Fenugreek: a naturally occurring edible spice as an anticancer agent

Shabana Shabbeer1,§, Michelle Sobolewski3, Sushant Kachhap1,*, Nancy Davidson3, Michael A. Carducci1, and Saeed Khan2,*

1Prostate Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD 21231, USA
2Chemical Therapeutics Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD 21231, USA
3Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD 21231, USA

Abstract

In recent years, various dietary components that can potentially be used for the prevention and treatment of cancer have been identified. In this study, we demonstrate that extract (FE) from the seeds of the plant *Trigonella foenum graecum*, commonly called fenugreek, are cytotoxic in vitro to a panel of cancer but not normal cells. Treatment with 10-15 ug/mL of FE for 72h was growth inhibitory to breast, pancreatic and prostate cancer cell lines (PCa). When tested at higher doses (15-20 ug/mL), FE continued to be growth inhibitory to PCa cell lines but not to either primary prostate or htert-immortalized prostate cells. At least part of the growth inhibition is due to induction of cell death, as seen by incorporation of Ethidium Bromide III into cancer cells exposed to FE. Molecular changes induced in PCa cells are: in DU-145 cells: down regulation of mutant p53, and in PC-3 cells up regulation of p21 and inhibition of TGF-β induced phosphorylation of Akt. The surprising finding of our studies is that death of cancer cells occurs despite growth stimulatory pathways being simultaneously up regulated (phosphorylated) by FE. Thus, these studies add another biologically active agent to our armamentarium of naturally occurring agents with therapeutic potential.

Keywords

Dietary bioactive agents; fenugreek; diosgenin; prostate cancer; phosphorylation

Introduction

Many agents purified from dietary sources are under evaluation for their therapeutic efficacy in the treatment of cancer. For example, when the soy isoflavone genistein was purported to be a chemopreventive agent, its therapeutic efficacy was also examined. It was found that genistein induces apoptosis and inhibits growth of both androgen-sensitive and androgen-independent prostate cancer (PCA) cells in vitro. Then in a clinical trial that followed, soy isoflavones were found to decrease the rate of the rise of serum Prostate Specific Antigen

---

*To whom correspondence should be addressed: khansa@jhmi.edu, 1650 Orleans Street/Room 1M46; Baltimore, Maryland 21231 USA; Tel.: +410.614.0200; Fax: +410.614.8397.
§NCI SPORE Grant P50CA58236, Prostate Cancer Foundation
†AEGON International Fellowship in Oncology

Some plant derived phenolics have also been shown to regulate neoplastic cell growth and survival when used at physiological concentrations [2].

Despite wide spread uses in traditional Indian medicine for the treatment of hypercholesterolemia and hypertriglycerideremia, fenugreek seeds have received little attention as an agent in the treatment of cancer. Some studies have identified active agents amongst the saponins and sapogenins that make up a considerable portion of the crude extracts of fenugreek (FE). One such active agent is the sapogenin, diosgenin. Diosgenin inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induce apoptosis in HT-29 human colon cancer cells [3]. 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 [4]. It is also the active component responsible for the anti-diabetic and hypocholesterolemic activity of fenugreek [5]. Another active agent identified in FE is Protodioscin. It induces cell death and morphological change indicative of apoptosis in the leukemic cell line H-60, but not in gastric cancer cell line KATO III [6]. However, none of these studies have examined whether the effects of diosgenin are specific to cancer and not normal cells.

Hence the aim of this study was to determine the therapeutic window of FE in malignant as well as primary and immortalised cells and also compare the efficacy of FE with diosgenin. We also compared cytotoxicity of FE and diosgenin with sulforaphane - an active ingredient isolated from another dietary source and thoroughly studied as a chemopreventive agent. Here we note that besides cytotoxicity caused by the detergent-like properties of the saponin / sapogenin content, FE causes molecular changes in key proteins involved in apoptosis and the signaling pathways. The changes induced by the whole extract are distinct from those caused by the pure agent diosgenin.

**Materials and Methods**

**Cell culture and reagents**

The PCa cell lines DU-145, LNCaP and PC-3, the breast cancer cell lines MDA-MB-231, MCF-7, T47D and SKBR3, and the pancreatic cancer cell lines MiaPaCa, HS766T, Panc1, L3.6PL and BXPC3, were obtained from American Type Culture Collection (Rockville, MD, USA). hTert-immortalised prostate cells (957 E/hTERT) called hTert-PrEC and primary prostate cells called PrEC were a kind gift from Dr.J.Isaacs (Baltimore, MD). The cell lines were tested and found to be free of Mycoplasma. All cells and cell lines were maintained in 5% CO2 at 37°C. The PCa and pancreatic cancer cells were cultured in RPMI 1640 (Invitrogen, Grand Island, NY), supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY). PrECs were grown in Keratinocyte-SFM and primary prostate cells in PrEGEM media (Lonza, Switzerland). The MDA-MB-231, MCF-7 and T47D cells were cultured in DMEM containing 5% FBS and 1% L-alanyl-L-glutamine (Mediatech, Herndon, VA). The SKBR3 cell line was cultured in McCoys media containing 15% FBS and 1% L-alanyl-L-glutamine (Invitrogen). TGF-β was obtained from R&D Systems (MN, USA) and stock solution prepared in ethanol. Sulforaphane was obtained from LKT laboratories (Pelham, NH) and diosgenin from Fluka Biochemicals (Buchs, Switzerland). A stock solution of sulforaphane in acetonitrile and diosgenin in ethanol was prepared and diluted in culture media before use. All stock solutions were stored at -20°C until use. Phospho-RTK Array Kit was purchased from R&D Systems (MN, USA).

**Isolation of fenugreek seed extract**

Fenugreek seeds originating from Saudi Arabia were purchased from a commercial source. The air-dried seeds were ground multiple times with an electric grinder. The powder
obtained (500g) was soaked in 1 liter of ethanol and refluxed for 24h at 50°C. The resulting supernatant was filtered and evaporated. The seed residue was soaked and refluxed with 1 liter each of hexane, petroleum ether, ethyl acetate and chloroform respectively. The resulting filtered supernatants were combined and evaporated.

**MTT assay of growth inhibition**

All cancer cells were cultured in their respective media as described above till mid-log phase. Cells were harvested and resuspended in growth media to make a stock cell suspension containing 100,000 cells/ml. 100μl of this stock cell suspension was added to the wells of a 96-well plate. The cells were allowed to attach and grow for 24h. FE was weighed and diluted with DMSO to make a 100mg/ml stock solution. This stock solution was further diluted with culture media to make a secondary working solution of 100ug/mL. The working solution was added to the wells such that final concentrations of 0-100ug/mL of FE were obtained. Further DU-145 and PC-3, PCa cells and hTert-PrEC cells only, were treated with 5 doses of diosgenin ranging from 10 – 20uM and sulforaphane from 0.5 – 2uM. Each experiment was performed in triplicates in parallel for each concentration. Controls were performed in which only culture media and DMSO were added. The cells were then incubated at 37°C in a 5% CO₂, 95% air atmosphere. After 72h of incubation, the culture medium was removed and the cells washed twice with phosphate buffered saline (PBS). Then 20μl of 5mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was added to each well. The cells were further incubated at 37°C for 4h. The supernatant was discarded and 100μl of DMSO was added to each well. The mixture was shaken on a micro-vibrator for 5min and the absorbance was measured at 570nm (A) that served as a measure of cell viability. Inhibition ratio (%I) was calculated using the following equation:

\[
\text{Formula } \%I = \frac{(A_{\text{control}} - A_{\text{treated}})}{A_{\text{control}}} \times 100
\]

**Cell cycle analysis**

LNCaP and PC-3 PCa cells were plated in 100mm dishes such that they were 30% confluent at the start of treatment. Cells were incubated with 0.1mg/mL of FE for 48h. Cells were harvested 48h after drug treatment. Aliquots of 10,000 harvested cells were analyzed by flow cytometry for their cell cycle profile following a modification of the method of Vindelov [7]. Briefly, cells were suspended in 100μl phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol and stored at -20°C until further analysis. At the time of analysis, cells were thawed and resuspended in 100μl citrate buffer. Diluted cells were treated with 50ug/ml RNaseA for 30min. Propidium iodide (PI) was then added to a final concentration of 20ug/ml. Cells were analyzed on a BD-LSR flow cytometer equipped with a UV laser. Fluorescent light emitted by PI stained cells was detected on the FL-2 channel. Emitted light in the FL2-W vs. FL2-A area was gated for single cells with different DNA content and a consequent histogram of the gated cell population was drawn.

**Detection of cell death**

To confirm cell death, the Ethidium bromide-calcein cytotoxic assay [providing a two-color fluorescent staining of live and dead cells using two probes: Calcein AM stains live cells green while EthD-III stains dead cells red] was performed as per manufacturer’s instructions. In brief, DU-145 and PC-3 PCa cells, and hTert-PrEC and PrEC were plated in 6-well dishes and incubated with or without agents: sulforaphane (100nM), diosgenin (20uM) and FE (15ug/ml) - doses that are cytotoxic for the cancer cells as determined by the MTT assay. After 96h incubation, the wells were washed with PBS. A mixture of Calcein AM and EthD-III was added to the cells and allowed to incubate for 15min at 37°C. The cells were then visualized within the hour with a fluorescence microscope.
Treatment of cells with FE and TGF-β

Cells were plated in 6-well dishes for Western blotting and in T175 flasks for hybridization to the Proteome Profiler™ array. They were cultured for 48h with 15mM diosgenin or 15ug/ml FE, doses determined from the MTT assay to have a %I for cancer cells of at least 50. To mimic signaling in a cancer microenvironment, cells were incubated simultaneously with 1ng/ml TGF-β [8] and 15ug/ml FE, for 48h. At the end of the incubation, cell lysates were prepared as described below and Western blotting performed.

Western blotting and phospho-protein array hybridization

Cells were lysed with a 1% NP-40 containing buffer supplemented with a 1× cocktail of protease inhibitors (Complete Mini®, Roche, Mannheim, Germany) and phosphatase inhibitors (Phosphatase inhibitor cocktail I and II, Sigma) at 4°C for 20min. Lysates were centrifuged at 10,000g at 4°C for 15min and supernatants collected. The protein concentration of the supernatant was determined using the BCA assay (Pierce, Rockford, IL). Samples were mixed in a ratio of 1:2 in Laemmli buffer and denatured by heating at 98°C for 5min. Fifty μg of protein was separated on 4%-15% Tris-SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) at 100V for 1h. For Western blotting, the separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) at 380mA for 1h. Western blot analysis was carried out using specific primary antibodies for p21 (BD Pharmingen, San Diego, CA), p53 (Calbiochem, San Diego, CA), p-EGFR (Upstate, Lake Placid, NY) and p-Akt (Cell Signaling Technology, Danvers, MA). The membranes were blocked with TBS plus 5% nonfat milk (20mM Tris-HCl, pH 7.6, 137mM NaCl) followed by incubation overnight with primary antibodies diluted in either 5% BSA for phospho-Akt or blocking solution for all other antibodies (100-1000-fold). This was followed by incubation again for 1h in the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, UK). For detection, an ECL kit was used according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). The Proteome Profiler™ array – human phospho-RTK array kit was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) except that 200μg protein was loaded / array.

Results

Effects of FE, diosgenin and sulforaphane on the inhibition of cell proliferation

Exponentially growing DU-145, PC-3, and LNCaP PCa cells and hTert-PrEC, breast cancer cells and pancreatic cancer cells were cultured continuously in the absence or presence of different concentrations of FE, diosgenin and sulforaphane. The effects of these agents on cell growth were assessed by the MTT assay after 72h of incubation with the different agents. As shown in Figure 1, FE treatment significantly inhibited the growth of all the cancer cells tested but not the hTert-PrEC. At concentrations as low as 10ug/ml for 72h, FE had a dose-dependent cytotoxic effect on three of the five pancreatic cancer cell lines tested, with complete elimination of all cells at 10ug/ml (Figure 1a). At concentrations as low as 10ug/ml, FE had a cytotoxic effect when tested on the prostate cancer cell lines, LNCaP and DU-145; PC3 cells were growth inhibited even at 5ug/ml FE (Figure 1b). Three of the four breast cancer cell lines tested were growth inhibited at 1ug/ml, FE (Figure 1c); the MDA-231 cells were the most resistant, showing growth inhibition only at 10ug/ml. The degree of growth inhibition is dose dependent. On the other hand, the pure agents, diosgenin and sulforaphane, were growth inhibitory, in a dose dependent manner, to both the PCa cells as well as the hTert-immortalised prostate cells (Figures 1d and 1e). At the higher doses tested, the effect of the treatments are significantly different from the control untreated group (P < 0.01, unpaired t-test).
Induction of cell death and G2/M cell cycle arrest in PCa cell lines

After 48h of FE treatment, a significant peak was seen in the sub-G1 population of the cell cycle profile of PC-3 and LNCaP cells. In the case of FE treated PC-3 cells, an increased G2/M peak compared to untreated cells, was also seen. Based on these observations (Figure 2), it can be concluded that depending upon the cancer cell type, FE induces cell death and may also cause some cells to arrest in the G2/M phase of the cell cycle.

Different effect of crude FE compared to pure agents

Results from the MTT assay suggest that FE but neither its purified component diosgenin nor sulforaphane has differential effect on malignant vs. immortalized prostate cells. This effect was verified by a cytotoxicity assay utilizing incorporation of Calcein AM to stain live cells green and EthD-III to stain dead cells red. The assay verified that at the doses that were growth inhibitory in the MTT assay and after incubation for 96h, FE was cytotoxic to the cancer cells but not to either hTert-PrEC or PrEC (Figure 3a). Again, the purified agents, sulforaphane and diosgenin, were cytotoxic to both the cancer cells and hTert-PrEC or PrEC at the same dose (Figure 3b).

Effect of FE treatment on the expression of apoptotic proteins and mediators of signaling

Many cancer cells are characterized by expression of mutant p53. We observed that FE caused a down regulation of mutant p53 in DU-145 cells (Figure 4). There was no inhibition of wild-type p53, as seen by the lack of change in p53 protein expression in hTert-PrEC or PrEC. On the other hand, in PC-3 but not DU-145 cells, FE inhibited phosphorylation of EGFR (Figure 4).

Simulating signaling in an in vivo situation

In order to simulate signaling in an in vivo microenvironment, PC-3 and tert-immortalised prostate cells were grown in culture in the presence of 5ng/mL TGF-β. In both cell types, TGF-β induced phosphorylation of EGFR (Figure 5). This induction was inhibited by FE. In PC-3 cells only, FE inhibited TGF-β induced phosphorylation of the kinase domain of the growth stimulatory Akt pathway. FE also induced p21 in PC-3 cells, and this induction was not lost even in the presence of TGF-β. hTert-PrEC cells showed constitutive expression of p21 with no change to this protein by either FE or TGF-β (Figure 5). Thus, by blocking a central anti-apoptotic pathway - Akt signaling - induced by TGF-β, FE potentially counteracts the growth promoting effects of TGF-β signaling in the microenvironment.

Cell growth promoting signaling pathways stimulated by FE

Lysates prepared from DU-145 cells and hTert-PrEC cells were incubated with 15ug/mL of FE for 48h. In FE treated DU-145 cells, the phospho-RTK array revealed phosphorylation of growth stimulatory proteins - Insulin receptor and IGF-1R and Dtk after treatment (Figure 6). These proteins are already present in their phosphorylated forms in hTert-immortalised prostate cells. Besides these receptors, FE also induced phosphorylation of FGF-R3, Axl and EphA2, also proteins reported to have a growth promoting effect. On the other hand, the phosphorylation pattern seen on incubation with diosgenin was similar in DU-145 and hTert-PrEC cells.

Discussion

In a limited number of preclinical studies, extracts of fenugreek seeds and some of their constituents have shown anticarcinogenic potency [6, 8]. In an Ehrlich ascites carcinoma model in BALB/c mice, fenugreek seed extract produced a 70% inhibition of tumor cell growth when compared to controls [8]. Some other in vitro studies, have evaluated
diosgenin – a sapogenin present in fenugreek with varying levels depending upon the source of fenugreek, with respect to the anticarcinogeneic properties. Our study demonstrates that FE is selectively cytotoxic to cancer and not normal cells, while diosgenin is not capable of making such a differentiation. For comparison, we treated cancer and normal cells with sulforaphane, another biologically active agent purified from a dietary source (broccoli). When used at the concentration that is cytotoxic to cancer cells, sulforaphane also does not differentiate between cancer and normal cells. This suggests that the whole product may have certain advantages over purified and isolated constituents of extracts. Some other studies too have shown the potent effects of whole extracts as opposed to individual components of the extracts [9, 10]. Curcumin - isolated from turmeric – is one example. Curcumin did not have the same ability to inhibit basal CYP1A1/1A2 enzymes in tissues of mice as did turmeric [11, 12].

Many tyrosine kinase genes in cancer are deregulated [16]. We evaluated the ability of FE to target some of the overactive kinases in PCa cells. Contrary to expectations, FE caused the phosphorylation of many receptor tyrosine kinases that promote cell growth. The pattern of receptor phosphorylation induced by FE is distinct from that seen by diosgenin.

The tyrosine kinase receptors that are upregulated on fenugreek treatment are a perplexing combination. Axl and Dtk are receptor tyrosine kinases of the same receptor family. They have anti-apoptotic properties [13]. DU-145 cells treated with 15ug/mL FE, resulted in phosphorylation of these domains. Eph RTKs and their ligands, the ephrins, are frequently overexpressed in a variety of cancers and tumor cell lines. In particular, EphA2, is overexpressed in breast, prostate, lung, and colon cancers [15]. The Insulin and IGF-1R, FGF and EphA2 receptors are activated in DU-145 cells treated with FE. There is no data in the literature to show that activation of these receptors may be contributing to cell death. However, there is ample data suggesting the contrary, that phosphorylation of FGF receptors leads to stimulation of intracellular signaling pathways that control cell proliferation, cell differentiation, cell migration, cell survival and cell shape [14]. Thus, FE contains agents that are pleiotropic in effect, capable of growth stimulation as well as growth inhibition. Depending upon the cell type, either one pathway dominates and results in the differential observations seen in this study.

As the results show, FE results in varied effects in different cell lines. In the mutant p53 expressing cell lines, FE down regulates p53 expression. Considering our results with TGF-β and the fact that p53 acts as an integration node between Ras/MAPK and TGF-β pathways [16], we predict that a growth inhibitory effect of FE will be seen in vivo, where TGF-β influences the tumor microenvironment. In PC-3 cells, a cell line expressing wild type p53, FE induces p21 expression. Another naturally occurring antitussive alkaloid, noscapine, also caused a dose-dependent accumulation of p21 in cancer cells that correlated with a cytotoxic response, suggesting a proapoptotic role for induced p21 [17].

It must be borne in mind that the concentration of biologically active agents in any extract prepared from a naturally occurring substance will vary based on factors like geographical region of harvest, condition of soil, harvest season, etc. For example, in a Canadian study, diosgenin levels from mature fenugreek seeds ranged from 0.28 to 0.92% (28-92 ug/10 mg) [18]. Analysis of variance revealed that accession, accession x year, and site x year effects were significant for diosgenin content, whereas site, year, and site x accession effects were not. This variance may make it difficult to extrapolate and generalize results obtained from one study to another. In such a scenario, it may be important to determine the presence and percentages of a panel of constituents in extract from a particular source with another, before drawing conclusions about a particular plant or plant product(s).
Since fenugreek is widely consumed in many parts of the world, and in view of our current studies, we hypothesize that fenugreek may possess cancer chemoprotective properties. Hence, we have begun an in vivo study to examine the chemoprotective activity of fenugreek against PCa. This involves the long-term administration of fenugreek seed powder mixed with regular mouse diet in the hi-myc mouse mouse model of PCa – a model that recapitulates many stages of progression of human PCa [19]. It is hypothesized that fenugreek may be able to selectively kill developing PCa cells in this mouse while leaving the normal cells unharmed.

In summary, fenugreek seeds possess potent anti-cancer properties. The potential to differentiate between the malignant and hTert-immortalised or primary prostate cells is a function of the crude extract and not its individual components like diosgenin. In fact, the therapeutic window provided by the whole crude extract is lost when some of its individual components are used. This effect may not be limited to prostate cells in that potent anti-tumor effect of FE are also seen in a panel of pancreatic and breast cancer cell lines.

References


**Abbreviations**

- **PCa**: Prostate Cancer
- **FE**: Fenugreek Extract
- **MTT**: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
- **%I**: Percent Inhibition
- **PSA**: Prostate Specific Antigen
- **A**: absorbance
- **PI**: Propidium iodide
- **BCA**: Bovine Serum Albumin
- **BSA**: Bovine Serum Albumin
Fig. 1.
MTT assay showing effect of FE on proliferation of cancer and hTertPrEC cells in vitro. Dose-dependent cytotoxic effect on all pancreatic cancer cell lines (Fig. 1a), PCa cell lines, (Fig. 1b), and breast cancer cell lines (Fig. 1c), treated with FE. Dose dependent growth inhibition with the pure agents, diosgenin and sulforaphane, in both the PCa cells as well as the hTertPrEC cells (Figs. 1d and 1e). The effect of all the treatments are statistically significant as compared with the DMSO-treated control group (P < 0.01, unpaired t-test). *, P < 0.05
Fig. 2.
Effect of FE on cell cycle arrest of LNCaP and PC-3 cells *in vitro*. Incubation of LNCaP and PC-3 cells with 100ug/ml FE for 48h causes cell death and G2M phase cell cycle arrest of PC-3 cells (left) and cell death in LNCaP cells (right).
Fig. 3.
Double staining of cells with ethidium homodimer and calcein-AM. FE treatment is cytotoxic to PCa but not hTert-immortalised or primary prostate cells. Diosgenin and sulforaphane are cytotoxic to hTertPrEC and PrEC cells. Green and red staining represent viable and dead cells, respectively.

Cancer Biol Ther. Author manuscript; available in PMC 2011 May 16.
Fig. 4.
Western blotting for markers of effect in vitro. FE treatment caused down regulation of mutant p53 in DU-145 cells, inhibition of phosphorylation of EGFR in PC-3 cells and stimulation of phosphorylation of EGFR in hTert-immortalised prostate cells.
Western blotting for markers of effect in a simulated *in vivo* situation. TGF-β induced phosphorylation of EGFR in hTert-PrEC cells as well as PC-3 cells. This phosphorylation was inhibited in the presence of FE. Similarly, phosphorylation of Akt was induced by TGF-β in PC-3 cells, which was counteracted by FE. In PC-3 cells, p21 was up regulated by TGF-β and remains up regulated by FE.
Fig. 6.
Phospho-RTK Array with DU-145 and hTert-PrEC cells. Images of Phospho-RTK Array with DU-145 and hTert-immortalised prostate cells before and after FE treatment, and after treatment with diosgenin; DU-145 (left) and hTert-PrEC cells (right). The black dots indicate phosphorylation of receptors. The phosphorylation pattern for FE treated cells is distinct from the pattern for diosgenin treated cells. However, there is greater similarity within the cell lines treated with diosgenin.