Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(2): 2024-2030
Received: 17-01-2019
Accepted: 20-02-2019

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Synergistic effect of galangin and TNF inhibits the cell proliferation and induces cell apoptosis through activating caspases pathways in breast cancer

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Abstract
Breast cancer is reported as the most frequent tumor with limited treatments among the female worldwide. Galangin, bioflavonoids, has been shown anti-cancer properties in various cancer cells including breast cancer. Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that has been linked to breast cancer development. It strives multiple functions such as cell proliferation, differentiation and apoptosis. In this study, we investigated whether galangin could enhance TNF-mediated apoptosis in breast cancer cells. We further focused to investigate the molecular mechanism of inhibition of proliferation and apoptosis induced by the synergistic effect of galangin and TNF against human breast cancer cells. Cytotoxicity induced by galangin and TNF alone and in combination was measured by MTT assay. Apoptosis was detected by DNA fragmentation assay and measured by real time PCR and western blotting. The study clearly showed the dose dependent cytotoxic effect of galangin in combination with TNF in all the breast cancer cells. The dying cells showed characteristics of apoptosis such as, DNA fragmentation in combined treatment with galangin and TNF in human breast cancer cells, compared to single treatments. Furthermore, we also found that treatment with galangin enhances TNF-induced apoptosis by increasing expression of apoptosis-related proteins including caspase-3, 8, 9 and Bax whereas by decreasing the expression of Bcl-2. The data in the present study clearly demonstrates that galangin sensitizes human breast cancer cells to TNF mediated apoptosis and could have a potential therapeutic significance in treating cancer.

Keywords: Galangin, TNF, caspases, apoptosis, BCL-2, MTT

Introduction
Breast cancer is the most frequent cancer among women in world and approximately 30% of all new cancer cases in women with a greater incidence in female over the age of 60 years [1–3]. However, a various procedure of treatments such as surgical resection, immuno chemotherapy, hormonal therapy and radiotherapy have been shown to reduce the risk of tumor reoccurrence [1–3]. Therefore, chemotherapy and radiotherapy have toxic effects on both normal and cancer cells patient [1, 3]. In spite of various advances in the treatment of breast cancer, the prognosis for patients with metastatic disease remains poor. However, there is an important finding to minimize the dose of chemotherapy and radiotherapy, without affecting their therapeutic efficiency specific to cancer treatment.

Flavonoids are well known antioxidants, which can protect cells from being harmed by free radicals, and are believed to exert inhibitory exerts on cancer cells [4, 5]. Galangin (GG) (3,5,7-trihydroxyavone) is a natural flavonol, extracted widely from the Alpinia galangal root and used in Asia as a herbal medicine, which is known to possess biological activities, including anti-oxidant and anti-inflammatory [6, 7], anti-obesity, and anti-tumor exerts, anti-microbial and anti-viral activities in a variety of *in vitro* and *in vivo* systems [8-10]. Galangin shows anti-tumorigenic effects in various cancer cells, including colon cancer 8, human mammary tumor cell [11], hepatocellular carcinoma cells [12], melanoma [13], ovarian cancer cells [14], promyelocytic leukemia [15] and prostate cancer cells [16]. Previous reports elucidated that G0/G1 arrest in cell cycle by down-regulation of cyclins D3, E, and A [11] and apoptosis via the mitochondrial pathway [12] are mechanisms underlying anti-tumor effect of galangin. Galangin induces autophagy and apoptosis in various concentrations through upregulation of p53 in HepG2 cells [17]. In particular, glangin influences several processes and plays a crucial roles in regulating various molecular targets, including NF-κB, Smads, peroxisome, proliferator-activated receptor γ (PPARγ), transcription factors, tumor necrosis factor-alpha (TNF-α),

interleukins, intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), JNK, p38, and ERK [18-20]. However, the molecular mechanism by which galangin suppresses tumor progression, especially in breast cancer is not fully understood.

Tumor necrosis factor alpha (TNF-α) is a classical member of a ligand family of cytokines that include TNF, lymphotoxin-α (LTα), Fas ligand (FasL), CD40 ligand (CD40L), and TNF-related apoptosis-inducing ligand (TRAIL) [21]. TNF-α is a pleiotropic pro-inflammatory cytokine inducing a broad range of cellular responses, ranging from inflammatory cytokine production, cell survival, cell proliferation, cell differentiation and cell death. TNF-α can trigger different forms of programmed cell death that are morphologically distinguished as apoptosis and necroptosis [22]. Lots of studies have confirmed that TNF-α can recruit a variety of signaling molecules to induce apoptosis and necroptosis to exert its cytotoxicity through binding to TNF-α receptors, including TNF receptor-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein kinase 1 and 3 (RIP1 and RIP 3) [23]. Therefore, TNF-α has been used as an antitumor agent because of its broad spectrums of cytotoxic effects against a variety of cancer cells [24-26]. However, the clinical application of TNF-α has been limited, largely due to its induction of pro-inflammatory and anti-apoptotic gene transcription mainly via activating the NF-κB signal pathway, which leads to systemic toxicity. Recently, studies have shown that combination with low-dose TNF-α could enhance therapeutic effects of chemotherapeutic drugs [27]. For instance, Galangin combined with TNF to suppress the proliferation of lung cancer cells, sensitizing cancer cells to TNF treatment [28]. However, the molecular mechanisms of galangin-induced TNF sensitization are enough to understand. In this study, we investigated whether galangin sensitize TNF-mediated apoptosis in breast cancer (MCF-7, T47D & ZR-75) cell lines. We demonstrate that TNF could enhance the cytotoxicity against breast cancer cells when given in combination to galangin. We also found that galangin sensitized TNF mediated apoptosis through down-regulation of anti-apoptotic factors, including Bcl-2 and up-regulation of pro-apoptotic protein Bax. The mechanisms involved in the synergistic anticancer effect were also investigated.

Material and methods

Materials

TNF and Galangin were purchased from Sigma (St Louis, Missouri, USA). Tissue culture medium, antibiotic, trypsin were purchased from Himedia (India) and FBS from Gibco, (South America). All other chemicals were of analytical grade. The water used in all experiments was Millipore.

Procurement of Cell lines and maintenance

Breast cancer cell lines (MCF-7, T47D & ZR75) was procured from NCCS Pune, India. MCF-7 T47D & ZR75 were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM), whereas supplemented with 10% Fetal Bovine Serum and antibiotics in a humidified atmosphere of 5% CO2 at 37 °C in culture dishes/flasks. Stock culture was maintained in the exponential growth phase by passaging as monolayer culture using in 0.02% EDTA. The dislodged cells were suspended in complete medium and reseeded routinely.

Cell metabolic assay by MTT

3-(4, 5-dimethyl-2-yl)-2,5-diphynyl tetrazolium bromide (MTT) is metabolic substrate which is reduced by the mitochondrial succinate dehydrogenase enzyme and forms formazan crystal. Cellular metabolic assay was carried out in galangin and TNF alone and combined treated breast cancer cell line (MCF-7, T47D & ZR-75) by MTT assay [29, 30, 31]. Cells were seeded over night at the number of 1×10^4 per wells and then incubated with various concentrations of curcumin and curcumin formulation for 24, 48 and 72 hrs respectively. At the end of the treatment, medium was removed and cells were incubated with 20µl of MTT (5 mg/ml in PBS) in fresh medium (50µl) for 4 hrs in CO_2 incubator. After four hours formazan crystal, formed by mitochondrial reduction of MTT were solublized in DMSO (150µl/well) and the absorbance was read at 570 nm after 10 min incubation on the *i*Mark Microplate Reader (Bio-Rad, USA). Percent cytotoxicity was expressed as IC_{50}. Percentage of cell viability was calculated and result was expressed as % mean of viability ± standard error of mean (SEM).

DNA Fragmentation Analysis

The breast cancer cell lines MCF-7, T47D & ZR-75 cells (5.0×10^4 cells per well) were treated with concentrations (200µM) of galangin and TNF alone and combined and DMSO control for 48 hr. After treatment, the cells were scraped and subjected to DNA fragmentation assay [32]. Briefly, cells were pelleted by centrifugation for genomic DNA isolation. The DNA was electrophoretically separated on 2% agarose gel and visualized by EB (0.5 µg/ml) staining under UV trans-illuminator.

Western blot analysis

The level of expression of various proteins Bcl2 and Bax were determined by western blotting for treated with galangin and TNF alone and combined at various time intervals, as described earlier [33]. In brief, the cells were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein quantification of the lysates was quantitated by Bradford's method. Equal amounts of protein extracts has been electrophoresed on 10–15 % SDS–Polyacrylamide gel depending upon the molecular weight of the protein, transferred to nitrocellulose membrane, and nonspecific binding blocked with 5 % BSA and 5 % FCS in Tris-buffered saline (TBS) for 2.5 h at 37°C. The blot was washed with 0.05 % Tween-20 in TBS and then TBS for 10 min each. The blot incubated with primary antibodies at 4°C overnight against the protein of interest and then incubated with secondary antibody conjugated to alkaline phosphatase (AP) for 2 h at room temperature, rinsed with 0.05 % Tween-20 in TBS, then with TBS. This was followed by addition of AP buffer and the bands visualized by adding BCIP and NBT. The bands were analyzed.

Real time-polymerase chain reaction analysis

Total cellular RNA was harvested from the tissue samples taken from a subset of individual's cell line in the study using Triazol reagent according to the manufacturer's instructions and quantitated by spectrophotometric absorbance at 260 nm. Synthesis of complementary cDNA was carried out using first strand H minus cDNA synthesis kit as per manufacturer's protocol [30]. Real Time PCR will be done using gene specific primers. The comparative CT method was used to evaluate the differential gene expression in breast cancer cell lines.

Statistical analysis

The differences of index are evaluated by means ± SEM using the ff data. Treated tissue and the corresponding controls were

compared using Graph Pad PRISM (version 6.0; Graph Pad Software, USA) by a one-way ANOVA with Dunn's least significance difference tests. Differences between groups were considered significant at 0.05.

Results

Galangin and TNF potentially suppresses the proliferation in breast cancer cells

Before demonstrating the synergistic role of galangin and TNF, the cytotoxicity of galangin, and TNF was monitored in human breast cancer cell (MCF-7, T47D & ZR-75). MTT assay revealed that at the concentration of 100 uM and 200 uM of galangin alone showed significant anti-cancer role in MCF-7 in compared to T47D and ZR-75 (Fig.1A). But TNF alone showed significant cytotoxicity at concentration of 200uM with ZR-75 (Fig.1B). The combination effect of galangin and TNF were also explored in similar breast cancer cell line MCF-7, T47D & ZR-75. As shown in Fig. 1C, galangin combined with TNF significantly decrease the cell proliferation at the concentration of 100 uM in MCF-7 and ZR-75 than T47D. In human normal breast cell, cytotoxicity was observed over a dose of 200 uM after galangin, TNF alone and combined treatment. Therefore, it is possibility for toxicity at higher concentrations. The results above revealed that galangin, TNF alone and with combined at a higher toxic concentration, possessed a promoted effect on suppressed cancer cell proliferation without cytotoxicity to normal breast cells. In our study, we applied the concentrations of galangin and TNF alone did not show significantly inhibitory proliferation in in T47D cells. Thus, the galangin (100 uM) and TNF (100 uM) were used for the combinational therapeutic potential in breast cancer.

Galangin and TNF causes DNA fragmentation within breast cancer cells

DNA fragmentation analysis was performed by galangin, TNF alone and with combined mediated cell death. The cells treated with combined galangin and TNF showed significantly DNA apoptosis in MCF-7 and ZR-75 in compared to T47D (Fig. 2). Galangin and TNF alone also showed slightly apoptosis in MCF-7and ZR-75 than T47D, whereas in the untreated cells did not display apoptosis. Cells treated with combined with galangin and TNF showed maximum chromatin condensation.

Galangin and TNF treatments enhanced apoptosis through suppression of bcl-2 in breast cancer cells

The balance of pro- and anti-apoptotic molecules, such as Bax/Bid and Bcl-2 are a main causing of apoptotic response through caspases-dependent pathway [32, 28]. The release of Cyto-c from mitochondrial to cytoplasm is a main reason for mitochondrial-regulated apoptosis. Pro- and anti-apoptotic molecules expressions are necessary for the signal of apoptosis. Western blot analysis revealed that in breast cancer cell lines, galangin and TNF alone treatment showed lower influence the expression of Bax protein levels in MCF-7 and ZR-75, but more influence in the expression level of Bax protein in T47D. Similarly, in galangin and TNF combination, Bcl-2 was moderately up-regulated of Bax in MCF-7 and ZR-75 but increased up-regulation in T47D, suggesting that pro-apoptotic response was enhanced in T47D cells (Fig. 3A). Galangin and TNF alone showed higher expression of anti-apoptotic protein Bcl- in MCF-7 than T47D and ZR-75 respectively. Similarly, the synergistic effect of galangin and TNF revealed significantly lower expression of Bcl-2 in breast cancer cells. The cleavage of Caspases exhibited that Caspase-3, Caspase-8 and Caspase-9 activities revealed slightly change in expression treated with galangin and TNF alone in MCF-7 and ZR-75, but more expression in T47D, while combined with galangin and TNF, they showed highly up-regulated. However, cell apoptosis was also induced by galangin and TNF combinational treatment in breast cancer cell (Fig. 3B). The results indicated that caspase dependent signaling pathway was activated by galangin and TNF combinational treatment in breast cancer cells to induce cell apoptosis.

Galangin and TNF treatments enhanced expression of pro-apoptosis through suppression of anti-apoptotic bcl-2 mRNA in breast cancer cells

RT-qPCR analysis was carried out to further confirm Bax and Bcl-2 gene alterations. As shown in Fig. 3, combined effect of galangin and TNF markedly enhanced pro-apoptotic gene Bax, while suppressed anti-apoptotic gene Bcl-2 mRNA levels. As shown in Fig. 3D, although the synergistic effect of galangin and TNF augmented the expression of cleaved Caspase-3, Caspase-8 and Caspase-9 elevated the activity in breast cancer cells. Normal human breast cell did not show any expression after the treatment of galangin and TNF alone or even a combined effect.

Taken together, the results here demonstrated that synergistic effect of galangin and TNF enhanced breast cancer cells to induce apoptosis through suppressing Bcl-2 anti-apoptotic protein.

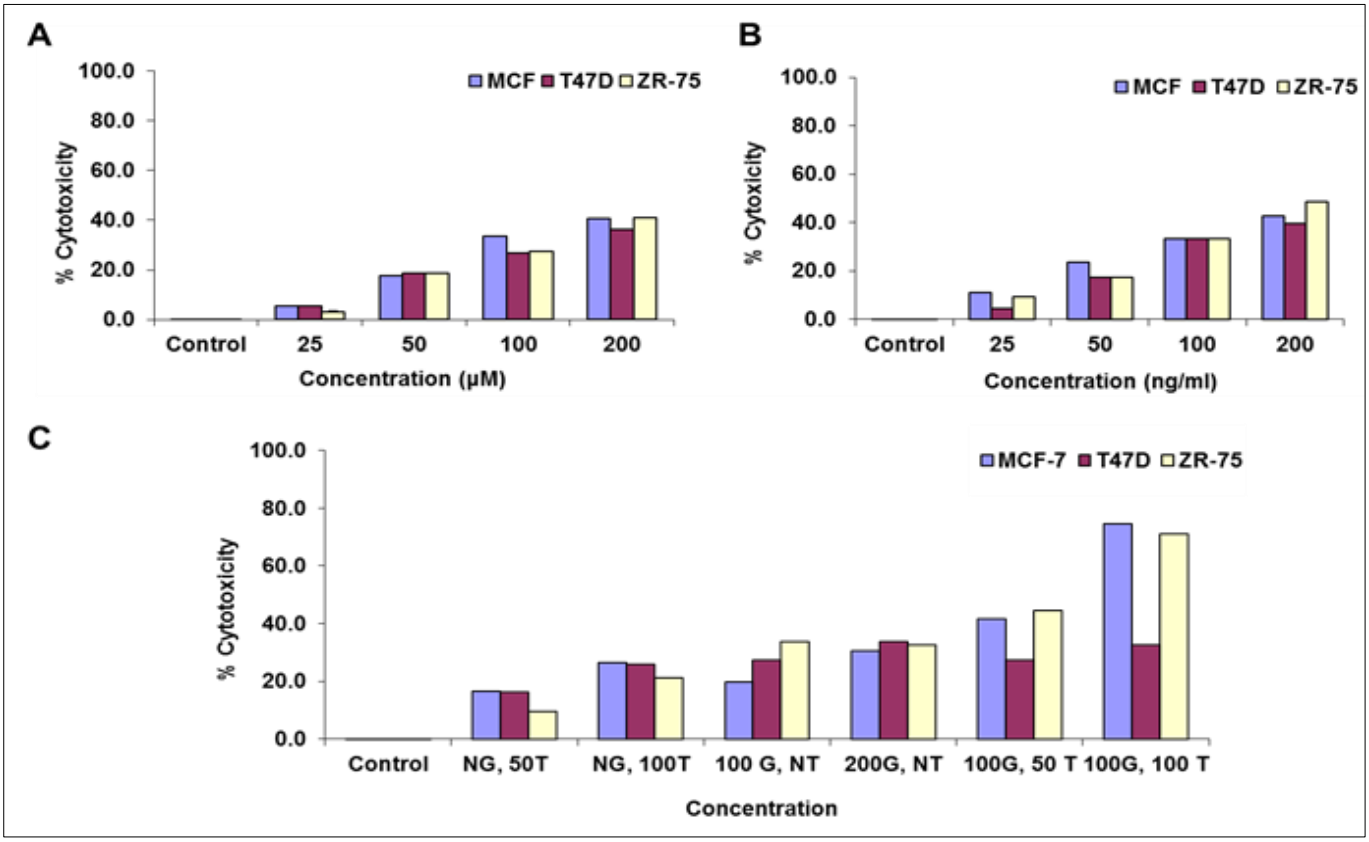

Fig 1: Galangin sensitizes TNF induced cytotoxicity in breast cancer cells: Cells were treated with galangin, TNF and in combination at an indicated concentration for 48 hr and MTT was performed. A: Cells were treated with galangin alone at indicated concentration and cytotoxicity was measured. B: Cells were treated with TNF alone at indicated concentration and cytotoxicity was measured. C: Cells were treated with galangin and TNF at indicated concentration and cytotoxicity was measured.(NOTE: NG: No Galangin, 50T: 50ng/ml TNF, 100T: 100 ng/ml TNF, 100G: 100µM Galangin, NT: No TNF, 200G: 200µM Galangin)

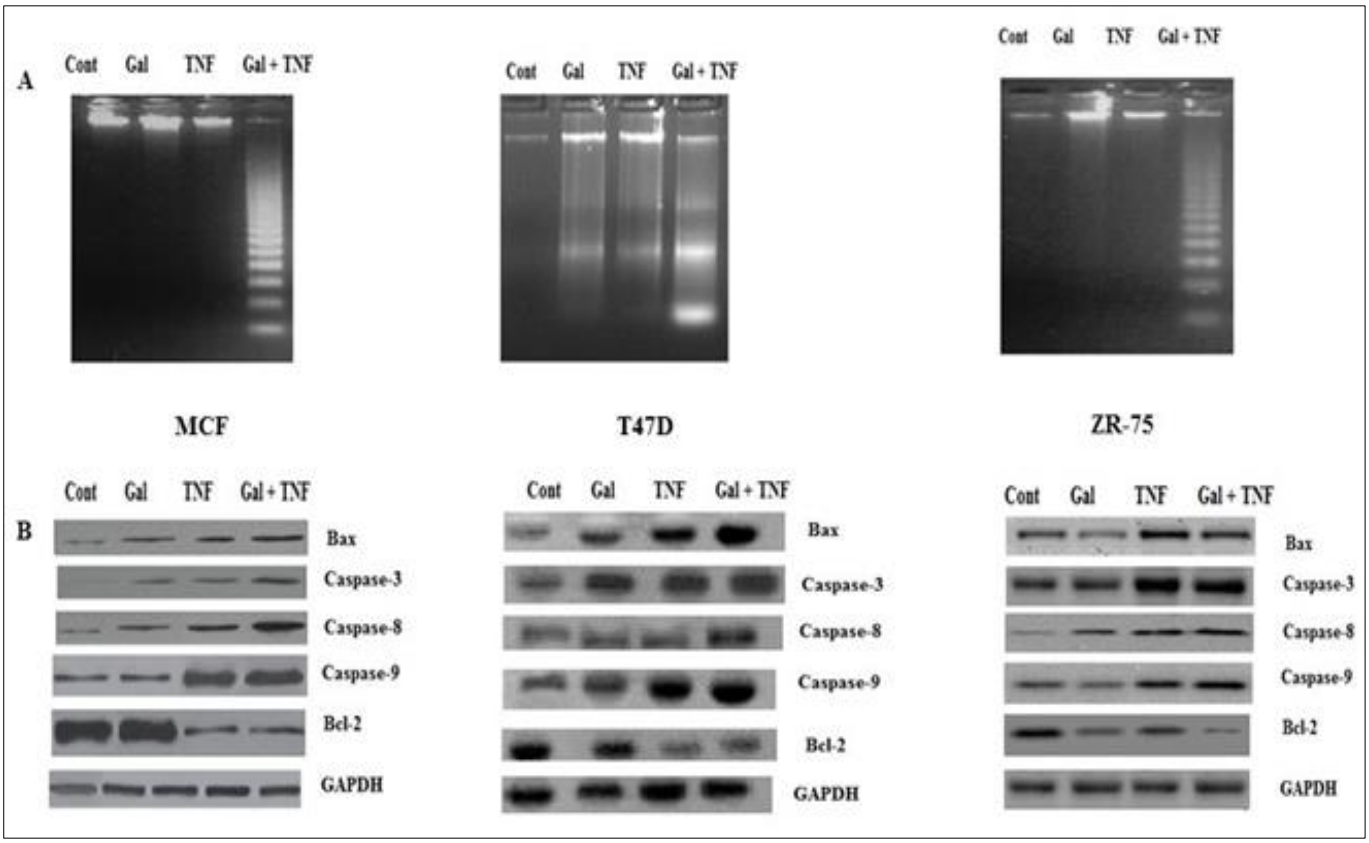

Fig 2: Galangin modulates TNF induced apoptosis in breast cancer cells: Cells were treated with galangin, TNF and in combination for 48 hr. A: DNA fragmentation analysis in galangin-TNF induced apoptosis in human breast cancer cells. B: Immunoblotting showing the effect of galangin on TNF induced caspase-3, caspase-9, caspase-8, Bax and Bcl-2 expression in human breast cancer cells

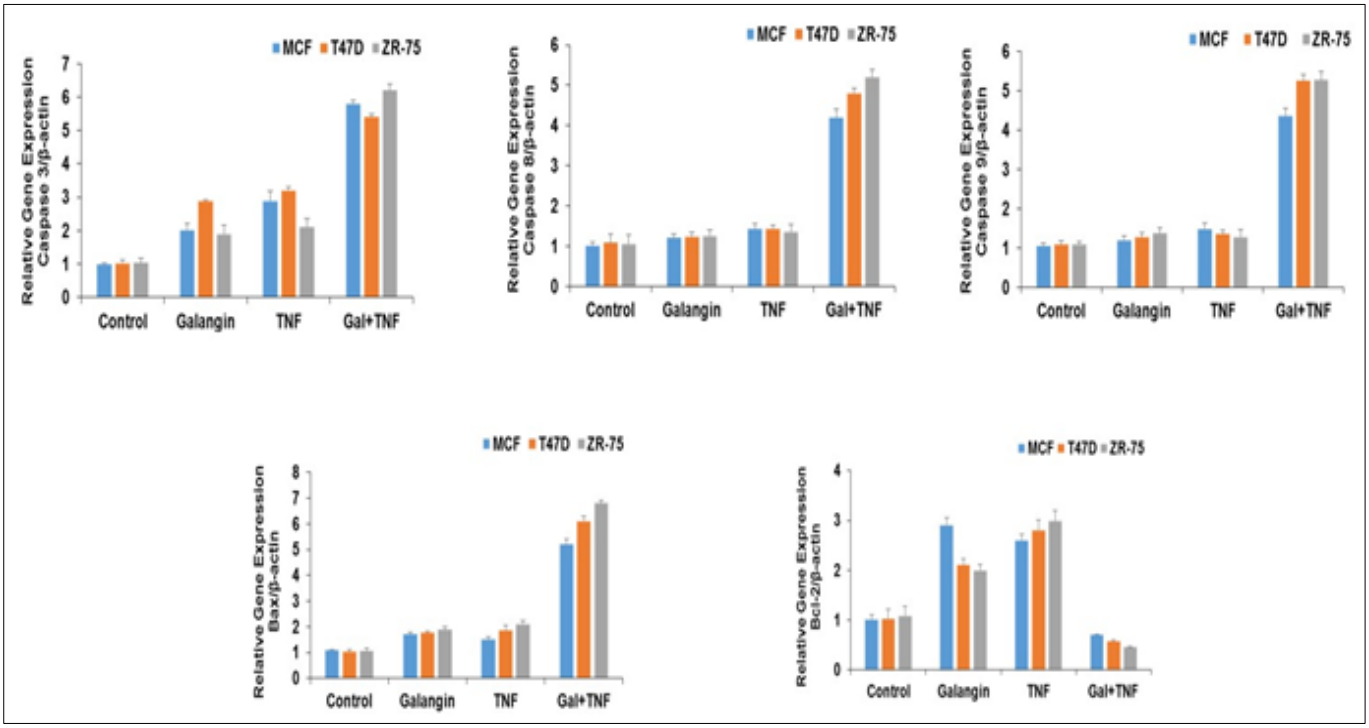

Fig 3: Galangin modulates TNF induced apoptosis in breast cancer cells: Cells were treated with galangin, TNF and in combination for 48 hr. Quantitative Real Time PCR showing the effect of galangin on TNF induced caspase-3, caspase-9, caspase-8, Bax and Bcl-2 expression in human breast cancer cells.

Discussion

In the present study, we showed that galangin significantly sensitizes the TNF induced apoptosis in breast cancer cells at a very minimal concentration. We further revealed the mechanism involved in dose effect of galangin in TNF induced apoptosis in breast cancer cells and found that caspase dependent signaling pathway was activated by galangin and TNF combinational treatment in breast cancer cells to induce cell apoptosis which results in enhanced expression of pro-apoptotic proteins, where as anti-apoptotic proteins are suppressed.

TNF-α is one of the most important inflammatory mediators of the cancer-associated inflammatory networks. It is a pleiotropic cytokine that regulates multiple cellular responses, including inflammation, cell survival, and cell death (apoptosis) [34, 35]. TNF-α has two types of receptors: TNF-α receptor 1 (TNFR1) and TNFR2. TNFR1 is ubiquitously expressed, whereas TNFR2 is expressed on specific types of cells such as immune cells, endothelial cells, and microglia [36]. TNFR1 has a death domain in its cytoplasmic region, and TNF-α binding to TNFR1 triggers the opposing biological responses; that is, cell survival via NF-κB activation and apoptosis via caspase-8 activation. Different cancer cells including breast cancer differ in their sensitivities to TNF- α. Previous studies have shown that several cancer cells are quite resistant to TNF- α induced cytotoxicity such as prostate cancer, non-small lung cancer *etc.* [37-39]. However, there is no such study has been made to elucidate TNF- α sensitivity in breast cancer cells. Therefore, we aimed to evaluate TNF- α induced apoptosis in breast cancer cells. Further, we aimed to study how galangin sensitizes the TNF- α induced apoptosis in breast cancer cells.

Consistently, we found no significant effect of TNF- α on breast cancer cells. All the breast cancer cell lines are resistant to TNF- α and showed resistance even at higher concentration. Treatment of TNF- α in combination with galangin induces significant cytotoxicity even at lower concentration in 2 of the cell lines. We also found that synergistic effect of galangin and TNF enhanced breast cancer cells to induce apoptosis through suppressing Bcl-2 anti-apoptotic protein.

Taken together, data from this study reveal a novel function of galangin on TNF- α activity and suggest that galangin can be considered as an anticancer compound to enhance TNF- α induced apotosis in breast cancer cells.

Acknowledgement

The authors would like to acknowledge the Department of Chemistry for providing the research facility.

Informed consent

Informed consent was obtained from all the individuals participants included in the study

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