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ORIGINAL ARTICLE

Anticancer Effects of *Calotropis procera* Latex Extract in MCF-7 Breast Cancer Cells

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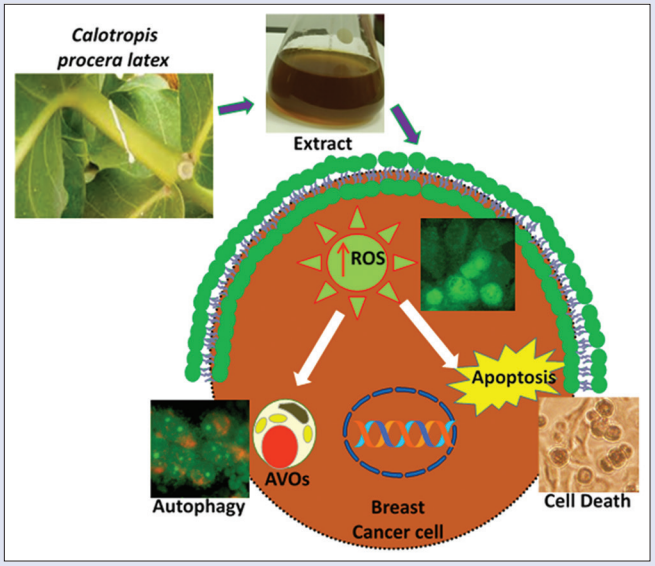
ABSTRACT

Background: *Calotropis procera* is a wild growing medicinal plant with many pharmacological properties, arising mainly from its latex, which contains many biologically active compounds, including cardiac glycosides. **Objectives:** The present study was conducted to isolate a cardiac glycosidal (CG) extract from the latex of *C. procera* and to assess its potential in inducing anticancer effects on breast cancer cells (MCF-7). **Materials and Methods:** Cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and morphological changes observations. The generation of intracellular reactive oxygen species (ROS) was evaluated both qualitatively and quantitatively. Flow cytometry technique was used to evaluate apoptosis and autophagy was determined by fluorescence microscopy and Western blotting. **Results:** The extract significantly ($P < 0.05$) inhibited the proliferation of MCF-7 cells and that this effect increased in line with concentration. Systemic changes in the morphology of treated cells when compared with control cells were observed. ROS levels were increased by about 1.5 and 1.95-fold at the highest concentration of 75 µg/ml after 12 and 24 h of treatment, respectively. A significant ($P < 0.05$) increase in the percentage of early and late apoptotic cells were recorded. Autophagy induction in treated MCF-7 was confirmed with the presence of acidic vesicular organelles. Finally, the change in the intracellular localization of light chain 3 (LC3) protein was determined by Western blotting using primary antibodies. A maximum of a 1.76-fold increase was observed in the expression level of the LC3 marker protein. **Conclusion:** These findings suggest that CG extract increased the levels of intracellular ROS resulting in the induction of cytotoxicity, apoptosis, and autophagy in MCF-7 cells.

Key words: Apoptosis, autophagy, breast cancer, *Calotropis procera,* oxidative stress

SUMMARY

- CG extract from latex of *C. procera* was isolated
- A significant cytotoxicity and morphological changes were observed post treatment with extract in MCF-7 breast cancer cells
- The CG extract at sublethal concentrations were able to enhance intracellular reactive oxygen species and induced apoptosis and autophagy
- For breast cancer, CG extract from *C. procera* could be a potential drug candidate.



Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS: Reactive oxygen species; PBS: Phosphate-buffered saline; MEM: Minimum essential medium; CG: Cardiac glycoside; AVO: Acidic vesicular organelles; FBS: Fetal bovine serum; LC3: Light chain 3; DMSO: Dimethyl sulfoxide; FITC: Fluorescein isothiocyanate; SEM: Standard error of mean; PI: Propidium iodide; DCFH-DA: 2′,7′-Dichlorofluorescin Diacetate; PVDF: Polyvinylidene difluoride.

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INTRODUCTION

Cancer remains one of the leading causes of morbidity and mortality worldwide and breast cancer is the most common and prevalent cancer among Saudi women.[1] Since traditional methods for the treatment of cancer have well-known undesirable effects and alternative treatment options are very limited, the development of new drugs from natural products that possess better effectiveness and fewer harmful events has become desirable and is the focus of much research.[2] This study contributes to that effort by examining the anticancer potential of *Calotropis procera* latex extract.

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C. procera (Ait.) R. Br. (*Asclepiadaceae*) is a wild growing tropical plant widely distributed across Asia, Africa, and the Northeast of Brazil. This plant possesses various medicinal properties and has accordingly been used in traditional systems of medicine.[3] In this context, the stems, flowers, latex, and leaves of plants from the family Asclepiadaceae contain compounds known as cardiac glycosides and a review of the literature indicates a surprising variety of plants whose extracts and isolated cardiac glycoside compounds have been cited for their antiproliferative effects.[4] Some of these studies have reported that *C. procera* extract, in particular, exhibits *in vitro* and *in vivo* antiproliferative activities.[5] Furthermore, *C. procera*'s anticancer and cytotoxic potential has been demonstrated in mice,[6] as well as *in vitro* cytotoxicity against various human cancer cell lines.[7-9]

Reactive oxygen species (ROS) have roles in cell signaling, homeostasis and shown to regulate autophagy.[10,11] Cardiac glycosides have been found to induce apoptosis in several types of cancer cells,[12] including the ability to suppress tumors in humans.[4] However, the mechanisms by which this is accomplished are still not fully understood. In this context, the present study explored the potential of cardiac glycosidal (CG) extract from the latex of *C. procera* to induce oxidative stress, autophagy, and apoptosis in MCF-7 breast cancer cells.

MATERIALS AND METHODS

Plant material and extraction

The plant material (latex) of *C. procera* was collected from the natural habitat in the vicinity of Riyadh (Saudi Arabia). To isolate CG from the latex of *C. procera* we applied the method previously described in Al-Rajhy *et al.*[13] In brief, latex collected from *C. procera* shrubs was stirred with ethanol (1:1). After vacuum filtration and concentration, the resulting solution was treated with aqueous lead acetate (50%); the filtrate was then treated with H_2S and filtered again. The filtrate was extracted twice with petroleum ether to remove lipids and then further extracted three times with chloroform. The chloroform extract was washed with water, before being evaporated to dryness over anhydrous sodium sulfate to obtain the final extract used in this study. The extract was stored at −20°C until use.

Evaluation of cytotoxicity in MCF-7 Cells

MCF-7 (ATCC® HTB22™) breast cancer cell lines were cultured in a Minimum Essential Medium containing 15% fetal bovine serum and 1% antibiotics (Invitrogen™ GIBCO®) in specialized cell culture incubator (BINDER®, Germany) at 37°C, maintaining a humidified atmosphere containing 5% CO_2. Meanwhile, the crude latex extracts were initially dissolved in dimethyl sulfoxide (DMSO) and finally diluted into complete cell culture medium to obtain the six solutions of different concentrations (i e. 50, 100, 200, 300, 400, and 500 µg/ml). 96-well flat bottom culture plates were used to grow MCF-7 cells (1×10^4 cells/well) and then exposed to the above concentrations of *C. procera* extract for 24 h, to determine the inhibitory concentration (IC_{50}). At the end of the desired treatment, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Invitrogen, USA), solution was added to each well and further incubated for 3 h at 37°C.[14] Finally, MTT solution was removed with medium and 200 µl of DMSO (Sigma Aldrich, St. Louis, MO, USA) was added to each well and further incubated for 20 min. A microplate reader (Synergy, BioTek, USA) was used to measure the optical density of each well at 550 nm.

In addition, morphological changes were observed under a phase contrast inverted microscope to determine the alterations induced by CG extract in MCF-7 cells treated for 24 h with three sublethal concentrations (25, 50, and 75 µg/ml).

Intracellular reactive oxygen species measurement

Spectrofluorometry was used to estimate intracellular ROS quantitatively. For this, 96-well black-bottomed culture plates were seeded with 1×10^5 cells/ml (6 replicates for each treatment and control) and these cells were then treated with three different concentrations of latex extract (25, 50, and 75 µg/ml) and incubated for 12 and 24 h at 37°C. After washing twice with phosphate-buffered saline (PBS) the cells were incubated with 20 µM working solution of 2',7'-dichlorofluorescin diacetate (DCFH-DA) in a serum free medium at 37°C for 30 min in the dark. Finally, the green fluorescence intensity was recorded (wavelengths: Excitation-485 nm, emission-528 nm) in a Synergy microplate reader (BioTek, Winooski, VA, USA). The values were expressed as a percentage of fluorescence intensity relative to the control wells after taking averages of all 6 wells used for each experimental group. For qualitative analysis of intracellular ROS, fluorescence microscopic images were captured after staining the cells. A cover-slip loaded six well plate was seeded with 1×10^5 cells/ml and these cells were then exposed to extract and processed as indicated above. Finally, the stained cells were mounted onto a microscope slide in a mounting medium and images were collected using a compound fluorescence microscope (Olympus BX41, Japan).

Apoptosis assay by flow cytometry

During early stages of apoptosis, phosphatidylserine is transported into the outer portion of the membrane which is usually located in the inner membrane of cells, and this can be detected by its strong affinity for annexin V, a phospholipid binding protein.[15] For this study an annexin V fluorescein isothiocyanate apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used as reported previously[16] applying flow cytometry. Briefly, MCF-7 cells were grown overnight and then treated with 25, 50, and 75 µg/ml latex extract of *C. procera* for 12 h and 24 h. The cells were removed from the culture flask by trypsinisation and washed twice with PBS. Samples were prepared following the manufacturer's instructions. A BD FACSCalibur flow cytometer (BD Biosciences) was then used to analyze the annexin V/propidium iodide (PI) fluorescence. The data for 10,000 events from each sample were analyzed using Cell Quest Pro software (BD Biosciences).

Detection of autophagy by acridine orange staining

Autophagy was detected qualitatively using fluorescence microscopic imaging with acridine orange staining (AO). 1×10^5 cells/ml were seeded on a cover-slip loaded six-well plate overnight. Next, the cells were exposed to *C. procera* extract (25, 50, and 75 µg/ml) and incubated for 12 and 24 h at 37°C. After being washed twice with PBS the treated cells were stained with working solution of AO (5 µg/ml) in a serum free medium at room temperature for 15 min in the dark. Finally, the stained cells were mounted onto a glass slide and observed in a compound fluorescence microscope (Olympus BX41, Japan).

Western blot analysis of autophagy marker protein light chain 3

Western blot analysis was used to determine the level of the light chain 3 (LC3) marker protein for autophagy. Cells previously treated with *C. procera* extract were washed with PBS (pH 7.4) and lysed in a buffer (50 mM HEPES, pH 7.4, containing 30 mM $Na_4P_2O_7$, 10 mMNaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1% Triton X-100 (v/v) and 20 µl/ml protease inhibitor cocktail) for 20 min at 4°C. The homogenate was then subjected to ultrasonication for three times for 30 s each to rupture the plasma membrane and centrifuged at 14,000 rpm at 4°C for 20 min and the supernatant was stored in aliquots at −80°C until use. The Bradford protein assay was performed to estimate the protein

level, with bovine serum albumin being used as the standard.[17] The R^2 value for the standard curve was estimated as 0.954. Protein samples were then denatured in a Laemmli sample buffer containing 100 mM DTT at 95°C and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were then transferred electrophoretically to polyvinylidene difluoride blotting membranes using a semidry transfer device (Bio-Rad). The membranes were then blocked by incubation in 5% nonfat dry milk and the blots were incubated with LC3 primary antibodies (Abcam, UK), with beta-actin primary antibody being used as loading control. Horseradish peroxidase-conjugated secondary antibodies (anti-IgG) were used for immunodetection, following the ECL protocol. The intensity of the resulting protein bands was quantified using the ChemiDoc system (Bio-Rad) for densitometric analysis, supported by Image Lab® 1-D analysis software (Bio-Rad).

Statistical analysis

All the above experiments were performed three times completely independently and the results of these three replicates are presented in this study as the means ± standard errors of the mean. MTT assay data were presented as linear graphs and tables. Microsoft Office Excel was used for calculations and for plotting the estimated means and standard errors in graphs. Statistical analysis of results was performed by applying the Student's *t*-test for comparison between the means using a significance level of $P < 0.05$.

RESULTS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay for antiproliferative effects of *Calotropis procera* extract on MCF-7 cells

In this assay, the toxicity of CG extract from the latex of *C. procera* was evaluated *in vitro* against the human MCF-7 breast cancer cell line. It was found that treatment with the extract was associated with a significant reduction in the proliferation of MCF-7 cells ($P < 0.05$). The inhibitory effect was observed after 24 h incubation with the extract. Figure 1 shows how the proportion of viable cells changed in response to treatment with different concentrations of CG extract (50–500 µg/ml) compared to the control cells. A concentration dependent decrease in cell viability was observed. At 500 µg/ml, only 31% of cells were viable, whereas at the lowest concentration (50 µg/ml) 79% of cells were viable. The IC_{50} value was estimated to be 135 µg/ml [Figure 1]. Hence, three sublethal concentrations of CG extract (25 µg/ml, 50 µg/ml, and 75 µg/ml) were selected and used in the subsequent experiments. Investigation of the morphological features of MCF-7 cells using an inverted phase contrast microscope revealed clear changes in the cell morphology at all concentrations when compared with the untreated cells. It could be clearly observed that control cells [Figure 2a] exhibited a normal shape (polygonal or spindle-shaped) and were attached to the surface of culture flask with well-developed nuclei, reaching about 95%–100% confluence. Conversely, systemic changes were evident in the morphological features of the cells in the treated group [Figure 2b-d]. These included cell shrinkage, loss of cell adhesion, decreased cell density, and increased intracellular space. At the highest concentration (75 µg/ml), more cells were detached and become round or irregular instead of spindle-shaped.

Qualitative and quantitative determination of intracellular reactive oxygen species

The DCFH-DA assay was used to investigate the role of CG extract in inducing oxidative stress, both qualitatively using fluorescence microscopy and quantitatively using spectrofluorometry. For the qualitative assessment, Figure 3a-d shows that after 12 h, exposure levels of ROS increased in a concentration dependent manner, as observed by the greater intensity of the green fluorescence in the treated compared to the control cells. A similar pattern of increased green fluorescence level was evident after 24 h treatment [Figure 3e-h]. The quantitative assessment through spectrofluorometry confirmed this pattern, with results revealing a statistically significant increase ($P < 0.05$) in the ROS level [Figure 4]. Compared to the control group, treatment with CG extract at a concentration of 75 µg/ml was associated with a 1.5 and 1.95-fold increase in ROS generation after 12 h and 24 h treatment, respectively.

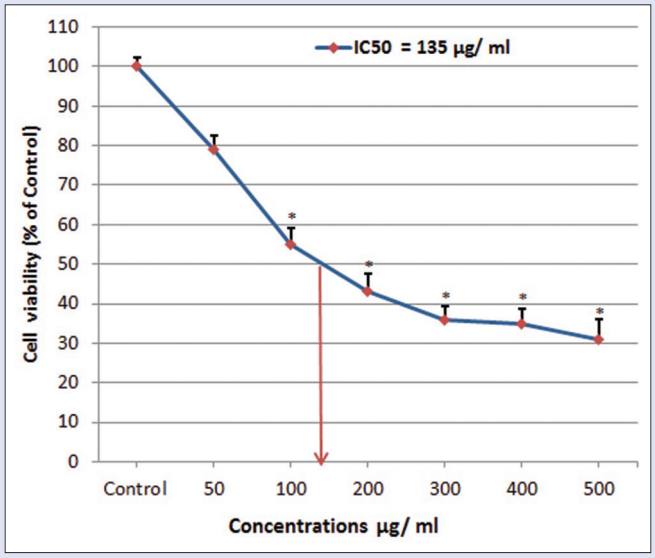

Figure 1: Cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. MCF-7 cells were exposed to the indicated concentrations of extract for 24 h. A significant ($P < 0.05$) concentration dependent cytotoxic effect was observed with increasing concentration of extract. An IC_{50} value of 135 µg/ml was estimated

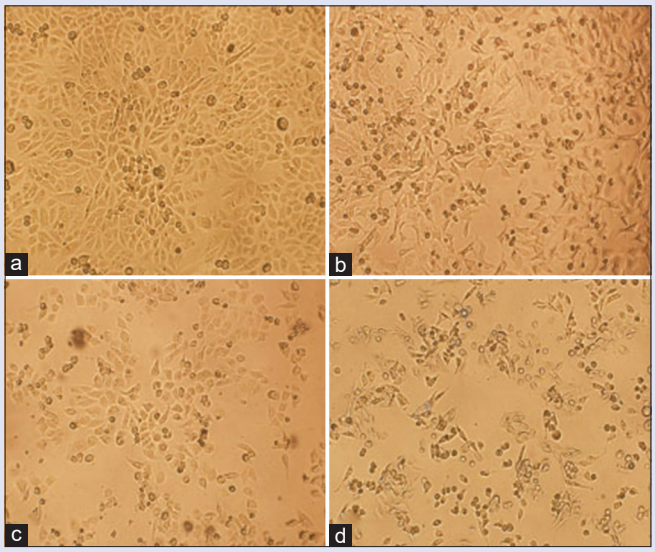

Figure 2: Morphological changes observed as a sign of toxicity in MCF-7 cells. Untreated cells (a) had normal shape with about 95%–100% confluence. A concentration dependent loss of cell adhesion, decreased cell density along with many detached cells were visualized in treated groups. (b) 25 µg/ml (c) 50 µg/ml and (d) 75 µg/ml (×100)

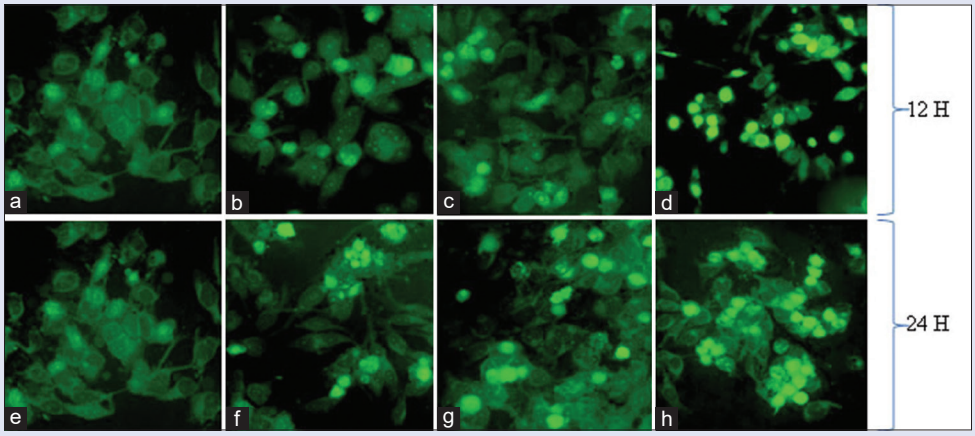

Figure 3: Fluorescence microscopic images showing detection of intracellular reactive oxygen species (a-h). Representative images from three independent experiments were shown. (a and e) control cells showing basal level of reactive oxygen species. A concentration dependent increase of green fluorescence intensity was observed after indicated durations: (b and f) 25 µg/ml (c and g) 50 µg/ml and (d and h) 75 µg/ml (×400)

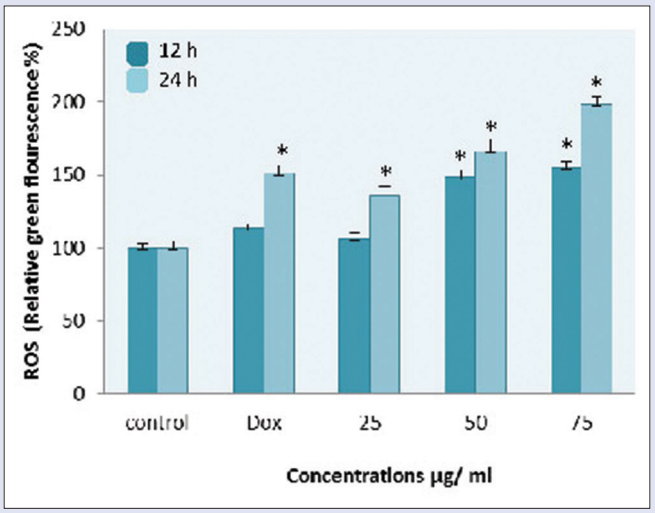

Figure 4: Quantitative detection of intracellular reactive oxygen species by spectrofluorometry. MCF-7 cells were exposed to different concentrations of cardiac glycosidal extract for 12 and 24 h and green fluorescence intensity was recorded (Excitation = 485 nm, emission = 530 nm). The values were shown as mean ± SE, from three independent experiments. A concentration dependent relative increase in intensity of green fluorescence was observed in treated groups

Analysis of apoptosis by flow cytometry

We quantified the extent of apoptosis in MCF-7 cells labeled with annexin V/PI staining using flow cytometry. Representative dot plots showed that only 2%–6% cells in the control group were dead or undergoing apoptosis, which is a normal level for cells growing in cultures. In the absence of extract (control), only 2% of cells were in early apoptosis (annexin V+/PI-) and 2.44% cells were in late apoptosis (annexin V+/PI +), while <2% cells exhibited necrosis (annexin V-/PI+) [Figure 5a and e]. When the cells were treated with the extract for 12 h, however, there was a significant ($P < 0.05$) increase in the percentages of early apoptotic, late apoptotic and necrotic cells. The proportion of cells in early apoptosis were 7.22%, 13.55%, and 19.45% at concentrations of 25, 50, and 75 µg/ml, respectively. Cells in late apoptosis also increased to 4.15%, 10.58%, and 16.75% respective to the above three concentrations, while necrosis was observed in between 2.33% and 4.33% of cells [Figure 5b-d]. A similar trend of apoptosis induction was also observed when the cells were treated for 24 h. In this case, for concentrations of between 25 µg/ml and 75 µg/ml of extract the percentage of early apoptotic cells was between 9.54% and 23.8%, while that of late apoptotic cells was between 5.23% and 21.55% [Figure 5f-h]. 24 h of treatment with these three different concentrations of CG extract resulted in between 2.90% and 4.76% of the cells appearing necrotic.

Detection of autophagy by acridine orange staining

Based on the results showing the role of apoptosis in the CG extract-induced cytotoxicity, we next checked whether autophagy was involved in the anticancer mechanisms of this extract. Fluorescence microscope images revealed untreated cells appeared with limited acidic vesicular organelles (AVOs) in the cytoplasm and showed evenly distributed green fluorescence with only traces of red fluorescence [Figure 6a and e]. In contrast, the cells treated with CG extract for 12 h exhibited more AVOs in the perinuclear region of the cytoplasm [Figure 6b-d]. After 24 h exposure, AVO formation and this increased as concentrations increased. At the highest concentration (75 µg/ml), there was a >40% increase in the red fluorescence indicating autophagy [Figure 6f-h].

Western blot analysis of light chain 3 marker protein

Microtubule associated protein LC3 is now widely used to monitor autophagy.[18] Specifically, when autophagy is initiated, LC3 is converted from LC3-I (17 kD) to LC3-II (15 kD), accumulating on the autophagosome membrane and appearing as punctae. This change in the intracellular localisation of LC3 protein have been used as a molecular marker for detecting autophagic activity. Western blotting was performed using anti-LC3 primary antibodies with lysates from MCF-7 cells receiving different concentrations of CG extract (25, 50, and 75 µg/ml). Densitometric scanning of the immunoblots was performed to quantify the levels of LC3 in treated and control samples. After 24 h of treatment, the expression levels of both LC3-I and LC3-II proteins increased significantly [Figure 7]. An increase of up to 1.76-folds was observed in the treatment with the highest concentration of 75 µg/ml. Similarly, elevations of 1.16-folds and 1.51-folds were registered in the case of the 25 and 50 µg/ml concentrations, respectively. The amount of β-actin served as the loading control where no change was observed in control and treated groups.

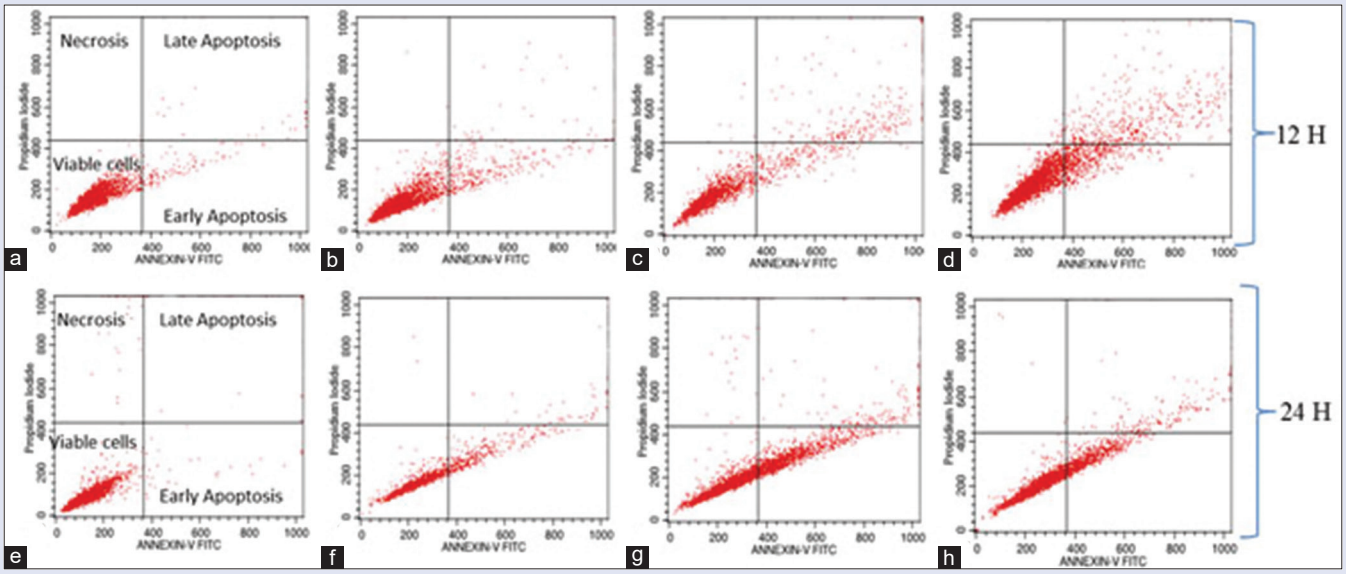

Figure 5: Flow cytometry analysis to determine the phosphatidyl serine translocation in MCF-7 cells (Annexin V– fluorescein isothiocyanate assay). MCF-7 Cells were harvested after desired treatment (as indicated for 12 h and 24 h) and incubated with Annexin V/PI and analyzed by flow cytometry. The percentage of viable cells, early apoptosis, late apoptosis, and necrosis cells is shown in the representative dot plots. The data were represented as mean ± SE from three independent experiments. A dose-dependent increase in apoptosis were observed in treated cells ($P < 0.05$). (a and e) Control, (b and f) 25 µg/ml (c and g) 50 µg/ml and (d and h) 75 µg/ml

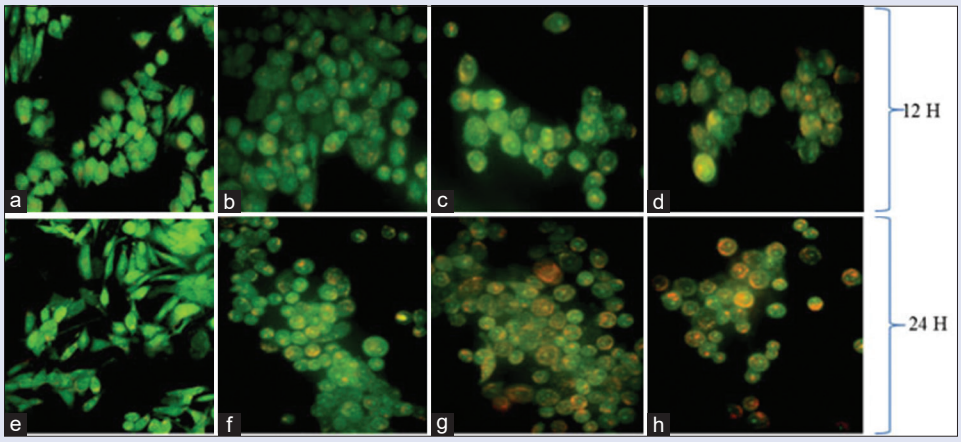

Figure 6: Induction of autophagy was determined by fluorescence microscopy after acridine orange staining in MCF-7 cells treated with extract for 12 h and 24 h. (a and e) control cells with negligible red fluorescence while treated cells (as indicated for 12 h and 24 h) displayed a concentration dependent increase in red fluorescence in the cytoplasm which represent acidic vescicles organalles. (b and f) 25 µg/ml (c and g) 50 µg/ml and (d and h) 75 µg/ml (×400)

DISCUSSION

The latex of *C. procera* has high pharmacological activity arising from its biologically active compounds, of which the focus for this study are cardiac glycosides.[19,20] In the present study, CG extract was isolated from the latex of *C. procera* following the method described in Al-Rajhy *et al.*[13] in which lead acetate precipitation is used to clean-up the ethanolic extract. Previously, phytochemical investigation has revealed fifteen cardiac glycosides in the latex of *C. procera.*[20] Moreover, recently, a bioassay-directed fractionation of the chloroform extract of *C. procera* latex led to the isolation of three new cardenolides derivatives along with eight known ones.[21] All of these isolated cardenolides have previously been evaluated using MTT colorimetric assay for their antiproliferative effects. Specifically, Mohamed *et al.*[21] showed that *C. procera* latex had a strong inhibitory effect on the growth of human lung (A549) and cervix (Hela) cancer cell lines, with an IC_{50} value of 3.37 µM for the in A549 cell line and 6.45 µM for the Hela cell lines. In addition, the cytotoxicity of *C. procera* latex fractions has been tested against four tumor cell lines: HL-60, Ovcar-8, HCT-116 and SF-295, again using the MTT assay. In that work, the hydrophobic fractions hexane, dichloromethane and ethyl acetate were all found to be cytotoxic, with IC_{50} values that ranged from 0.05 to 6.5 µg/ml.[22] Moreover, Meena *et al.*[23] demonstrated that three anticancer cardenolides (2"-Oxovoruscharin, uscharin and voruscarin) extracted from *C. procera* were extremely cytotoxic to various cancer cell lines, while Choedon *et al.*[6] showed that the methanolic extract of *C. procera* exhibits chemopreventive activity *in vitro* and *in vivo* in hepatocellular carcinoma and Juncker *et al.*[24] showed the potential for a hemisynthetic cardenolide, UNBS1450, to inhibit cancer cell proliferation by inducing cell death.

The present study extends this prior work to MCF-7 breast cancer cells, demonstrating that a CG extract from the latex of *C. procera* induced

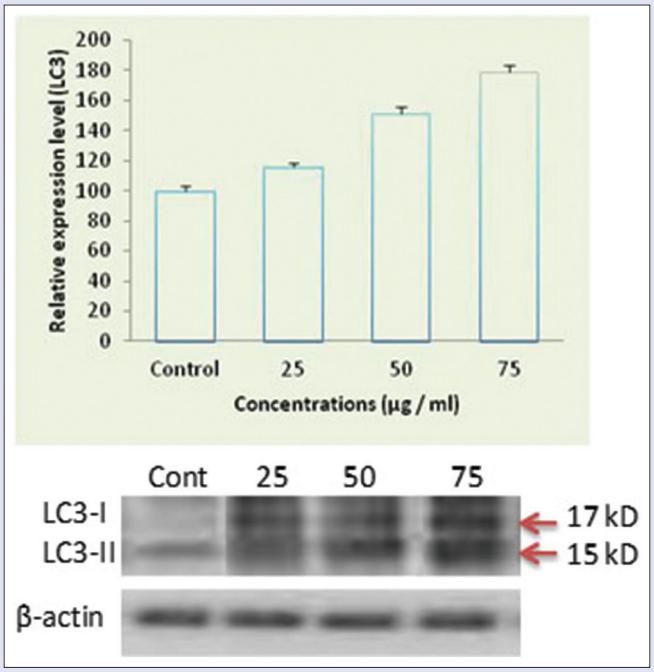

Figure 7: Western blot analysis of LC3I and LC3-II expression level in MCF-7 cells treated with the cardiac glycosidal extract at 25, 50, and 75 µg/ml for 24 h. The cell lysates were subjected to immunoblotting using anti-LC-3 primary antibody. Densitometric scanning of the immunoblots was performed to quantitate the level of LC-3 in treated and control cells. The amount of β-actin served as the loading control. Each image is a representative of at least three independent experiments

significant cytotoxicity in these cells. Specifically, after 24 h of treatment, cell viability was reduced to 31% at the highest concentration used and the IC_{50} value was therefore estimated to be 135 µg/ml. Morphological changes in MCF-7 cells exposed to CG extract also confirmed the cytotoxic effects, e.g., detachment of the adherent cells and reduced confluency with increasing CG extract concentrations.

While the mechanisms behind CGs' anticancer activity are not completely clear, several studies have suggested that CGs inhibit the plasma membrane Na^+/K^+-ATPase and behave as potential oestrogen receptor antagonists. Therefore, Na^+/K^+-ATPase in combination with oestrogen receptors could serve as valuable targets for cardiac glycosides to be developed as promising antibreast cancer drugs.[25,26] Chen *et al.*[25] further suggest that CGs could be developed into antibreast cancer drugs by making use of the sodium pump as an oncology target. A number of studies have reported that the CGs bufalin and ouabain can generate ROS in human colon or lymphoma cancer cells[27] and the present study provides further evidence along the same lines by showing that ROS are generated in human breast cancer cells upon treatment with CG extract. Furthermore, the study shows that CG extract could induce MCF-7 cell apoptosis by increasing the intracellular ROS concentration. In terms of how CG extract works to enhance the production of ROS, it is possible that it reduces the glycolysis level of MCF-7 cells, decreasing the production of Nicotinamide–Adenine Dinucleotide Phosphate (NADPH). NADPH is necessary to eliminate ROS in tumor cells. It has been reported that DNA damage and apoptosis could be induced with the increasing intracellular ROS levels.[27] The low concentration of ROS induces the permeability transition pore of mitochondria opening and promotes the release of greater quantities of ROS. In addition, the increasing intracellular ROS concentration increases the intracellular Ca^{2+} concentration.

There is increasing evidence to show that ROS generation can trigger autophagy.[10] Based on the above evidence showing the role of ROS and apoptosis in the CG extract-induced cytotoxicity, we further analyzed whether autophagy was involved in the anticancer mechanisms of this extract. The role of autophagy in cancer has been intensively explored and depends on the experimental conditions and the different tumor stages.[28,29] Expression levels of autophagy-related genes are reduced or even completely absent in certain cancer cells, indicating that autophagy may function as a tumor suppressive pathway. On the other hand, autophagy may be necessary for tumor progression, acting as a pro-survival pathway that allows cells to tolerate hypoxic conditions and/or chemotherapy. We examined the expression of LC3-II by immunoblotting, which serves as a good indicator for the formation of autophagosomes.[30] An increment of LC3-II was observed to emerge after 24 h which was consistent with the observation of AVOs by fluorescence microscope. Wang *et al.*[31] showed that when NSCLC cell lines were treated with digoxin or ouabain autophagic flux was induced. In accordance with these observations, the finding that CG extract induces apoptosis and autophagy in tumor cells may provide a mechanism for how cardiac glycosides affect cancers.

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Conflicts of interest

There are no conflicts of interest.

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