytometer (BD FACSCanto™ II, San Jose, CA, USA).

Caspase analysis

The caspase-3, caspase-8, and caspase-9 colorimetric assays were performed using a commercial kit (R&D Systems Inc, Minneapolis, MN, USA). The cells were seeded and treated with the IC_{50} concentration in 25 mL flasks for 24, 48, and 72 hours. Cells that had been stimulated for apoptosis were collected and centrifuged at 1,600 rpm for 10 minutes. The cell pellet was lysed by addition of lysis buffer. Prior to centrifugation, the lysate cells were incubated on ice for 10 minutes and then centrifuged at $10,000\times g$ for 1 minute. The assay was carried out in a 96-well flat bottom microplate. Next, 50 µL of cell lysate and 50 µL of 2× reaction buffer 3, 8, or 9 were added in each well; 5 µL of caspase-3, caspase-8, or caspase-9 colorimetric substrate (LEHD-pNA) was then added to each reaction well and incubated at 37°C for 1 hour. The plate was read on a luminescence microplate reader (Infinite M200 PRO, Tecan, Männedorf, Switzerland) at a wavelength of 405 nm.

Multiple cytotoxicity assays

Multiple cytotoxicity assays were carried out using the Cellomics® Multiparameter Cytotoxicity 3 kit (Thermo Scientific, Pittsburgh, PA, USA). The assay was performed using a 96-well microplate. The cells were seeded in the plate at a concentration of 5×10^3 cells per well. The cells were then treated with liriodenine at concentrations of 20, 30, and 40 µM, respectively, and incubated overnight at 37°C and 5% CO_2 saturation. Briefly, several solutions were added successively in each well containing 50 µL of live cell staining, 100 µL of fixation solution, 100 µL of 1× permeabilization buffer, and 100 µL of 1× blocking buffer for an incubation duration of 30, 20, 10, and 15 minutes, respectively. Two antibodies solutions (primary and secondary antibody) were used, whereby 50 µL of each solution were

added to the wells. The plate was then read and the results were evaluated on an ArrayScan HCS reader.

Analysis of DNA fragmentation

The CAOV-3 cells were plated at a density of 5×10^4 cells/mL in a 25 mL flask and incubated for 24 hours. The cells were then treated with liriodenine for 24, 48, and 72 hours. A Suicide-Track™ DNA Ladder isolation kit (Calbiochem KgaA, Darmstadt, Germany) was used to analyze the DNA fragments (mononucleosome and oligonucleosomes) formed during apoptosis. In brief, the cells were trypsinized and centrifuged at 1,800 rpm for 5 minutes. The pellet was gently resuspended in 55 µL of solution 1, 20 µL of solution 2, and 25 µL of solution 3 (kit components). After incubation overnight at 50°C, the mixture was mixed with 500 µL of resuspension buffer. DNA laddering was detected by preparing 1.5% agarose gel in 1× TAE buffer with the staining reagent supplied in the kit. The gel was run consistently at 50 V until the dye front was 1–2 cm from the bottom of the gel. The DNA was visualized by ultraviolet light transilluminator and photographed.

Western blot analysis

The CAOV-3 cells were seeded in a 75 mL culture flask and treated with liriodenine at 24, 48, and 72 hours. The cells were then harvested by centrifugation at 13,000 rpm for 10 seconds and resuspended in 400 µL of Pro-Prep™ solution. Cell lysis was induced by incubation at −20°C for 20 minutes.

After incubation, the cell pellet was centrifuged at 13,000 rpm (4°C) for 5 minutes. Total proteins were taken and mixed with loading dye before separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 90 minutes. The gel was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to run for another 90 minutes. The membrane was blocked with 5 mL of 5% bovine serum albumin and incubated overnight. The appropriate primary antibodies for β-actin (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), and survivin (1:1,000) were used (Abcam, Inc, Burlingame, CA, USA) and conjugated with secondary antibody (anti-rabbit) for 1 hour at room temperature. The bound antibody was identified using peroxidase-conjugated and exposed for a few minutes to allow the appearance of bands. The polyvinylidene difluoride membrane was finally viewed and photographed using an ultraviolet light transilluminator.

Cell cycle analysis

The CAOV-3 cancer cells were seeded into a 25 mL culture flask at a concentration of 1×10^6 cells/mL and treated with

liriodenine for 24, 48, and 72 hours. The cells were then centrifuged at 1,800 rpm for 5 minutes. The supernatant was removed and the pellet was washed with phosphate-buffered saline. The cells were fixed with fixation solution (700 μL of 90% cold ethanol [EtOH]) and kept at 4°C overnight to restore the integrity of the cells. EtOH was discarded by centrifuging at 1,800 rpm for 5 minutes. The cells were then rinsed, and 600 μL of phosphate-buffered saline was added. A total of 25 μL of RNase A (10 mg/mL) and 50 μL of PI (1 mg/mL) were added to the fixed cells, which were then incubated for 1 hour at 37°C. Next, the DNA content of the cells was analyzed by flow cytometer (BD FACS Canto™ II).

Statistical analysis

Each test was performed as three separate experiments, and the results are the reported as mean ± standard deviation. The data were evaluated by one-way analysis of variance followed by Dunnett's test using Statistical Package for the Social Sciences version 17.0 software (Chicago, IL, USA) to determine statistical significance and GraphPad Prism version 4.0 (GraphPad Software Inc, La Jolla, CA, USA) to determine the IC_{50} values.

Results

Identification of liriodenine

Liriodenine (Figure 1) appeared in the form of yellow needles. The ^{1}H and ^{13}C nuclear magnetic resonance spectroscopic data of compound in this study was compared with liriodenine structure that has been identified in other species.[18] However, this current study reported nuclear magnetic
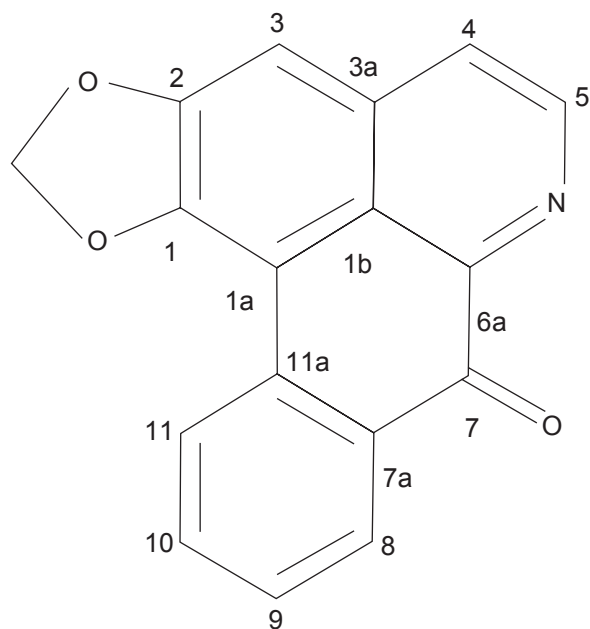

Figure 1 Structure of liriodenine.

Table 1 ^{1}H (600 MHz) and ^{13}C (150 MHz) nuclear magnetic resonance data for liriodenine

C/H	δ_H (J in Hz)	δ_C type	[a]HMBC
1	–	147.9, C	–
1a	–	108.1, C	–
1b	–	131.3, C	–
2	–	151.7, C	–
3	7.09, s	103.2, CH	3a 2, 1
3a	–	123.2, C	–
4	7.69, m	124.2, CH	3a, 3
5	8.82, d (4.86)	144.9, CH	4, 6a, 7a
6a	–	145.4, C	–
7	–	182.4, C=O	–
7a	–	135.7, C	–
8	8.52, d (7.02)	128.8, CH	9, 10
9	7.51, t (6.72)	128.5, CH	–
10	7.66, m	133.9, CH	11, 11a
11	8.55, t (7.44)	127.3, CH	1a
11a	–	132.3, C	–
-OCH$_2$O-	6.31, s	102.5, CH$_2$	2, 1

Note: [a]Assigned by HMBC. s, d, t, and m represent singlet, doublet, triplet, and multiplet of the proton peaks, respectively. C/H represents carbon and proton numbers in the structure, respectively. –, not applicable.
Abbreviation: HMBC, heteronuclear multiple-bond correlation.

resonance spectroscopic data in one- and two-dimensional nuclear magnetic resonance, which has never been reported before (heteronuclear single quantum coherence and heteronuclear multiple-bond correlation) techniques and electrospray ionization mass spectrometry (Table 1).

Liriodenine inhibited CAOV-3 cells in vitro

The cytotoxic effects of liriodenine were studied in CAOV-3, SKOV-3, and normal ovarian (WRL-68 and SV-40) cell lines. Two standard drugs, paclitaxel and cisplatin, were used as positive controls in this experiment (Table 2). The IC_{50} results show that liriodenine inhibited CAOV-3 cell growth at a concentration of 37.3±1.06 μM after 24 hours of exposure. However, the IC_{50} concentration decreased after the cells were exposed to liriodenine at 48 and 72 hours, with values of 26.3±0.07 μM and 23.1±1.62 μM, respectively (Figure 2). The effect of liriodenine on CAOV-3 was significant when compared with cisplatin as a standard drug. Meanwhile,

Table 2 Inhibitory effects of study compounds at 24 hours

Compounds/ cell lines	IC_{50} ± SD (μM)			
	CAOV-3	SKOV-3	WRL-68	SV-40
Liriodenine	37.3±1.06	68.0±1.56	>100	>100
Paclitaxel	0.91±0.01	5.5±0.31	>30	>30
Cisplatin	62.81±0.35	66.7±0.42	>100	>100

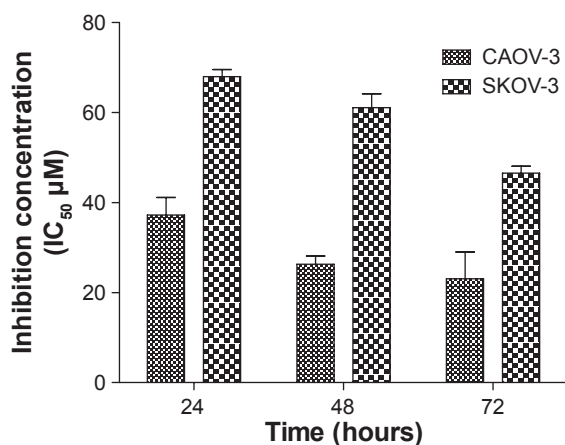**Abbreviation:** SD, standard deviation.

Figure 2 IC$_{50}$ value of liriodenine at different time points in a CAOV-3 cell line.

liriodenine exhibited less activity in SKOV-3 cells, with IC$_{50}$ values of 68.0±1.56, 61.1±3.09, and 46.5±1.55 μM at 24, 48, and 72 hours, respectively. Additionally, both the normal ovarian cell lines displayed no evidence of cytotoxicity at a concentration higher than 100 μM.

Determination of changes in morphology using AO/PI double staining

Morphological changes in CAOV-3 cells were observed under the fluorescence microscope after exposure to liriodenine. These changes could be seen at 24, 48, and 72 hours (Figure 3). In comparison with untreated CAOV-3 cells, the treated cells showed an intact green nuclear structure with a round shape and no disruption. After 24 hours of treatment with liriodenine, the morphology of CAOV-3 cells showed cell membrane blebbing and fragmented DNA with bright green fluorescence. The changes could be clearly observed after 48 and 72 hours of treatment, with clear growth inhibition, increased cell membrane blebbing, presence of more apoptotic bodies, and also the appearance of a reddish-orange color due to PI being bound to denatured DNA cells, indicating dead cells. Furthermore, the numbers of CAOV-3 cells that underwent early and late apoptosis increased in a time-dependent manner.

Annexin V analysis

CAOV-3 cells undergoing early apoptosis after being treated with liriodenine was detected by Annexin V-FITC assay. The Annexin V-FITC assay showed an initial stimulation of apoptosis in a time-dependent manner (Figure 4). The treated CAOV-3 cells exhibited a significant increase ($P<0.05$) of early apoptosis by 22.0%, 30.1%, and 36.6% at 24, 48, and 72 hours, respectively. Conversely, the percentages of viable cells were reduced from 54.7%, 41.3%, and 29.8%. The exposure of
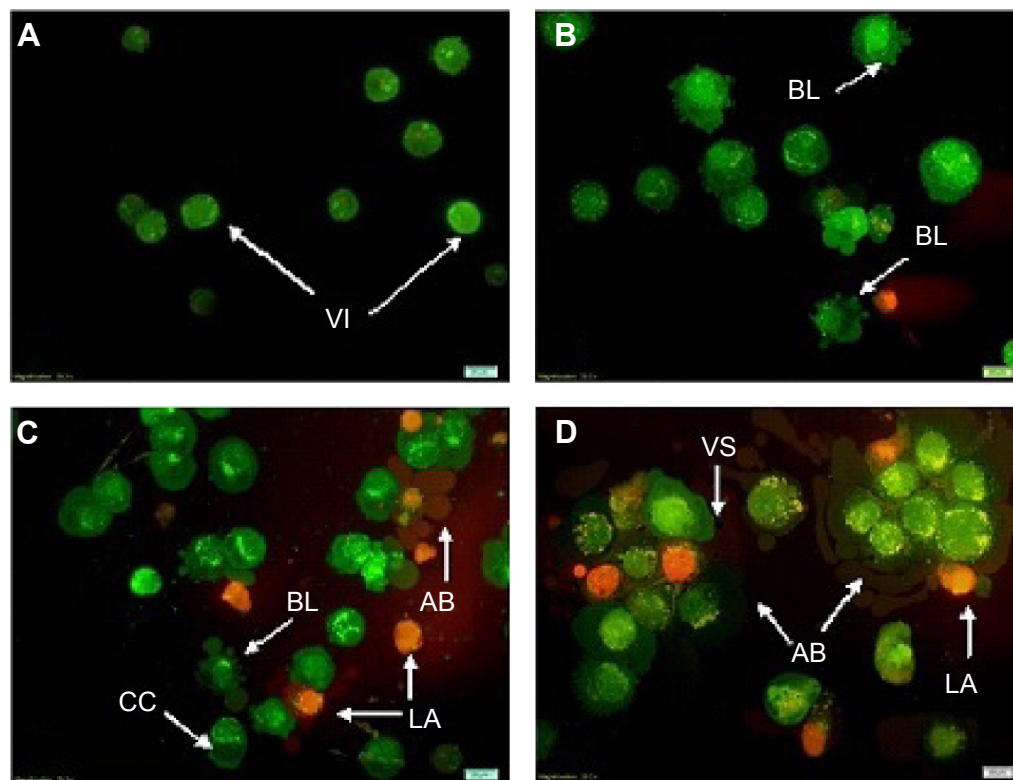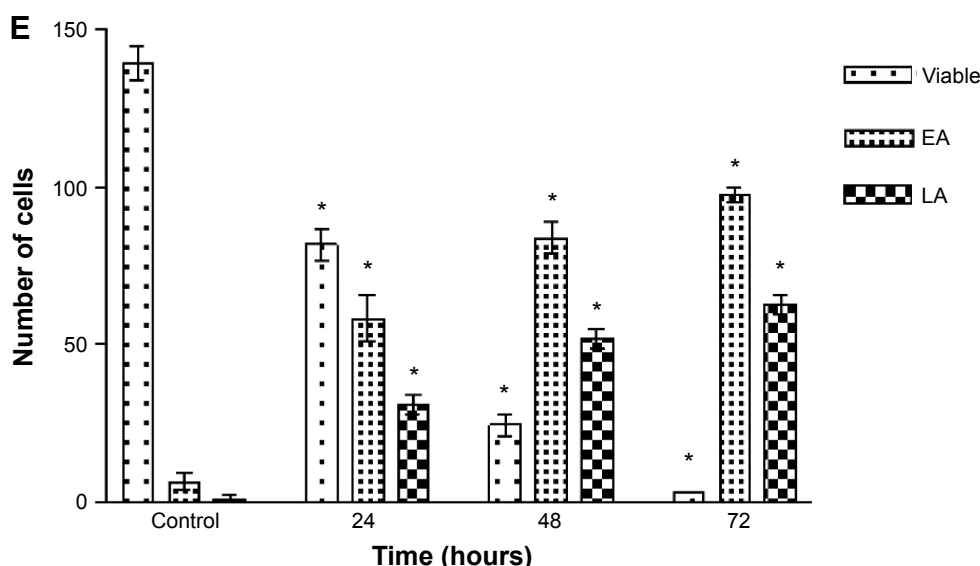

Figure 3 (*Continued*)

Figure 3 Fluorescent micrographs and quantitative analysis of CAOV-3 cells double-stained with acridine orange and propidium iodide. Cells were treated with liriodenine in a time-dependent manner.
Notes: (**A**) Untreated cells without any morphological changes. (**B**) Cells treated for 24 hours show early apoptosis by the presence of bright green color (acridine orange). (**C**) Cells treated for 48 hours show more membrane cell blebbing and chromatin condensation. (**D**) Cells treated for 72 hours show more orange-colored staining, indicating late apoptosis. (**A–D**) Magnification 40×. (**E**) Histogram of three cell phases. *$P<0.05$ indicates a significant difference from control.
Abbreviations: Vl, viable cells; BL, blebbing of cell membrane; CC, chromatin condensation; EA, early apoptosis; LA, late apoptosis; AB, apoptotic bodies; VS, production of vesicles.

liriodenine in CAOV-3 cells at a longer period (24 to 72 hours) caused decrease in viable cells and increase in cell death.

Liriodenine induced caspase activity

CAOV-3 cells treated with liriodenine were analyzed for induction of caspase-3, caspase-8, and caspase-9. The results showed that liriodenine stimulates both caspase-3 and caspase-9, as indicated by increasing values for luminescence units over time (Figure 5). There was a significant difference ($P<0.05$) between treated and untreated cells for caspase-3 and caspase-9 during the three stipulated time periods, while the luminescence unit for caspase-8 decreased. Stimulation of caspase-3 and caspase-9 indicates that the apoptosis triggered by liriodenine involves the intrinsic pathway.

Liriodenine induced apoptosis on mitochondrial disruption

The Multiparameter Cytotoxicity 3 assay (Cellomics) was carried out to investigate four parameters that are important in the apoptosis process, ie, total nuclear intensity, mitochondrial membrane potential, cell permeability, and cytochrome c release (Figure 6). The results indicated a reduction in total nuclei (Hoechst dye), which refers to the number of CAOV-3 cells present after being treated with liriodenine for 24 hours. The same situation occurred in the mitochondrial

membrane potential, whereby there was a significant reduction in intensity ($P<0.05$) in cells treated with liriodenine 30 and 40 µM, respectively, at 24 hours. Meanwhile, cell permeability and cytochrome c were significantly increased ($P<0.05$) when compared with untreated CAOV-3 cells at the same concentrations.

Liriodenine triggered DNA fragmentation in CAOV-3 cells

CAOV-3 cells treated with liriodenine at concentrations of 37 µM showed the presence of DNA laddering after 72 hours of treatment (Figure 7). The presence of laddering indicated DNA fragmentation in CAOV-3 cells. The positive control used in this study comprised HL-60 cells treated with actinomycin D, which showed formation of a clear ladder and had close similarities to the marker.

Western blot analysis

Involvement of the mitochondria in apoptosis was confirmed at the protein level by Western blotting. There were three proteins involved, ie, Bax, Bcl-2, and survivin. We observed upregulation of Bax protein in CAOV-3 cells treated with liriodenine when compared with untreated CAOV-3 cells. In contrast, Bcl-2 and survivin showed downregulation of protein expression after exposed to liriodenine at 24, 48, and 72 hours (Figure 8). Moreover, there were significant
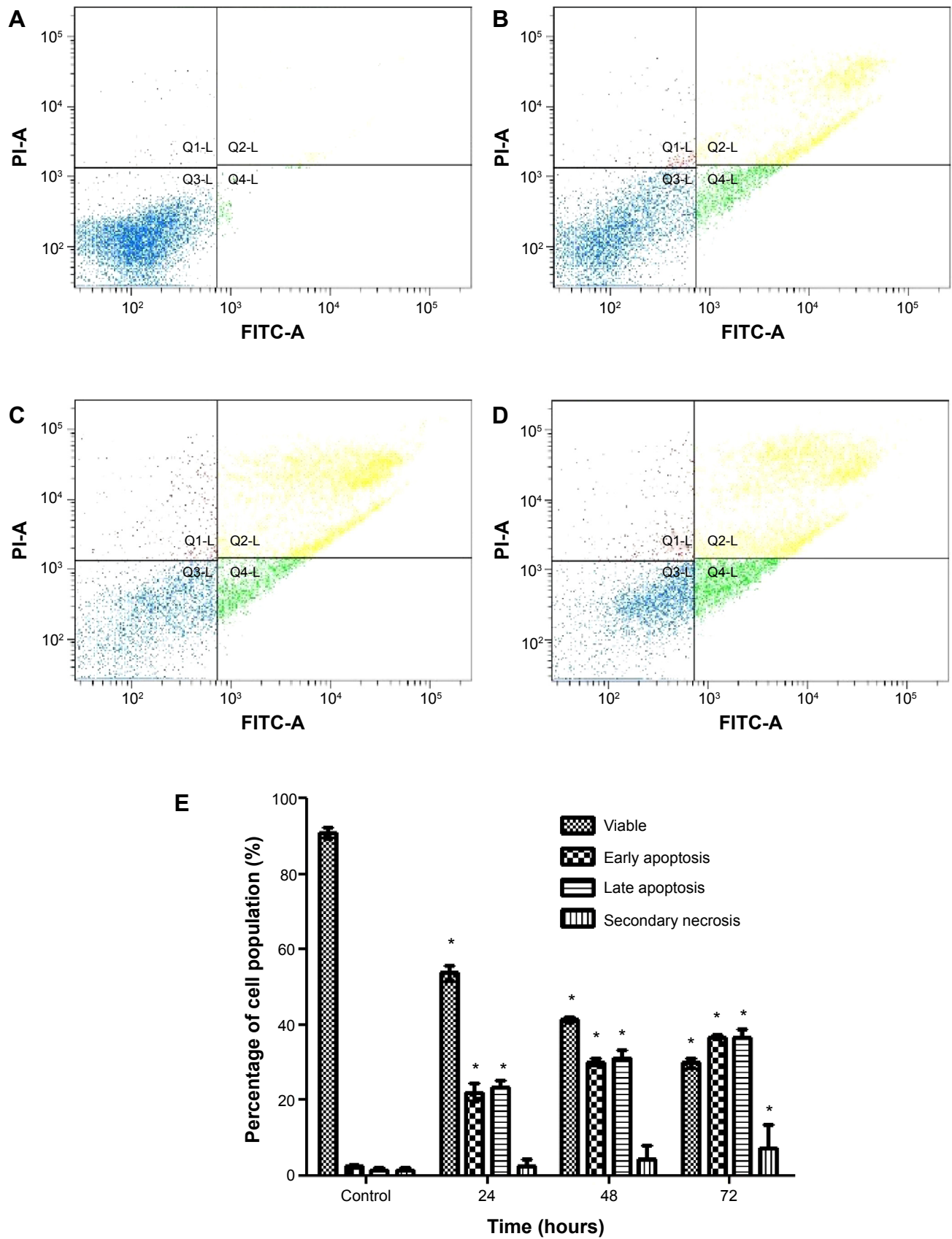
Figure 4 Flow cytometry analysis of Annexin V-fluorescein isothiocyanate in CAOV-3 cells treated with liriodenine in a time-dependent manner.
Notes: (**A**) Controls (untreated), (**B**) 24 hours, (**C**) 48 hours, (**D**) 72 hours, and (**E**) histogram. Results are shown as the mean ± standard deviation of three replicates.
*$P<0.05$ indicates a significant difference from control.
Abbreviations: PI-A, propidium iodide; FITC, fluorescein isothiocyanate; Q, quadrant; Q1, secondary necrosis; Q2, late apoptosis; Q3, viable cells; Q4, early apoptosis.
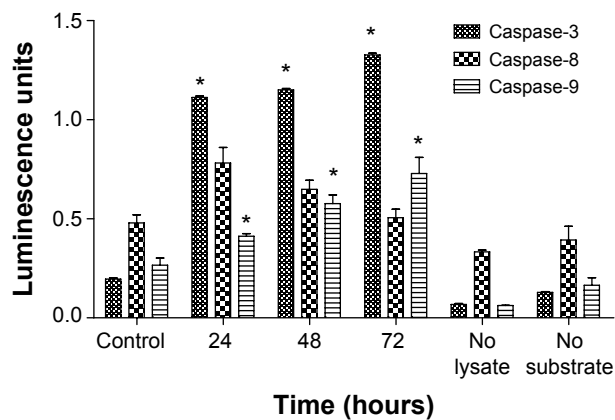
Figure 5 The colorimetric analysis of caspase-3, caspase-8, and caspase-9 in untreated and treated CAOV-3 cells with liriodenine at 24, 48, and 72 hours.
Notes: Values are reported as the mean ± standard deviation of three independent experiments. *$P<0.05$ indicates a statistically significant difference.

differences ($P<0.05$) in all proteins at 72 hours between CAOV-3 cells treated or not treated with liriodenine.

Liriodenine inhibited proliferation of CAOV-3 cells and arrested the cell cycle at S phase

Analysis of cell cycle arrest by liriodenine in CAOV-3 cells found that this alkaloid inhibits cell proliferation at S phase (Figure 9). The results showed a significant ($P<0.05$) increase in the number of treated cells in S phase at 24–72 hours when compared with untreated cells. However, uneven changes occurred at the three time points for G0/G1 and G2/M phases, indicating that division of CAOV-3 cells halted during S phase.
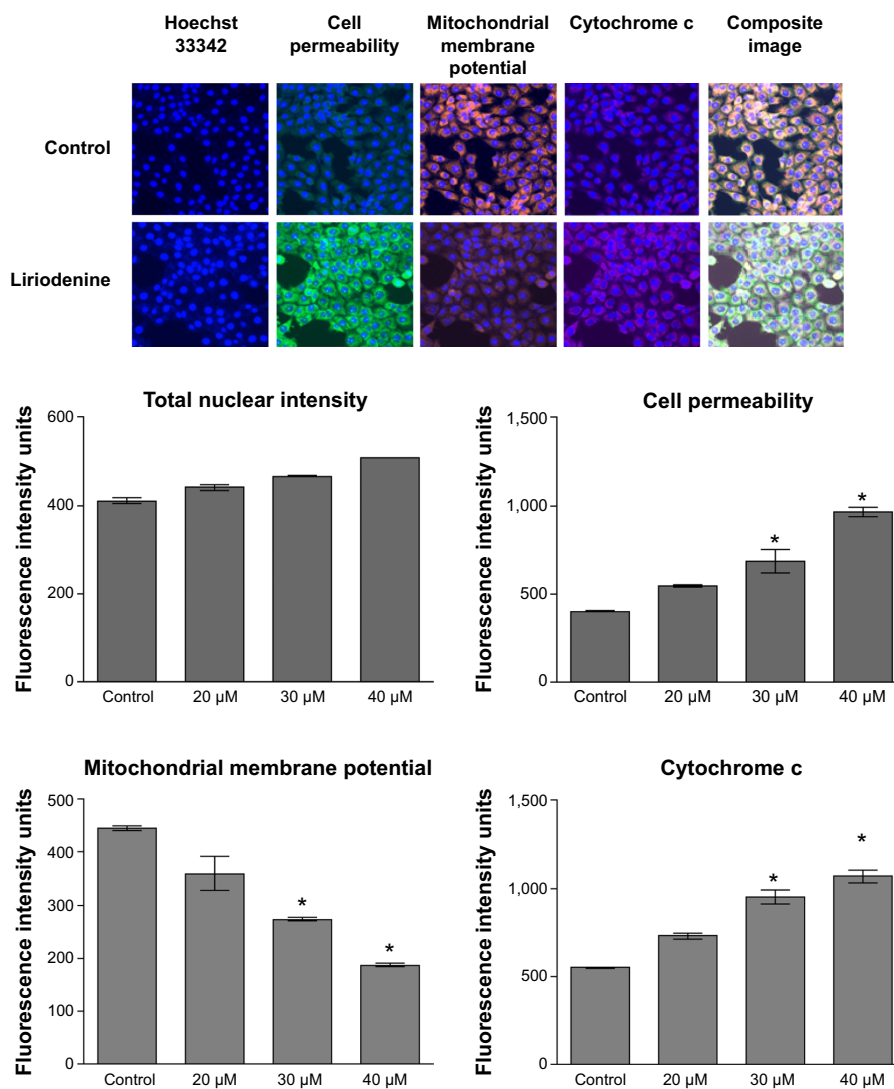

Figure 6 Representative images and quantitative analysis of CAOV-3 cells treated or not treated with liriodenine at 24 hours.
Notes: The cells were stained with Hoechst, fluorescein isothiocyanate, mitochondrial membrane potential, and cytochrome c dyes. The treated cells stained with Hoechst, fluorescein isothiocyanate, and cytochrome c dyes showed an increase in intensity that was dependent on the increase of liriodenine concentration, suggesting cell permeability was disrupted, allowing release of cytochrome c into the cytosol, while the mitochondrial membrane potential and cell numbers showed a reduction in fluorescence intensity. Average fluorescence intensities were observed simultaneously in CAOV-3 cells for all parameters. All data are shown as the mean ± standard deviation. Statistical significance was expressed at *$P<0.05$ (magnification 20×).
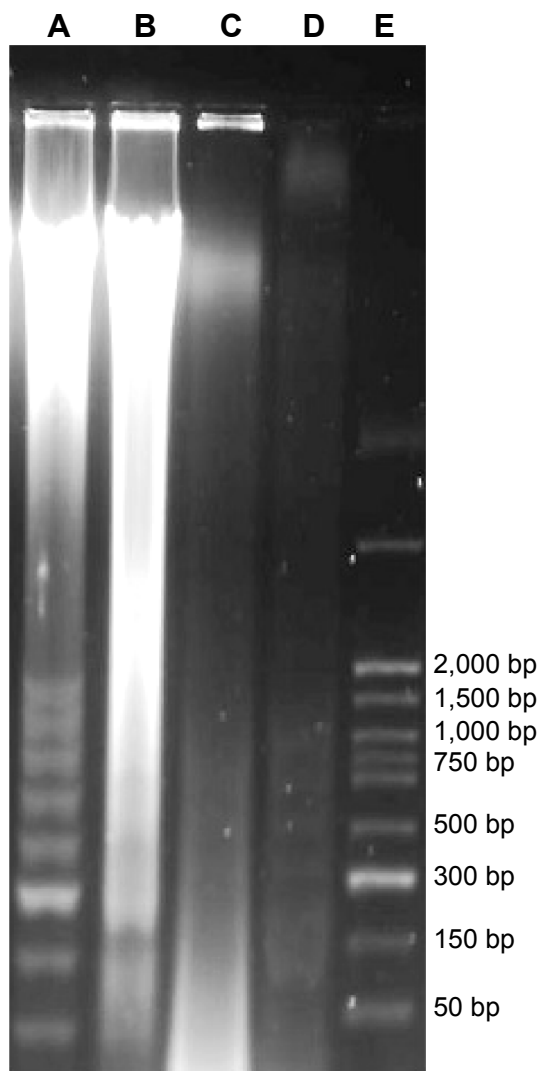
Figure 7 Electrophoretic separation of fragmented DNA for CAOV-3 cells treated or not treated with liriodenine.
Notes: Lane **A** is the positive control, where HL-60 cells were treated with actinomycin **D**. Lanes **B–C** are cells treated with liriodenine for 72 and 48 hours, respectively. Lane **D** shows untreated cells. Lane **E** shows the DNA marker (50 bp).
Abbreviation: bp, base pairs.

Figure 8 Effect of liriodenine on level of apoptosis in regulatory proteins at 24, 48, and 72 hours, with β-actin as a loading control.
Notes: All data are shown as the mean ± standard deviation. *$P < 0.05$ indicates a statistically significant difference.

Discussion

The main aim of this study was to evaluate liriodenine, which was isolated through the modern technique of preparative high-performance liquid chromatography, in the search for potential anticancer drugs particularly for human ovarian cancer. The effect of liriodenine on CAOV-3 cells in vitro was observed by investigation of the mechanisms of induction of apoptosis. The ability of liriodenine to inhibit 50% growth of CAOV-3 cells at concentrations as low as 37 μM after 24 hours of treatment showed that liriodenine and cisplatin are both comparable in terms of their activity. However, the findings of this study demonstrate that liriodenine appears to be better than cisplatin, an existing drug that is used extensively in the treatment of ovarian cancer. In light of
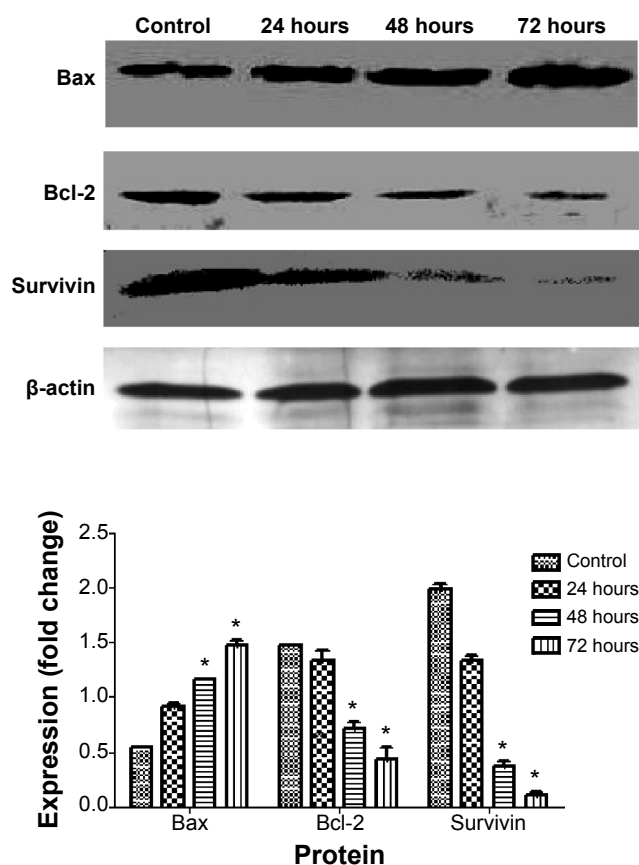
the current findings, liriodenine can potentially overcome the resistance problems faced by some drugs.[19]

Detection of apoptosis by morphological changes in CAOV-3 cells was observed using the AO/PI assay under fluorescence microscopy. Changes in the treated cells were similar to those described by previous researchers,[20,21] which can be seen via formation of a dense and packed cytoplasm as well as chromatin condensation.[19] In addition, longer exposure of the cells to liriodenine can lead to aggressive changes in cell morphology, such as the presence of extensive cell membrane blebbing. After extensive cell membrane blebbing occurs, it will be followed by the formation of apoptotic bodies through the separation of cell fragments.[22] Annexin V-FITC is an important marker for confirmation of apoptosis in cells due to the presence of an intact cell membrane in the early phase of apoptosis.[22] This condition allows Annexin V-labeled FITC to be bound to the outer membrane of the cell, whereby the presence of translocated phosphatidylserine can later be detected by flow cytometry.[23,24] This action happened as early as 24 hours, as shown in Figure 4. Induction of apoptosis by liriodenine through the intrinsic pathway was
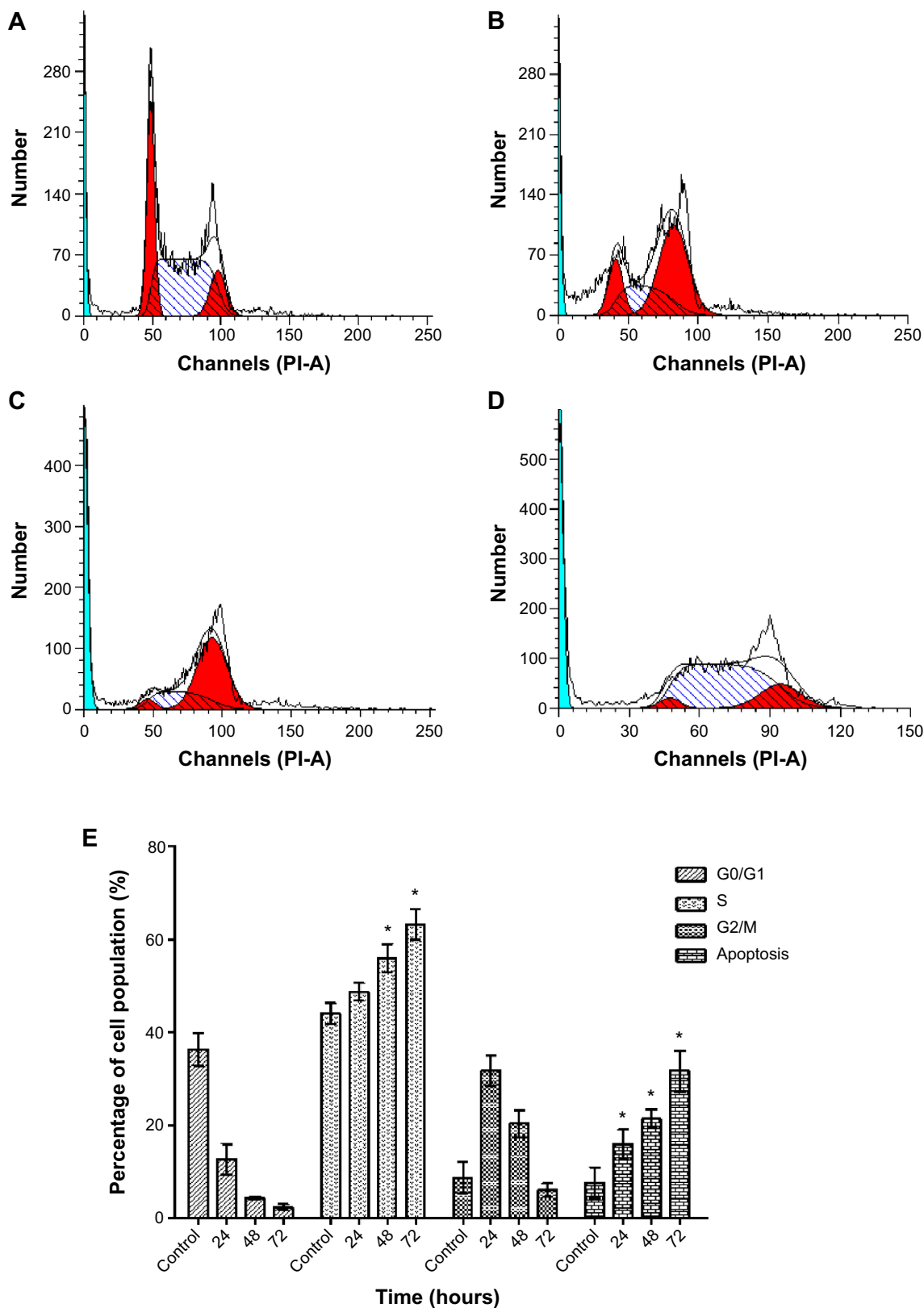
Figure 9 Flow cytometric analysis of cell cycle distribution of CAOV-3 cells treated with liriodenine in a time-dependent manner.
Notes: (**A**) Untreated cells, (**B**) 24 hours, (**C**) 48 hours, (**D**) 72 hours, and (**E**) graphical analysis of cell cycle arrest in CAOV-3 cells. The results are shown as the mean ± standard deviation of three replicates. *$P<0.05$ indicates a statistically significant difference from the control in each phase. G0/G1 is Gap 0 or 1 in which G0 indicated resting phase where the cell has left the cycle and stop dividing. G1 indicated that cells increase in size. S represents synthesis where the DNA replication occurs during this phase. Meanwhile the G2/M represents Gap2 or mitosis where the cell will continue to grow.
Abbreviation: PI-A, propidium iodide.

also seen in activation of caspase-9. The intrinsic pathway is one of the crucial apoptotic pathways, as opposed to the extrinsic pathway.[22,25] Once this pathway has been activated, several factors will cause the mitochondria to be involved in downstream apoptotic signaling.

Factors involved in downstream events of apoptosis can be explained as chaos in the inner mitochondrial membrane, leading to opening of the mitochondrial permeability transition pore and damage to the mitochondrial transmembrane potential, which finally allows cytochrome c to be released from the mitochondria into the cytoplasm.[26] Involvement of that mitochondria can also be supported through several proteins that trigger apoptosis, including Bax and Bcl-2. The Bcl-2 and Bax proteins play a vital role as antiapoptotic and proapoptotic factors, respectively. Both of these proteins have special significance in determination of apoptosis or otherwise.[27] In the present study, both proteins exhibited an opposing physiology action on cancer cells after exposure to liriodenine, suggesting that apoptosis was induced in CAOV-3 cells.

One of the common processes that is widely used in the determination of apoptosis is DNA fragmentation.[22] This phenomenon occurs when the cell enters the final phase of apoptosis. As shown in Figure 7, the formation of a DNA ladder indicates DNA fragmentation occurring in treated-CAOV-3 cells after 72 hours of exposure. The existence of the DNA ladder provides evidence of DNA fragmentation as a result of endonuclease cleavage when caspase-3 breaks down.[28,29]

The effects of liriodenine on proliferation of CAOV-3 cells were also examined by cell cycle analysis and expression of survivin protein. This protein not only inhibits apoptosis but is also involved in regulation of cell division and enhancement of angiogenesis.[30] Survivin is expressed at high levels on many cancer cells, including ovarian cancer,[31] making this protein a good marker for use in this study. In the present study, liriodenine successfully decreased the expression of surviving, as shown in Figure 8. A similar situation was observed in the cell cycle analysis, where inhibition of survivin caused the CAOV-3 cells to stop dividing due to arrest in S phase (Figure 9). When the cell cycle is disturbed, DNA becomes damage and apoptosis is initiated.[32] Based on all the evidence and the results shown, liriodenine does positively induce apoptosis in CAOV-3 cells.

Conclusion

Liriodenine induced apoptosis in CAOV-3 cells via the mitochondrial signaling pathway by involvement of caspase-3 and caspase-9. Similarly, liriodenine was able to inhibit proliferation of CAOV-3 cells. This study demonstrates that liriodenine is a promising potential drug candidate for the treatment of ovarian cancer.

Disclosure

The authors report no conflicts of interest in this work.

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