

## Gecko Skin Extract Induce Cytotoxicity and Apoptosis in Human Breast Cancer Cell Line

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### Abstract

Gecko has been used against malignant tumors in medicinal practice in china, especially digestive system tumors such as esophageal cancer, gastric cancer, and liver cancer. Reduce inflammation, active against allergic responses, swelling having low toxicity are other activity of Gecko skin extract, has been mentioned in chinese folk medicine. The aim of this study was to examine the alcoholic skin extract of Gecko species induced cytotoxicity and apoptosis activity in breast cancer (MCF7) cell line. *in vitro*, the inhibitory effect of gecko skin alcohol extract (GSAE) on cytotoxicity of human breast cancer cells was measured by MTT assay and trypan blue exclusion assay which resulted in MCF7 cell proliferation was significantly inhibited in both dose- and time- dependent manner ( $P < 0.01$ ). High percentage (71.19%) early cell apoptosis in GSAE-treated group ( $IC_{50}$  dose) exhibited selective cytotoxic action of GSAE confirmed by PE-Annexin V/FITC assay. Flow cytometry study showed that MCF7 cell cycle was arrested at G1 phase. Immunocytochemical study revealed that GSAE down regulated nuclear proliferative antigen Ki-67. Gecko alcohol extract decreased the potential of mitochondrial membrane and increased the expression of Bax, cytochrome c, caspase9, caspase3 and  $Ca^{2+}$  in cytosol. GSAE also downregulated the expression of Bcl2, HSP70 and HSP90. Although the level of VEGF did not change significantly, the expression of MMP9 was remarkably down-regulated in GSAE treated group. Thus GSAE showed significantly inhibitory effect in MCF7 cell line and acts through mitochondrial intrinsic pathway of cell apoptosis with downregulation of nuclear proliferative protein and heat shock protein.

**Keywords:** Gecko skin extracts; Breast cancer; Cytotoxicity; Apoptosis; Extrinsic pathway

### Introduction

Natural Products of animal and plant has afforded a rich source of compounds that have found many applications in the fields of biology, pharmacy and biomedical science [1]. Cancer has emerged as an important public health problem worldwide and an increasing interest from pharmaceutical companies and research institutions for new drug discovery [2,3]. The study of the animal toxin has elicited great interest as a source of materials for the design of new drugs and therapies [4,5]. Many reptiles and amphibians such as *Naja naja*, *Naja kaouthia*, *Bungarus fasciatus*, *Daboia russelli*, and toad skin effectively exhibit antitumor activity [6] and offer a new horizon of research in biomedical sciences [7]. Lizards are also the group of reptiles whose flesh is believed to have curative properties [8]. The Tokay Gecko (*Gekko gekko*) is one such reptile that mainly use in traditional Chinese medicine found to restrain inflammation, allergic response with high activity and low toxicity [9,10]. Gecko has been increasingly concerned by the medical world because of its strong antitumor effect. In 1999, Wu reported 105 cases of esophagus tumor treatment by compound recipe of gecko [11]. Protein isolated from gecko, exerted antitumor effect against cervical cancer cells via PI3-kinase/Akt pathway [12]. Several processing methods are used in clinical practice, such as, living and drying whole Gecko. There are some studies describing the action of lyophilized powder of fresh Gecko in inhibiting H22 hepatocarcinoma and C6 glioma cells [13-15], and antitumor effect of whole dry Gecko powder on human esophagus carcinoma cell lines *in vitro* and xenografted sarcoma 180 in Kunming mice [16]. But there is still no report on anticancer effects of dried skin of Gecko. Therefore, in this work *Gekko gekko* skin was selected for experiment objectives.

Breast cancer is by far the most common cancer diagnosed in women and it ranks second according to incidence of cancer in the world. Breast cancer is increasing both in young (11% per decade) and old women (16% per decade) [17]. There are an estimated 1,25,000 new breast cancer cases in India every year. An increasing trend in incidence

has been reported from various registries of national cancer registries project and now India is a country with largest estimated number of breast cancer deaths worldwide [18].

In the present study, Gecko skin alcohol extract (GSAE) was used to explore the cytotoxic effect and mechanism of apoptotic on breast cancer (MCF7) cell line.

### Method and Materials

#### Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sodium bi carbonate ( $NaHCO_3$ ), RNase A, Propium Iodide, Trypan Blue, De- Methoxy sulphoxide (DMSO) were purchased from Sigma- Aldrich co. (St. Louis, MO , USA). Dulbecco's modified Eagle's medium (DMEM) medium, fetal bovine serum, penicillium-streptomycin was purchased from invitrogen, (USA). Annexin V-FITC, Cell cycle kit, JC-1 Mito potential kit, MMP9, (MIB-1) Ki-67 anti human antibody kits were purchased from BD-Bioscience, USA. Primary antibody (cytochrome c, Bax, Bcl2, HSP70, HSP90, Caspase3, Caspase9 and  $\beta$ -actin), VEGF ELISA kit and HRP-conjugated goat anti-rabbit secondary antibody were procured from Santa Cruz, CA, USA.

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All other chemicals were purchased locally and were of analytical grade.

### Collection of gecko skin and preparation of extract

Two dead *Gekko gekko* samples were collected from the forest area of south Tripura, India during the month of April-June, 2014. The dried skin were removed, dipped in 70% alcohol for 15 days of 37°C. The extract was centrifuged at 1500 rpm in 15 min, dried in a rotary evaporator. The dried extract was designated as GSAE and expressed in tenure of dry weight. Before express, it was dissolved in RPMI 1640 media (mg/ml).

### Cell culture

MCF7 (Human Breast adenocarcinoma), HEK-293 (Non-cancerous human kidney epithelial cell) and NIH-3T3 (Non-cancerous mouse fibroblast cell) were purchased from National facility for Animal Tissue and cell culture, Pune India. Cells were cultured in DMEM supplemented with 10% heat inactivated FBS, NaHCO<sub>3</sub> (1.5 g/L), Penicillin (100 units) and streptomycin (10 µg/ml). Cells were grown to confluence at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> inside an incubator (Heal Force, China).

### Cell proliferation study by direct count using trypan blue

MCF7, NIH-3T3 and HEK-293 cell line (1x 10<sup>6</sup>) were seeded in 96-well sterile plates and were treated with different concentrations (0.1mg/ml - 0.5 mg/ml) of GSAE for 24 h, 48 h and 72 h of incubation. The cell growth inhibition studies were done using 0.2% trypan blue with direct count under light microscope (Olympus, Tokyo) using Haemocytometer chamber [19].

### Cytotoxicity studies on MCF7 cells, HEK 293 and NIH-3T3

Effect of GSAE on cell proliferation was determined by MTT based colorimetric assay where cells (MCF7 cells, HEK293 and NIH-3T3 cells (1x 10<sup>6</sup>/ml)) were treated with different concentrations (0.1 mg/ml - 0.4 mg/ml) of GSAE for 24 h and 48 h [19].

### Detection of apoptosis by flow cytometric analysis

Flow cytometric analysis was done to assess the apoptotic activity induced by the GSAE [20]. In brief, MCF7 cells (1x10<sup>6</sup>) were treated with GSAE (250 µg/ml) for 24 h. Cells (pellets) were dissolved in Annexin V-FITC binding buffer containing annexin V- FITC and Propidium iodide. After 15 min incubation in dark at room temperature flow cytometric analysis was done. All data were acquired with a Becton-Dickinson FACS Verse double laser cytometer.

### Study of cell cycle arrest by flow cytometric analysis

Seeded MCF7 cells were grown to about 50% confluency then treated with GSAE (250 µg/ml) and incubated for 24 h. The cells were washed and fixed respectively with cold PBS and 70% ethanol and stored at -20° C overnight. Cells finally dissolved in PBS treated with RNase A for 30 min at 37°C and stained with propidium iodide (50 µg/ml) and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm laser light source and 623 nm band pass filter [21].

### Mitochondrial permeability potential assay

Detection of mitochondrial permeability transition event provides an early indication of the initiation of cellular apoptosis [22]. To study the disruption of mitochondrial membrane potential and resulting mitochondrial permeability transition, MCF7 cell and HEK-293 cell were treated with GSAE (0.25 mg/ml) for 24 h. Following the end of incubation period, cells were stained with 3 µl of the cationic dye JC-1 (1

mg/ml in DMSO) which exhibited potential dependent accumulation in mitochondria and ten thousand cells were examined on a FL-1 (530 nm) versus FL-2 (585 nm) dot plot on FACS Verse flow cytometer (FACS Verse: Becton Dickinson, San Jose, CA, USA).

### Analysis of intracellular Ca<sup>2+</sup> concentration

Changes in intracellular Ca<sup>2+</sup> concentrations were determined using a calcium kit (OCPC method) [CREST BIOSYSTEMS]. MCF7 cells (1x10<sup>6</sup>) were treated with 0.25 mg/ml GSAE for 24 h and 48 h. GSAE treated and control cell culture media were collected after proper time of incubation. Media (0.5 ml) were mixed with o-cresolphthalein complexon (0.5 ml) and incubated for 10 min in RT. Optical density were measured at 570 nm wavelength by spectrophotometer [23,24].

### Western blot analysis of pro and anti-apoptotic protein expression of MCF7 cells

MCF7 cell lysate of GSAE (0.25 mg/ml) treatment were prepared by cell lysis buffer. Equivalent amount of proteins were electrophoresed in 12% SDS-PAGE and transferred to a PVDF membrane which was then blocked with 5% skimmed milk in PBS-T. Incubation with primary antibodies of Bax, Bcl2, HSP 70, HSP 90, Cytochrome C, Caspase 9, Caspase 3 and β-actin were done followed by subsequent wash with PBS-T (phosphate buffer saline with 0.1% Tween 20). Then membrane was incubated with horse-redox peroxidase conjugated goat antirabbit secondary antibody followed by a detection reagent (Amersham Bioscience) [25].

### Immunocytochemistry and flow cytometry analysis Ki-67

Immunostaining was done as described previously [26,27], with some modification. MCF7 cells were treated with GSAE (0.25 mg/ml) for 12 h. Briefly, the cells attached to the cover slips were washed thrice with PBS and fixed using 4% paraformaldehyde in PBS solution for 30 min. The cells were then permeabilized with cold 0.5% Triton-X-100 in PBS for 10 min at room temperature and washed with cold PBS. PBS supplemented with 10% FBS and 5% nonfat milk was used as a blocking solution (30 min, 25°C). After removal of the blocking solution, the cells were incubated at 4°C overnight in primary human anti-ki-67 monoclonal antibody, MIB-1 (1:100; BD-Bioscience, CA, USA). After washing with PBS, the cells were incubated at 37°C for 30 min with horseradish peroxidase-labelled secondary rabbit anti-goat IgG antibodies. The cells were then stained with diaminobenzidine, and examined under a microscope.

MCF7 cells (1x10<sup>6</sup>) were treated with IC<sub>50</sub> dose and of GSAE for 12 h. Cells were washed with PBS, and fixed with ice-cold 70% ethanol and kept at -20°C for overnight. Cell were washed with wash buffer (PBS with 1% FBS, 0.09% NaN<sub>3</sub> pH-7.2) twice and resuspended the cells in PBS. 20 µl/100 µl of cell of Ki-67 antibody were added [21]. After mixing it gently, kept it 20 min at room temperature in the dark. After centrifugation at 1000 rpm for 5 min, dissolved 500 µl of PBS and added 10 µl (50 µg/ml) of PI staining solution and incubated for 15 min at room temperature in dark place. Then the sample were analysed with FACS verse (BD Bioscience).

### Determination of expression of MMP9

The enzymatic activity of MMP-9 was determined by Flowcytometry analysis. Briefly, MCF7 cells were treated with GSAE with varying concentration and with various time scales. Cells were processed as per manufacture protocol and analysed with FACS [28].

### Measurement of the cytokine VEGF by ELISA

GSAE treated cell lysate was prepared in cell lysis buffer. The expression of VEGF secreted by MCF7 cell was measured by ELISA according to manufacture protocol [29].

### Statistical analysis

All values are represented as arithmetic mean  $\pm$  SEM. Statistical analysis was done by student's t test. A probability value of less than 0.05 was chosen as the criterion of statistical significance.

### Results

#### GSAE treatment resulted in a significant and selective inhibition of MCF7 cell (1) Proliferation and (2) Increase cellular toxicity

GSAE at concentrations of 0.1 mg/ml -0.5 mg/ml significantly inhibited the growth of the MCF7 cells compared with the control cell and normal fibroblast cell (mouse) and epithelial cell (human) in a time and concentration dependent manner which were showed by trypan blue exclusion direct count assay (Figure 1). The cytotoxicity activity of GSAE in the two cell lines examined was both dose dependent and time dependent. The  $IC_{50}$  values of the GSAE in human breast adenocarcinoma (MCF7) were  $0.25 \pm 0.022$  mg/ml and  $0.2 \pm 0.014$  mg/ml respectively after 24 h and 48 h of incubation. But such potent cytotoxicity were not observed in human non-tumorigenic epithelial cell (HEK-293) and mouse non-cancerous fibroblast cells (NIH-3T3) even after incubation for 48 h. With this same concentration (0.25 mg/ml and 0.2 mg/ml after 24 h and 48 h) only 10- 12% (in case of HEK-293) and 8-11% (NIH-3T3) cell death of epithelial/fibroblast cells which was 5-6 times less than that of the cancer cell lines (Figure 2).

#### Induction of apoptosis by GSAE in breast cancer cell

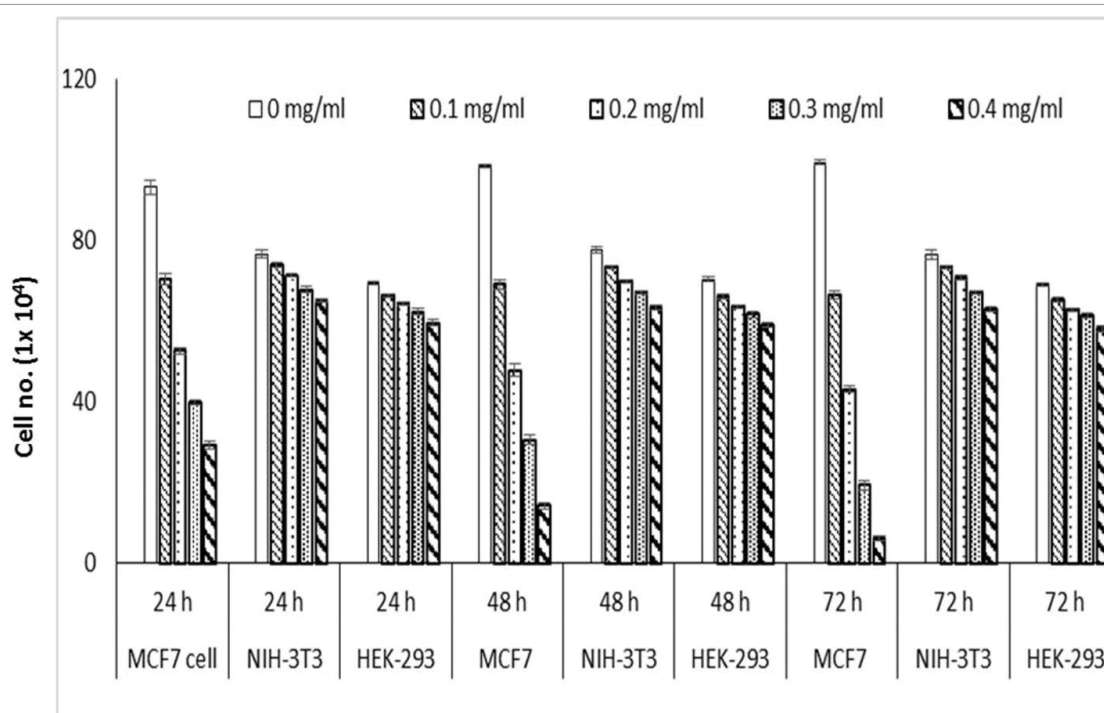
In Figure 3, left- lower quadrant represented living cells, the right- lower quadrant represented early apoptotic cells and right upper quadrant represented late apoptotic or necrotic cells. In In order to confirm whether the cause of cell death induced by GSAE was apoptosis, Annexin V/PI analysis was performed. Results showed that GSAE treatment exhibited 71.19% in early apoptotic, 7.89% in late apoptotic cell (necrotic cells) in MCF7 cell at 0.25 mg/ml concentration and 24 h incubation. 25-30% increase of late apoptosis when treated with GSAE with 0.25 mg/ml at 48 h.

#### GSAE arrested the MCF7 cell in G0/G1 phase of cell cycle

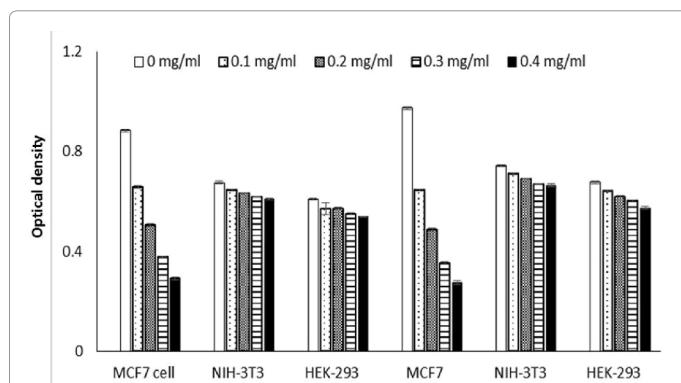
Cell cycle deregulation has been recognized as the hallmark of cancer progression in most malignant tumors, so its inhibition is regarded as an effective strategy for eliminating cancer cells. The cell cycle analysis of untreated and treated cells revealed marked differences in the proportion of cells in respective phases of the cell cycle. Where GSAE treated MCF7 cell showed 60.71% G1/G0, 28.72% S and 16.24% G2+M, the control cell showed 47.71% G1/G0, 34.54% S and 16.24% G2+M. Tamoxifen treated MCF7 cell exhibited 59.72% G1/G0, 34.72% S and 3.24% G2+M cell in respective phase. GSAE (0.25 mg/ml) increased in 31.16% the number of cells in G0/G1 phase followed by reduction of cells in S phase of 16.85% and in G2/M phase 42.61% ( $P < 0.05$ ). These results suggested that GSAE inhibited cell cycle proliferation associated with cell cycle arrest in G0/G1 phase. Tamoxifen (100  $\mu$ m) treated cells used as a positive control, and also arrested cells in G0/G1 with decrease of cell count in S phase (Figure 4).

#### Loss of mitochondrial membrane potential of MCF7 cells after treatment with GSAE

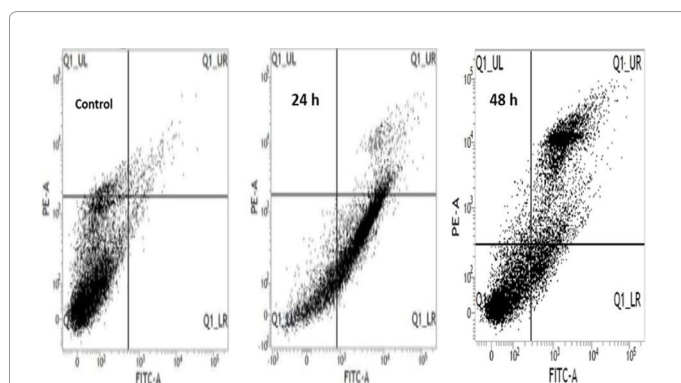
In order to determine a time dependent loss of mitochondrial membrane potential preceding apoptosis, GSAE treated MCF7 cells were incubated with the membrane potential sensitive dye, JC-1 which



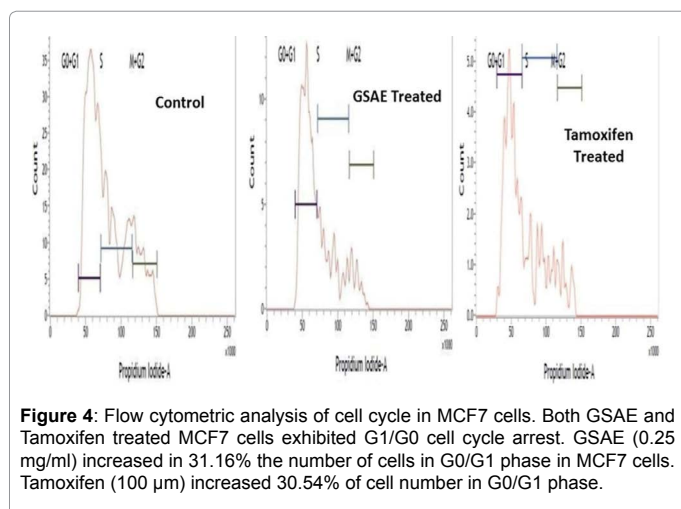
**Figure 1:** Direct count of cell proliferation by Trypan blue exclusion assay. MCF7 cell exhibited 24.5%-68.6% cell inhibition (24 h), 29.8%-85.5% cell inhibition (48 h), 33%-93.8% cell inhibition (72 h); NIH-3T3 cell showed only 4%-17.6% cell inhibition (72 h) and HEK-293 cell showed 5.5%-16% (72 h) cell inhibition at 0.1 mg/ml-0.4 mg/ml concentration of GSAE.



**Figure 2:** Cytotoxicity effect of GSAE on MCF7 cell, NIH-3T3 cell and HEK-293 cell showed by MTT assay. IC<sub>50</sub> dose of GSAE after 24 h and 48 h on MCF7 cell was 0.25 mg/ml. and 0.2 mg/ml respectively. The bar displays the mean  $\pm$  sem of quadruplicate experiments ( $P < 0.05$  compared to untreated controls).



**Figure 3:** Determination of apoptosis by flow cytometry. MCF7 cells were treated with 0.25 mg/ml GAE for 24 h and 48 h. Annexin-V and Propidium iodide stained cells were acquired under the FACS Verse flow cytometer. Cells were categorized into four groups based on dye uptake. The quadrant (Q) showing Q1UL: PI positive, Q1UR: Annexin V/PI positive, Q1LL: Annexin V/PI negative and Q1LR: Annexin V positive. Early apoptotic cell and late apoptotic cells were increased time dependently.



**Figure 4:** Flow cytometric analysis of cell cycle in MCF7 cells. Both GSAE and Tamoxifen treated MCF7 cells exhibited G1/G0 cell cycle arrest. GSAE (0.25 mg/ml) increased in 31.16% the number of cells in G0/G1 phase in MCF7 cells. Tamoxifen (100  $\mu$ m) increased 30.54% of cell number in G0/G1 phase.

easily gets sequestered by energized mitochondria without inducing any cytotoxic effects. This result showed that GSAE treatment led to 20% loss of the membrane potential initially (24 h). On longer

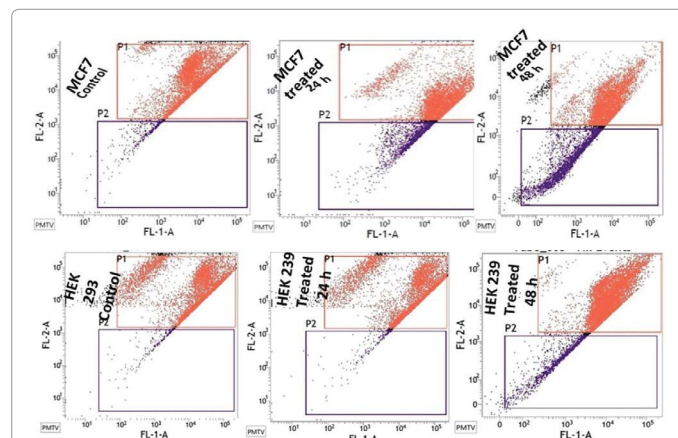
exposure (48 h) MCF7 cells became slightly more sensitive (35% loss) to GSAE treatment (Figure 5). In the same time of interval HEK-293 cell experienced with only 1.2% and 10.4% loss of mitochondrial membrane potential after treatment with GSAE.

### MCF7 cell membrane potential loss by GSAE modulated by increase level of intracellular Ca<sup>2+</sup> concentration

Calcium with o-cresolphthalein complexone formed purple colored complex. Intensity of the color formed is directly proportional to the amount of calcium present in the sample. GSAE exhibited that  $4.8 \pm 0.22$  mg/dl and  $11.4 \pm 0.23$  mg/dl Ca<sup>2+</sup> at 24 h and 48 h respectively whereas control cell media cell media contain only  $2.1 \pm 0.15$  mg/dl Ca<sup>2+</sup>. GSAE induced MCF7 cells showed 2.28 fold and 5.42 fold enhanced intracellular Ca<sup>2+</sup> level at 24 h and 48 h respectively (Figure 6).

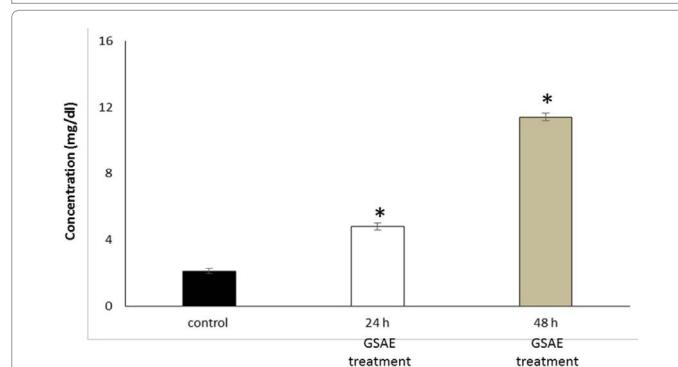
### GSAE treatment resulted in (i) Cytochrome C release from breast cancer cell, (ii) apoptotic induction by modulation of Bcl-2 family proteins and (iii) apoptotic cell death signaling through caspase 3

Loss of Mitochondrial membrane potential leads to Cytochrome c release into cytosol from mitochondria. Estimation of cytochrome

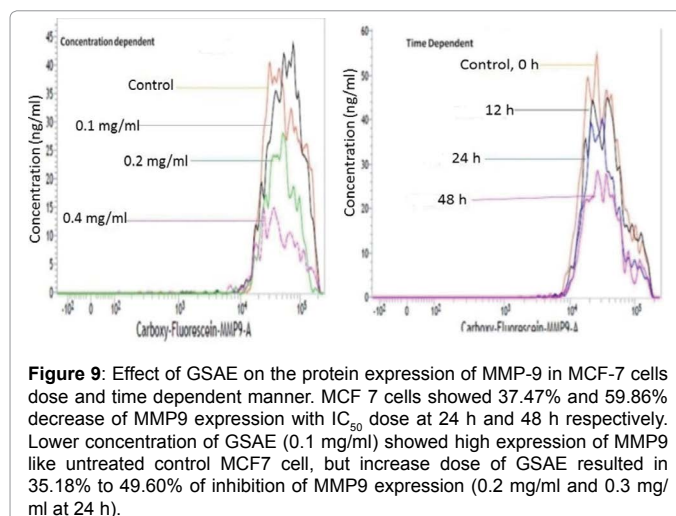
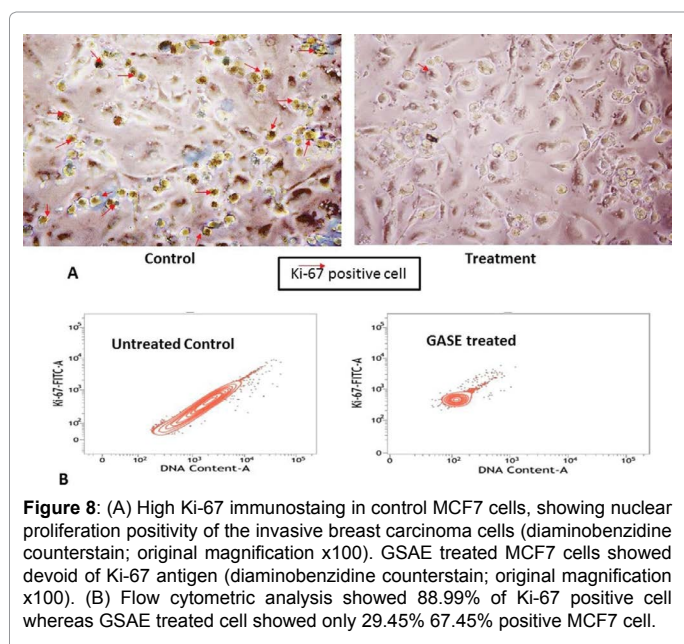
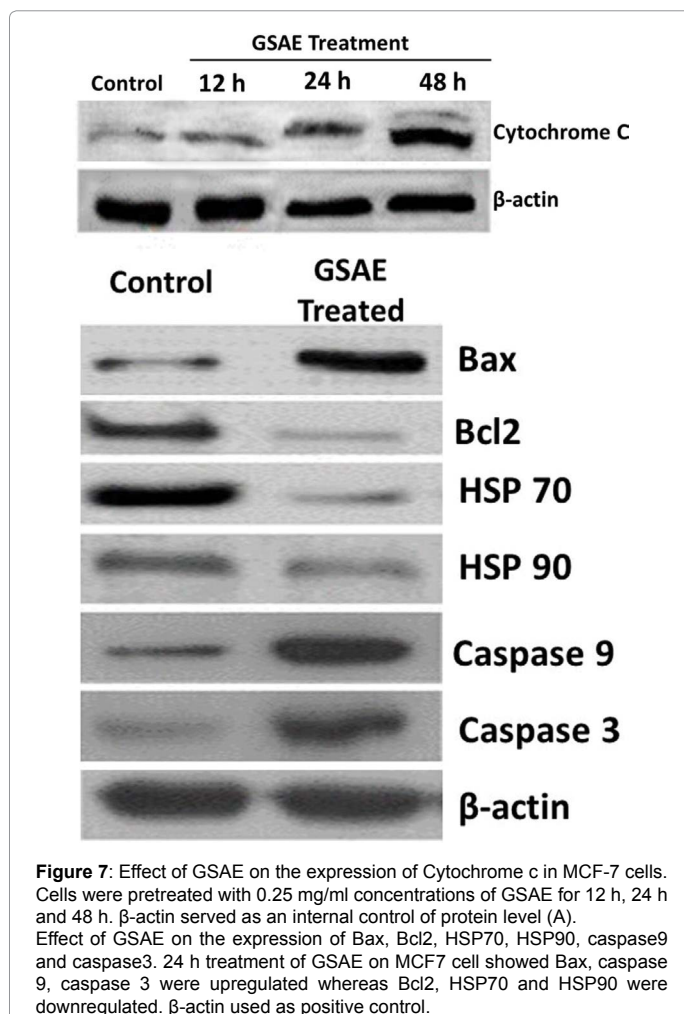


**Figure 5:** Flow cytometric analysis of mitochondrial membrane potential

( $\otimes$ mito $\Psi$ ) on MCF7 cells. GSAE treated MCF7 cells exhibited with decreased mitochondrial potential with time dependent manner. GSAE treatment led to 20% loss of the membrane potential initially (24 h). 48 h incubation showed MCF7 membrane potential lost by 35%. HEK-293 cell showed only 1.2% and 10.4% loss of mitochondrial membrane potential after treatment with GSAE



**Figure 6:** Ca<sup>2+</sup> concentration measurement in cytosol of MCF7 cell after treatment with GSAE using spectroscopy. GSAE exhibited that  $4.8 \pm 0.22$  mg/dl and  $11.4 \pm 0.23$  mg/dl Ca<sup>2+</sup> at 24 h and 48 h respectively whereas control cell media contain only  $2.1 \pm 0.15$  mg/dl Ca<sup>2+</sup>.



c release revealed that GSAE treatment (0.25 mg/ml) increased the cytosolic cytochrome c expression by 3-4 folds in time dependent manner (Figure 7A).

Western blot analysis showed that, GSAE treatment up regulated the pro-apoptotic protein Bax and down regulated the expression of anti-apoptotic protein Bcl2. GSAE also up regulated the caspase 9, and caspase 3 expression in MCF 7 cell. The present data also showed expression of HSP70 and HSP90 decreased significantly by GSAE. HSP are often expressed in range of cancers including breast cancer. Downregulation of HSP70 and HSP90 in MCF7 cell were significant clinical sign of GSAE.

### Cell proliferation induction by GSAE was mediated by loss expression of Ki-67 on MCF7 cells

Strong nuclear staining was considered a positive reaction. Comparison of the number of Ki-67 antigen in untreated control group and GSAE treated group, immuno-cytochemical study showed GSAE decreased the expression of Ki-67 MCF7 cell. Flow cytometric analysis showed 88.99% of Ki-67 positive cell whereas GSAE treated cell showed only 29.45% 67.45% positive MCF7 cell (Figure 8).

### GSAE induces the anti-angiogenesis with downregulation of MMP9 expression in breast cancer cells

Cell metastasis, angiogenesis was stopped by decreased expression of matrix metalloproteinase 9 (MMP9). Flow cytometry analysis exhibited that GSAE down-regulated the expression of MMP9 by time and dose dependent manner. MCF 7 cells showed 37.47% and 59.86% decrease of MMP9 expression with  $IC_{50}$  dose at 24 h and 48 h respectively. Though lower concentration of GSAE (0.1 mg/ml) showed high expression of MMP9 like untreated control MCF7 cell, but increase dose of GSAE resulted in 35.18% to 49.60% of inhibition of MMP9 expression (0.2 mg/ml and 0.3 mg/ml at 24 h) (Figure 9).

### VEGF (other antiangiogenic protein) expression not changed significantly after treatment with GSAE in MCF7 cells

After 48 h of treatment with GSAE, MCF7 cell showed no significant change of expression of vascular-endothelial growth factor with respect to control cell line. ELISA estimation revealed that untreated MCF7 cell expressed 58.09 pg/ml VEGF at 48 h ( $1 \times 10^5$  cell/ml), whereas GSAE (0.25 mg/ml) cell showed very slight decrease of VEGF protein (54.05%).

## Discussion

Tumor formation is triggered by disruption of the balance between cell proliferation and cell death, which is maintained through regulation of various molecular signalling pathways [30]. Through the discovery of non-toxic and apoptosis inducing anticancer drugs, chemotherapy is regarded as tumor regressive way followed by extended life span. In recent years, scientists have emphasized on the anticancer activity of natural products from plant, animal, marine organisms and microorganisms. With comparison to synthetic drug, natural antitumor therapeutic have the advantages of high activity, low side effects and they do not usually induce drug resistance easily [31]. This study elicited that GSAE showed time and dose dependent cytotoxic effect on MCF7 cells with  $IC_{50}$  dose 0.2 mg/ml. Similar study on BMP1, a high molecular weight protein (MW-79 KDa), isolated from the Indian toad (*B. melanostictus*) skin aqueous extract, exhibited dose dependently inhibited U937 and K562 cell growth having  $IC_{50}$  values of 49  $\mu$ g/ml and 30  $\mu$ g/ml respectively [32]. Though, this  $IC_{50}$  value is much higher (may be due to the crude nature of extract) than other purified product from animal (Snake Venom, Bee Venom) protein but it showed selective toxicity against target cell lines with reduced toxic effect on normal human fibroblast. Cancer cell death is tightly regulated processes which involve two major pathway- apoptosis and antiangiogenesis. Apoptosis induction depends on a balance between two major genes proapoptotic (e.g. Bax) and antiapoptotic (e.g. Bcl-2) [33]. Upregulation of Bax can promote its translocation to outer membrane of mitochondria, triggering the release of cytochrome c,  $Ca^{2+}$  and loss of mitochondrial membrane potential ( $\Delta\psi_m$ ). In this study, GSAE showed loss of mitochondrial membrane potential and increasing concentration of cytochrome c and  $Ca^{2+}$  in the cytosol. GSAE treatment also increased the expression of Bax and downregulated the anti-apoptotic protein Bcl2. Bax show some structural similarities with pore forming proteins. Hence, it is believed that Bax can form transmembrane pores across the outer mitochondrial membrane, which leads to a loss of membrane potential. The localization of Bax has been shown to change from the cytosol to the mitochondria during apoptosis. Bcl2 located in the outer mitochondrial membrane and it is especially enriched at contact sites where the inner and outer membranes come in close proximity [34]. It acts on mitochondria to counteract the action of pore-forming Bax protein and downregulation of that (by GSAE) failed to do so.

Cytochrome c and apoptosis protease activating factor-1 (Apaf-1) acted as cohorts in the activation of caspase-9. Pro-caspase-9, ATP, Cyt C, and Apaf-1 together form apoptosome complex. Thus cytochrome c release from the mitochondria is considered as a key signal that initiates the irreversible events in cell death. Interestingly, caspase-9 activated via the mitochondrial pathway and participates independently in the activation of pro-caspase-3, which is the active form of central executioner of apoptosis [35]. In this study, both upregulation of caspase9 and caspase3 by GSAE seemed that apoptosis in MCF7 cell was triggered through caspase3 pathway. Hyperpolarization of cytosolic  $Ca^{2+}$  is known to contribute to the opening of the mitochondrial permeability transition pore, which depolarizes the mitochondria [36]. Depolarization of mitochondria can occur increase of cytosolic pH (ROS generation). HSP70 is a powerful anti-apoptotic protein that acts at different key points, affecting both the extrinsic and intrinsic pathway of apoptosis. HSP70 was reported to inhibit the important apoptotic mediator, Bax translocation, thus preventing mitochondrial membrane permeabilization. It also interacts with apoptosis protease activating factor-1 (Apaf-1), thereby inhibiting recruitment of pro-caspase9 to the apoptosome complex and the consequent caspase3 activation. HSP90 is also very important molecular chaperon that regulates the

stability and activity of numerous client proteins covering almost all cellular processes [37]. HSP90 constantly updated HER2 and C-SRC which are essential for breast cancer progression. HSP90 also has a role in angiogenesis owing to its stabilizing properties on the transcription factor HIF-1 $\alpha$  [38]. In this study, GSAE acted on both HSP70 and HSP90 with decrease expression and signalled the breast cancer cell (MCF7) to follow apoptosis through caspase 3 intrinsic pathway. Cell cycle regulated genes are repressed to increase the duration of G1 phase under this oxidative condition [39]. In order to assess the amount of macromolecular alteration, a cell cycle arrest is required and if necessary, cell enters the apoptotic pathway. The Ki-67(MIB-1) has proved to be a reliable marker of proliferating cells in large variety of human neoplasms, including cancers of the lung, breast, gastrointestinal tract, kidney, prostate and soft tissue sarcomas [40]. The antigen detected by Ki-67 begins to be expressed in the mid G1 phase and is present through the M phase. Non-cycling cells or apoptotic cells or early G1 phase arrested cells do not demonstrate immunostaining. In agreement with this work, it was documented that the antiproliferation effect of *Synadenium umbellatum* Pax in EAC cells was exhibited by arresting cycle in the early G1 phase inducing apoptosis [24]. Tumor-induced angiogenesis begins with the dissolution of basement membrane surrounding a pre-existing blood vessel, a process aided by MMPs produced by tumor cells and supporting cells. The dissolution of extracellular matrix also facilitates the release of sequestered angiogenic factors. Degradation of basement membrane and extracellular matrix, a process known to be associated with MMPs which is very important for the initial stage of cancer and metastasis at distant sites [41]. Upregulation of MMP2, MMP7 and MMP9 has been reported during tumor invasion and metastasis. In the present study, GSAE showed reduced protein expression level of MMP9 in MCF7 cells. This result is in conformity with earlier evidences which revealed that cardiotoxin III from *Naja naja atra* also inhibited EGF induced MMP9 expression, suggesting the potential role of cardiotoxin-III in blocking cell metastasis [42]. The present study also elicited that GSAE only regulated the MMP9 gene not the other angiogenic factor like vascular endothelial growth factor (VEGF). The obligatory neovascularization, a rather uncommon process under normal conditions for tumor growth and metastasis, makes angiogenesis a prominent target for therapeutic intervention. Most of the antitumor agents used in cancer therapy are cytotoxic in nature, designed mainly to prevent cancer growth. The most significant effect of GSAE was its both cytotoxic and antiangiogenic effect and it might be used for prevention and treatment of breast cancer in future. The major concern of the GSAE was its high  $IC_{50}$  dose, though in this concentration no such toxicity in normal epithelial and fibroblast cell but it may cause severe organ toxicity.

To conclude, it may be report that GSAE triggered apoptosis in MCF7 cells at different levels involving increase in  $Ca^{2+}$ , alteration of mitochondrial membrane permeability, downregulation of Ki-67 antigen, reduced MMP9 expression in cell death. Its selective effect on MCF7 cell and reduced toxicity suggest a potential role in breast cancer chemotherapy. Further details study are warranted as purification of bioactive compound of GSAE.

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