Antiproliferative activity and induction of apoptosis in estrogen receptor-positive and negative human breast carcinoma cell lines by *Gmelina asiatica* roots

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ABSTRACT

Low risk of breast cancer has been proposed to be associated with high intake of lignans. We have reported the presence of lignans in *Gmelina asiatica* roots. There are no scientific reports on the antiproliferative activity of *G. asiatica* roots. The objective of the present study was to evaluate the effect of ethyl acetate extract from *G. asiatica* roots (EGAR) on estrogen receptor-positive (MCF-7) and negative (MDA-MB-231) human breast cancer cell lines. The effects of 50% inhibitory concentrations (IC_{50}) of EGAR on MCF-7 and MDA-MB-231 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit. The mode of cell death caused by EGAR was determined using dual apoptosis assay kit by observing the cells under fluorescent microscope. The quantification of apoptosis and necrosis in cells caused by EGAR was determined using cell death detection kit through ELISA. Down-regulation of the proliferative activity occurred in a clear dose-dependent response with IC_{50} values of 32.9 ± 3.8 μg/mL in MCF-7 and 19.9 ± 2.3 μg/mL in MDA-MB-231 cell lines. Treatment of breast cancer cells with EGAR resulted in significant apoptosis. The EGAR contain lignans and flavonoids. The antiproliferative activity of the extract is attributed to the presence of these secondary metabolites. The results suggest the efficacy of *G. asiatica* roots as antiproliferative agents on human breast cancer cells, supporting the hypothesis that plants containing lignans have beneficial effects on human breast cancer.

Key words: Annexin-V, caspase-3, enrichment factor, lignans, MCF-7, MDA-MB-231, phosphatidylserine

INTRODUCTION

Plant lignans are commonly classified as phytoestrogens. Some of the plant lignans, such as secoisolariciresinol, pinoresinol, lariciresinol and matairesinol, are converted by gut microbiota to mammalian lignans enterodiol and enterolactone, which are suggested to be the biologically active lignan forms in mammals.[1-3] Consumption of fiber-rich diet is linked with a lower risk of breast cancer. It has been suggested that this may be related with high intake of fiber-associated plant lignans, which are converted to enterolactone.[4] A recent epidemiological study from Germany reported the potential role of lignans in breast cancer prevention.[5]

Gmelina asiatica Linn. (syn. Gmelina parvifolia) (family

Verbenaceae) is commonly known as 'Asian Bush beech'. The plant has been used in gonorrhea, catarrh of the bladder, rheumatism and as a blood purifier.[6] We have reported the presence of (+) sesamin, (-) pinoresinol, (-) piperitol, sakuranetin and ovalifolin in the roots of *G. asiatica*.[7] We have also reported the antimicrobial activity of *G. asiatica* roots.[8] The root powder of *G. asiatica* showed potent anti-inflammatory activity. This activity is attributed to its potent antioxidative actions.[9]

In spite of the clear evidence that lignans play a major role in the prevention of breast cancer, until now little attention has been paid to the possible antiproliferative activity of plants containing lignans. Since *G. asiatica* roots contain lignans and there has been no scientific report on its possible antiproliferative effect on breast cancer, we have investigated the antiproliferative activity *G. asiatica* roots. The aim of the present study was to evaluate the antiproliferative effect of ethyl acetate extract from *G. asiatica* roots (EGAR) on human breast carcinoma cell lines. We examined its antiproliferative effects and the mode of cell death (apoptosis) after treatment of estrogen receptor-

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positive (ER +ve) MCF-7 and estrogen receptor-negative (ER -ve) MDA-MB-231 breast cancer cell lines.

In the present study, we have shown that the antiproliferative effect of EGAR on both (ER +ve) and (ER -ve) breast cancer cell lines could be related to its apoptosis-inducing activity as detected by the adhesion of annexin V to phosphatidylserine (PS) on the outer leaflet of the cell membrane and activation of caspases.

MATERIALS AND METHODS

Chemicals, reagents and test kits
MTT assay kit and dual apoptosis assay kit were purchased from Biotium, USA. Cell death detection ELISA[PLUS] kit was purchased from Roche Applied Sciences, Germany. All the chemicals used in this experiment were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated.

Preparation of ethyl acetate extract from *Gmelina asiatica* roots
Fresh roots of *Gmelina asiatica* were collected from the Zoo Park, Visakhapatnam, Andhra Pradesh, India, and identified by Prof. M. Venkaiah, Department of Botany, Andhra University, Visakhapatnam. The voucher specimen (no. 135 C) was placed in Andhra University's herbarium. The roots were cut into small pieces and dried in a hot-air oven at a temperature not more than 50°C. The dried roots were powdered using an electric blender. Ethyl acetate was chosen as a solvent for extraction as we found that ethyl acetate can extract all lignans and flavonoids present in *G. asiatica* roots. Powdered roots (50 g) were extracted with ethyl acetate using soxhlet extractor. The ethyl acetate extract was concentrated in a rotary evaporator at a temperature not more than 50°C. The concentrated extract was dried using freeze dryer at −33°C.

According to the National Cancer Institute (NCI), USA, a crude extract may be considered as potent cytotoxic if its IC$_{50}$ ≤ 20 µg/mL.[10] So in the present study, the highest concentration of extract used was 100 µg/mL. Dried extract was dissolved in 30% (v/v) dimethylsulfoxide (DMSO) in ultra-pure water to obtain the final concentrations of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, 1.25 and 0.625 µg/mL. For proliferation and apoptosis assays, solutions of different concentrations of the extract were sterilized by passing them through 0.22-µm membrane filters.

Estimation of total phenol content
Total phenol content (TPC) was determined by using Folin-Ciocalteu method[11] with minor modifications. To each 1 mL of sample, 5 mL of distilled water was added along with 0.5 mL of Folin-Ciocalteu reagent (2 N), vortexed and allowed to incubate at 37°C for 5 minutes. Thereafter, 1 mL of 5% (w/v) sodium carbonate solution was added to each sample, vortexed and incubated at 37°C in the dark for 1 hour. After incubation, samples were vortexed and absorbance was measured at 765 nm in triplicate using a spectrophotometer. A standard curve was generated using gallic acid with concentrations ranging from 20 to 500 µg/mL. The calibration equation for gallic acid was y = 0.0098 × − 0.0152 (R^2 = 0.9998). TPC was expressed as gallic acid equivalents (GAE) in mg per 100 g of dry weight of *G. asiatica* roots.

Cell lines and culture condition
Breast-adenocarcinoma cell lines MCF-7 (ER +ve) and MDA-MB-231 (ER -ve) from ATCC (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycin (PenStrep) under a fully humidified atmosphere, 5% CO$_2$ at 37°C. For experiments, cells were collected from subconfluent monolayers with accutase. The studies were carried out using cells from passages 3-7.

Proliferation assay
The effect of EGAR on the viability of the cancer cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide) assay. Briefly, 99 µL of the cell suspensions were plated in 96-well flat-bottomed tissue culture plates (Nunc, Denmark) at a concentration of 1 × 10^4 cells/well. After incubating the plates for 24 hours at 37°C in a humidified incubator, different concentrations of sterilized EGAR solutions were added to the respective wells of the plate. The final volume in each well was 100 µL. Each solution was placed in triplicate into wells. The whole assay was done in triplicate. The plates were further incubated for 48 hours. Five microliters of MTT reagent was added to each well and incubated for 4 hours, after which the plates were centrifuged at 600 g for 5 minutes at 4°C. MTT solution and medium were aspirated from the wells, and100 µL of buffered DMSO was added to each well. The plates were shaken for 5 minutes, and the absorbance (OD) was recorded on a microplate reader at a wavelength of 570 nm and a reference wavelength of 630 nm. The effect of EGAR on proliferation of cells was assessed as percent cell viability where vehicle (0.3%v/v DMSO in water)-treated cells were taken as 100% viable. Percentage of cell viability in each well was calculated using the following formula:

$$\text{Percentage of viable cells} = \left(\frac{\text{OD of the extract - OD medium control}}{\text{OD vehicle control - OD medium control}} \right) \times 100$$

The IC$_{50}$ values were calculated from the dose-response curves.

Untreated and vehicle-treated cells were incubated as

controls. The final concentration of DMSO in each well did not exceed 0.3% (v/v). This concentration did not affect the apoptosis or cell proliferation of the investigated cell lines.

The mode of cell death induced by EGAR at its IC_{50} concentrations on MCF-7 and MDA-MB-231 were also studied. Apoptosis was qualitatively determined using dual apoptosis assay and quantified using cell death detection by enzyme-linked immunosorbent assay (ELISA).

Dual apoptosis assay

This assay was carried out using dual apoptosis assay kit with NucView™ 488 caspase-3 substrate and sulforhodamine 101-annexin V (Texas Red®-annexin V) according to instructions in the product protocol. This kit detects two important apoptosis events — caspase-3 activation and phosphatidylserine (PS) translocation — in a single experiment. The MCF-7 and MDA-MB-231 cell lines were incubated for 24 hours in a humidified CO_2 incubator on cover slips that were previously coated with poly-L-lysine. The cell lines were challenged with the extract, and the negative control cell lines were challenged with the 0.3% (v/v) DMSO in water. The cells were incubated further for 6 hours and the apoptotic changes were observed at 3 hours and 6 hours . After the respective incubation periods, the culture medium was aspirated and the cells were washed with annexin V–binding buffer. Then annexin-binding buffer (100 μL), 0.2 mM NucView™ 488 caspase-3-substrate (5 μL) and sulforhodamine 101-annexin V (5 μL) were added to each cover slip and incubated for another 45 minutes. The cover slips were washed with annexin V–binding buffer and mounted in annexin V–binding buffer onto slides. The apoptotic events in the stained cells were observed under a fluorescence microscope using FITC and Texas-Red filters. The positively stained apoptotic cells were counted, and the apoptotic index was calculated as the number of apoptotic cells relative to the total number of cells. The whole assay was done in triplicate.

Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death, i.e., apoptosis or necrosis, was quantitatively determined using the cell death detection ELISA[PLUS] assay[12] as recommended by the manufacturer. This kit can detect and quantify both apoptosis and necrosis. Briefly, 99 μL of the cell suspensions (MCF-7 and MDA-MB-231) were plated in 96-well flat-bottomed tissue culture plates at a concentration of 1×10^4 cells/well. After incubating for 24 hours at 37°C in a humidified incubator, sterilized solutions of EGAR were added to the respective wells of the plate. The final volume in each well was 100 μL. The plate was centrifuged at 600 g at 4°C for 10 minutes. The DNA fragments released from the cells

due to necrosis were present in the supernatant layer. The supernatant was carefully transferred without disturbing the cell pellets into a glass vial and stored in a refrigerator at 4°C until further analysis for necrosis. The cell pellet containing the apoptotic bodies was resuspended in lysis buffer and incubated for 30 minutes at room temperature. The plate was centrifuged and cell lysate was transferred into a glass vial and stored in a refrigerator at 4°C until further analysis for apoptosis.

Supernatant and cell lysate solutions (20 μL) were placed in triplicate into wells of streptavidin-coated microplate, to which was added 80 μL of the immunoreagent containing a mixture of anti–histone-biotin and anti–DNA-POD. The plate was covered with an adhesive cover foil and incubated for 2 hours at 25°C in a shaking incubator at 300 rpm. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. At the same time, the anti–DNA-POD antibody reacts with the DNA component of the nucleosomes. The unbound antibodies were washed with incubation buffer. The amount of nucleosomes retained by the POD in the immunocomplex, corresponding to the extent of apoptosis and necrosis, was quantitatively determined photometrically with ABTS (2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid) as substrate using microplate reader at a wavelength 405 nm and reference wavelength of 490 nm.

Statistical analysis

All the data were represented as mean $\pm$ SD (standard deviation) of triplicate. Statistical analyses were conducted using SPSS version 15.0 software. The significance of difference between control and treated groups was determined by Student *t* test, and *P* values less than 0.05 were taken as significant.

RESULTS

To evaluate the effect of activity of EGAR on the proliferation of mammary cancer cells, the MCF-7 cells, a well established model for the *in vitro* investigation of estrogenic activities; and MDA-MB-231 (ER -ve) cells were employed.

Total Phenol content of EGAR

Total Phenol content of ethyl acetate extract from *G. asiatica* roots was determined using the Folin-Ciocalteu method and was found to be 4270 $\pm$ 362 mg GAE/100 g of dried roots.

Antiproliferative activities of EGAR

The effect of EGAR was studied as a dose-response

experiment for 48 hours at dilutions of 0.25-100 μg/mL. Both cell lines exhibited significant EGAR-induced suppression of proliferation. A dose-dependent inhibition of cell growth was observed between 4 and 80 μg/mL (data not shown). The antiproliferative effect of EGAR was more pronounced in MDA-MB-231 cells, which was mirrored in its IC_{50} concentrations. The IC_{50} values of extract, representing the effect on MCF-7 and MDA-MB-231, were found to be 32.9 ± 3.8 μg/mL (MCF-7) and 19.9 ± 2.3 μg/mL (MDA-MB-231). The proliferation of both cells was inhibited almost completely by 100 μg/mL of EGAR. No effect on proliferation of cancer cells was observed up to 2 μg/mL.

EGAR-induced apoptosis in MCF-7 and MDA-MB-231 cell lines

In principle, a reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these two mechanisms. We also investigated whether the extracts can induce apoptosis by monitoring the two important apoptosis markers: phosphatidylserine (PS) translocation on cell membrane (identified by sulforhodamine 101-annexin V)[13] and caspase-3 activation (identified by NucView™ 488 caspase-3 substrate).[14] The morphological changes were inspected by microscopy. Some cells were beginning to detach from the plate and become rounded after 3 hours of treatment by EGAR (30 μg/mL for MCF-7 and 20 μg/mL for MDA-MB-231). Because loss of adhesion to the culture dishes of tumoral epithelial cells has been described as an apoptosis-related event,[15] we examined the morphological apoptotic changes on slides under bright field after 3 hours and 6 hours. In contrast to good spread cells in the negative control, a morphological change with

cell shrinkage was detected in cell lines treated with EGAR extract. Necrosis was less prominent than apoptosis. In Figures 1a-e, the representative photomicrographs of MDA-MB-231 cells after treatment with 20 μg/mL EGAR extract in comparison to solvent-treated control are shown.

To further substantiate the antiproliferative effects of EGAR, the apoptotic cells were monitored by annexin V adherence and caspase-3 activation. In viable cells, PS is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS. Annexin V is a Ca^{2+}–dependent phospholipids-binding protein with high affinity for PS. The binding of sophorodamine101-annexin V probe to PS that has translocated to the outer membrane cell produces red border around the cell under fluorescent microscope using red filter [Figure 1c]. Caspase-3 (CPP32) is a cytosolic protein that normally exists as a 32-kDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis.[16] The cleavage of NucView™488 caspase-3 substrate by activated caspase-3 stains the cell nucleus green [Figure 1d]. The induction of apoptosis by EGAR was time dependent [Figure 2]. In MDA-MB-231 cells, an elevation in apoptosis-positive cells up to 26.5% was found after 3 hours and reached 48.3% after 6 hours of exposure to 20 μg/mL EGAR. In MCF-7, there were fewer apoptotic cells, EGAR at concentration of 30 μg/mL inducing apoptosis in 28.1% and 42.9% after 3 and 6 hours of treatment, respectively. The solvent controls did not increase the spontaneous apoptotic rate in the two malignant cell lines tested.

To quantitate and further support the finding that EGAR
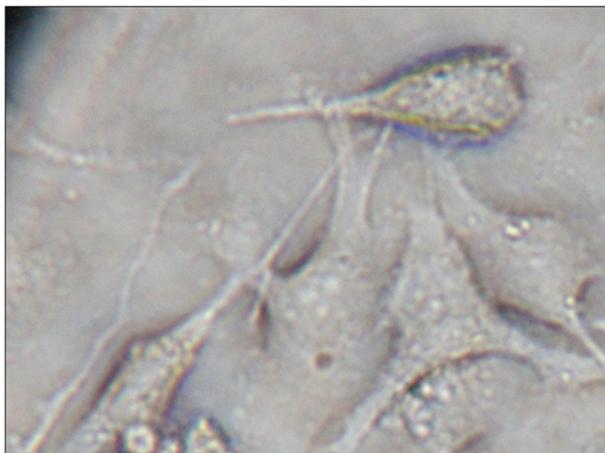
Figure 1(a): Photomicrograph of MDA-MB-231 cell lines after 6 h treatment with 0.3% v/v DMSO (control). Original magnification is x 400 viewed under bright field using fluorescent microscope (Nikon eclopse 80 i). The control (0.3% w/v DMSO) did not cause the changes in the morphology and integrity of MDA-MB-231 cells
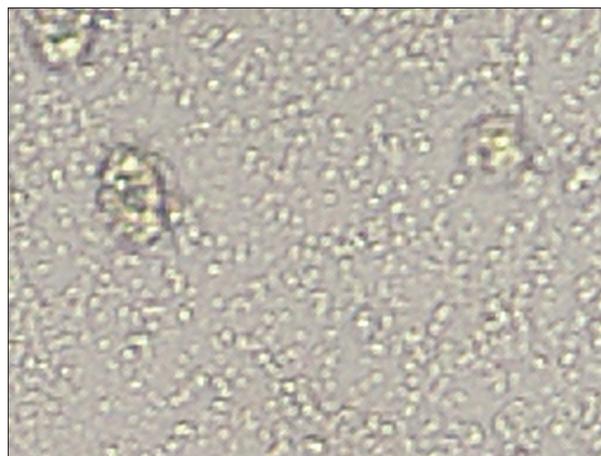
Figure 1(b): Photomicrograph of MDA-MB-231 cell lines after 6 h treatment with 20 μg/mL of ethylacetate extract from *G. asiatica* roots. Original magnification is × 400 viewed under bright field using fluorescent microscope (Nikon eclopse 80 i). The ethylacetate extract from *G. asiatica* roots at 20 μg/mL caused the MDA-MB-231 cells to detach from the surface and the cells become rounded
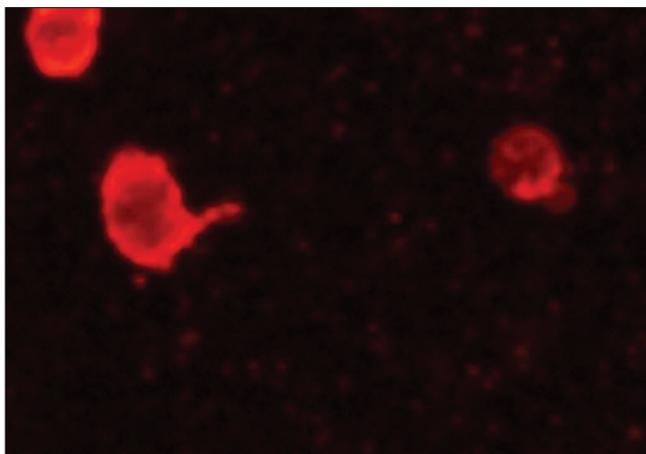
Figure 1(c): Photomicrograph of MDA-MB-231 cell lines stained with annexin-V substrate after 6 h treatment with 20 µg/mL of ethylacetate extract from *G. asiatica* roots. Original magnification is x 400. The cells were stained with fluorescently labeled sulforhodamine 101 annexin-V and the cells were viewed under Texas-red filter (510-560 nm) using fluorescent microscope (Nikon eclopse 80 i). Red border around the MDA-MB-231 cells indicating phosphatidylserine translocation from inner to the outer leaflet of the cell membrane
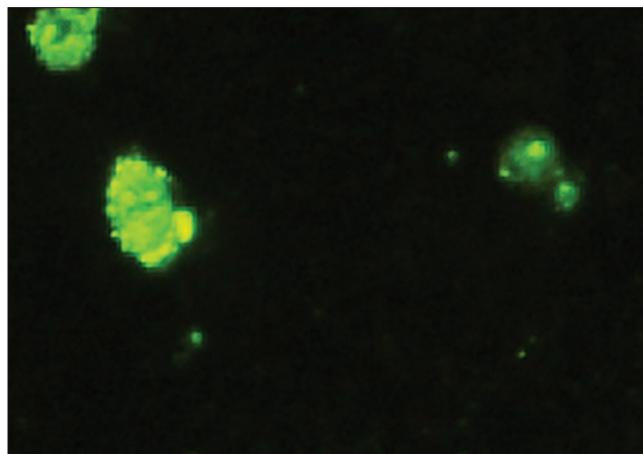
Figure 1(d): Photomicrograph of MDA-MB-231 cell lines stained with caspases-3 substrate after 6 h treatment with 20 µg/mL of ethylacetate extract from *G. asiatica* roots. Original magnification is x 400. The cells were stained with fluorescently labeled Nuc View™ 488 caspase-3 substrate and the cells were viewed under Fluorescein isothiocyanate (FITC, 450-490 nm) using fluorescent microscope (Nikon eclopse 80 i). Green nucleus in the MDA-MB-231 cells indicating caspase-3 activation
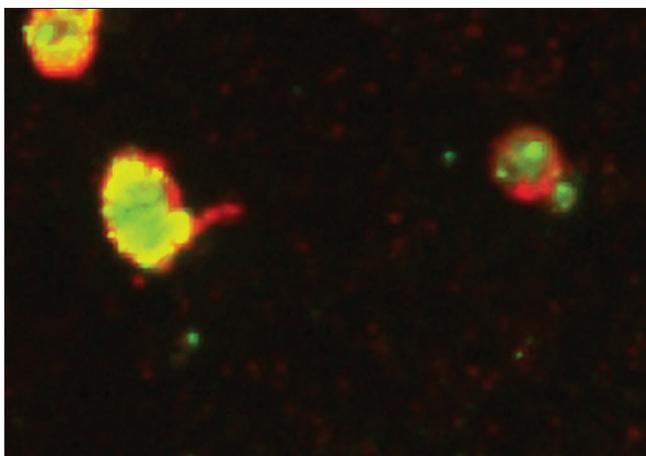
Figure 1(e): Merged photomicrograph of stained MDA-MB-231 cell lines after 6 h treatment with 20 µg/mL of ethylacetate extract from *G. asiatica* roots. Green nucleus surrounded by red border indicating the apoptoic MDA-MB-231 cells
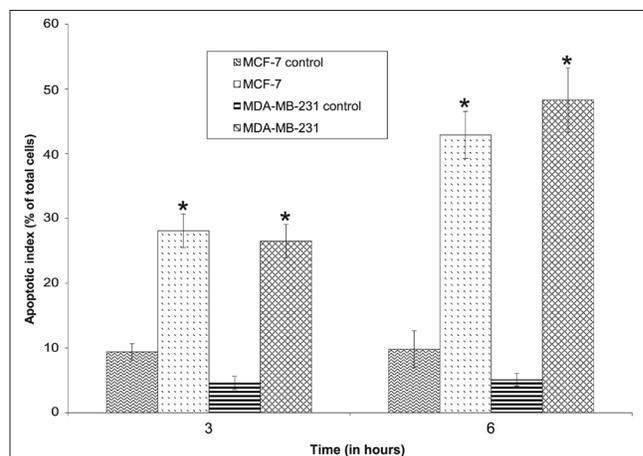
Figure 2: Time dependent apoptosis of MCF-7 and MDA-MB-231 cell lines induced by ethylacetate extract from *G. asiatica* roots. *Values are statistically significant at *P*<0.05 with respect to corresponding controls

exposure causes apoptosis in cancer cells, we performed cell death detection by ELISA. Compared to solvent-treated control, exposure of MCF-7 and MDA-MB-231 cells to EGAR at 30 and 20 µg/mL concentrations resulted in 4.3- and 3.5-fold increases in induction of apoptosis in MCF-7 and MDA-MB-231 cells, respectively, while necrosis induced by EGAR was negligible [Figure 3].

DISCUSSION

The results reported herein reveal that the ethyl acetate extract of *G. asiatica* roots exerts antiproliferative action on MCF-7 and MDA-MB-231 breast cancer cell lines. The dying cells exhibit the ultrastructural and biochemical features that characterize apoptosis, as shown by the loss of viability, PS translocation and capase-3 activation. Like many other plants, EGAR is a multicomponent mixture with pharmacologically active substances. The antiproliferative activity may be attributed to a number of lignans and flavonoids.

We first determined IC_{50} values of the extracts using MTT assay. The MDA-MB-231 (ER -ve) cell lines were significantly more sensitive than MCF-7 (ER +ve) cell lines, with an approximately 2-fold variation in the IC_{50}
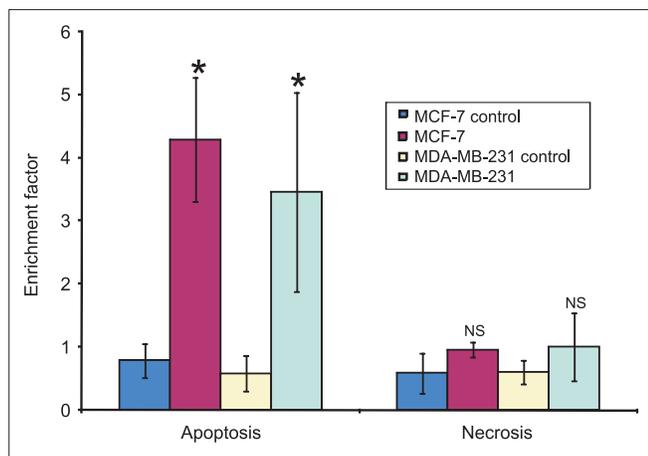
Figure 3: Quantification of apoptosis and necrosis in MCF-7 and MDA-MB-231 cell lines induced by ethylacetate extract from *G. asiatica* roots. *Values are statistically significant at *P*<0.05 with respect to corresponding controls, NS: Values are not statistically significant (*P*>0.05) with respect to corresponding controls

concentration of the EGAR. The antiproliferative action of EGAR on breast cancer cell lines is dose dependent and probably evoked by ER-mediated and non–ER-mediated mechanisms because of the various physicochemical properties of individual components of EGAR. Discovery of active compounds from natural products with apoptosis-inducing ability rather than cytotoxic ability is of great interest for cancer treatment. Screening for anti-cancer substances is commonly conducted using viability assays. An inherent problem with this approach is that all compounds that are toxic and antiproliferative, irrespective of their concentration-dependent mechanism of action, will score positive. Apoptosis is not only essential for normal physiological development but is also critical in eliminating any abnormal cells after exposure to genotoxic or DNA-damaging agents.

Therefore, we investigated the apoptotic changes in mammary cancer cells induced by EGAR, using dual apoptosis assay kit. The morphological changes occurred early, after 3 hours treatment, with loss of adhesion. The apoptotic changes in MDA-MB-231 cell lines, as shown in Figure 1a-e, characterized by PS exposure (detected by annexin V adherence and caspase-3 activation) are representative for both cell lines . The initial apoptotic rate of MCF-7 cells in comparison to MDA-MB-231 cells was higher [Figure 2], probably due to the estrogen-deprived test conditions. About 43% of ER +ve cells and about 48% of ER -ve cells had undergone apoptosis after 6 hours of treatment with 30 and 20 μg/mL of EGAR, respectively. These findings correspond with the results from proliferation assays and suggest that EGAR cytotoxicity appears to be explained in part by the induction of apoptosis. Further, it was interesting to note that there

was a significant increase in the level of apoptosis compared to necrosis in both mammary cancer cell lines induced by EGAR [Figure 3].

The major secondary metabolites present in ethyl acetate extract of *G. asiatica* roots are lignans; (+) sesamin, (-) piperitol and (+) pinoresinol and flavonoids; sakuranetin and ovalifolin. Among these secondary metabolites, sesamin is known to suppress mammary tumor multiplicity in 7,12-dimethylbenz[a]-anthracene–induced rat mammary carcinogenesis model.[17] Further work is in progress to identify the role of other secondary metabolites and their molecular mechanisms in antiproliferative effect on human breast cancer cells.

In conclusion, to our knowledge this is the first report showing that *G. asiatica* roots exhibit an antiproliferative effect by induction of apoptosis that is associated with phosphatidyl serine translocation and caspase-3 activation in MCF-7 and MDA-MB-231 cancer cell lines. As apoptosis has become a promising therapeutic target in cancer research, these results confirm the potential of *G. asiatica* roots as an agent of chemotherapeutic and cytostatic activity in human breast cancer cells. However, more detailed studies are required to determine the exact mechanism(s) of action of major secondary metabolites present in *G. asiatica* roots, specifically evaluating their effects on epigenetic and signal transduction pathways.

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