IJBC 2009; 4: 139-146

Antineoplastic Effect of Fenugreek (Trigonella Foenum Graecum) Seed Extract against Acute Myeloblastic Leukemia Cell Line (KG-1)

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Abstract

Background: Treatment of cancer patients using conventional chemotherapy causes serious side effects and, at best, merely extends the patient's lifespan by a few years. The potential of alternative therapies may therefore be of great benefit in cancer control. The effect of Trigonella Foenum Graecum seed extract has been previously reported on some neoplastic cells. Here, its effect is evaluated on human acute myeloblastic leukemia cell lines.

Materials and Methods: The cell line KG-1 was treated with various concentrations of Fenugreek seeds extract with various durations. Cellular enumeration, viability test, staining and light microscopy, and apoptosis induction were evaluated.

Results: Results showed significant cytotoxic effect of Fenugreek seeds extract against this cell line which resulted in growth inhibition, cell death and morphological changes. Apoptosis induction was not considerable. Fenugreek seeds extract did not change the count and morphology of normal lymphocytes.

Conclusion: Applying herbal medicines could be an effective and safe treatment for leukemia. To our knowledge, this is the first study that suggests significant chemotherapeutic effects of Fenugreek seeds against these cell lines.

Keywords: Trigonella, Acute myeloblastic leukemia, Apoptosis.

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Introduction

Reports on cytotoxic activity of some herbal extracts against tumor cells are available.[1] Fenugreek (Trigonella Foenum Graecum) is an herb belonging to the family Leguminosea.[2] It is a widely grown plant in India, Egypt, and Middle East countries. The name Fenugreek comes from foenum-graecum meaning Greek hay.[3] Fenugreek seed, popularly known as Methi, is commonly used in Indian spices. Its use in the Ayurvedic system of medicine is well documented, and has been shown to possess a hypocholesterolemic effect in rats and dogs.[4,5] The extract of the seed is also useful for the treatment of diabetes and hypercholesterolemia in Ayurvedic (Indian), Unani (Arabic), and Chinese medicine.[6-8] There are some reports on using the extract of fenugreek seed as an anti-inflammatory agent in rats.[9] Induction of natural killer cell activity against tumor cells by fenugreek and some other wild plants from has been reported.[1]

Several compounds extracted from plants were reported to have antitumor activity and the ability to induce cell death and morphological changes indicative of apoptosis in leukemic cell lines CCRF-HSB-2 and HL-60,[10] but not in gastric cancer cell line KATO III.[11] Considerable attention has been focused on the sequence of events referred to as apoptosis, and the role of this process in mediating the lethal

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effects of antineoplastic agents in leukemia cells.[10,12] Apoptosis as a highly regulated process is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder with multiple fragments of 180-200 bp caused by inter-nucleosomal DNA cleavage.[13] The extraction of protodioscin (PD) from Fenugreek was followed by revealing inhibitory effects of PD on the growth of human leukemia HL-60 cells through apoptosis induction.[11]

Fenugreek protective effect against breast cancer development was observed using the DMBA-induced mammary tumor model in rats.[14] It is of interest to explore the possibility of using phytochemicals as chemopreventive/therapeutic agents.[15]

Furthermore, investigating biological effects of phytochemicals at cellular level provides the molecular basis for their anti-tumor effects, and helps establish the platform to produce more potent chemopreventive and chemotherapeutic agents.[16] Theoretically, cancer chemoprevention can be defined as an intervention in the carcinogenic process by a chemical that either blocks neoplastic induction or prevents transformed cells from progressing into malignant phenotype. It may also encompass a reversal of the process of progression.[14,17] Practically, a chemopreventive agent must enhance the physiological processes protecting humans against preneoplastic cell progression or neoplastic cell growth.[15] This background promoted us to look for the antitumor effect of Fenugreek seeds against leukemic cell line KG-1.

Materials and Methods

Leukemic cell line and cell culture

KG-1 cell (a myeloblastic cell line) was obtained from the bone marrow of a 59 year-old Caucasian male with acute myeloid leukemia and passaged eight times through new born Syrian hamsters. This cell line is supplied by the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran.

KG-1 cells were grown in RPMI 1640 medium (Sigma, USA) with 10% fetal calf serum (FCS) (Gibco, USA) at 37º C under a humidified atmosphere consisting of 95% air and 5% CO_2 for 48 hours.

Plant extract

Fenugreek seed was provided by Agriculture Department of Tehran University, and then extracted by percolation (lixiviation) method. The hydroalcoholic extract obtained was then filtrated with a non-pyrogenic filter (pore size: 0.2 µm, maximum pressure:7 bar) (Schleicher & Schuell, Germany) and distilled in vacuum. At this stage, a thick and sticky tar-like substance was produced. This extract was dried by further evaporation.

Preparation of lymphocyte cells (control)

Lymphocyte separation medium (3 ml) was aseptically transferred to a centrifuge tube, and diluted blood (heparinized blood: physiologic saline= 1:1) was layered over the lymphocyte separation medium in the tube. The tube was centrifuged at 400 g at room temperature for 20 minutes. The top layer of clear plasma was removed, and the lymphocyte layer was transferred to a new centrifuge tube. An equal volume of phosphate buffer saline (PBS) without Ca^{++} was added to the lymphocyte layer in the tube and centrifuged for 10 minutes at room temperature and 260 g. The precipitated lymphocytes were then washed again with PBS, and suspended in RPMI 1640 medium containing 10% FCS.

Count and viability of the cells

Following exposure of the extract on samples, lymphocyte (control) and KG-1 myeloid (test) tubes were centrifuged for 2 minutes at 1500 g. Cells were counted, after removing the supernatant, using hemacytometer. Counting was repeated with trypan blue (Sigma, USA) dissolved in normal saline (1/2 diluted), and the percentage of viability for each group was assessed.

LD50 determination

The KG-1 cells were treated with different doses of extract for 24 hours to determine the lethal dose by which 50% of cells are killed (LD50). Results showed that LD50 for these cells was 10 µg/ml. The viability test was also applied in different doses and incubation times, using trypan blue. According to the calculated LD50, tests were done with different doses of extract at various time intervals (6, 12, 24, 48 hours). Most doses were selected below LD50 to decrease direct toxicity effect. After optimization of methods, seed extract concentrations of 1, 2, 4, 8,

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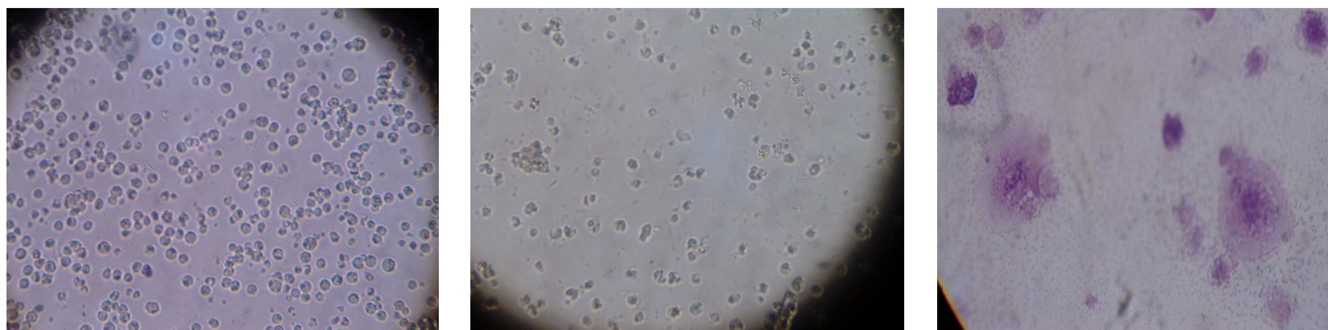

Figure 1. *(a) KG-1 cells in culture flask before treatment with extract (40X) are alive, sharp, and with a high count. (b) KG-1 cells in culture flask after treatment with 16.0µg/ml extract for 24 hours (40X) show a severe reduction in count. (c) KG-1 cells after treatment with 16.0µg/ml extract for 24 hours stained with wright-Geimsa (100X) demonstrate a severe reduction in count, and morphologic apoptosis markers including blebbing and changes in the cell membrane such as loss of membrane asymmetry, cell shrinkage, and nuclear fragmentation.*

Table 1. *Growing KG-1 cells cultivated and counted after exposure to plant extract in various concentrations and time intervals.*

Extract (µl)*	RPMI+10% FCS (µl)	Final dose (µg/ml)	Count/µl (24 hours)	Viability (24 hours) (%)	Dead cells (24 hours)	Apoptotic cells (24 hours) (%)	Count/µl (48 hours)	Viability (48 hours) (%)
0	1000	Control	210	88.3	12.7	9.4	290	91
0.5	999.5	1	210	88.3	6.7	5.4	273	86
1.0	999	2	180	87.5	12.5	9.5	190	83
2.0	998	4	160	88.7	11.3	7.6	135	72
4.0	996	8	150	90.0	9.5	9.5	110	78
8.0	992	16	140	75.0	25	11.0	87	61

* Concentration of consumed extract: 2000 µg/ml.

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and 16µg/ml and incubation times of 24 and 48 hours were selected for final test.

Staining and light microscopy

The smear from control and tested cells were prepared after incubation with Fenugreek seed extract, and stained with Wright–Giemsa. Cells were then studied microscopically to detect destroyed/apoptotic elements and other morphological changes. Processes of disposal of cellular debris which does not damage the organism differentiate apoptosis from necrosis.

Apoptosis detection by flowcytometry

We used Annexin V, a 35-36 kd Ca-dependent phospholipid binding protein with a high affinity for the membrane phospholipid phosphatidylserine (PS). One of the earliest features of apoptosis is the translocation of PS from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V. The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. Annexin V can be conjugated to biotin or to a fluorochrome such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), cyanine dyes (Cy5, Cy5.5, etc), and PE-Cy5 or PerCP-Cy5.5 and used for the flow identification of cells in the early stages of apoptosis.

In our study, we used Annexin V conjugated with FITC for the flowcytometric (Partec GMBH, Germany) identification of apoptotic cells. Since PS translocation also occurs during necrosis, Annexin V is not an absolute marker of apoptosis. It is

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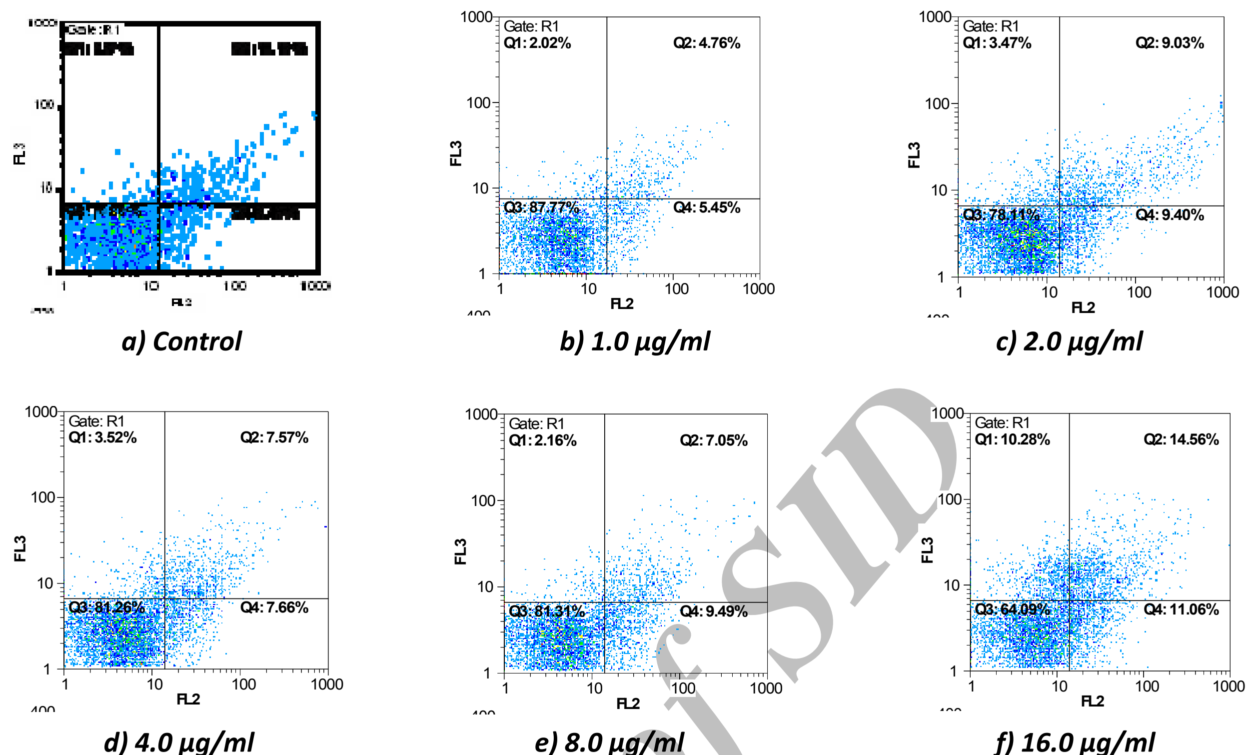

Figure 2. *Flowcytometric patterns of KG-1 cells treated by various extract concentrations. Bivariate annexin V-FITC (FL2: flourochrome 2): PI (FL3: fluorochrome 3) analysis of exponentially growing KG-1 cells after 24 hours culture in the absence (a) or presence of 1.0 µg/ml (b), 2.0 µg/ml (c), 4.0 µg/ml (d), 8.0 µg/ml(e), and 16.0 µg/ml (f) Fenugreek seed extract. The lower left quadrant contains the living population (double negative), the lower right quadrant contains the apoptotic population (annexin V+: PI-), the upper right quadrant contains dead cells (annexin V+: PI+), and the upper left quadrant contains damaged cells (annexin V-: PI+).*

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therefore used in conjunction with vital dyes such as 7-amino-actinomycin (7-AAD) or propidium iodide (PI). Such vital dyes bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached as occurs in the later stages of apoptosis or in necrosis. In fact, PI was used to differentiate necrosis from early steps of apoptosis. Cells positive for both PI and Annexin V were considered necrotic, and cells positive for Annexin V were considered apoptotic.

Results

Toxicity of Fenugreek extract and assay for growth inhibition

Normal lymphocytes were treated with the hydroalcoholic extract of Fenugreek with various concentrations ranging from 1.0µg/ml to 16µg/ml for 48 hours. The treatment did not show any toxic effect, neither in terms of morphological changes nor in the cell count.

Exponentially growing KG-1 cells were seeded at 4-5x10^5 cells/ml in a tissue culture flask and cultivated in the presence of a control, or Fenugreek extract. After cultivating for certain times, cells were counted by a hemocytometer. Presence of different concentrations of Fenugreek seed extracts led to growth inhibition and cell death. Although the effect of extract increased with increasing doses and incubation times, it was not increases in a dose-dependent manner. Apoptosis rate was not linear either. The results are shown in table 1 and figure 1(a, b, and c).

Induction of apoptosis

The significant growth inhibitory activities of Fenugreek extract led us to investigate the

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induction of apoptosis. Morphological changes included apoptotic body, fragmentation of nucleus, and vacuolization in some cells. However, evaluation of apoptosis induction by Annexin V-FITC kit showed that apoptosis occurs only in a few cells (figure 2).

Data analysis

Based on the result of Kolmogorov-Smirnov (KS) test, the distribution of data was normal. To assess correlation of dosage and cell count, correlation coefficient and ANOVA tests were used (0.854, 0.030 respectively). It showed that the two variables had significant correlation. Totally, with the probability of 95% the difference between the results was significant (p-value <0.05).

Discussion

Trigonella Foenum Graecum has primarily been introduced as a cholesterol-reducing plant, as well as an anti-hyperglycemic herb in humans in laboratory animals.[4,5,18-20] Some reports have described the overall stimulatory effect of Fenugreek on specific and non-specific immune functions of mice.[21] Various herbal chemicals are extracted from Fenugreek. Fibers, flavonoids, polysaccharides, and saponins are the main chemical constituents of T. Foenum Graecum.[22,23] One or more of these constituents may have mitogenic effects leading to stimulatory effects on the immunocompetent cells. Some of the constituents also possess antioxidant properties, and they may induce immunostimulant effects.[24-30]

Apoptosis is a type of cell death, so called programmed cell death. In fact the agents capable of inducing apoptosis in tumors have the potential to be used for anti-tumor therapy. The apoptosis-inducing activities of flavonoids have been identified in several studies.[11,31,32] The effect of Fenugreek extract on several noplastic diseases was investigated. Flavonoids and catechins were shown to be apoptotic in lung tumor cell lines,[33] human carcinoma cells,[34] colon and prostate cancer cells,[35] breast cancer cells,[15,35,36] stomach cancer cells,[37] brain tumor cells,[38] head and neck squamous carcinoma,[39] leukemia,[10,11] cervical cancer cells,[40] and melanoma.[41] It appears that these flavonoids can also differentially induce apoptosis in cancer cells, but not in their normal counterparts.[42]

Annexin V recognizes the early stages of apoptosis, based on exposure of PS on the cell surface. According to table 1 and figure 2, number of dead cells after 24 hours treatment with the extract is much higher than number of apoptotic cells. Therefore we think mechanisms other than apoptosis may cause growth inhibition and cell death. Alternative mechanisms of cell death have been shown in epithelial cells, including autophagy and para-apoptosis.[43-45] A process called type II cell death may contribute to the programmed cell death. The mechanism can be activated in parallel with apoptosis. There is considerable correlation between the apoptotic and alternative death pathways.[46]

Sur and colleagues evaluated antineoplastic effect of Trigonella Foenum Graecum seed extract in the Ehrlich Ascites Carcinoma (EAC) model in Balb-C mice. Intraperitoneal administration of the alcohol extract of the seed led to more than 70% inhibition of tumor cell growth compared to the control.[47] Their results were consistent with ours, however, they could also show the anti-inflammatory effect of the extract because their study was performed in mice (in vivo).

Jayadev et al indicated the preventive effect of dietary Fenugreek seed and its major steroidal saponin constituent, diosgenin, on azoxymethane-induced rat colon carcinogenesis during initiation and promotion stages. They also indicated that diosgenin inhibits cell growth, and induces apoptosis in the HT-29 human colon cancer cell line in a dose-dependent manner. Furthermore, diosgenin induced apoptosis in HT-29 cells, at least in part, by inhibition of bcl-2 and by induction of caspase-3 protein expression. On the basis of these findings, Fenugreek constituent, diosgenin, seems to have potential as a novel colon cancer preventive agent.[48] The inconsistency of our results with this study might be related to the different cell line and extract type used in our work.

The current study suggests that T. Foenum Graecum has appreciable anticancer activity. In our in-vitro study, we showed that count of dead cells and apoptotic cells is increased in parallel with increasing extract concentration. More studies are needed to investigate the possible effects of Fenugreek extract in cancer treatment. Administration of Fenugreek is simple, since its

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seeds and leaves are used as common dietary constituents in many parts of the world. The most effective anticancer constituent of T. Foenum Graecum is not known yet. However, based on the published studies, flavonoids seem to be the most likely candidates eliciting antitumorigenic effects.[31,41] We suggest using other evidences of apoptosis such as DNA fragmentation to detect the potential apoptotic effect of the plant.

Further investigations are underway to uncover the molecular mechanisms mediating the anticancer effects. Akt signaling is a highly active pathway in most malignancies. In 2006, Bharat and Shishiar mentioned that diosgenin suppresses activation of Akt pathway.[49] In 2009, Srinvasan and colleagues evaluated the effect of diosgenin on Akt signaling and its downstream targets (NFkB, Bcl-2, survivin and XIAP), and showed its ability to inhibit Akt signaling. Additionally, they found that diosgenin caused G1 cell cycle arrest, inhibition of cell proliferation, and induction of apoptosis.[50]

Fenugreek seeds contain components such as saponins (diosgenin, yamogenin), alkaloids (trigonellin), unsaturated oils, and proteins (tryptophan and 4-hydroxy isoleucine). Further studies are underway to isolate and characterize the Fenugreek's active ingredients that contribute to its preventive effects on cancers. A strategy to selectively induce apoptosis of leukemia cells without altering healthy cells is a major goal, and we think that fenugreek may have a great potential to achieve this goal.

Acknowledgements

We appreciate the assistance of Reza Safdari, Mohamad Kazem Sharifi Iazdi, and Mohamad Akrami in performing this research. We acknowledge the financial support provided by the Faculty of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran.

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