Chapter 2

Apoptosis-Inducing Effect of Artonin E in Breast Cancer

Imaobong Etti, Rasedee Abdullah and Arifah Kadir

Additional information is available at the end of the chapter

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Abstract

Breast cancer is among the principal cause of cancer fatalities in women. Notwithstanding the use of chemotherapy and advances in drug delivery techniques, cancer-related morbidity and mortality are still increasing with the increase in resistance to known therapeutics. This dilemma has drastically reduced the rate of survival from this deadly disease, creating a dire need for new drugs, especially from natural sources that would exhibit similar or better anticancer properties while imparting minimal adverse side effects. Among the bewildering hallmark capabilities of cancer is the evasion of apoptosis while in its course to immortality. Thus, induction of apoptosis in cancerous cells is an important breakthrough that cannot be ignored. There are, however, two major pathways of apoptosis in mammals, viz., the death receptor–mediated pathway (extrinsic pathway) and the mitochondrial-mediated pathway (intrinsic pathway). Artonin E, a prenylated flavonoid isolated from the stem bark of *Artocarpus elasticus*, was seen to induce apoptosis in MCF-7 breast cancer cells, hence halting the breast cancer cells in their journey to immortality.

Keywords: Artonin E, breast cancer, apoptosis, MCF-7 cells

1. Introduction

Breast cancers, like other forms of cancer, possess the ability for uncontrolled proliferation while resisting cell death [1]. This undue proliferation is a consequence of apoptosis evasion as well as loss of normal cell cycle control. Apoptosis is an efficient and uniquely regulated mode of cell death, involving the interplay of many factors. In mammals, there are two major pathways of apoptosis viz., the death receptor mediated pathway (extrinsic pathway) and

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the mitochondrial mediated pathway (intrinsic pathway). Caspases are the key regulatory proteins for both pathways [2]. The extrinsic pathway requires the binding of ligands such as tumor necrosis factor α (TNFα), Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) to the death receptor on cell surfaces and corresponding transduction of signals leading to apoptosis [3]. The intrinsic pathway, on the other hand, is mediated by the release of cytochrome c by the mitochondrial following DNA damage. The released cytochrome c then induces the formation of apoptosomes composed of apoptotic protease activating factor 1 (Apaf-1), procaspase 9, and either ATP or dATP [4]. Caspase 9 is a downstream factor that activates the executioner caspase-3, which cleaves substrates such as Poly (ADP-ribose) polymerase (PARP) [5]. The activated caspase 3 then initiates the caspase cascade that culminates in the demolition of the cell [6].

In this study, the colorimetric microculture tetrazolium assay [7] was employed to access the cells viability while assays including morphology studies, Annexin V-FITC Assay and DNA fragmentation were thereafter utilized to ascertain the mode of the cell death.

2. Materials and methods

The chemical and reagents used in the study were; MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (PhytoTechnology Laboratories, USA), DMSO (dimethylsulfoxide) (Fisher Scientific, UK), Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI 1640) powders with L-glutamine (GIBCO, New Zealand), fetal bovine serum (FBS), horse serum, trypsin–EDTA, phosphate-buffered saline (PBS) tablets and penicillin (10,000 U/mL)–streptomycin (10 mg/mL) (Sigma-Aldrich, USA), acridine orange (AO) and propidium iodide (PI), annexin V-FITC (BD Pharmingen), and gel red nucleic acid stain and apoptotic DNA ladder detection kit (Abcam, USA).

2.1. Cell culture

The cell culture procedures were carried out in an aseptic condition in a Class II biohazard cabinet according to good cell culture practice (GCCP) guidelines. The MCF-7 breast cancer cell line (ATCC, USA) were grown in 25 cm^2 tissue culture flasks (TPP, Switzerland) and incubated in an incubator (Binder, Germany) at 37°C under a humidified atmosphere of 5% CO_2. The cells were maintained in RPMI supplemented with 10% FBS and 1% penicillin–streptomycin. Upon reaching about 80% confluency, the cells were washed twice with PBS and 1 mL of trypsin–EDTA solution was added to detach the monolayer cells. The trypsin was inactivated by the addition of the complete cell growth medium and the cells were collected by centrifugation at 1000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min before discarding the medium. The cell count was determined using a hemocytometer counting chamber (Marienfeld, Germany) and about 0.5 to 1 × 10^6 cells were subcultured into a fresh 25 cm^2 tissue culture flask containing 6 ml of fresh medium at the subcultivation ratio of 1:4. The cultures were incubated at 37°C under 5% CO_2 and 95% air.

2.2. Cryogenic preservation and recovery

The cells were preserved in liquid nitrogen to avoid loss of their original characteristics. Briefly, the cells growing in the exponential phase were subcultured. Following detachment and centrifugation, the cells were resuspended in freezing medium containing 90% FBS and 10% DMSO, as a cryoprotective agent, to yield a final cell density of 2 to 5 × 10^6 cells/mL. Subsequently, 1 mL of cell suspension was transferred into each labeled cryotube and allowed to stand at −20°C for 2 hours and at −80°C overnight before storage in liquid nitrogen (−196°C) (CBS Cryosystem). When needed, the cells were recovered by thawing with gentle agitation in water bath at 37°C for approximately 2 min. The cell suspension was transferred into a sterile 15 mL centrifuge tube (TPP, Switzerland) containing prewarmed complete growth medium in a biosafety cabinet and centrifuged at 1000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min at room temperature to remove the cryoprotective agent (DMSO). Finally, the cell pellets were resuspended in culture media and transferred to a 25cm^2 culture flask (TPP, Switzerland), incubated at 37°C in a CO_2 incubator. After 2 days, the medium was replaced with fresh complete growth medium.

2.3. Plating

At about 80% confluency, the cells were collected and the concentration was determined using a hemocytometer. Briefly, 0.5 × 10^5 cells/mL were dispensed into each well of a 96-well flat bottom tissue culture plate. To obtain the desired plating concentration of 0.5 × 10^5 cells/mL, the initial cell concentration was adjusted with culture medium using the following formula:

$$M_1 V_1 = M_2 V_2 \quad (1)$$

where M_1 is the initial cell concentration, V_1 is the initial cell suspension volume, M_2 is the final cell concentration, and V_2 is the final cell suspension volume.

Using a multichannel pipette, 100 μL of the cell suspension was dispensed into each well, except the blank wells, which received 200 μL of culture medium. The cell-seeded plate was incubated overnight at 37°C to facilitate attachment.

2.4. Preparation of the test agents

Artonin E was kindly donated by Dr. Najihah Hashim of the Department of Pharmacy, Faculty of Medicine, University of Malaya. The compound was isolated from *Artocarpus elasticus*, characterized and identified as reported by [8]. The standard agents, Tamoxifen and Paclitaxel were purchase from Sigma Aldrich, St. Louis, MO, USA. These agents were dissolved in DMSO and normal saline respectively and then diluted with respective medium with highest final DMSO concentration of 0.1% for *in vitro* cell culture studies. They were diluted serially using culture medium to obtain concentrations in the range of 1.56–100 μM. About 100 μL of each concentration of Artonin E, Tamoxifen and Paclitaxel solutions was added into appropriate wells in four replicates.

2.5. Microculture tetrazolium assay

The colorimetric microculture tetrazolium assay ([7] was used to assess breast cancer cell viability. The tetrazolium dye is converted into an insoluble formazan by the action of nicotinamide adenine dinucleotide hydrogenase present in metabolically active cells. This cellular conversion into a purple-colored formazan is directly proportional to the number of viable cells. Briefly, exponentially growing cells were seeded into a 96-well flat bottom tissue culture plate at a density of approximately 0.5×10^4 cells/well and allowed to adhere to the plate by incubating at 37°C under 5% CO_2 and 95% air overnight. Following cell attachment, the cells were incubated with the tested compounds at concentrations ranging from 1.56 to 100 μM. Control cells were treated with 0.01% of DMSO, which was equivalent to the amount of DMSO used as vehicle. After each of the 24, 48 and 72 h treatment time period, 20 μL of 5 mg/mL of MTT solution was added to each well and the plate was reincubated for 4 h to facilitate catalysis by mitochondrial dehydrogenases. Next, 100 μL DMSO was added to each well to solubilize the formazan crystals. The absorbances of the resultant solutions were determined colorimetrically at 570 nm. The experiment was performed in triplicate.

We conducted a nonlinear regression analysis and utilized the GraphPad Prism software to fit a dose-response curve. The concentration of the compound that triggered a 50% growth inhibition was indicated at different time interval in **Figure 1**. The percentage viability used in fitting the dose-response curve was calculated using the following formula:

$$\% \textit{ of cell viability } = \frac{A_T}{A_C} \times 100 \tag{2}$$

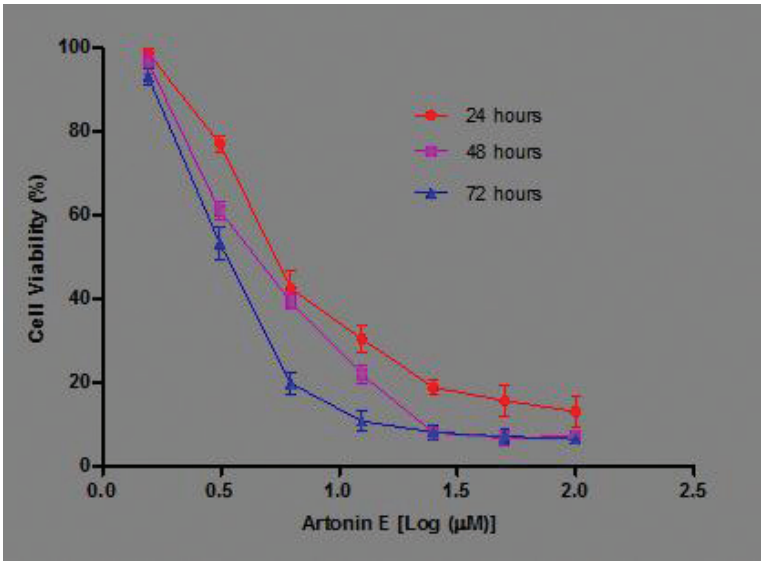


Figure 1. Viability of MCF-7 cell line treated with Artonin E. The half maximum growth inhibitory concentrations (IC_{50}) were: 6.90, 5.10 and 3.77 μM Artonin E at 24, 48 and 72 h, respectively.

Where the absorbance reading of treated samples is indicated as A_T while the absorbance of control samples treated with 0.01% of DMSO equivalent to the concentration used in dissolving the tested agent is depicted as A_C.

2.6. Cell morphological study

Acridine Orange (AO) and propidium iodide (PI) double staining assay was employed 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 exposed to various treatment concentrations of Artonin E (1.56–100 μM) at varying time points (24–72 h). The adherent MCF-7 cells were trypsinized, and centrifuged at 2000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min. The pellet was washed with ice-cold PBS, re-centrifuged before suspending in 20 μL of PBS. The cells were thereafter stained on ice with 20 μL dye containing 10 μg/mL of AO with 10 μg/mL of PI. Carl Zeiss Axioskop plus-2 fluorescence microscope was used to visualize aliquots of 20 μL of the cell suspension. Three fields each containing at least 200 cells were immediately assessed for viability, early and late apoptosis as well as necrosis [9]. The experiment was performed in triplicate.

2.7. Annexin V-FITC assay

Apoptosis was also investigated by detection of externalized phosphatidylserine using the Annexin V Kit (BD Pharmingen, USA). The cell preparation was carried out in accordance with the manufacturer's protocol. The prepared 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). The data were analyzed using the Summit V4.3 software.

2.8. DNA fragmentation analysis

Qualitative DNA fragmentation (Roche) kit was used in this analysis. Briefly, after trypsinization, 1×10^6 cells were collected and washed twice with PBS. The cells were then pelleted by centrifugation at 3000 rpm for 5 min, the supernatant removed and replaced with 200 μL of fresh PBS. The sample was thereafter lysed with 200 μL binding/lysis buffer consisting of 6 M guanidine-HCl, 10 mM urea, 10 mM Tris–HCl, 20% Triton X-100 (v/v), pH 4.4 and incubated for 10 min at 15–25°C. After the incubation, 100 μL of isopropanol was added and the mixture was filtered through a high pure spin filter tube combined with a clean collection tube and centrifuged at 8000 rpm for 1 min. The flow-through was discarded and the filter tube was combined again with the collection tube before washing with 500 μL of washing buffer consisting of 20 mM NaCl, 2 mM Tris–HCl, pH 7.5 in 20 mL with the addition of 80 mL ethanol. This washing step was done twice before spinning the tube dry at 13,000 rpm (Eppendorf 5424 microcentrifuge, USA) for 10 s to remove any residual washing buffer.

The extracted DNA was eluted into a fresh collection tube with 200 μL of prewarmed (70°C) elution buffer (10 mM Tris) and recentrifuged at 8000 rpm for 1 min. The quality of the

extracted DNA was assessed with a nanodrop spectrophotometer (nanodrop lite spectrophotometer, Thermo Scientific, USA). The DNA sample was mixed with 1× loading dye and electrophoresed on 1% agarose gel 75 V for 1 h and stained with GelRed™ nucleic acid gel stain. The fragmented DNA was visualized under UV transilluminator and photographed with a chemiluminescence image analyzer system (Chemi-Smart, Vilber Lourmat, Germany).

2.9. Statistical analysis

All data collected were analyzed using the GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). One way analyses of variance was performed, followed by Turkey's *post ho*c tests to compare replicate means of treatment and control groups. The significance was set at $p < 0.05$.

3. Results

3.1. Growth inhibitory effect of Artonin E on MCF-7 breast cancer cells

Artonin E, at concentrations of 6.9, 5.10 and 3.77 μM induced a half maximal growth inhibitory effect on estrogen receptor-positive breast cancer cells (**Figure 1**) at 24, 48 and 72 h respectively.

3.2. Artonin E treated MCF-7 breast cancer cells displayed morphology of apoptosis

The results of the AO and PI double staining analysis showed that Artonin E treated breast cancer cells displayed morphological features typical of apoptosis. These features included chromatin condensation and membrane blebbing. The control breast cancer cells which were not exposed to Artonin E had their normal nuclear structure displayed as green fluorescence. Early apoptotic cells were observed as bright green fluorescence resulting from the interposition of acridine orange with the fragmented DNA whereas late stage apoptosis (**Figure** 2**C** and **D**) was observed as reddish-orange, resulting from the binding of propidium iodine to denatured DNA. The apoptosis inducing effect of Artonin E was observed to be time and concentration dependent.

Challenging the cancer cells with Artonin E caused a significant ($p < 0.05$) loss in the proportion of viable MCF-7 breast cancer cells (**Figure 3**) from 94.5% to 67, 38, and 21.5% after treatment with 3, 10, and 30 μM Artonin E respectively. There was no significant ($p \geq 0.05$) increase in necrotic breast cancer cells except after exposure to 30 μM of Artonin E. This percentage decreased. There was a concentration and time dependent significant ($p < 0.05$) increase in the proportion of cells that entered the apoptotic phase.

3.3. Annexin V-FITC assay flowcytometric analysis

The externalized phosphatidyl serine was detected with Annexin V-FITC dye as an indication of apoptosis induced upon treatment of breast cancer cells with Artonin E. From the results, there was a significant ($p < 0.05$) shift in the population of the breast cancer cells from viability

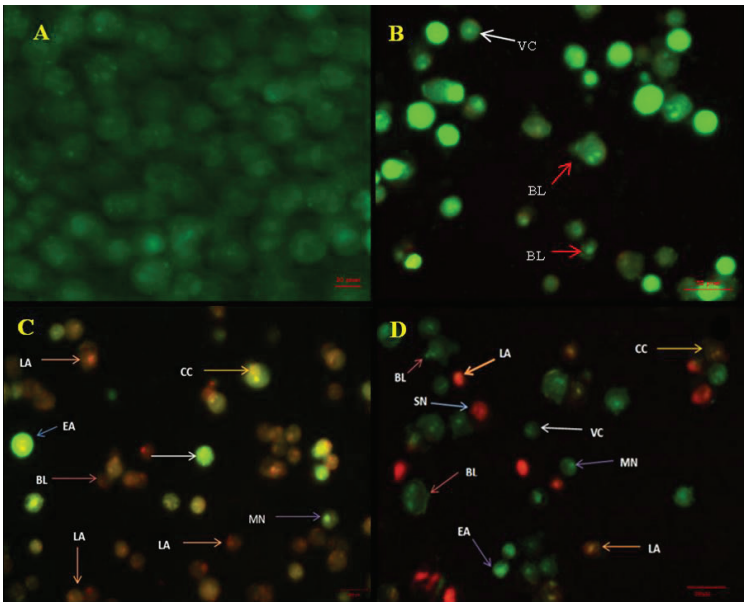


Figure 2. Acridine orange/propidium iodide double staining of MCF-7 cells after 24 h exposure. (A) Control, (B) 3, (C) 10, (D) 30 μM Artonin E. VC = viable cells; BL = cell membrane blebbing; CC = chromatin condensation; EA = early apoptosis; LA = late apoptosis; MN arginated nuclear chromatin; SN secondary necrosis. Magnification: 200×.

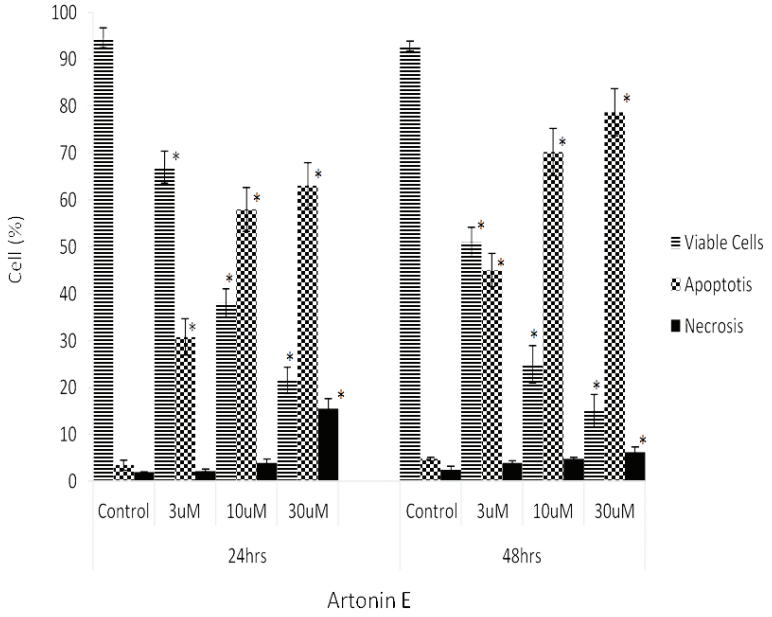


Figure 3. Quantification of early and late apoptotic MCF-7 cells after AO/PI double staining analysis. All values are mean ± standard deviation. *at each time point, means significantly different for control at $p < 0.05$.

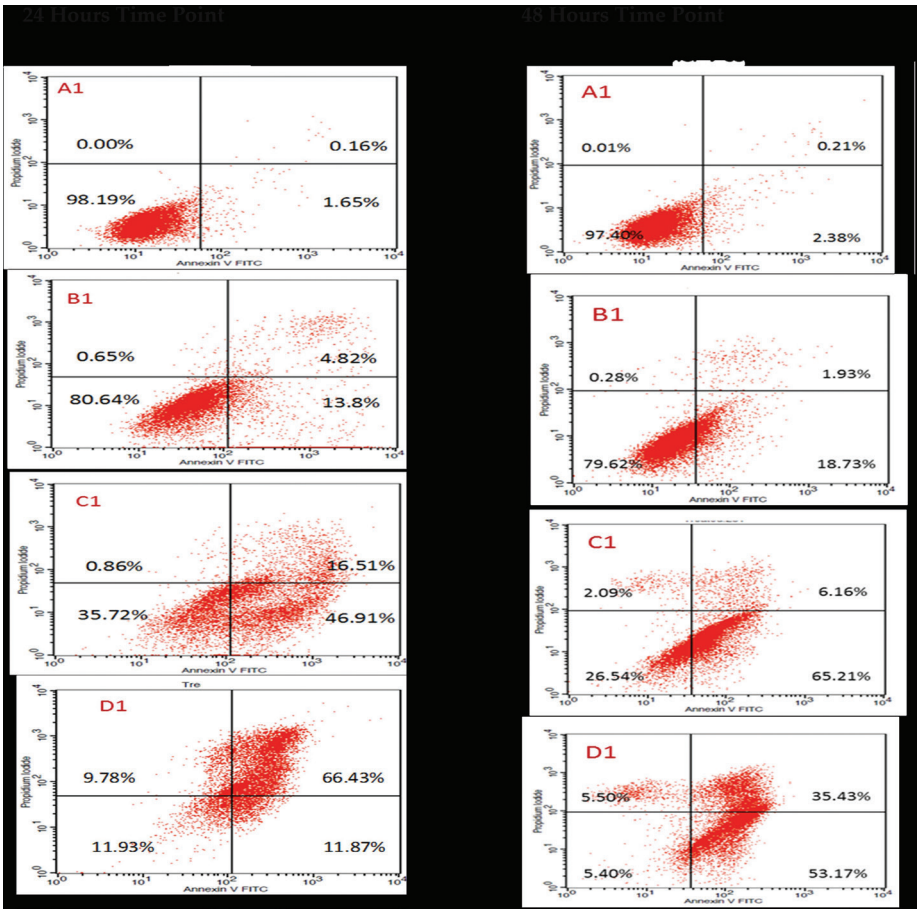


Figure 4. Representative histogram analysis of the Annexin V assay in MCF-7 cells treatment with Artonin E at 24 and 48 h. Viable cells population are shown in the lower left quadrant, while those undergoing early apoptosis are in the lower right quadrant. The upper right quadrant shows cells at late stage of apoptosis, and in upper left corner, cells at the necrotic stage are shown.

to late apoptosis (**Figure 4**). This effect was concentration as well as time dependent. After 24 h exposure of the cancer cells to Artonin E, the percentage viability decreased from 97.78% to 81.27, 36.33 and 12.0 6% when treated with 3, 10 and 30 μM of Artonin E respectively.

3.4. DNA fragmentation analysis

Another unique feature of apoptosis is DNA fragmentation which can be visualized by agarose gel electrophoresis [10]. From the results, nuclear DNA fragmentation occurred after treatment with Artonin E. This was observed in the breast cancer cells (**Figure 5**) in comparison to the untreated control group which showed intact DNA.

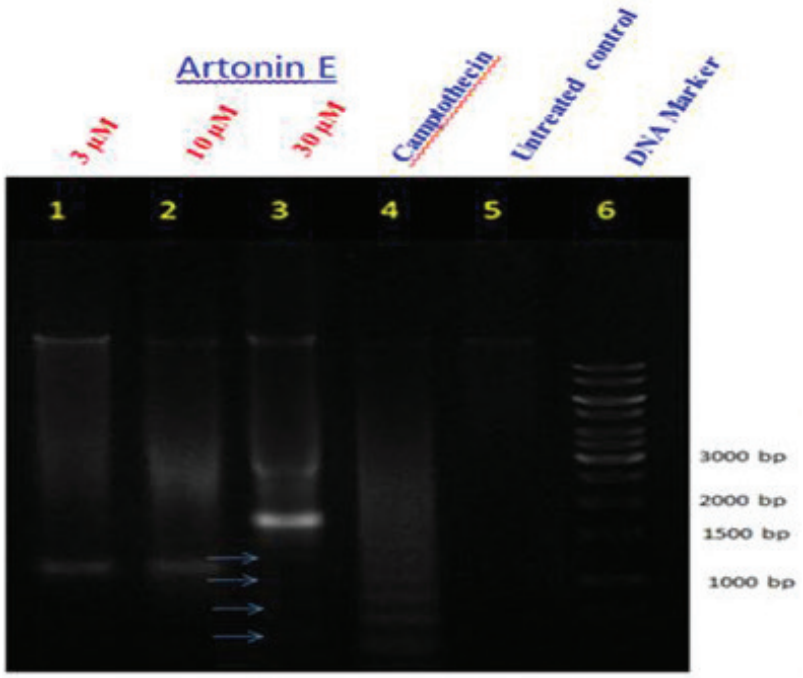


Figure 5. DNA fragmentation in MCF-7 cells treated with Artonin E after 24 h. The positive control cells were treated with 4 μg/mL camptothecin, the DNA marker is a 1 kb ladder.

4. Discussion

The death of cancer cells in a tumor is the ultimate goal of cancer drug discovery. Unlike normal cells, cancer cells possess the ability to proliferate uncontrollably while evading apoptosis. Apoptosis induction is thus a valuable characteristic of a potential anticancer drug. Hence, the cytotoxic effect of a compound can be accessed by investigating its growth inhibition on the target cancer cells. It is also vital to examine the mode of cell death induced by the compound. In this study, Artonin E, previously shown to be drug-like with a better *in silico* growth inhibitory properties when compared with similar structural analogues [11], was evaluated for its *in vitro* growth inhibition and mode of cell death induced in MCF-7 breast cancer cell line.

Artonin E was found to significantly inhibit the proliferation of this breast cancer cells in a dose and time dependent manner. At 24, 48 and 72 h, Artonin E showed half maximal inhibitory concentrations of 6.90, 5.10 and 3.77 μM, respectively. MCF-7 is estrogen receptor positive, progesterone positive and HER2 (human epidermal growth factor 2) positive and has wild type p53 [12]. Etti et al. [11] reported the *in silico* affinity of Artonin E to the human estrogen receptor α and pin pointed Artonin E as having greater binding affinity for the estrogen receptor α among the reported structural analogues from the *Artocarpus*.

It was also reported that the prenylation together with the 4^1, 5^1 vicinal diol groups in Artonin E had enhanced its affinity to the human estrogen receptor α. This affinity of Artonin E to the human estrogen receptor can be said to be mostly responsible for its better growth inhibition in MCF-7 cells observed in this study. Consistent with our findings, Obiorah et al. [13] discovered that ERα was exclusively responsible for the apoptosis induction of genistein, equol, and coumestrol, compounds which are structurally similar to Artonin E. They confirmed the phenomenon by a gene knockdown of ERα which prevented growth inhibition and apoptosis induced by these phytoestrogens. These support the involvement of the estrogen receptor in the growth inhibitory potential of Artonin E. Similarly, Turner et al. [14] also reported that prenylflavones show selectivity to estrogen receptors. Thus, it is of no doubt, that the affinity of Artonin E for the estrogen receptor is a possible basis for the observed sensitivity of MCF-7 breast cancer cells to the compound.

Various modes of cell death include apoptosis, necrosis and autophagy. However, from the results of this study, Artonin E provoked morphological features typical of apoptosis in the Artonin E treated breast cancer cells. In support of this, Carou et al. [15] and Gerl and Vaux [16], reported that apoptosis results in unique morphological changes including cell shrinkage, membrane alteration, DNA fragmentation and nuclear condensation. These features were observed in this study after treating the breast cancer cells with Artonin E. Succinctly, agents that trigger apoptosis are very essential in the management of cancer, giving that a unique hallmark of cancer cells is apoptosis evasion which is also implicated in its pathogenesis [17]. Hence, induction of apoptosis becomes a strategy for cancer drug discovery [18].

A compromise in the phospholipid membrane asymmetry of a cell, results in the externalization of phosphatidylserine. To further strengthen the assessment of the apoptotic mode of cell death, annexin V FITC in combination with a DNA binding fluorochrome, PI were utilized [9, 19, 20]. In this study, Artonin E has been seen to significantly reduce the population of viable breast cancer cells in a concentration and time dependent manner while increasing the population undergoing apoptosis. Thus, treatment of breast cancer cells with Artonin E distorted the integrity of the lipid bilayer of the cancer cells, exposing their phospholipid as detected in this study. These observations qualifies apoptosis as the mode of cell death induced by Artonin E.

Apoptotic endonucleases in the course of apoptosis, degrades chromosomal DNA into fragments [21]. This fragmented DNA can be visualized in a gel electrophoresis. In this study, after treating the cancer cells with Artonin E, its DNA was seen to have degraded into fragments in comparison to the untreated control. This DNA fragment induction by Artonin E, confirms apoptosis as the mode of cell death [22] which was also seen in the morphology of Artonin E treated breast cancer cells and the Annexin V-FITC flowcytometric assays.

5. Conclusion

Artonin E inhibited the unregulated growth of breast cancer cells and induced apoptosis in the cancer cells. It produced enhanced cytotoxicity on the estrogen receptor positive MCF-7 cells thus, halting its progression to immortality.

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Conflict of interest

The authors declare no conflict of interest.

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