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Original Article

# The Calotropis Gigantea Methanolic Extract Induces Apoptosis in Human Breast Carcinoma Cells

Kiran R. Kharat[1], PhD;
Arun S. Kharat[2], PhD

[1]Center for Advanced facility for Life Sciences, Deogiri College, Aurangabad, India;
[2]School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

**Correspondence:**
Arun S. Kharat, PhD;
School of Life Sciences, Jawaharlal Nehru University, Pin code: 110067, New Delhi, Inida
**Tel:** +91 11 26704530
**Fax:** +91 11 26742558
**Email:** arunkharat2007@gmail.com
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## Abstract

**Background:** *Calotropis gigantea* (family: Asclepiadaceae) has been known to contain cardiac glycosides. The *C. gigantea* extracts have been reported as cytotoxic to a few cancer cell lines. The present study was designed to examine the effect of *Calotropis gigantea* methanolic extract (CGME) on the growth and apoptosis in human breast carcinoma cell line (MCF-7 cells).
**Methods:** The study was conducted in Aurangabad (India) from 16 February to 10 June 2015. CGME treated MCF-7 cells were analyzed for growth inhibition and apoptosis. The exhibition of phosphatidylserine was analyzed with the Annexin-V Fluorescein isothiocyanate flow cytometry (FITC) method. Accumulated poly-caspases were determined with carboxyfluorescein poly-caspase assay, Apo-BrdU™ tunnel assay for DNA fragmentation and pro/anti-apoptotic gene expression with real-time polymerase chain reaction. The high-performance liquid chromatography analysis indicated the presence of two unknown cardenolides along with known cardenolides such as calactin, calatropagenin, usharin, afroside, calatoxin, and gamphoside. The Kruskal-Wallis and Wilcoxon tests (GraphPad Prism version 7.0) were used for statistical analyses.
**Results:** Upon treatment with 40 µg/ml CGME, about 56.9% of the cell population underwent apoptosis. Compared to paclitaxel, the accumulation of active caspases in CGME treated with MCF-7 cells was found to be dose-dependent, whereas the G2/M cell cycle arrest was time-dependent. The Apo-BrdU™ tunnel assay confirmed that CGME treatment caused DNA fragmentation and RT-PCR analyses indicated elevated transcription for pro-apoptotic gene expression. Kruskal-Wallis test results were significant; Bcl-2 (P=0.00193), Bak-1 (P=0.00021), and Bax (P=0.0019).
**Conclusion:** CGME treatment caused the accumulation of phosphatidylserine on the cell membrane, recruitment of poly-caspases, DNA fragmentation, and enhanced transcription of pro-apoptotic gene expression.

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**Keywords** ● *Calotropis* ● Apoptosis ● Caspases ● Apocynaceae

### What's Known

- The *Calotropis gigantea* exhibits medicinal potential such as anti-bacterial and anti-cancer activities. Amongst many phytochemicals, cardiac glycosides are known to exist in the plant.

### What's New

- Treatment with *Calotropis gigantea* methanolic extract caused the accumulation of phosphatidylserine on the cell membrane, recruitment of poly-caspases, DNA fragmentation, and enhanced transcription of pro-apoptotic gene expression in human breast carcinoma cells.

## Introduction

The *Calotropis gigantea* (L.) W.T.Aiton is a family member of Asclepiadaceae found in major parts of the world.[1] The plant has been reported to contain medicinally important secondary

metabolites.[2-4] The compounds include cardiac glycoside such as calotropin, uscharin, calatoxin, calactin, calatropagenin, calatropagenin glycosides I, II, III and IV, and aschepin.[5-9] Cardiac glycosides have been known to inhibit Na,K-ATPase.[8, 9] In addition to chemicals, the *Calotropis gigantea* proteins have been known to suppress breast tumor growth through suppression of NF-κB pathway.[10] Cytotoxic activity of cardenolides, which was isolated from *C. gigantea* leaves, was reported to act against MCF-7 cancer cells, KB skin cancer cell, and NCL-H18 lung cancer cell.[11] Dichloromethane extract of *C. gigantea* leaves has shown cytotoxic activity against MCF-7 cancer cell, MDA-MB-231 cancer cell, HeLa cell, HT-29 cancer cell, SKOV-3 cancer cell, and Hep-G2 cancer cells.[12] Calotropin, which was isolated from its roots, had cytotoxic activity in K562 leukemia and stomach cancer 790.[13] Methanol extract and chloroform fraction of the root has antitumor activity on ascites carcinoma.[14] Anhydrosophoradiol-3-acetate (A3A) isolated from the flower could inhibit Ehrlich ascites carcinoma (EAC) growth effectively.[15]

Malignant transformation is characterized by a significant increase in the activity of Na, K-ATPase. Cardiac glycosides can induce apoptosis in cancer cells by inhibiting Na, K-ATPase activity, and UNBS1450 (a steroid cardiac glycoside synthesized from *Calotropis procera*) also induces apoptosis in various types of cancer cells.[16-18] The Bcl-2 family proteins regulate entry into apoptosis through controlling mitochondrial membrane permeability.[19] Upon activation, the BH3-proteins only promote apoptosis through neutralizing apoptosis inhibitory proteins such as Bcl-2 and Bcl-xL and also by promoting the opening of mitochondrial pores comprised by the death-promoting members of the Bcl-2 family, Bax and Bak.[19]

The present study is the first report on the effect of CGME on MCF-7 cells demonstrating apoptosis induction and its mechanism. We aimed to examine the effect of CGME prepared from the stem and leaves on growth and apoptosis in MCF-7 cells. The effect was compared to the commonly used cytotoxic drug, paclitaxel. The exposure of membrane phosphatidylserine (PS) was studied as a major cause of apoptosis in MCF-7 cells. Comparative studies between paclitaxel and CGME while inducing apoptosis in MCF-7 are discussed.

## Material and Methods

The present study was carried out from 16 February to 10 June 2015 in Aurangabad, Maharastra, India.

### *Collection of Plants and Preparation of Crude Extract*

Leaves and stems of *Calotropis gigantea* were collected from the Marathwada region of the State Maharastra (India). A specimen voucher was deposited at the Herbarium, Department of Botany, Deogiri College, Aurangabad, India (specimen voucher number: 19108). Exactly 500 gr of shed dried leaves and stems were crushed into fine powder and used for the preparation of methanolic extracts. The methanolic extracts were prepared according to procedures as described in a previous study.[20] The dried methanolic extract was dissolved in water for further experimentation.

### *HPLC Analysis of Cardenolides in CGME*

High-Performance Liquid Chromatography (HPLC) analysis was carried out using a UV/VIS detector and manual sampler. The Thermo C18 column (150×4.6 mm, 5 µm) was used. The mobile phase consisted of methanol:water (55:45). The flow rate was maintained at 1 ml/min and effluents were monitored at 222 nm.

### *Cell Culture Conditions*

MCF-7 cells were purchased from the Cell Repository, NCCS, Pune (India). The cells were maintained in Glutamax-DMEM (Life Technologies Inc., USA) containing 10% heat-inactivated FBS (Life Technologies Inc., USA) and 100 U/ml penicillin plus 100 µg/ml streptomycin (HiMedia, India). The cells were grown in a 37 °C humidified incubator supplied with 5% $CO_2$. At 70%-85% confluent growth, the cells were trypsinized with 0.25% TPVG (HiMedia, India); counted and aliquots at the desired density for growth assays were made and/or processed. For each experiment, the cells were freshly grown by diluting the stock with phosphate-buffered saline (PBS, pH7.2).

### *Determination of Cell Viability and $IC_{50}$*

The viability of CGME treated MCF-7 cells was determined with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The cells were plated at ~$1\times10^3$ cells in each well of 96-well plate in 100 µl DMEM medium (Life Technologies Inc., USA) and 0, 5, 10, 20, 40, and 60 µg/ml CGME was respectively added to each well. Each concentration of CGME was repeated in 8 wells (8 repeats). Cell viability was determined after 24 hours and 48 hours of incubation in a $CO_2$ incubator at 37 °C. The MTS (5 mg/ml in PBS) was added to each well and incubated for 4 hours. The procedure was

followed in accordance with the manufacturer's instructions. The absorbance was recorded at 490 nm using a 96-well Multiscan Ascent (Thermo Inc., USA). The inhibitory effect of CGME on cell growth was assessed as percent cell viability, where cells without treatment were considered 100% viable. Paclitaxel (Merck, USA) was used as a control drug for the assay. The Wilcoxon test was also used to compare the data from the 24 hours and 48 hours incubation in the presence of CGME.

*Detection of Phosphatidylserine on Membranes of Apoptotic Cells*

The FITC apoptosis detection kit-I (Life Technologies Inc., USA) was used in accordance with the manufacturer's instructions for the analysis of phosphatidylserine exposure. Briefly, the cells were inoculated with ~$1\times10^5$ cells in each well of 6-well plates in 500 µl DMEM medium supplemented with 10% v/v FBS and 0 and 40 µg/ml CGME. Paclitaxel at 20 µg/ml was used as the control drug and 40 g/ml of CGME as under test concentration was used. After 0, 1, 3, 6, 12 and 24 hours, harvested MCF-7 cells were washed with cold PBS and re-suspended in a 200 µl Annexin binding buffer [50mM HEPES, 700 mM NaCl, 2.5mM $CaCl_2$, pH 7.4]. Annexin V-FITC 5 µl and 1 µl PI (100 µg/ml) were then added to the 100 µl cells suspensions. After 15 minutes of incubation at room temperature and prior to analysis with an Attune flow cytometer (Life Technologies Inc., USA), a 400 µl of the Annexin binding buffer was added and phosphtidylserine on the membrane of apoptotic cells was calculated with Attune cytometric software, version 2.1 (Life Technologies Inc., USA).

*Analysis of Poly-caspases Activity in MCF-7 Cells*

To prove that the cytotoxic effect of CGME mediated caspase activation, we used Carboxyfluorescein poly-caspase (FAM) assay (Life Technologies Inc., USA.).[21] The MCF-7 cells treated with CGME (40 µg/ml) for 0, 1, 3, 6, 12 and 24 hours were used for the poly-caspases analysis. The Vybrant® FAM Poly Caspases Assay Kit (Life Technologies Inc., USA.) was used to detect active caspases in apoptotic cells, based on the fluorescent inhibitor of caspases (FLICA™) methodology. The assay was performed with the Attune flow cytometer in accordance with the manufacturer's instructions.

*Cell Cycle Analysis*

The MCF-7 cells were treated for 24 hours with 40 µg/ml CGME and then washed twice with PBS. The cells were fixed in 70% (v/v) ice-cold ethanol at 4 °C for 24 hours. The fixed cells were stained with propidium iodide (Life Technologies Inc., USA) and filtered through a 50 µm filter. The samples were then analyzed using the Attune flow cytometer.

*Analysis of DNA Fragmentation of MCF-7 Cells*

Live studies of the cellular DNA content and cell cycle distribution are useful for studying tumor behaviors and tumor suppressor gene mechanisms, to monitor apoptosis.[22] The DNA fragments generated by endonuclease recruitment in apoptotic cells were analyzed using the APO-BrdU™ TUNEL Assay Kit (Life Technologies Inc., USA). Procedures were followed as per manufacturer's instructions; both positive controls (cells fixed in alcohol after exposure to DNase 1) and negative controls (cells fixed without exposure to DNase 1). The analysis was performed immediately after the staining reaction using the Attune flow cytometer equipped with a 488 nm laser. Apoptosis was quantified by following the increase in FITC dUTP labeling against control cells.[23, 24] The cell cycle kinetics of MCF-7 cells, either treated or left untreated, were determined using flow cytometry analyses of PI nuclear staining in accordance with the instructions given by the manufacturer (Life Technologies Inc., USA).

*Expression of Pro-Apoptotic and Anti-Apoptotic Genes*

Expression levels of RNA transcripts were quantified by real-time PCR, as described in the previous study.[25] The cDNA was mixed with SYBR Green PCR master mix (Biorad Inc., USA) containing gene-specific primers and real-time PCR was performed in triplicates in accordance with the manufacturer's instructions (Biorad Inc., USA). For each single-well amplification reaction, a threshold cycle (CT) was observed in the exponential phase of amplification and relative expression level quantification was achieved using standard curves for both the target and endogenous controls. The primers used are listed in table 1.

**Table 1:** The primers used for the amplification of genes

| Primer | Forward | Reverse |
|---|---|---|
| Bcl2 | ATGTGTGTGGAGAGCGTCAA | ACAGTTCCACAAAGGCATCC |
| Bax | TTTTGCTTCAGGGTTTCATC | CAGTTGAAGTTGCCGTCAGA |
| Bak-1 | GCCTTTGCAGTTGGACTCTC | GGGTTGGGAGCAAGTGTCTA |

*Statistical Analysis*

Data were reported as mean±SD All experiments were done at least in triplicates, and three or more independent observations were made on each occasion. Statistical significance was compared using the Kruskal-Wallis and Wilcoxon tests, GraphPad Prism version 7.0.[26]

## Results

*Analysis of Cardenolide Content of CGME*

The presence of cardenolides in CGME was detected using the HPLC method. We used isolated compounds characterized by liquid chromatography-mass spectrometry as the standard for the analysis of the cardenolides by HPLC. In the mobile phase that consisted of methanol:water (55:45), there were known cardenolides (e.g. calactin, calatropagenin, usharin, afroside, calatoxin, gamphoside) and two unknown cardenolides; as listed in figure 1.

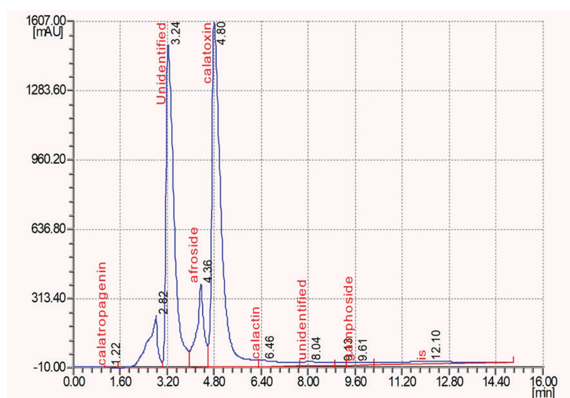


Figure 1: High-performance liquid chromatography analysis of the cardenolides present in CGME.

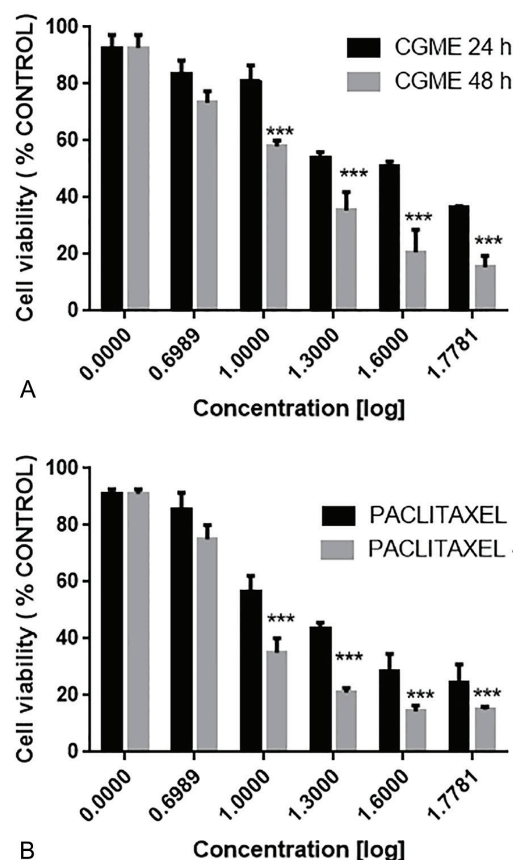


Figure 2: The cytotoxic effect of CGME on MCF-7 cells is illustrated. Cell viability was estimated using the MTS assay. (A) CGME treated cells after 24 and 48 hours and (B) paclitaxel-treated cells after 24 and 48 hours. Cells were treated with concentrations of CGME 0, 5,10,20,40,60 µg/ml for 24 hours. P=0.10 vs. drug-untreated group. Data represent the mean±SD of three different experiments with triplicate sets in each assay. The P value was determined using the Kruskal-Wallis test.

*Inhibition of Growth of MCF-7 Cells*

To analyze the effect of CGME on cell growth, cells were treated with CGME and paclitaxel (as control) for 24 hours and cell growth was assessed using the MTS assay. Results shown in figure 2 demonstrated that as the CGME concentration increased (0, 5, 10, 20, 40, 60 µg/ml), the cell viability gradually decreased. Interestingly, the control showed 60.3±5% inhibition of cell proliferation at 40 g/ml in 24 hours while the effect of CGME at 40 g/ml was comparable to paclitaxel.

*CGME Induced Apoptosis in MCF-7 Cells*

Cell membrane associated with phosphatidylserine was measured in CGME treated cells using the flow cytometry method with Annexin V-FITC/propidium iodide (PI) staining. The CGME treatment with 40 µg/ml was found to induce apoptosis in growing MCF-7 cells. As shown in figure 3, the apoptotic cell population after 1 hour of incubation was 12.4%, while after 24 hours of incubation it was 56.9%. As the duration of CGME 40 g/ml exposure was enhanced from 1 to 24 hours, the apoptosis was found to be progressive (figure 3B to figure 3F) as the measure of incubation time. Apoptosis seen in MCF-7 cells with CGME was comparable to paclitaxel (figure 3).

*Role of Caspases in Induction of Irreversible Apoptosis in MCF-7 Cells*

Results shown in figure 4 denote a marked dose-dependent increase in caspases upon the treatment of MCF-7 cells with CGME. CGME treatment with 40 g/ml (figure 4B) and 60 g/ml (figure 4C) caused the exhibition of active caspases in 20.4% and 27.4% of the cells, respectively. Paclitaxel treatment resulted in the exhibition of caspases in 34.1% of the cells (figure 4D).

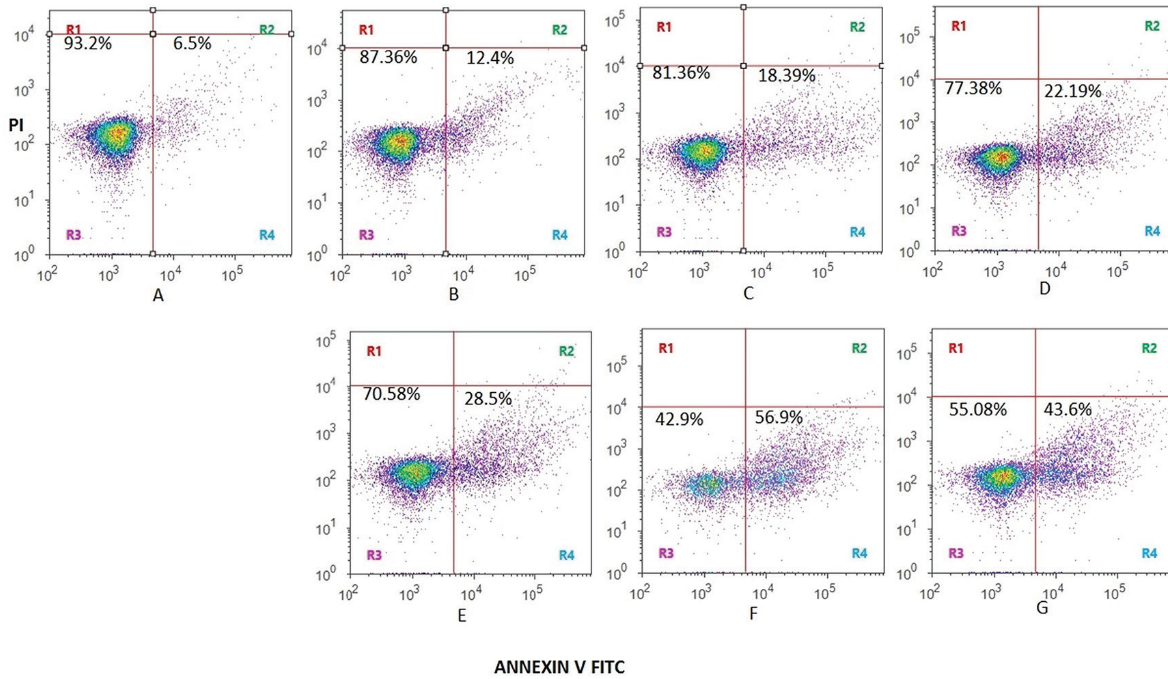


**Figure 3:** CGME treatment induced time-dependent apoptosis of MCF-7 cells.
Apoptosis in MCF-7 cells was assessed after treatment with CGME 40 µg /ml or paclitaxel 20 µg/mL, (A) MCF-7 cells without treatment, (B) CGME 1 hour treatment, (C) CGME 3 hours treatment, (D) CGME 6 hours treatment, (E) CGME 12 hours treatment, (F) CGME 24 hours treatment, (G) Paclitaxel 24 hours treatment. Numbers indicate the percentage of cells in each quadrant. The lower left quadrant (R3) shows viable cells, the lower right quadrant (R4) shows cells in early stages of apoptosis, the upper right quadrant (R2) shows cells in the later stage of apoptosis, and the upper left (R1) quadrant shows necrotic dead cells.
The P value was determined using the Kruskal-Wallis test (P=0.10).

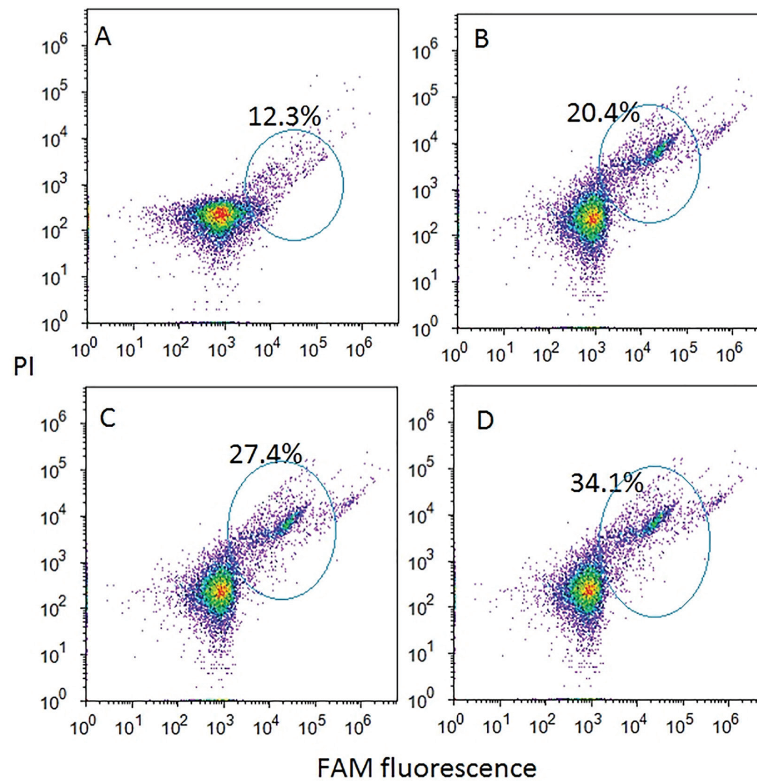


**Figure 4:** Flow cytometric analysis of the poly-caspases in CGME treated MCF-7 cells. (A) MCF-7 cells, (B) MCF-7 cells with CGME 40 g/ml, (C) MCF-7 cells with CGME 60 µg/ml, (D) MCF-7 cells with 20 µg/ml paclitaxel.

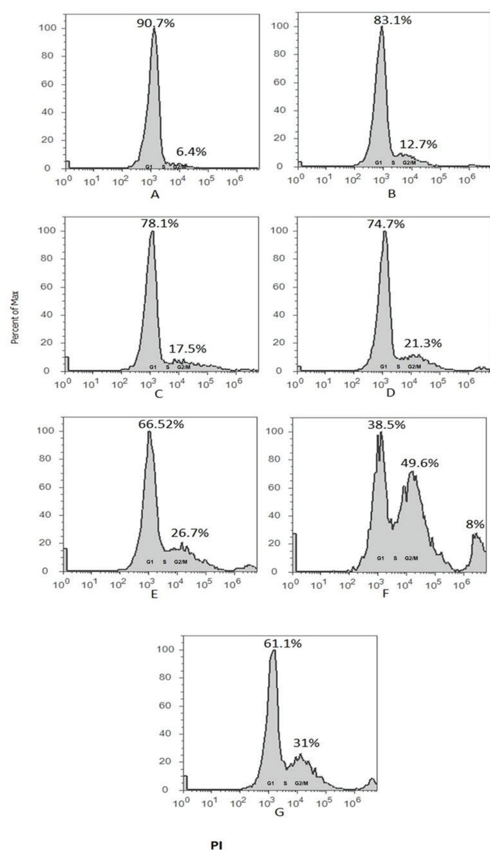


**Figure 5:** Cell cycle analysis in MCF-7 cells. G2/M phase and Sub-G1 arrest in MCF-7 cells by CGME. (A) Untreated MCF-7 cells. (B to F) MCF-7 cells with CGME 40 μg/ml, (B) 1 hour, (C) 3 hours, (D) 6 hours, (E) 12 hours, (F) 24 hours, (G) MCF-7 cells treated with paclitaxel 20 μg/ml 24 hours.

*Cell Cycle Arrest in MCF-7 Cells*

CGME treatment caused an accumulation of cells in the G2/M phase of the cell cycle. Treatment with 40 g/ml of CGME for 0, 1, 2, 6, 12 and 24 hours resulted in an increase in the percentage of the cells in the G2/M phase from 6% to 49.6% (figure 5A to figure 5F), and a decrease in the percentage of cells in the G1 phase from 90% to 38.5%, whereas 31.0% of cells had accumulated in the G2/M phase when treated with paclitaxel (figure 5G). Our results showed that CGME treatment resulted in a significant increase of G2/M arrest, accompanied by a marked increase in cell death (figure 5).

*TUNEL Assay*

The results demonstrated that the DNA fragmentation due to CGME treatment was 52% (figure 6C). DNA fragmentation after treatment with paclitaxel was 68% (figure 6D). CGME exerted strong growth inhibitory effects on MCF-7 cells by perturbation in the G2/M phase of the cell cycle.

*Expression of Anti-Apoptotic and Pro-Apoptotic Genes in CGME Treated Cells*

The expression of pro-apoptotic and anti-apoptotic genes we studied with the real-time qPCR using SYBR Green chemistry. As shown in figure 7, the anti-apoptotic Bcl-2 expression in MCF-7 cells was down-regulated in the presence of CGME. The expression of the Bak-1/Bax, pro-apoptotic genes, was found to

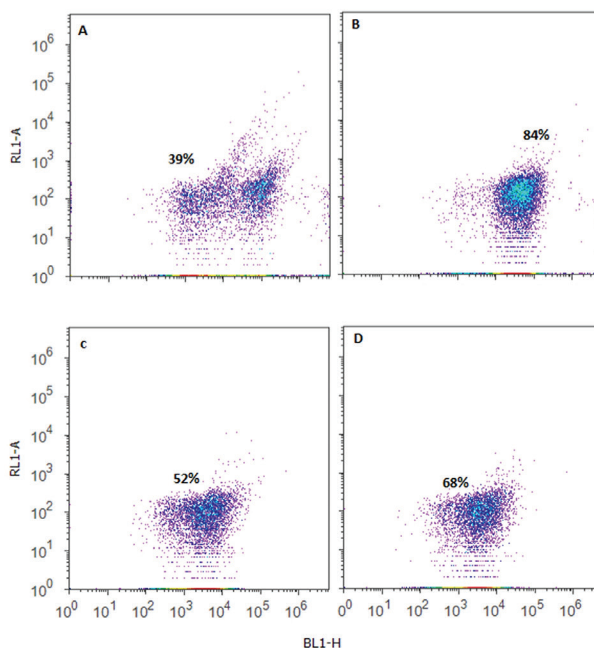


**Figure 6:** The figures demonstrated the TUNEL assay in CGME treated cells. (A) Positive control (degraded DNA) MCF-7 cells, (B) Negative control (intact DNA) MCF-7 cells, (C) CGME treated MCF-7 cells, (D) Paclitaxel treated MCF-7 cells.

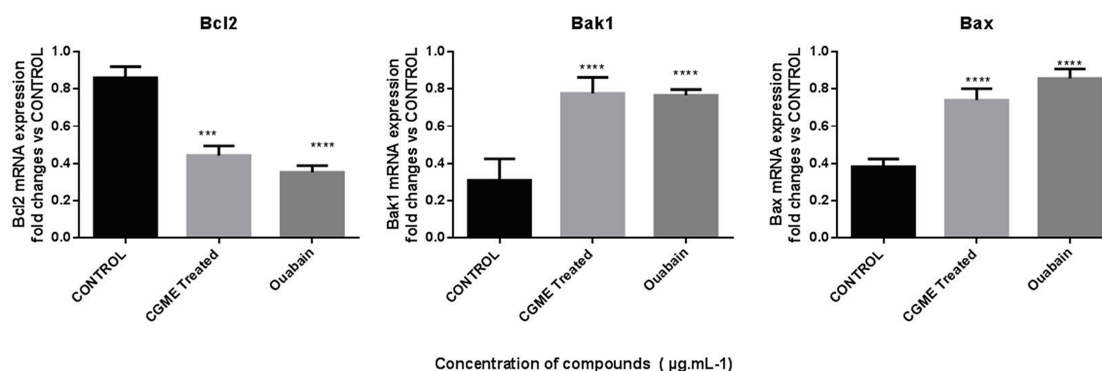


**Figure 7:** The anti-apoptotic proteins and proapoptotic proteins Bak-1 and Bax in MCF-7 cells in the presence of CGME were analyzed (error bars indicate SD). The Kruskal-Wallis test was used for the determination of P values; Bcl-2 (P=0.00193), Bak-1 (P=0.00021), and Bax (P=0.0019).

be more up-regulated in CGME treated MCF-7 cells. The CGME treatment down-regulated the expression of Bcl-2, whereas it up-regulated the expression of Bak-1/Bax-1 genes in MCF-7cells. After exposure of MCF-7 cells to the extract for 24 hours (figure 7), Bax-1 expression was found to increase (0.75 fold) in the presence of CGME. Expression of the anti-apoptotic proteins, Bcl-2, on the other hand, was found to be more down-regulated in the presence of CGME.

## Discussion

Earlier studies have reported on caspases dependent apoptosis induction by the cardenolides from *Ascelepia subulata*.[27] There have been 109 cardenolides from the *Apocynaceae* family identified so far of which 25% have been implicated in the regulation of cancer cell survival or death through various signalling pathways.[28] Recently, Parhira and colleagues reported that eight different cardenolides from *C. gigantea* have been shown to inhibit transcription by hypoxia-inducible factor-1 transcription. Interestingly, these cardenolides have exhibited strong cytotoxic effects against MCF-7 cells, but less on normal cells.[29] In line with Parhira and colleagues, our results systematically showed that cardenolides present in *C. gigantea* were able to induce transcription of pro-apoptotic genes, while repressing anti-apoptotic gene expression and impart apoptosis in MCF-7 cells. The $IC_{50}$ value for CGME was found to be 40.16±0.54 µg/mL in MCF-7 cells at 24 hours. Henceforth, 40 µg/mL of CGME concentration was used to detect molecular changes in MCF-7 cells. This $IC_{50}$ value is very low in comparison with previously reported effects of methanolic extract of *C. gigantea* against HepG2 and MCF-7 cell lines with average $IC_{50}$ values of 85.05 µg/ml and 92.4 µg/ml, respectively.[30] In addition to modulating gene expression externalization of phosphatidylserine, recruitment of caspase cascade, G2/cell cycle arrest, and DNA disintegration demonstrated with the TUNEL assay are in agreement that CGME was able to induce apoptosis of MCF-7 cells. The impact of *C. gigantea* extract on various carcinomas have been reported, one of the carcinoma cell lines utilized was MCF-7.[10-13] This report not only reiterated that CGME mediated cytotoxicity in MCF-7 cells, but also demonstrated the induction and mechanism of apoptosis; a step forward compared to earlier studies. The exposure of membrane phosphatidylserine (PS) was detected, a major indicator of apoptosis in MCF-7 cells. Similarly, Habib and colleagues reported the anti-tumor activity of ethyl acetate extract from the flower of the *C. gigantea* against EAC in Swiss mice. The flower extract exhibited a significant decrease in both viable tumor cells and body weight gain induced by the tumor burden and prolonged survival time.[14, 15] The PUMA family proteins involved apoptosis pathways through controlling mitochondrial membrane permeability and cell death.[19] This observation is in agreement with earlier findings that upon activation, the BH3-proteins only promote apoptosis through neutralizing apoptosis inhibitory proteins (Bcl-2 and Bcl-xL) and also by promoting the opening of mitochondrial pores comprised by the death-promoting members of the Bcl-2 family, Bax, and Bak.[19] We found a low expression of Bcl-2 and increased expression of other pro-apoptotic genes in the CGME treated cells. Similarly, Priya and colleagues evaluated anti-proliferative activity within the root extracts from *C. gigantea* on human hepatocellular carcinoma cells, HepG2, and MCF-7 cells. Gene expression

studies of Bcl-2 family of genes (Bax, Bcl-2, and p53) showed significantly ($P<0.05$) increased expression in Bax and p53 but significantly ($P<0.05$) reduced Bcl-2 expression.[30] Regulation level seen for Bcl-2 and Bax/Bak-1 was fairly similar in both paclitaxel and CGME treatment of MCF-7 cells. Our results agree with the earlier report by Priya and colleagues concluding that the expression pattern of Bax/Bcl-2 shifted as the cells received CGME treatment. The Bax levels were significantly elevated ($P<0.001$) in the treatment group, whereas the Bcl-2 expression significantly decreased ($P<0.05$) in methanolic extract treated HepG2 cells.[30] The ratio of Bax/Bcl-2 is thus a decisive factor and plays an important role in determining apoptosis under experimental conditions promoting cell death.[31] Our results also suggested that up-regulation of Bax/Bak-1 genes and down-modulation of the Bcl-2 gene could be another molecular mechanism through which CGME induces apoptosis.

Treatment with CGME caused cell arrest in G2/M phase of the cell cycle. CGME induced the caspase pathways and the DNA endonucleases were found to be active in both cell types. We sought to analyze whether CGME treatment of MCF-7 cells caused DNA fragmentation. We carried out the Apo-BRdU™ tunnel assay as described in the materials and methods section. The G2 checkpoint was of particular importance in cancer because many cancers acquired defects in the G1 checkpoint, which provide the transformed cells with a growth advantage over normal cells.[32] Defects in the G2-M arrest checkpoint may allow a damaged cell to enter mitosis and undergo apoptosis. Efforts to enhance this effect may increase the cytotoxicity of chemotherapy. Alternatively, efforts to increase G2-M arrest have also been associated with enhanced apoptosis. DNA damage is associated with many cellular events, including activation of Chk1, which in turn phosphorylates and inactivates Cdc25, allowing inactivation of the Cdc2-B1 complex and G2-M arrest. Agents capable of overriding this G2-M arrest were shown to enhance the cytotoxicity of DNA damaging agents. The Chk1 indolocarbazole inhibitor (SB-218078) abrogated gamma-irradiation and topotecan induced G2-M arrest in HeLa cells and enhanced cytotoxicity.[33] Similar to the case of the activation of caspases, the percentage of G2/M arrest induced by CGME was comparable to that of paclitaxel mediated G2/M arrest (figure 5E, figure 5G). The G2/M arrest after 24 hours treatment was found to be even more pronounced indicating that almost 50% of cell population exhibited G2/M arrest (figure 5F). Our observations were in line with the earlier report by Yan and colleagues that calotropin-induced cell cycle arrest was associated with a significant loss of cyclins, which resulted in G2/M phase cell cycle arrest.[22]

## Conclusion

The present study is the first report substantiating that CGME treatment of MCF-7 cells caused externalization of phosphatidylserine, recruitment of poly-caspases as key steps in apoptosis. The cell cycle arrest in G2/M, DNA fragmentation, and augmented pro-apoptotic gene expression for Bak-1 and Bax clearly indicated that CGME treatment induces apoptosis in MCF-7 cells.

## Acknowledgement

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**Conflict of Interest:** None declared.

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