INVESTIGATION OF ANTINEOPLASTIC EFFECTS OF TRIGONELLA FOENUM GRAECUM SEEDS EXTRACT ON SOME ACUTE LEUKEMIC CELL LINES

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ABSTRACT

Cancer is second cause of mortality all over the world. Treatment of the patients with cancer always is a concern because of serious side effect of the usage conventional chemotherapy. This treatment at best, extend their lifespan for few years, the potential of the alternative therapies may therefore be of great advantage in cancer control. Previously, the effect of Trigonella Foenum Graecum (Fenugreek) seed extract has been reported on some neoplastic cells. We study the effect of seed extract on myeloblastic leukemia (KG-1) and acute lymphoblastic leukemia (CCRFHSB-2) cell lines.

In this study these two cell lines were treated with different doses of the seed extract for different duration. The results were evaluated after 24–48 hours of incubation by cell count, viability, staining and light microscopy. Also, we evaluate the induction of apoptosis by using Annexin V – FITC flowcytometry kit. We demonstrated that fenugreek seeds extract has a significant cytotoxicity effect against these two cell lines. We observed a significant change in morphology in addition to effect on growth inhibition and cell death. Apoptosis induction by this extract was not considerable. Fenugreek seeds extract don’t change the count and morphology of normal lymphocyte as a control. The findings demonstrated the anti-neoplastic effect of the Trigonella on the cancerous cell lines.

Keywords: Fenugreek, acute lymphoblastic leukemia, acute myeloblastic leukemia, apoptosis

INTRODUCTION

It has been reported that medical herbs extracts which have been used in folk medicine, have cytotoxic activity(1). Fenugreek (trigonella foenum graecum) is an annual herb belongs to the family of leguminosea (2)which is widely grown in India, Egypt and middle east countries. The name fenugreek comes from, foenum graecum which means Greek hay (3). Fenugreek seeds, popularly known as “Methi”. These seeds in common used as a ingredient in Indian spices. It is, also, use in Ayurvedic system of medicine. It has been shown that this seeds have a hypocholesterolemic effect in rats and dogs (4, 5). Moreover, the extract of the seed is useful for the cure of diabetes mellitus and hypercholesterolemia in Ayurvedic (India), Unani(Arabic) and Chinese medicine (6, 7). It has been published that fenugreek extract has an anti-inflammatory activity in rats (8). The fenugreek seed extract evaluation of natural killer cell activity against tumor cells has been reported (1).

It has been reported that several extractions from plants have anti-tumor activity and the ability to induce cell death. The efficacy of anti-tumor
compounds seems to be related to propensity of tumor cells to respond to these compounds, by apoptosis (9). Many considerable attention has been focused on the sequence of events which referred to apoptosis and the role of this process in mediating the lethal effects of anti-neoplastic agents in leukemic cells. Apoptosis, which is the programmed cell death, is a highly regulated process that is characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of DNA ladder with multiple fragment with the size of 180-200bp which is caused by inter-nucleosomal cleavage (10). It has been declared that the extraction of Protodioicin (PD) from fenugreek was followed by showing inhibitory effects of PD on the growth of human leukemic cell line HL-60, Human promyelocytic leukemia cells, which causes by the induction of apoptosis. Trigonella protective effect against breast cancer development was demonstrated by using DMBA-induced mammary tumor models in rats (11). Chemo-protection research is to advance knowledge in identifying and characterizing entities that might reduce the risk of the human population developing cancer (12). So, it is of interest to explore the feasibility of using phytochemicals or other dietary compounds as chemo-protective agents (13).

Moreover, studying the biological influence of these phytochemical at cellular level provides the molecular basis for their anti-neoplastic effect, and helps to provide a platform to produce more potent chemo-protective and chemotherapeutic elements (14). Theoretically, a chemo-protective element can be defined as an intervention in the carcinogenic process by chemical which either blocks induction of neoplastic process or prevents transformed cells to progress into malignant phenotype. It may also induce a reversal of process of progression (15,16). Practically, a potential intervention chemical agent must increase the physiological process protecting humans against, pre-neoplastic cell progression or neoplastic cell growth.

This background promoted us to study the anti-neoplastic effect of fenugreek seed extracts against leukemic cell lines CCRF-HSB and KG-1.

METHODS AND MATERIALS:

Plant Extraction

Fenugreek seeds were supplied by agriculture department of Tehran medical university and then extracted by percolation (Lixiviation) method. The hydroalcoholic extract was obtained then filtered by a non pyrogenic 0.22 μm filter (Schleicher and Schuell Germany) and distilled in vacuum. At this step, the extract was dried by further evaporation and dried weight of the extract was 90%.

Leukemic cell line and cell culture

KG-1 (a myeloblastic cell line) and CCRF-HSB2 (the T lymphoblastoid cell line), which respectively was obtained from the bone marrow of a 59 years old Caucasian male with acute myeloid leukemia and the peripheral blood of an 11 years old boy with acute lymphoblastoid leukemia, were purchased from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. Both cell lines were cultured in RPMI 1640 (Sigma,USA) with 10% fetal Bovine serum (Gibco) at 37°C and 5% CO2 and 95% humidity. These cells passaged every 48 hours.

Preparation of human lymphocyte cells

Lymphocyte separation medium (3 mL) was aseptically transferred to a centrifuge tube and the diluted blood (heparinized blood: normal saline =1: 1) was layered over lymphocyte separation medium in the tube. The tube was centrifuged for 20 min at 400xg at room temperature. The upper layer of the clear plasma was removed and the lymphocyte layer was transferred to a new tube. An equal volume of Phosphate buffer saline (PBS) was added into the lymphocyte layer in tube and centrifuged for 10 min at room temperature. After centrifugation, the precipitated lymphocytes were washed again with PBS and suspended in RPMI 1640 supplemented with 10% FBS. These lymphocytes were used as a control.

Count and cell viability assay
Following exposure of the cells to seed extract, the lymphocyte and cell lines were centrifuged at 1500 rpm for 2 minutes. Then, cells were counted by using hemacytometer. Also, cells viability was analyzed by trypan blue (1:2 diluted) and the percentage of viability for each cell line and control was reported.

**LD50 determination**

The KG-1 and CCRF-HSB-2 cell lines were treated with different doses of fenugreek extract for 24 hours to determine the lethal dose by which 50 % of cells were killed (LD50). It was shown that LD50 for KG-1 and CCRF-HSB-2 cell lines were 10µg/ml and 7.5µg/ml respectively. The viability test was also applied in different doses and various incubation times by trypan blue to show the percent of viable cells. According to the calculated LD50, tests were applied in various doses and different time intervals (6,12,24,48 hours ). After optimization we showed that the best extract concentration and incubation times for KG-1 was 0.5,1,2,4,8µg/ml and 24 – 48 hours, respectively. These parameters for CCRF-HSB-2 cell line was 0.5,1,2,4µg/ml and 24 – 48 hours.

**Staining and light microscopy**

The prepared smear of lymphocytes was stained with Wright-Giemsa and then the microscopic observation was done to detect destroyed cells, apoptotic cell and other morphological changes.

**Apoptosis detection by flowcytometry**

Apoptosis is characterized by a series of morphological changes such as loss of membrane symmetry and attachment, condensation of the cytoplasm and nucleus, in addition to inter-nucleosomal cleavage of DNA. To detect the apoptosis, 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 signs of apoptosis is the translocation of PS from the inner to the outer leaflet of the plasma membrane. After exposure to the extracellular environment, binding sites on PS become available for Annexin V. Other apoptotic events such as loss of plasma integrity, DNA fragmentation and chromatin condensation happens following the translocation of PS. Annexin V have the ability to 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 flowcytometric identification of cells in the early stages of apoptosis. In this study we used annexin V conjugated with FITC. As, PS translocation also occurs during necrosis, Annexin V by itself is not an absolute marker for detection of apoptosis. Hence, annexin V is often used in conjugation with vital dyes such as 7-Amino-Actinomysin (7-AAD) or Propidium Iodide (PI), which bind to nucleic acids, but only could penetrate the plasma membrane when plasma membrane integrity branched as happens in the late stages of apoptosis or necrosis. Double positive cells for PI and Annexin V were considered as necrotic, and cells which were positive for Annexin V count take into count as apoptotic.

**RESULTS:**

**Toxicity of Fenugreek extract and growth inhibition assay**

Normal lymphocytes were treated with hydroalcoholic extract of fenugreek with various concentrations ranging from 1µg/ml to 16µg/ml for 48 hours. The treatment did not show any toxic effect. Any changes have not observed in morphology and cell count.

In an exponential manner growing of both cell lines were seeded at 4-5×10⁷ cells/ml in a tissue culture flask and cultivated in the presence of a control, or fenugreek extract. After cultivating for specified time, cells were counted by a hemacytometer. Presence of different concentrations of fenugreek seeds extract causes growth inhibition and cell death. All the data were shown in Tables 1, 2.
Table 1. Cell count and viability of CCRF-HSB-2 cells after 24 and 48 hours of incubation with various dosages of fenugreek seed extract

Table 2. Cell count and viability of KG-1 cells after 24 and 48 hours of incubation with various doses of fenugreek seed extract

Induction of apoptosis

The significant growth inhibitory activities of Trigonella extract guided us to explore the induction of apoptosis. Morphological changes included apoptotic body, fragmentation of nucleus, and vacuolization in some cells. Although, evaluation of apoptosis occurs only in few cells (Figure 1, 2).

A)
Figure1.

A) (1) CCRF-HSB-2 cells in culture flask before treatment with extract (40X). (2) CCRF-HSB-2 cells in culture flask after treatment with 4.0 μg/ml of extract for 24 h (40X) . (3) CCRF-HSB-2 cells after treatment with 4.0 μg/ml extract for 24 h, cell destruction and vacuolization is obvious (100X)

B) (1)KG-1 cells in culture flask before treatment with extract (40X) are alive, sharp, and with a high count. (2) KG-1 cells in culture flask after treatment with 16.0μg/ml extract for 24 hours stained with wright- Geimsa (100X) demonstrate a severe reduction in count. (3) KG-1 cells after treatment with 16.0μg/ml extract for 24 hours stained with wright- Geimsa (100X) show a severe reduction in count. (3) 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.
**Figure 2.** Flowcytometric patterns of CCRF-HSB-2 (A) and KG-1 (B) cells treated by various extract concentrations. Bivariate annexin V-FITC (FL2: fluorochrome 2); PI (FL3: fluorochrome 3) analysis of exponentially growing KG-1 cells after 24 hours culture in the absence (control) or presence of different concentrations of 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+).
Data analysis

The collected data was analyzed with SPSS software. The Kolmogorov-Smirnov (KS) test, the distribution of data was normal. To assess correlation of dosage and cell count, correlation coefficient and ANOVA test was used. For KG-1 the results was 0.854 and 0.030, respectively. This analysis for CCRF-HSB-2 was 0.966 and 0.007. It is shown that the two variables had significant correlation. Absolutely, with the probability of 95% the difference between the results was significant (P<0.05).

DISCUSSION:

Fenugreek has mainly been introduced as a cholesterol-reducing agent. Also, this herb had an anti-hyperglycemic effect in humans and laboratory animals (4,5,15-18). Some of the documents have described the extensive stimulatory influence of fenugreek on specific and non-specific immune functions of mice (19,20). The main chemical components, which are extracted from fenugreek, are fibers, flavonoids, polysaccharides, and saponins (21,22). One or more of these components may have mitogenic influence which causes stimulatory effects on the immunocompetent cells. Also, some of these elements have antioxidant properties which may induce immunostimulant effects (23-29). Apoptosis is a programmed cell death. In fact the agents, which have the ability to induce apoptosis in tumors, could be used for anti-tumor therapy. The apoptosis inducing activities of flavonoids have been reported in several studies (9,30,31). The effect of fenugreek seed extract was investigated on multiple neoplastic diseases. It was shown that flavonoids and catechins had apoptotic effect in lung tumor cell lines (32), human carcinoma cells (33), colon and prostate cancer cells (34), breast cancer cells (13,34,35) stomach cancer cells (36), brain tumor cells (37), head and neck squamous carcinoma (38), leukemia (9,12), cervical cancer cells (39), and melanoma (40). Also, it seems that these flavonoids can distinctively induce apoptosis in cancer cells, but not in their normal counterparts (41). Annexin V recognizes the early stages of apoptosis, based on exposure to of PS on the cell surface. Under the data which shown in table 1 and figure 2, number of dead cells after 24 hours treatment with the extract is much more higher than number of apoptotic cells. So, we think that they are may be other mechanisms which may lead to growth inhibition and cell death. Alternative mechanisms of cell death have been shown in epithelial cells including autophagy and para-apoptosis (42-44). A process called type II cell death may help to cause the programmed cell death. The mechanism can be activated in parallel with apoptosis and there is a considerable mutual relation between the apoptotic and alternative death pathways (45). The current study suggest that fenugreek has appreciable anticancer activity. In our in-vitro study we showed that count of dead cells is increased in parallel with increasing extract concentration. However, based on published studies, flavonoids appear to be most likely candidates eliciting anti-tumorigenic effect. Administration of trigonella to man is simple, since its seeds are used as common dietary constituents in many parts of the world.

REFERENCE


