RESEARCH ARTICLE

Fenugreek Induced Apoptosis in Breast Cancer MCF-7 Cells Mediated Independently by Fas Receptor Change

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Abstract

***Trigonella foenum* in *graecum* (*Fenugreek*) is a traditional herbal plant used to treat disorders like diabetes, high cholesterol, wounds, inflammation, gastrointestinal ailments, and it is believed to have anti-tumor properties, although the mechanisms for the activity remain to be elucidated. In this study, we prepared a methanol extract from *Fenugreek* whole plants and investigated the mechanism involved in its growth-inhibitory effect on MCF-7 human breast cancer cells. Apoptosis of MCF-7 cells was evidenced by investigating trypan blue exclusion, TUNEL and Caspase 3, 8, 9, p53, FADD, Bax and Bak by real-time PCR assays inducing activities, in the presence of FME at 65 μg/mL for 24 and 48 hours. FME induced apoptosis was mediated by the death receptor pathway as demonstrated by the increased level of Fas receptor expression after FME treatment. However, such change was found to be absent in *Caspase 3*, *8*, *9*, *p53*, *FADD*, *Bax* and *Bak*, which was confirmed by a time-dependent and dose-dependent manner. In summary, these data demonstrate that at least 90% of FME induced apoptosis in breast cell is mediated by Fas receptor-independently of either FADD, Caspase 8 or 3, as well as p53 interdependently.**

Keywords: *Fenugreek* - apoptosis - breast cancer - gene expression - Fas receptor

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Introduction

In spite of the advances made in cancer treatment, there is a continues need for intervention strategies, including chemopreventive agents that act as primary defensive agents by preventing, delaying or reversing preneoplastic lesions, as well as those that act on secondary or recurrent cancers as therapeutic agents (Kwon et al., 2007). Chemoprevention has been successfully achieved in numerous *in vitro* and as well as *in vivo* studies and has been validated in several human intervention trials (Lee et al., 2005). Chemopreventive agents have low side effects and toxicity and are involved in neutralization of carcinogenic agents as well as their effects on cells (Sun et al., 2006). In recent years, increasing attention has been focused on to identify the naturally occurring chemopreventive agents, particularly those present in dietary and medicinal plants due to their bioactive substances (Sebastian et al., 2007). Most of these bioactive substances exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumor cells has become an indicator of the tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Paschka et al., 1998; Cho et al., 2009; Lee et al., 2010). Thus, searching for new alternative agents for the prevention and treatment of breast cancer is in great need.

Fenugreek (*Trigonella foenum-graecum L.*) is an annual legume crop, due to its spice possessing amazing therapeutic and medical properties it is used in many parts of the world. It is one of the oldest medicinal plants known and has long been recognized as a traditional medicine in Asia, Africa and Mediterranean countries (Mebazaa et al., 2009; Naidu et al., 2011). Current research on *Fenugreek* has shown that it contains active beneficial chemical constituents, including steroidal sapogenins (Taylor et al., 1997), dietary fiber (Naidu et al., 2011), galactomannans (Wu et al., 2009), antioxidants (Naidu et al., 2011), and amino acids such as 4-hydroxyisoleucine which possess anti-diabetic (Kumar et al., 2005), hypocholesterolemic and hypoglycemic properties (Meghwal et al., 2012) which have potential to be used in the treatment of antileukemic (Acharya et al., 2011), antipyretic (Bhatia et al., 2006), antinociceptive, antifertility activity, cure leprosy galactogogue (Bhalke et al., 2009; Khoja et al., 2011), obesity, diabetes and cancer (Thomas et al., 2011).

One such active agent is the diosgenin, which inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon

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cancer cells (Raju et al., 2004). Diosgenin also inhibits osteoclastogenesis, invasion and proliferation through the down-regulation of Akt, I kappa B kinase activation and NF kappa B-regulated gene expression in tumor cells (Shishodia and Aggarwal, 2005). It has an antioxidant activity in HIV patients with dementia (Turchan et al., 2003). Another active agent identified in *Fenugreek* is Protodioscin, which induces cell death and morphological change indicative of apoptosis in the leukemic cell line HL-60 (Hibasami et al., 2003). The chemopreventive aspects and the potential protective effect of *Fenugreek* seeds against 7, 12-dimethylbenz[α] anthracene (DMBA) in rats has been reported (Amin et al., 2005). Some constituent of alkaloids, called 'trigonelline', has revealed potential for use in cancer therapy (Bhalke et al., 2009).

This study was aimed to evaluate the therapeutic window of methanol extract of *Fenugreek* plant on immortalized breast cells (MCF-7). The relative quantification of *Caspase 3, 8, 9, p53, Fas, FADD, Bax* and *Bak* gene expression on MCF-7 cell line with treatment of *Fenugreek* extract was examined by RT-PCR.

Materials and Methods

Preparation of plant extracts

Fenugreek plant was selected on the basics of ethanopharmacology. Whole plant was shade dried, grounded and soaked into methanol for extraction. The quantity of solvent was taken 10 times the quantity of plant material. Extraction was performed thrice and extraction was done for 24 hours. The filtrate extract were then evaporated to dryness at 30°C under reduced pressure. Further 100 mg of each extract was dissolved in 10 mL DMEM medium (10% FCS) to obtain stock solution and was further diluted in medium to 10, 25, 50, 75 and 100 μg/mL (Haraguchi et al., 2000; Hasan et al., 2011).

Maintenance of MCF-7 cells

The MCF-7 breast cancer cell line was a kind gift from Dr Akbarshah at the Mahatma Gandhi-Doerenkamp Center (MGDC) for alternatives to use of animals in life science education, Bharathidasan University, India. The cell line was tested and found to be free from Mycoplasma. The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM)+phenol red supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 Units/0.1mg, mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. All the studies done with the cell at ~70-80% confluence. Cells were harvested after being subjected to brief trypsinization. Cell viability was assayed by Trypan Blue exclusion test with slight modification (James and Warburton, 1999). The viability of cells was greater than 95%.

Cell Titer Blue® viability assays

Cell Titer Blue® viability assay (Promega) was performed to assess the toxicity of different concentrations of methanol *Fenugreek* extract (FME) on MCF-7 cells. The assay was performed according to the manufacturer's instructions. In brief, MCF-7 cells (2×10^4 cells/well) were plated in 96 well plates and treated with 0-100 μg/mL extract for 24 hours. Then 40 μL of the Cell Titer Blue solution was directly added to the wells and incubated at 37°C for 6 hours. The fluorescence was recorded with a 560/590 nm (excitation/emission) filter set using a Bio-Tek microplate fluorescence reader (FL×800™), and the IC_{50} was calculated. Quadruplet samples were run for each concentration of the FME in three independent experiments.

FME-Treatment for a concentration and time-dependent study

For a concentration and time dependent study, FME of 65 μg/mL was treated with MCF-7 cells for 24 and 48 h for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The cells were incubated with the same FME concentration for 24h for real-time quantitative PCR analysis.

TUNEL assay

The DeadEnd® TUNEL assay kit (Promega) was used for studying apoptosis in a time and dose dependent manner. The manufactures instructions were followed. Briefly, MCF-7 cells (1.5×10^6 cells/well) were cultured in 6 well plates to study apoptosis in adherent cells. Cells were treated with FME of 65 μg/mL for 24 and 48 h. After the incubation period, the culture medium was aspirated off, and the cell layers were trypsinized. The trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained according to the DeadEnd fluorometric TUNEL system protocol. The stained cells were observed using a Carl- Zeiss (Axiovert) epifluorescence microscope using a triple band-pass filter. To determine the percentage of cells demonstrating apoptosis, 1000 cells were counted in each experiment (Shafi et al., 2009; Hasan et al., 2013).

Real-time quantitative PCR analysis

The expression of apoptotic genes was analyzed by the reverse transcription-PCR (RT-PCR; Applied Biosystems 7500 Fast) using a real-time SYBR Green/ROX gene expression assay kit (QIAGEN). The cDNA was directly prepared from cultured cells using a Fastlane® Cell cDNA kit (QIAGEN), and the mRNA levels of *Caspases 3*, *8*, *9*, *p53*, *Fas*, *FADD*, *Bax* and *Bak* as well as the reference gene *GAPDH*, were assayed using gene-specific SYBR Green-based QuantiTect® Primer assays (QIAGEN). Quantitative real-time RT-PCR was performed in a reaction volume of 25 μL according to the manufactures instruction. In brief, 12.5 μL of master mix, 2.5 μL of primer assay (10×) and 10 μL of template cDNA (100 μg) were added to each well. After a short centrifugation, the PCR plate was subjected to 35 cycles of the following conditions: PCR activation at 95°C for 5', denaturation at 95°C for 5" and annealing/extension at 60°C for 10". All samples and controls were run in triplicates on an ABI 7500 Fast Real-time PCR system. The quantitative RT-PCR data was analyzed by the comparative threshold (Ct) method, and the fold inductions of samples were compared with the untreated samples. *GAPDH* was used as an internal reference gene to normalize the expression of the apoptotic genes. The

Ct cycle was used to determine the expression level in the control cells and MCF-7 cells treated with FME for 24h. The gene expression level was then calculated as described earlier. The results were expressed as the ratio of reference gene to target gene by using the following formula: ΔCt=Ct (apoptotic genes)-Ct (*GAPDH*). To determine the relative expression levels, the following formula was used: ΔΔCt=ΔCt (treated)-ΔCt (control). In short, the expression levels were expressed as n-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic genes using the expression of $2^{-\Delta\Delta Ct}$.

Results

Determination of FME toxicity on MCF-7 cells

The cytotoxic effect of 0 to 100 μg/mL concentration of different FME on MCF-7 cells was examined using the Cell Titer Blue® viability assay (Promega). A dose-dependent reduction in color was observed after 24 h of treatment with different FME. In brief, 71.8% of the cells were found dead at the highest concentration of FME tested (100 μg/mL), whereas the IC_{50} of FME was achieved at 65 μg/mL (Figure 1).

Quantification of apoptosis by a TUNEL assay

To determine whether the inhibition of cell proliferation by FME was due to the induction of apoptosis, a TUNEL assay was used. Figure 2 and 3 summarize the effect of FME on MCF-7 cells. A dose and time dependent increase in the induction of apoptosis was observed when MCF-7 cells were treated with FME. When compared to the control cells at 24 h, 46.1% of cells treated with 65 μg/mL of FME, respectively underwent apoptosis. Similarly, 58.9% of cells treated with 65 μg/mL of FME, respectively, for 48 h underwent apoptosis.

Quantification of mRNA levels of apoptotic related genes

To investigate the molecular mechanism of FME induced apoptosis in MCF-7 cells, the expression levels of several apoptosis related genes were examined for 24 h only. The relative quantification of *Caspase 3*, *8*, *9*, *p53*, *Fas*, *FADD*, *Bax* and *Bak* mRNA expression levels was performed by SYBR Green based quantitative real-time PCR (RT-PCR) using a 7500 Fast Real Time System

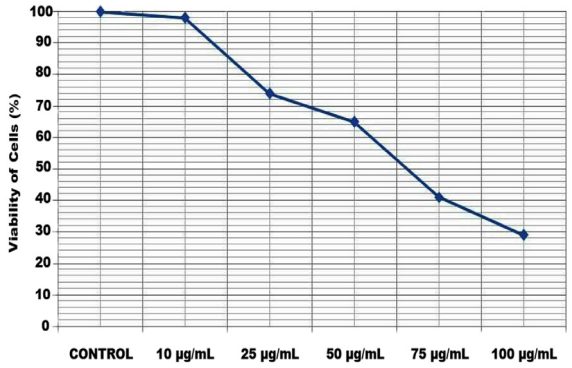

Figure 1. MCF-7 Cell Viability was Determined by the CellTiter Assay. MCF-7 cells were treated with various concentrations (10-100μg/mL) of FME and results are expressed as percentage of viability was normalized with untreated control (mean±SE)

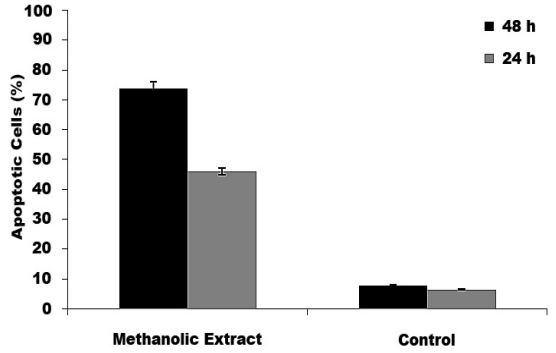

Figure 2. Percentage of TUNEL Positive Cells-Indication of Apoptosis after 24 and 48 Hours of Exposure of MCF-7 Cells With or Without FME (65μg/mL)

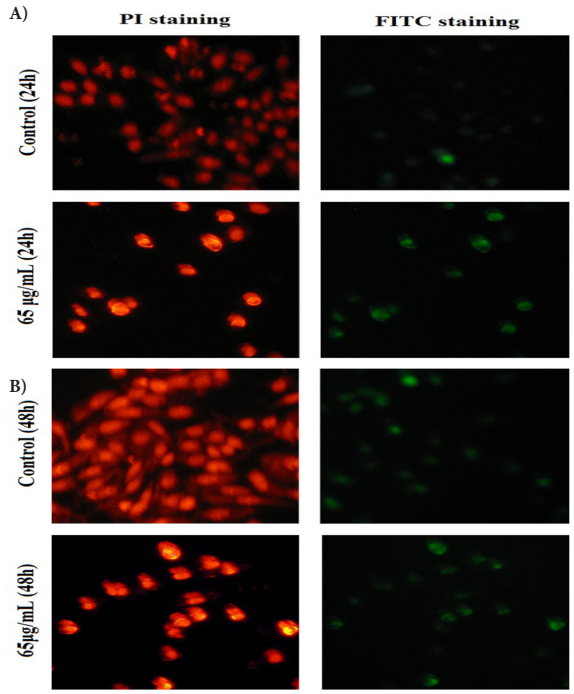

Figure 3. TUNEL Assay (microscopic) after 24 and 48 Hours Incubation of MCF-7 Treated against 65 μg/mL FME with Control. Red fluorescence is due to Propedium Iodide staining and observed under green filter while green fluorescence is due to FITC staining and observed under blue filter. Observations done at 200× magnification

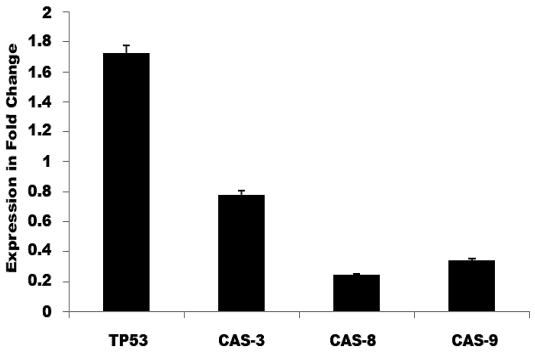

Figure 4. Comparison of the Change in the Expression of *p53*, *cas-3*, *8* & *9* Genes Expressed as the Fold Change (ratio of target:reference gene) in MCF-7 Cells after 24h of Exposure with FME (65μg/mL)

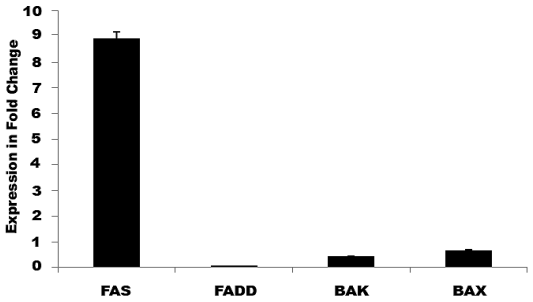

Figure 5. Comparison of the Change in the Expression of *FAS*, *FADD*, *BAK* & *BAX* Genes Expressed as the Fold Change (ratio of target:reference gene) in MCF-7 Cells After 24h of Exposure with FME (65*µ*g/mL)

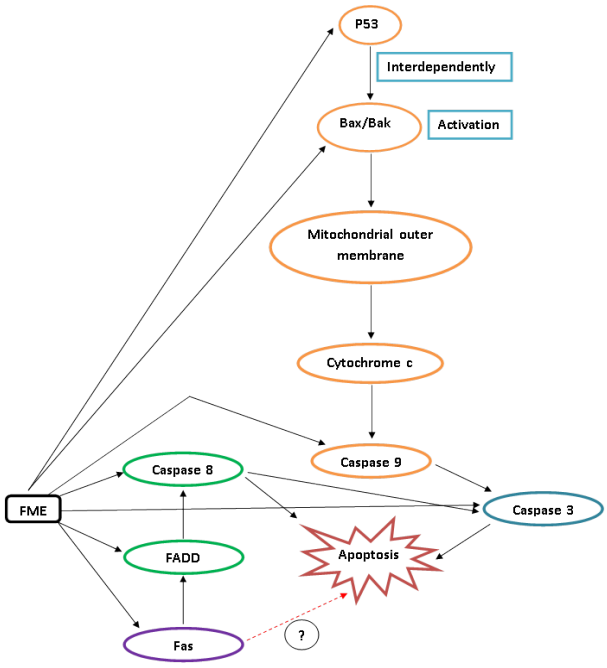

Figure 6. Proposed Pathway for FME Induced Breast Cell Apoptosis

(Applied Biosytem).

Figures 4 and 5 summarize the gene expression changes of *Caspase 3*, *8*, *9*, *p53*, *Fas*, *FADD*, *Bax* and *Bak*. In most of the expression FME lesser the transcripts of *Caspase 3*, *8*, *9*, *p53*, *FADD*, *Bax* and *Bak* by few fold, whereas the *Fas* has showed several fold when compared to the other genes. The expression levels of these genes in MCF-7 cells treated with 65 *µ*g/mL of FME for 24h increased by as follows: 0.9 fold in *Caspase-3*, 0.25 fold in *Caspase-8*, 0.3 fold in *Caspase-9*, 1.7 fold in p53, 8.8 fold in *Fas*, 0.12 fold in *FADD*, 0.4 fold in *Bax* and 0.7 fold in *Bak* respectively as compared to the levels in untreated control cells. All together these data advocates that these *caspases*, *p53*, *Fas*, *FADD*, *Bax* and *Bak* were induced by FME in dose and time dependent manner.

Discussion

The goal of this article was to determine the effect of apoptosis on MCF-7 cell line with the treatment of FME in *Caspase 3*, *8*, *9*, *p53*, *Fas*, *FADD*, *Bax* and *Bak* activation. The presented data in this paper demonstrate a time and dose dependent inhibition by FME of MCF-7 human breast cancer cell proliferation. There are various mechanisms through which apoptosis can be induced in cells such as the expression of pro and anti-apoptotic proteins. The mitochondrial apoptotic pathways and death receptor pathways are the two major pathways that have been characterized in mammalian cells. The mitochondria have a central role in regulating the *caspase* cascade and apoptosis (Shafi et al., 2009). *Caspases* have a central role in the apoptotic process in that they trigger a cascade of apoptotic pathways (Shah et al., 2003). The release of cytochrome-c from mitochondria leads to the activation of procaspase-9 and then *caspase-3* (Shafi et al., 2009). The activation of *caspase-3* is an important downstream step in the apoptotic pathway (Earnshaw et al., 1999; Alshatwi, 2010). In addition, the effector *caspase-3*, and the initiator *caspase-8* and *9*, are the main executors of apoptosis (Riedl et al., 2004). *Caspase-8* is in the death receptor pathway whereas *caspase-9* is in the mitochondrial pathway, and both pathways share *caspase-3* (Pommier et al., 2004). *Caspase-8* activates crosstalk between the death receptor pathway and the mitochondrial pathway by the cleavage of *Bid* to *tBid*, a pro-apoptotic member of the *Bcl-2* family. The activation of *caspase-8* has a central role in *Fas*-mediated apoptosis. Moreover, the cleavage of *Bid* has been shown to be associated with *caspase-8* activation (Malik et al., 2008). Further, *Bax* and *Bak* are the two key molecules in the mitochondrial pathway of apoptosis, were interdependently activated by *p53*, leading to cytochrome c release and followed by apoptosis, which may be indirect activation of *caspase 3* from *caspase 8*, *9* or direct by *caspase 9*.

According to the Americans recent survey estimates that between 12% and 17% have used herbal remedies and those women often use such medicine as hormone replacement therapy. Despite the widespread use of these herbs, little is known about their safety and efficacy (Hu et al., 2009). Considering this, the GC-MS data of methanolic extract of *Fenugreek* has been reported that there are major classes of compounds such as aldehydes, ketones, acids, alcohols, sulfur compounds, furans, monoterpenes, sesquiterpenes, and aromatic hydrocarbons are found, which can be used as a phototherapy or chemotherapy (Mebazaa et al., 2009). The unique amino acid, 4-hydroxyisoleucine stimulates the discharge of insulin thereby controlling blood sugar levels (Gupta et al., 2001; Broca et al., 2004; Haeri et al., 2009). It is rich in flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin and isovitexin (Shang et al., 1998, Blumenthal et al., 2000, Kaviarasan et al., 2007). These natural antioxidants help to strengthen the immune system, improve cellular health and diminish signs of ageing (Bacco et al., 1978). The spice seeds contain 0.1-0.9% diosgenin and are extracted on a commercial basis. Several coumarin compounds have been identified in *Fenugreek* seeds as well as a number of alkaloids (e.g., trigonelline, gentianine, carpaine), also contains 5.5-7.5% lipids constituting mainly of neutral lipids (85%) followed by phospholipids (10%) and glycolipids (5%). Unsaturated acids comprising mainly of linoleic (40%), linolenic (25%) and oleic (14%) acids dominate the fatty acid profile (Sulieman et al., 2000; Yang et al., 2012).

N-acylethanolamines and their precursors, N-acyl phosphatidylethanolamines have been identified as phospholipid constituents in desiccated seeds of diverse plant species. These minor membrane lipid components have been implicated in lipid signalling pathway that regulates an array of physiological processes in multicellular eukaryotes including plant defense response and seedling root development (Chapman, 2004). Oleamide an important member of this class is a sleep-inducing lipid with diverse action such as antinociceptive (pain reducing) properties and stimulates increased food uptake (Boger et al., 1998). Some of them have anti-inflammatory (Sindhu et al., 2012) and anti-cancer properties and help to control many physiological and pathological processes in the reproductive system. Oleoylethanolamine is an endogenous regulator of food intake and is suggested as a potential anti-obesity drug. Steroidal sapogenin is considered as an essential compound in the hemisyntheis of steroid drugs such as cortisone and sexual hormones (Brenac and Sauvaire, 1996).

Several compounds such as furanones, diosgenin, dioscin have been shown to have anticancer activity in mice, breast cancer, and colon cancer. Dioscin were also shown to include antifungal, antivirus and antitumor activities. In cell culture studies dioscin exerted apoptosis-inducing effects against human myeloblast leukemia HL-60 cells, human cervical cancer Hela cells, Caco-2, HCT-116, HepG2, K562 and A-549 (Yum et al., 2010). Diosgenin has also induces apoptosis in human rheumatoid arthritis, human osteosarcoma 1547 cell line, HT-29 human colon cancer cells (Raju et al., 2004), leukemic cell line HL-60 and prostate cancer cells PC-3 by various carcinogens in the form of cancer therapy (Hibasami et al., 2003; Chen et al., 2011).

According to Alshatwi et al (unpublished data, 2011) explains that some of the compounds of *Fenugreek* hexane extract has found to be more activate in intrinsic apoptotic pathway rather than extrinsic, which might be due to the presence/absence of methanolic soluble compounds. The data presented in this study suggest that FME induced apoptosis is mediated by the death receptor pathway as demonstrated by the increased level of change folds in Fas receptor expression after FME treatment which could be the presence of some the phytocompounds in methanolic extract alone. However, the change folds was found to be absence in *Caspase 3*, *8*, *9*, *p53*, *FADD*, *Bax* and *Bak*.

Consequently, it seems there is a second extrinsic Fas receptor-independent cell death pathway that induces many of the characteristics of apoptosis (Figure 6). Regardless of the mechanisms involved, data from this study demonstrate that less than 10% of FME induced apoptosis in dependent on caspases activation and Fas receptor activation. It will be interesting to determine whether these same results are seen in other cell types, especially fibroblast and tumor cells.

In conclusion, we have demonstrated the novel observation that FME induced breast cell apoptosis is mediated by Fas receptor-independently of either *FADD*, caspase 8 or 3 and as well as p53 interdependently. This signaling pathway has a major role in initial breast cell-apoptosis, accounting for more than 90% of apoptosis. As cell death progress a parallel and distinct mechanism results in an apoptotic-like cell death that has similar morphology and biochemical characteristics to apoptosis but it is not inhibited by the inhibitors of either p53 or FADD, caspase 3, 8, and 9.

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