

Artonin E induces p53-independent G1 cell cycle arrest and apoptosis through ROS-mediated mitochondrial pathway and livin suppression in MCF-7 cells

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Abstract: Artonin E is a prenylated flavonoid compound isolated from the stem bark of *Artocarpus elasticus*. This phytochemical has been previously reported to be drug-like with full compliance to Lipinski's rule of five and good physicochemical properties when compared with 95% of orally available drugs. It has also been shown to possess unique medicinal properties that can be utilized in view of alleviating most human disease conditions. In this study, we investigated the cytotoxic mechanism of Artonin E in MCF-7 breast cancer cells, which has so far not been reported. In this context, Artonin E significantly suppressed the breast cancer cell's viability while inducing apoptosis in a dose-dependent manner. This apoptosis induction was caspase dependent, and it is mediated mainly through the intrinsic pathway with the elevation of total reactive oxygen species. Gene and protein expression studies revealed significant upregulation of cytochrome c, Bax, caspases 7 and 9, and p21 in Artonin E-treated MCF-7 cells, while MAPK and cyclin D were downregulated. Livin, a member of the inhibitors of apoptosis, whose upregulation has been noted to precede chemotherapeutic resistance and apoptosis evasion was remarkably repressed. In all, Artonin E stood high as a potential agent in the treatment of breast cancer.

Keywords: Artonin E, breast cancer, apoptosis, cell cycle, livin

Introduction

Breast cancer has been the major cause of cancer-related death among women worldwide.¹ This disease is complex and heterogenous, bringing an untold burden to the world at large. The development of molecular profiling using DNA microarrays, gene expression profiling, and immunohistochemical analysis classifies breast cancers into five subtypes: luminal A, luminal B, HER2, basal-like breast cancer, and normal-like breast cancer² with each subtype having a different prognosis and treatment response. The luminal types are estrogen receptor-positive breast cancers with two subtypes, which are human estrogen receptors α and β . The estrogen receptor α has been implicated in ~70% of all breast cancer cases.³ In spite of several research done in this area with the view of lessening the burden of breast cancer, its survivability is generally low and existing therapies are frequently being overcome by resistance. This frailty incited the search into nature for a potential anti-breast cancer agent, which can abrogate the unique hallmarks of these cancers.

Livin, a novel member of the human inhibitors of apoptosis protein family, has been reported to be crucial for tumor progression and even the poor prognosis for several types of malignancies.⁴ This protein closely correlates key hallmarks displayed

by breast cancer: undue proliferation, evasion of apoptosis, and invasiveness. It is no doubt that the downregulation of this independent prognostic indicator for breast cancer, livin,⁵ will mitigate the burden of breast cancer.

In the past decades, >50% of the drugs approved by the US Food and Drug Agency were natural products.⁶ Artonin E is a prenylated flavonoid compound isolated from the stem bark of *Artocarpus elasticus*.⁷ It has been reported to possess a wide range of medicinal properties including the following: antibacterial, anticandidal,^{8,9} antimalarial,^{10,11} and arachidonate 5-lipoxygenase inhibition.¹² The compound has also been shown to induce anoikis in lung cancer and to cause dysregulation of the mitochondrial membrane of ovarian cancer cells.^{7,13} More recently, Artonin E was computationally shown to abrogate estrogen receptor signaling in breast cancer and was conferred with the potential of inhibiting breast cancer using an in silico approach,¹⁴ but the mechanism of the predicted anti-breast cancer inhibition of this compound has not been reported. Hence, this study is aimed at investigating the mechanism of breast cancer cell death induced by Artonin E in an estrogen receptor-positive breast cancer cell line, MCF-7, being the most abundant type in breast cancer cases in a clinical setting.^{3,15,16}

Materials and methods

Preparation of Artonin E

The compound, Artonin E (Figure 1), was kindly given to us by Dr Najihah Mohd Hashim of the Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala

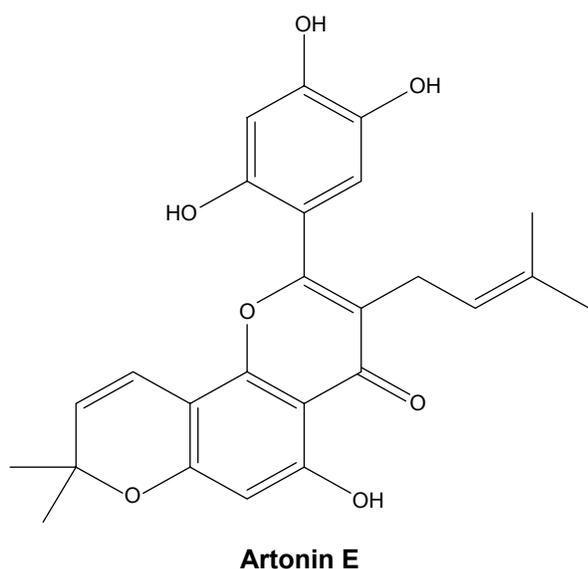


Figure 1 Chemical structure of Artonin E.

Lumpur, Malaysia. It was extracted from *A. elasticus* as reported by Rahman et al.⁷ For the in vitro assays, Artonin E was dissolved in dimethyl sulfoxide, with the volume of dimethyl sulfoxide administered to the cells being <0.1%.

Cell culture

The cell culture and growth inhibition assay were performed as previously reported.¹⁴ Briefly, the MCF-7 cells were acquired from the American Type Culture Collection (Manassas, VA, USA) and grown in Roswell Park Memorial Institute medium-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. The cell count for each of the assay was determined using the hemocytometer counting chamber (Marienfeld, Germany), and $0.5\text{--}1 \times 10^6$ cells were subcultured into a fresh 25 cm² tissue culture flask containing fresh medium (6 mL) at the subcultivation ratio of 1:4 while the cultures were incubated at 37°C under 5% CO₂ and 95% air.

Morphological assessment of apoptotic cells using DNA fluorochrome

Acridine orange (AO) and propidium iodide (PI) double-staining assay were used to determine the effect of Artonin E in the induction of breast cancer cell death. A total of 3×10^5 MCF-7 breast cancer cells were seeded in a six-well plate and allowed to adhere overnight before treatment with various concentrations of Artonin E (3, 10, and 30 μM) at various time points. After the incubation period, both the floating and adherent cells were collected and centrifuged at 2,000 rpm (Hettich Universal 32 R centrifuge; DJB Labcare Ltd, Interchange Park, Newport Pagnell, United Kingdom) for 5 minutes and the pellet was washed with ice-cold PBS, recentrifuged before resuspending in 20 μL of PBS. The cells were thereafter stained on ice with dyes (20 μL) containing 10 μg/mL AO and 10 μg/mL PI. Aliquots of cell suspension (20 μL) were examined under Carl Zeiss Axioskop plus-2 fluorescence microscope. At least 200 cells in each of three fields were immediately assessed for viability, early and late apoptosis, as well as necrosis.¹⁷ The experiment was done in triplicates.

Detection of apoptosis by Annexin V-FITC assay

The Annexin V-FITC assay was carried out using the Annexin V Kit (BD Pharmingen, San Diego, CA, USA). Briefly, after 24- and 48-h incubation of MCF-7 cells with Artonin E, both adherent and floating cells of treated and control samples were collected by centrifugation, washed with ice-cold PBS, and resuspended in 1× binding buffer before transferring

to BD Falcon flow cytometry tubes. PI solution (5 μL) and annexin V-FITC conjugate (5 μL) were added to the cell, and the sample was gently mixed and incubated for 20 minutes at room temperature in the dark. The cells were finally subjected to flow cytometric analysis using laser emitting excitation light at 488 nm and a BD flow cytometer equipped with an Argon laser (Cyan ADP, DAKO, Glostrup, Denmark). These data were analyzed using the Summit V4.3 software.

Caspases 8 and 9 Fluorimetric Assays

The activity of caspases 8 and 9 in the breast cancer cells was determined using a Fluorimetric Assay Kit (R&D System) based on spectrophotometric detection. Briefly, 1×10^6 of MCF-7 cells in media (6 mL) were seeded in a T25 flask. After attachment, the cells were incubated with 3, 10, and 30 μM of Artonin E for 24 h. The cells were thereafter trypsinized and collected by centrifugation at 2,500 rpm (Hettich Universal 32 R centrifuge; DJB Labcare Ltd) in a conical tube for 10 minutes. The cell pellet was lysed by the addition of cold lysis buffer (50 μL) containing 10 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin, and 10 $\mu\text{g}/\text{mL}$ pepstatin. The protein in the cell was extracted and quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). The protein (200 μg) in 50 μL solution from each of the samples was added to a 96-well flat black bottom microplate, followed by the addition of $2 \times$ reaction buffer 8 or 9 (50 μL), as appropriate, containing 10 μL dithiothreitol/mL reaction buffer. For each reaction well, 5 μL of either caspase 8 or 9 fluorogenic substrate (LEHD-AFC) was added, and the plate was incubated at 37°C for 2 h. Control wells were without treatment. Finally, the plate was read with a fluorescence microplate reader at an excitation of 400 nm and emission of 505 nm.

Measurement of total reactive oxygen species production

To evaluate the production of total reactive oxygen species (ROS), the ROS Assay Kit (eBioscience Inc., Affymetrix, San Diego, CA, USA) was utilized. Briefly, breast cancer cells at a density of 1×10^6 per T25 flask were treated with 3, 10, and 30 μM Artonin E for 24 h and trypsinized and centrifuged at 2,000 rpm for 5 minutes. The cells were resuspended in PBS and incubated in ROS (100 μL) assay stain in buffer solution at 37°C for 60 minutes before the flow cytometric analysis.

Cell cycle regulation by Artonin E

The flow cytometric cell cycle analysis was performed to investigate the breast cancer cell cycle regulation induced upon Artonin E treatment. The cells were seeded in a T25

tissue culture flask at a density of $1 \times 10^6/\text{flask}$ and allowed to stand overnight in the incubator for attachment prior to treatment with 3, 10, and 30 μM Artonin E for 12 and 24 h. Treated cells were trypsinized, collected, and centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded, and the cells were washed twice with buffer (1 mL) consisting of sodium citrate, sucrose, and DMSO. For the staining procedure, the cell suspension was transferred into a flow cytometry tube, and solution A (250 μL trypsin buffer) was added to each tube, mixed gently, and allowed to react for 10 minutes at room temperature. Solution B, consisting of trypsin inhibitor and RNase buffer, was thereafter added, gently mixed, and allowed to react for 10-minutes incubation. Ice-cold solution C (200 μL) comprising PI was then added to the tube, mixed, and incubated for 10 minutes on ice in the dark. The stained samples containing 1×10^6 cells were analyzed by flow cytometry using FACSCalibur flow cytometer (Becton Dickson, USA), and CellQuest Pro software was used to determine the cell cycle distribution.

Multiplex mRNA expression analysis using GeXP analysis system RNA isolation

A 3-mL suspension containing 3×10^5 MCF-7 breast cancer cells was seeded in each well of a six-well plate, treated with 10 μM of Artonin E and subjected to RNA extraction using the RNA isolation kit according to the manufacturer's manual. Following the RNA extraction, the purity and concentration of the isolated RNA were ascertained using a nanospectrophotometer (Beckman Coulter, Inc., USA).

Reverse transcription and polymerase chain reaction

Sample preparation was done in accordance with the procedure described by GenomeLab GeXP Start Kit (Beckman Coulter, Inc.). The concentrations of RNA were normalized to 100 ng/ μL using RNase-free water. One microliter of the reverse primer (Table 1) for each gene in RNase-free water (5.5 μL), reverse transcription buffer (2 μL), reverse transcriptase (0.5 μL), 100 ng/ μL sample (0.5 μL), and internal control (0.5 μL) was mixed. The reverse transcription reaction was set to run for 1 minute at 48°C , 60 minutes at 42°C , and 5 minutes at 95°C . The cDNA produced in the above reverse transcription reaction was amplified in a PCR reaction consisting of $5 \times$ PCR buffer (2 mL), magnesium chloride (2 μL), forward primers mixture (1 μL ; Table 1), Taq polymerase (0.35 μL), and cDNA (4.65 μL). The reaction was run on an XP Thermal Cycler (Bioer Technology) with an initial denaturation at 95°C for 10 minutes, followed by

Table 1 Accession number, gene name, and primer sequence used for the gene expression analysis

Genes	Accession number	Forward primers	Reverse primers
Bax	NM_004324	AGGTGACACTATAGAATAGCAAAGCTGGTGCTCAA	GTACGACTCACTATAGGGAAACCACCCTGG TCTTG
Bcl-2	NM_000633	AGGTGACACTATAGAATACTGTGGATGACTGA GTACCT	GTACGACTCACTATAGGGATCAGAGACAGCC AGGAG
APAF-1	NM_001160	AGGTGACACTATAGAATACATACTCTTTCACCA GATCA	GTACGACTCACTATAGGGAACAAGTTCTGTTT TTGCTTT
Cyt C	NM_018947	AGGTGACACTATAGAATAGAGCGAGTTTGGTTGC	GTACGACTCACTATAGGGAAAATCTTCTTGCTC TTCTC
MAPK 8	NM_139046	AGGTGACACTATAGAATACAGAAGCTCCACCAC CAAAGAT	GTACGACTCACTATAGGGAGCCATTGATCACT GCTGCAC
AKT1	NM_001014431	AGGTGACACTATAGAATAGAGGAGATGGACTTC CGGTC	GTACGACTCACTATAGGGAAGGATCTTCATGGC GTAGTAGC
ACTB	NM_001101	AGGTGACACTATAGAATAGATCATTGCTCCTCC TGAGC	GTACGACTCACTATAGGGAAAAGCCATGCCAAT CTCATC
Caspase 7	NM_033338	AGGTGACACTATAGAATAGCTTCTTATGTTACC CAGAT	GTACGACTCACTATAGGGAGGTGACATTTTTCTT CTTCT
Caspase 8	NM_001228	AGGTGACACTATAGAATAGTGCCTCCACTTTCTG	GTACGACTCACTATAGGGAGGCCAGATCTTCACTGT
Caspase 9	NM_032996	AGGTGACACTATAGAATAGCTGGTGAAGAGCTG	GTACGACTCACTATAGGGACTCTAAGCAGGAGAT GAACA
Cyclin D	NM_053056	AGGTGACACTATAGAATACCTGTGCTGCGAAGT	GTACGACTCACTATAGGGAGAAGCGGTCCAGGTAG
Cyclin E	NM_001238	AGGTGACACTATAGAATACAGGATCCAGAT GAAGAA	GTACGACTCACTATAGGGACCTTAAGTATGTCTT TTCCTT
p21	NM_000389	AGGTGACACTATAGAATAAGCTGAGG TGTGAGCAG	GTACGACTCACTATAGGGACCCAGGCGAAGTCAC

two-step cycles of 94°C for 30 s, primer annealing at 55°C for 30 s, and ending with a single-extension cycle at 68°C for 1 minute.

Separation by GeXP genetic analysis system

The PCR products were separated by capillary gel electrophoresis and analyzed using the GeXP machine (Beckman Coulter, Inc.). Briefly, each PCR product (1 µL) was mixed with sample loading solution (38.5 µL) and DNA size standard 400 (0.5 µL, GenomeLab GeXP Start Kit; Beckman Coulter, Inc.), and the mixture in the 96-well sample plate was overlaid with mineral oil before loading onto the machine.

Fragmentation analysis and express profiling analysis

The results were analyzed with the fragment analysis module of the GeXP system software and normalized with β-actin on the eXpress profiler software.

Proteome profiling of human apoptosis antibody array

To investigate the effects of Artonin E on the expression pattern of human apoptosis-related proteins, the proteome profiling was done (R&D Systems, USA). The proteins from treated and untreated MCF-7 breast cancer cells were

harvested and quantified using the BCA Pierce Protein Qualification Kit. The membranes were first blocked in the array buffer 1 in a four-well multi-dish array with 1 h of incubation on a rocking platform shaker. At the same time, 200 µg of proteins from each sample was incubated in array buffer 1 at room temperature for 60 minutes. After blocking the membranes, the array buffer 1 was aspirated and the prepared protein samples were added and incubated with the membrane at 4°C overnight on a rocking platform shaker. On the following day, each array was carefully removed and washed thrice with 1× washing buffer on a rocking platform. For each of the arrays, the reconstituted biotinylated detection antibody cocktail (15 µL) which was diluted to 1.5 mL with 1× array buffer 2 and 3 mixture was used to incubate each array for 1 h on a rocking platform. Thereafter, the arrays were again washed thrice with 1× array buffer. A 1:2,000 dilution of the streptavidin/horseradish peroxidase (HRP) in 1× array buffer 2/3 was used to incubate each of the membrane for 30 minutes on a rocking platform shaker. Following incubation, the arrays were washed thrice with 1× wash buffer. Finally, the membranes were carefully removed and placed in a sheet protector. These membranes were layered with evenly mixed chemiluminescence reagent (1 mL) and incubated for 1 minute before viewing them with the ChemiDoc XRS (Bio-Rad, USA) and the dot intensities quantified with the Image Lab software (BioTechniques, NY, USA).

Western blotting

Total protein lysates were obtained by lysing both the Artonin E-treated and Artonin E-untreated cells with RIPA buffer supplemented with phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The protein content was then measured using Pierce BCA Protein Assay Kit by Microplate Assay (Universal Microplate reader; BioTek Instruments, Inc., Winooski, VT, USA). Then, 20 µg of each sample was subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The proteins were then transferred to a PVDF membrane (Roche, Laval, Canada) using the Pierce Fast Semi-Dry Blotter (Thermo Scientific, Rockford, IL 61105 USA). Afterward, the membrane was blocked with 5% nonfat milk in 1× PBST for 1 h with agitation on a Belly Dancer® (Stovall Life Science, Inc., Waltham MA, USA). The membranes were then washed thrice in TBST and incubated overnight at 4°C with the respective specific primary antibody to subunit Bcl-2, Bax, p21, and p53 (Thermoscientific, Rockford, IL, USA) at 1:1,000 dilution in TBST with constant agitation. On the following day, the membrane was washed thrice in TBST with agitation and incubated with goat-anti-mouse IgG conjugated to HRP at 1:5,000 in TBST for 1 h with constant agitation followed by three washes using TBST. Finally, the immunoreacted protein bands were developed and detected using a chemiluminescence blotting substrate kit (ECL Western blot substrate; Abcam plc, Cambridge, England). A chemiluminescence image analyzer system (Chemi-Smart; Vilber Lourmat, Germany) was used to view the membranes and the intensities of band quantified with the Image Lab software (BioTechniques, New York, USA).

Statistical analysis

All experiments were done in triplicates, and the average values are presented as mean ± standard deviation. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance was performed, followed by Tukey's post hoc test.

Results

Artonin E suppresses cell viability and induces apoptosis in MCF-7 cells

We previously reported the half-maximal growth inhibitory concentration (IC₅₀) of Artonin E-treated (Figure 1) MCF-7 cells at 24, 48, and 72 h as 3.8, 5.1, and 6.9 µM, respectively.¹⁴ For subsequent in vitro analysis, concentrations of 3, 10, and 30 µM were chosen. Results of the AO/PI double-staining revealed the morphological features of apoptosis evident

in Artonin E-treated MCF-7 cells in comparison to the untreated control cells. These features that included membrane blebbing, chromatin condensation, and nuclear fragmentation were enhanced as the concentration of Artonin E increased (Figure 2).

The Artonin E treatment caused significant ($P<0.05$) loss in MCF-7 cell viability (Figure 2). The percentage of viable MCF-7 cells decreased from 94.5% in the control group to 67%, 38%, and 21.5% after treatment with 3, 10, and 30 µM Artonin E, respectively. The proportion of necrotic MCF-7 cells was only significantly ($P<0.05$) higher than control cells after treatment with 30 µM Artonin E. The proportion of MCF-7 cells that went into the apoptotic phase was most significant ($P<0.05$) at all concentrations of Artonin E used in this study. The effect was greater at 48-h treatment when compared with 24-h treatment.

During apoptosis, when the integrity of the cell's plasma membrane is lost,¹⁸ the phospholipid phosphatidylserine flips to the extracellular environment and was detected in this study using the Annexin V-FITC staining. From the results, after 24 h of treatment, the viability of MCF-7 cells was significantly ($P<0.05$) lower at 81.27%, 36.33%, and 12.06% with 3, 10, and 30 µM Artonin E treatment, respectively, in comparison to the control cells which showed 97.78% viability during the same time period (Figure 3). This effect was observed to be dose and time dependent.

Artonin E induces apoptosis in a caspase-dependent manner and enhances the intracellular level of ROS

Caspases are key players of apoptosis. Caspases 8 and 9 are initiators of the extrinsic and intrinsic pathways, respectively. After treating MCF-7 cells with Artonin E, the activity of caspase 9 increased significantly ($P<0.05$) and dose dependently. The activity of caspase 8 was observed to be unchanged at concentrations of 3 and 10 µM. However, it only increased significantly ($P<0.05$) at 30 µM (Figure 4).

After 24 h of treatment, the level of total ROS also increased in comparison to the untreated control in a dose-dependent manner (Figure 5). Upon increasing the concentration of Artonin E to 10 and 30 µM, the production of total ROS increased from 17.7% in the untreated control group to 47.80% and 66.26%, respectively.

Artonin E arrests breast cancer cell cycle

After incubation of MCF-7 cells with Artonin E, there was a significant ($P<0.05$) increase in the percentage of cells in the G0/G1 phase from 57.6% in the control cells to 60.8% and 68.7% after treatment with 3 and 10 µM Artonin E,

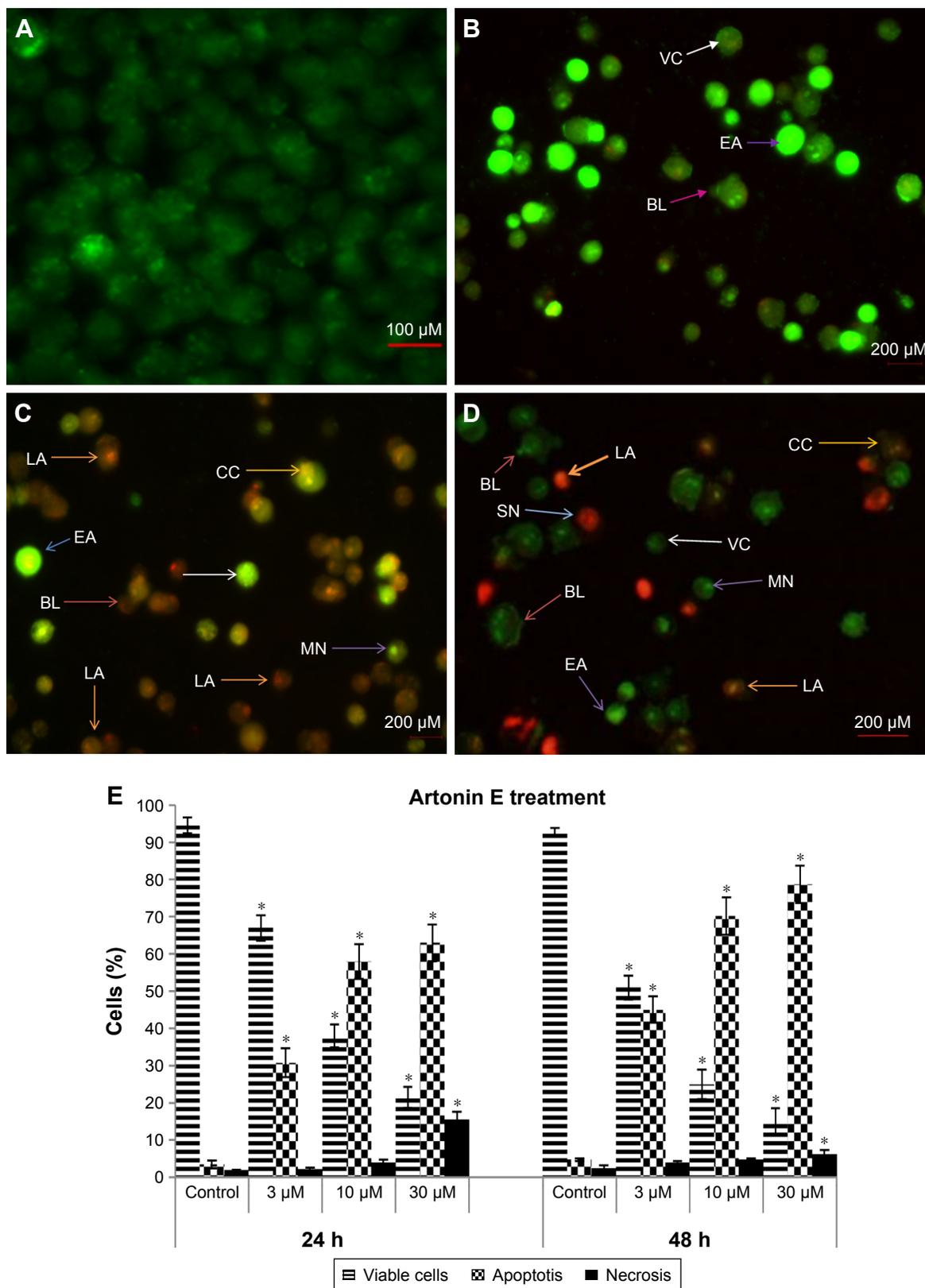


Figure 2 Artonin E induces morphological features of apoptosis in MCF-7 cells.

Notes: (A) Untreated breast cancer cells, (B) MCF-7 cells treated for 24 h with 3 μ M, (C) 10 μ M, (D) 30 μ M, and (E) quantification of viable, apoptotic, and necrotic cells at both 24 and 48 h. *Means differ significantly from that of control at $P < 0.05$. All values are expressed as mean \pm standard deviation of three independent experiments.

Abbreviations: BL, cell membrane blebbing; EA, early apoptosis; VC, viable cells; CC, cancer control; LA, late apoptosis; MN, margined nuclear chromatin; SN, secondary necrosis.

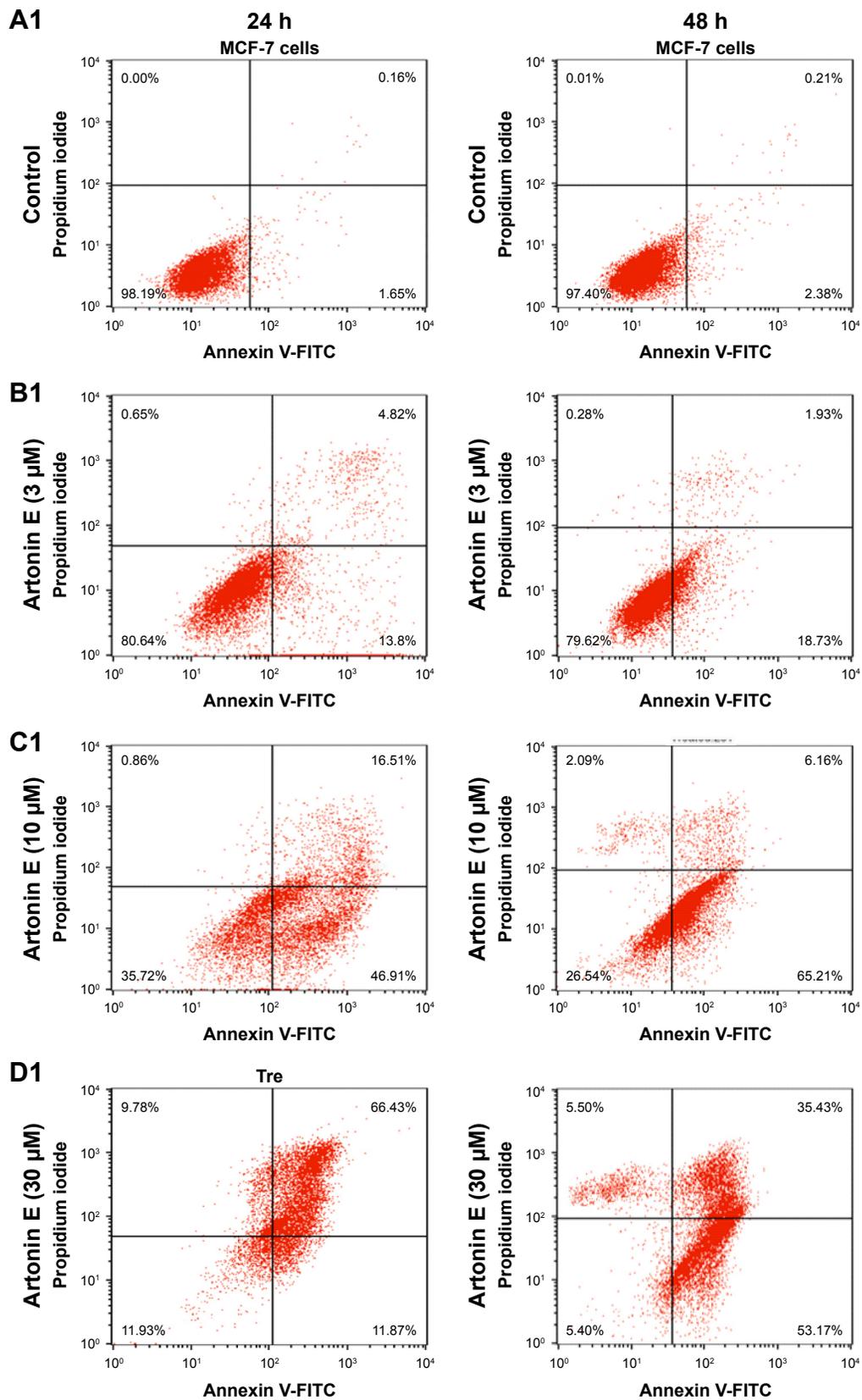


Figure 3 Effect of Artonin E on viability of MCF-7 cells after 24- and 48-h treatment.

Notes: Cell population in lower left quadrant is viable, in lower right quadrant is in early apoptosis, in upper right quadrant is at late stage of apoptosis, and in upper left corner is at the necrotic stage. **A1**, **B1**, **C1**, and **D1** represent MCF-7 cells treated accordingly. The image is a representative histogram of three independent experiments.

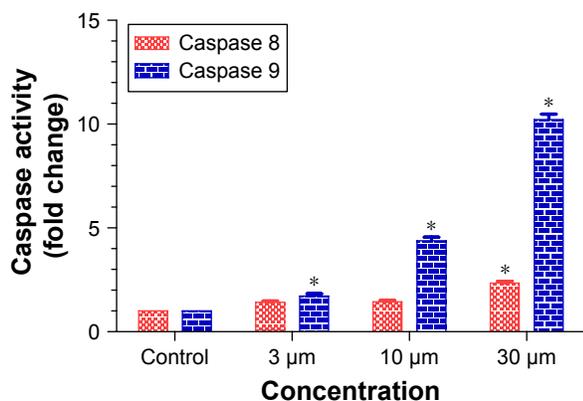


Figure 4 Caspase activity in Artonin E-treated MCF-7 cells after 24-h incubation with different concentrations of Artonin E. The experiment was done in triplicate and the data are expressed as mean \pm standard deviation, * $P < 0.05$.

respectively. However, there was no significant increase ($P > 0.05$) at a concentration of 30 μM (Figure 6). A dose-dependent increase was observed in population of cells in the sub-G0/G1 phase when the exposure time was increased to 48 hours (Figure 7).

Artonin E alters the expression of apoptosis and cell cycle-related genes

The alteration in the expression of apoptosis- and cell cycle-related genes was determined at the mRNA level. The results showed that the expression of cytochrome c, Bax, caspases 7 and 9, and p21 genes were significantly ($P < 0.05$) upregulated

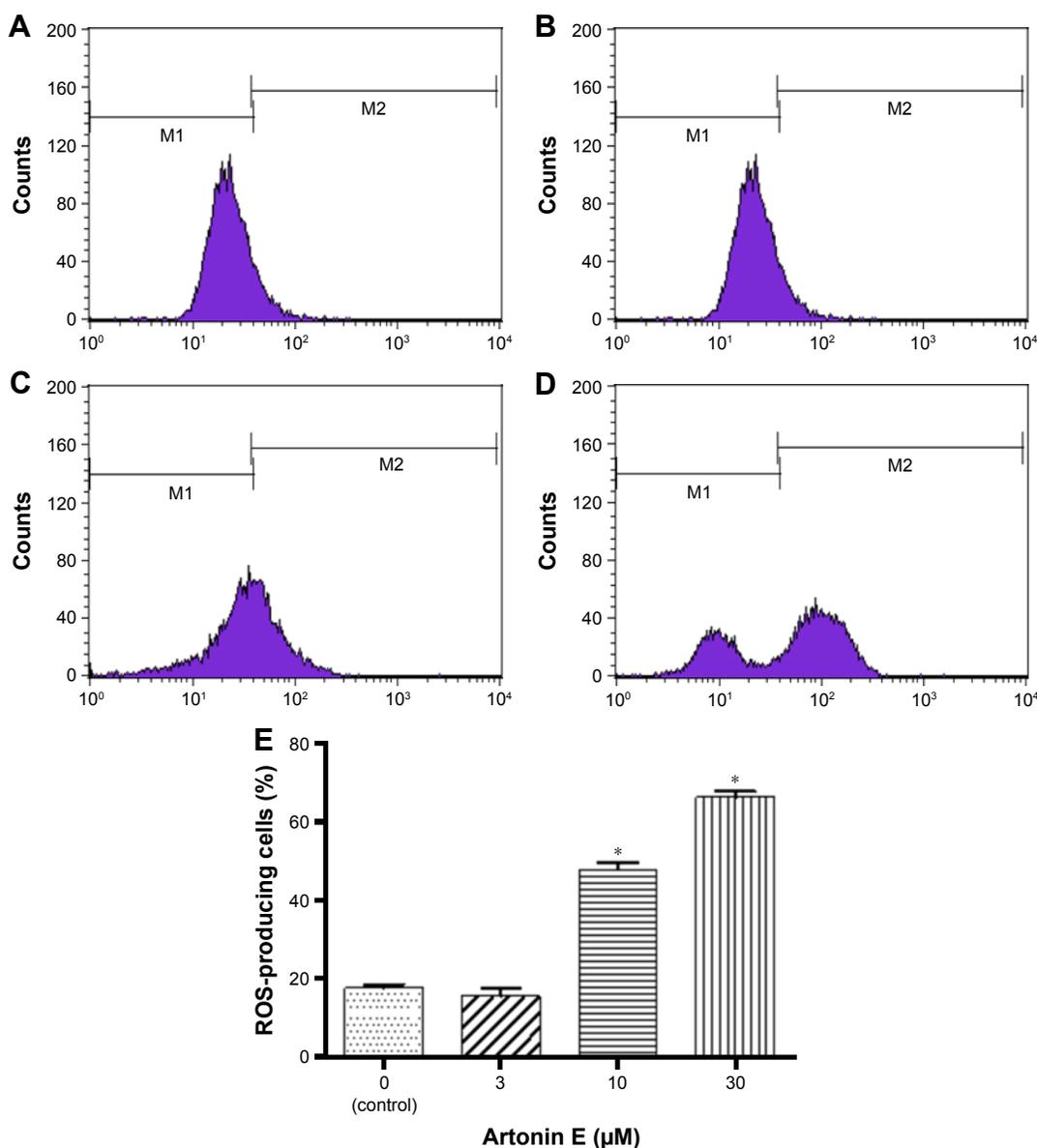


Figure 5 Total reactive oxygen species (ROS) production by MCF-7 cells after 24-h treatment with Artonin E.

Notes: (A) Untreated control and (B, C), and (D) are treatment with 3, 10, and 30 μM Artonin E, respectively, (E) The analysis of the flow cytometric data. Values are expressed as mean \pm standard deviation of three independent experiments. *Means differ significantly ($P < 0.05$) from that of untreated control. M1 = non-ROS, M2 = ROS-producing cell population.

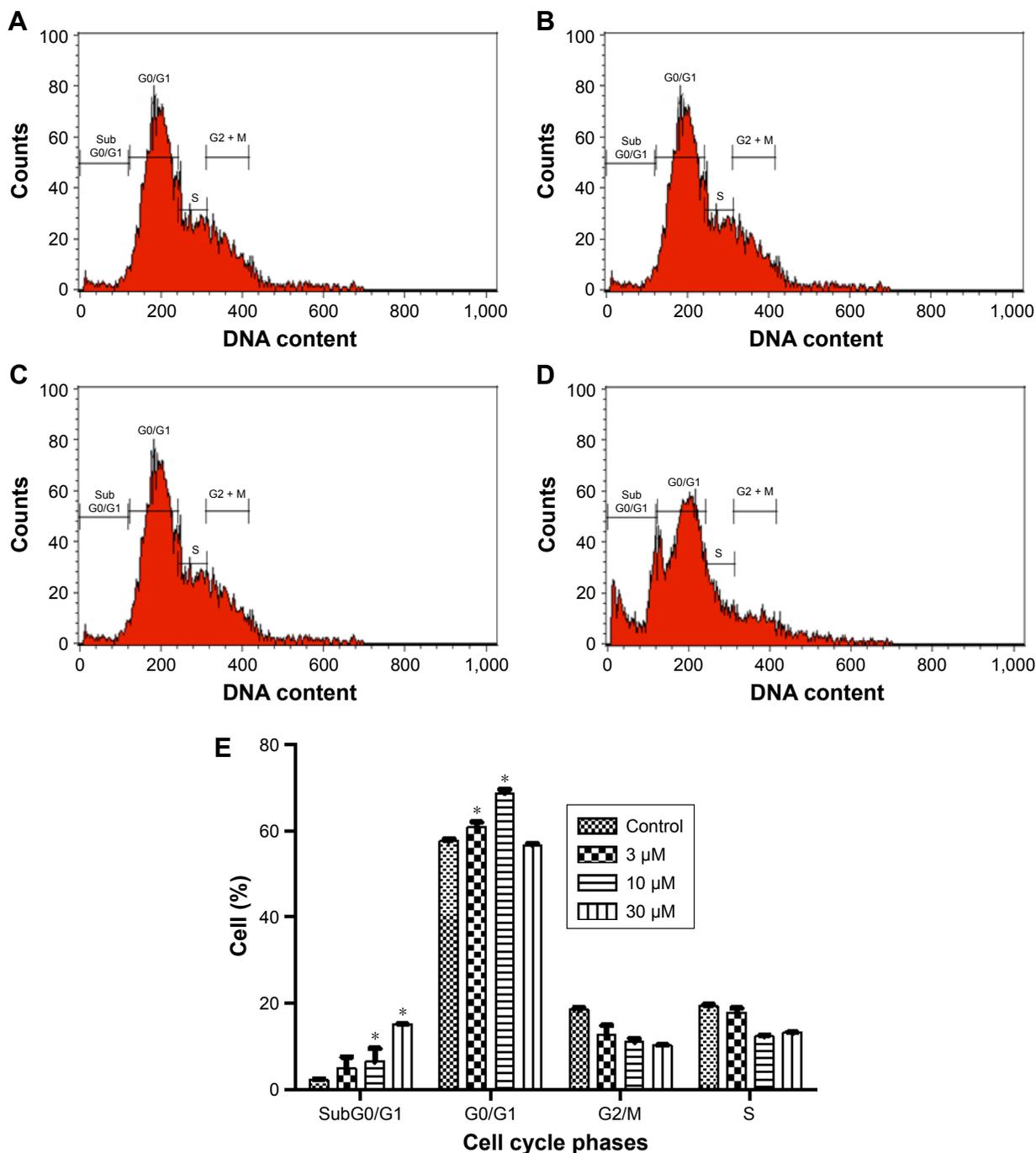


Figure 6 Artonin E arrests MCF-7 cell cycle after 12-h treatment with (B) 3, (C) 10 μM, and (D) 30 μM Artonin E. (A) Is untreated control. (E) Analysis of cell population in the cycle phases. Values are expressed as mean ± standard deviation.

Note: *Means in each cell cycle phase differ significantly ($P < 0.05$) from that of control.

in Artonin E-treated MCF-7 cells, while MAPK and cyclin D were significantly downregulated (Table 2).

Artonin E regulates the expression of apoptosis-related proteins in breast cancer cells

Artonin E downregulated the expression of paraoxigenase genes (PON2), Bcl-x, xIAP (x-linked inhibitor of apoptosis),

Bad, and Bcl-2 (Table 3). Survivin, the smallest member of the inhibitor of apoptosis protein family, was also downregulated as well as livin and heat shock protein 27. An upregulation of heat shock proteins 60 and 70 as well as the cell cycle regulatory protein p21 was also observed. Western blot analysis also revealed the upregulation of Bax, p21 with a corresponding downregulation of livin, an inhibitor of apoptosis (Figure 8).

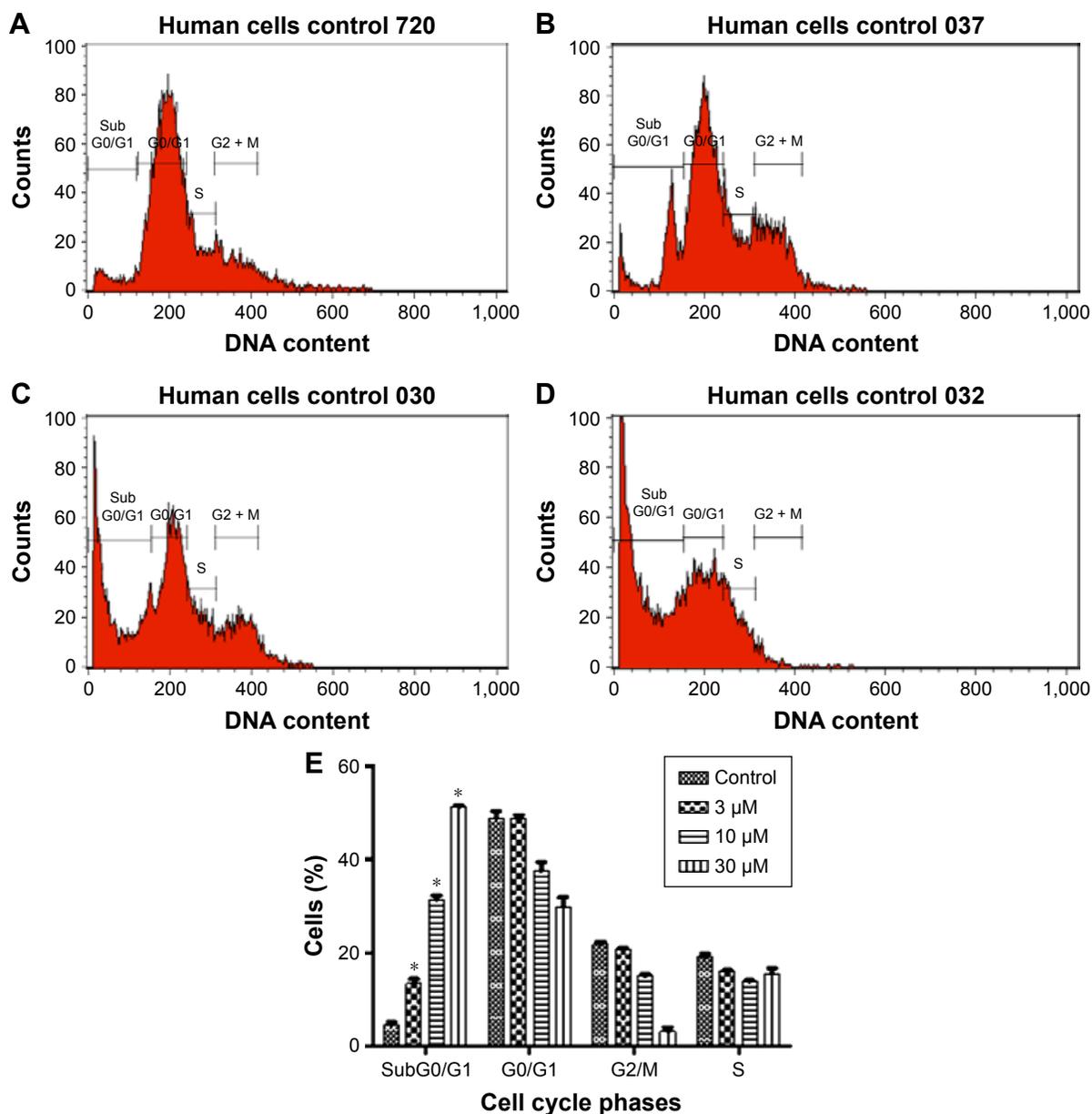


Figure 7 Artonin E arrest MCF-7 cell cycle after 24-h treatment with (B) 3 μ M, (C) 10 μ M, and (D) 30 μ M Artonin E. (A) is untreated control. (E) Analysis of cell population in the cycle phases. Values are mean \pm standard deviation.

Note: *Means in each cell cycle phase differ significantly ($P < 0.05$) from that of control.

Discussion

Breast cancer is still a threat to the female population and a burden to the world at large. Among all breast cancers, the estrogen receptor-positive breast cancer has been implicated in ~70% of the reported cases.^{3,15,16} Hence, the need for compounds that can inhibit the replicative immortality of this cancer. One of the hallmarks of cancer cells is its ability to evade apoptosis while proliferating uncontrollably.¹⁹ Hence, apoptosis in cancer cells is vital and highly sought, making it of great essence to investigate the apoptotic signaling pathways targeted by any potential anti-cancer

agent.²⁰ In this study, Artonin E was investigated for its molecular mechanism of apoptosis induction in MCF-7 breast cancer cells.

From the results, Artonin E-treated MCF-7 breast cancer cells displayed characteristic features of apoptosis,^{21,22} including the following: cell shrinkage, membrane alteration, DNA fragmentation, and nuclear condensation in a dose-dependent manner. These morphological characteristics of the breast cancer cells revealed the involvement of apoptosis. Upon quantification, the percentage of viability decreased significantly ($P < 0.05$) with a corresponding

Table 2 Relative mRNA level of expression of apoptosis and cell cycle-related genes in MCF-7 cells after treatment with 10 μ M Artonin E for 24 h

No	Gene	Relative expression (% Log [fold change])
1	Bcl-2	-13.8 \pm 0.09
2	Cyc	19.0 \pm 0.01
3	Bax	15.5 \pm 0.04
4	MAPK 8	-21.1 ^b \pm 0.06
5	Apaf1	6.4 \pm 0.04
6	Akt1	-6.8 \pm 0.05
7	Caspase 7	51.8 \pm 0.03
8	Caspase 8	8.8 \pm 0.01
9	Caspase 9	17.3 \pm 0.02
10	Cyclin D	-23.7 ^b \pm 0.19
11	Cyclin E	-15.3 \pm 0.09
12	p21	23.7 \pm 0.05

Notes: Values are expressed as mean \pm standard deviation of three replicates at $P < 0.05$. ^aSignificantly upregulated, ^bSignificantly downregulated.

increase in the percentage of cells undergoing early and late apoptosis. The loss of membrane asymmetry, which is evident during apoptosis, always leads to the externalization of phosphatidylserine.²³ In this study, Annexin V FITC and DNA-binding fluorochrome PI were utilized to further strengthen the assessment of the apoptotic mode of cell death and to examine the progression of apoptotic cells.^{24,25} Here, Artonin E was seen to significantly reduce the viability of MCF-7 cells while increasing its rate of apoptosis in a concentration-dependent manner. The percentage of cells undergoing apoptosis after Artonin E treatment was greater than MCF-7 cells undergoing apoptosis after treatment

Table 3 Apoptosis pathway-related protein expression in Artonin E-treated MCF-7 cells

No	Protein	Relative expression (% Log [fold change])	Regulation	Full name
1	PON2	-15.9	Down	Protein paraxonase-2
2	p21	15.5	Up	Cyclin-dependent kinase inhibitor
3	Survivin	-33.2	Down	Apoptosis Inhibitor
4	Bad	-20.3	Down	Bcl-2 antagonist of cell death
5	xIAP	-15.6	Down	X-linked inhibitor of apoptosis
6	Hsp 27	-34.2	Down	Heat shock protein 27
7	Hsp 60	11.7	Up	Heat shock protein 60
8	Hsp 70	3.0	Up	Heat shock protein 70
9	Livin	-3.7	Down	Inhibitor of apoptosis protein
10	Bcl-2	-20.0	Down	B-cell lymphoma 2
11	Bcl-x	-29.5	Down	Bcl-2-associated X protein

with Tamoxifen, a standard agent as previously reported by Subramani et al.²⁶

Caspases are the major players in the execution of apoptosis and are critical in the apoptosis signaling pathway. There are two pathways of apoptosis initiated via either the mitochondria or the cell surface receptors. These pathways of apoptosis can be recognized and distinguished by the initiator caspases.²⁷ To begin the investigation into the apoptosis signaling pathway mediated by Artonin E, the activities of caspases 8 and 9 which are initiators of the extrinsic and intrinsic pathways, respectively, were investigated.²⁸ From the results, Artonin E at all concentrations, stimulated the activity of caspase 9 dose dependently in MCF-7 cells, but not the activity of caspase 8 except at a concentration of 30 μ M. This confirmed that Artonin E predominantly initiated apoptosis in MCF-7 via the intrinsic pathway. The mitochondria is a major source of ROS in cells, and these chemicals regulate cell viability or mortality.^{29,30} Although oxygen metabolism is central to life, beyond the cellular antioxidant capacity, it has been reported to damage macromolecular structures and to induce apoptosis of cancer cells.^{17,31,32} In this study, ROS production in Artonin E-treated MCF-7 cells was significantly ($P < 0.05$) enhanced. The level of ROS escalated from 17.7% in the untreated control cells to 47.80% and 66.26% in cells treated with 10 and 30 μ M Artonin E, respectively, at 24 h. This upregulation of ROS indicated that one of the mechanisms of the apoptotic cell death induced by Artonin E treatment is via oxidative insult, an observation similar to that of Mounjaroen et al.^{33,34}

Deregulation of cell cycle control has been evidently implicated in cancers.^{35,36} This is often mediated by alterations in the activities of cyclin-dependent kinases that result in proliferation and chromosomal instability. Targeting the cell cycle and discovering inhibitors of the cyclin-dependent kinases are beneficial strategies in drug discovery.³⁷ In this study, the growth of MCF-7 cells was transiently arrested in the G0/G1 phase of the cell cycle upon treatment with 3 and 10 μ M Artonin E. This G0/G1 arrest was attributed to the upregulation of p21 that led to the downregulation of cyclin D as evident by the gene expression studies. To elucidate the molecular basis of the apoptotic signaling pathways in Artonin E-treated cancer cells, the gene and protein expressions were investigated. From the results, the mRNA expression of cytochrome C, Bax, caspases 7 and 9, and p21 were significantly upregulated in comparison to the control when treating MCF-7 cells with 10 μ M Artonin E for 24 h. A concomitant and significant downregulation of cyclin D and MAPK 8 was

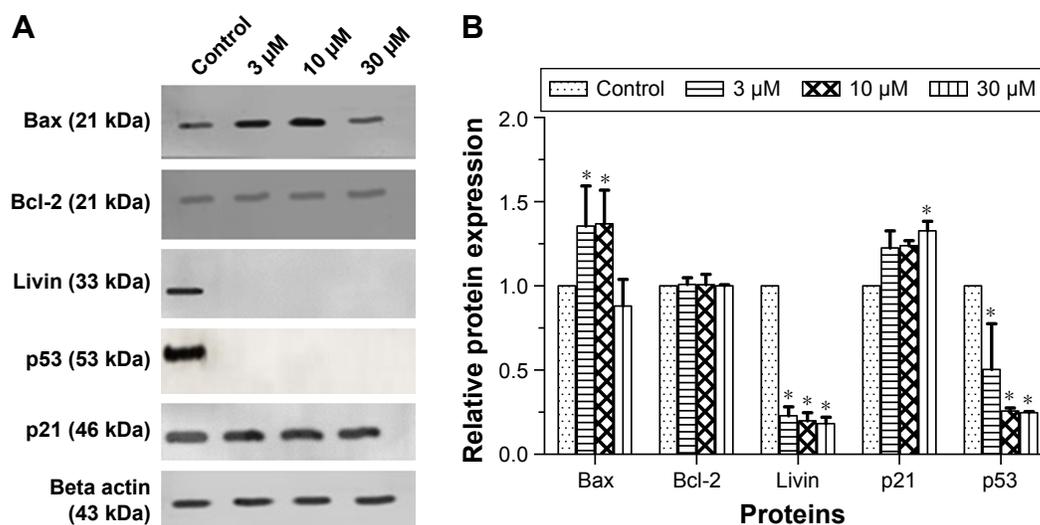


Figure 8 Western blot protein expression of apoptosis- and cell cycle-related proteins in Artonin E-treated MCF-7 cells at 24 h.

Notes: (A) Western blot and (B) analysis of protein expression. There was an upregulation of Bax and p21 and a downregulation of livin and p53 proteins, * $P < 0.05$.

also observed. The upregulation of cytochrome C, Bax, and caspases 7 and 9 are further evidences that Artonin E induced apoptosis in MCF-7 cells via the mitochondrial pathway. Consistent with the induction of the intrinsic pathway, it is postulated that the oxidative insult prompted by Artonin E greatly compromised the mitochondrial membrane integrity of the estrogen receptor-positive breast cancer cells, leading to the activation and release of cytochrome c (Figure 9). There was also an elevated expression of Bax, which can also directly affect the polarity of the mitochondrial membrane, compounding the rate of the release of cytochrome c by the mitochondria.³⁸ The consequence of these effects of Artonin E was apoptotic MCF-7 cell death.^{39,40} Artonin E was also observed to significantly inhibit the expression of antiapoptotic proteins and inhibitors of apoptosis. Utilizing the human apoptosis proteome profiling, Artonin E-treated MCF-7 cells showed downregulation of Bcl-x, PON2, xIAP, survivin, and Hsp 27, all being inhibitors of apoptosis. These antiapoptotic proteins had been reported to be overexpressed in tumors and, by doing so, cancer cells could develop resistance to chemotherapy while evading death.⁴¹ The observed downregulation of Hsp 27 was similar to the report of Ma et al,⁴² in which the knockout of Hsp 27 gene resulted in caspases 3 and 7 cleavage that eventually sensitized breast cancer cells to undergo apoptosis after chemotherapeutic treatment. In another study, short-term silencing of Hsp 27 augmented proteasome activity, abrogated metastatic potential, and induced regression of established breast tumors.⁴³ These observations showed that by downregulating Hsp 27 with Artonin E, the MCF-7 cells had greater tendency to undergo apoptosis.

Surprisingly, p53 protein expression was observed to be suppressed after treatment with Artonin E. Although most of the cancers harboring wild-type p53 do not have normal p53 function,⁴⁴ it is possible that other intrinsic molecular alteration had played a role. A similar observation was reported by Wu et al,⁴⁵ in which a specific downregulation of p53 in MCF-7 led to apoptosis when Smad4, a unique marker in breast cancer and an important suppressor gene in tumorigenesis, was elevated. Hence, it is highly probable that Smad4 is also involved in the apoptosis of Artonin E-treated breast cancer cells and is, therefore, recommended for further studies. Livin, a novel member of apoptosis which has been reported as a marker of breast cancer progression and invasiveness,^{4,5} was observed to be significantly ($P < 0.05$) repressed after treatment with Artonin E. We propose that this repression of livin might have been responsible for the downregulation of MAPK observed in the gene expression pathway. This downregulation of livin is responsible for the antiproliferative effect displayed by Artonin E. In an earlier experiment reported by Ou et al,⁴ knockdown of livin was shown to inhibit proliferation and invasiveness of cancer cells. Artonin E inhibition of livin had played a crucial role in the inhibition and apoptosis induction of the estrogen receptor-positive breast cancer.

Conclusion

Breast cancer is indeed a threat to human existence, especially to the female population. Emergence of resistance to existing therapies without a corresponding reduction in the intriguing havoc of estrogen receptor-positive breast cancer cells, encourage more research into finding potential agents for breast cancer. In this regard, Artonin E was evaluated for its

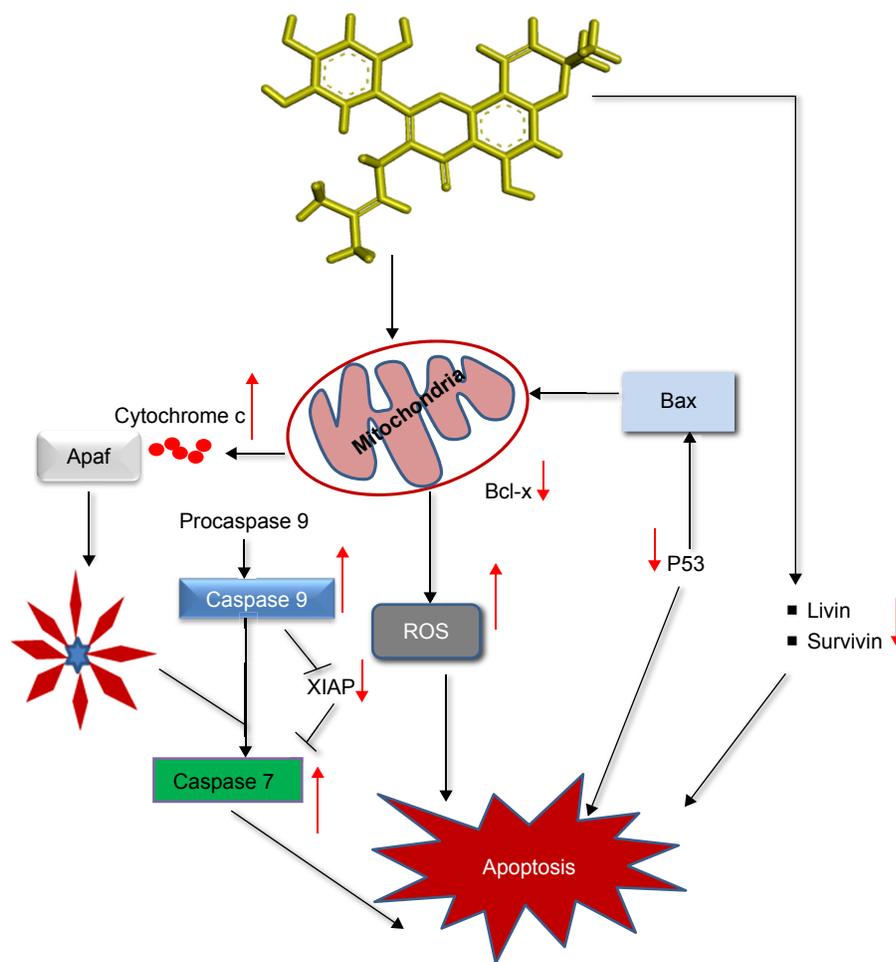


Figure 9 Mechanism of action of Artonin E on MCF-7 breast cancer cells.
Abbreviation: ROS, reactive oxygen species.

mechanism of apoptosis in MCF-7 cells. The mode of cell death induced by Artonin E was primarily apoptosis with the breast cancer cells also showing cell morphology and DNA fragmentation typical of apoptosis along with quantitative evaluation of apoptotic stages shown with Annexin V-FITC stain. The antibreast cancer effect of Artonin E was dependent on concentration and time. Artonin E was also seen to regulate the cell cycle whose control is usually deregulated in cancers.³⁵ A G0/G1 MCF-7 cell cycle arrest was induced independent of p53. This effect of Artonin E was in addition to its significant upregulation of pro-apoptotic proteins with the downregulation of inhibitors of apoptosis. Interestingly, Artonin E inhibited livin, which has been reported to promote invasiveness of breast cancer as well as being responsible for chemoresistance. In conclusion, this study showed that Artonin E inhibited breast cancer growth and proliferation through initiation of the intrinsic apoptotic pathway while suppressing the antiapoptotic mechanism, thus circumventing the relative immortality of cancer cells.⁴⁶ Artonin E also

seemed to have an additional effect of induction of cell cycle arrest, which is inhibitory to cancer growth. Interestingly, Artonin E-treated MCF-7 cells showed significant downregulation of livin expression while upregulating p21 expression. These effects may have increased the effectiveness of breast cancer inhibition by Artonin E. Thus, Artonin E has the potential to be developed into an anticancer agent.

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Disclosure

The authors report no conflicts of interest in this work.

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