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The Effects of Fenugreek on Radiation Induced Toxicity for Human Blood T-Cells in Radiotherapy

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ABSTRACT

Many cellular damages either in normal or cancerous tissues are the outcome of molecular events affected by ionizing radiation. T-cells are the most important among immune system agents and are used for biological radiation dose measurement in recommended standard methods. The herbs with immune modulating properties may be useful to reduce the risk of the damages and subsequently the diseases. The T-cells as the most important immune cells being targeted for biological dosimetry of radiation. This study proposes a flowcytometric-method based on fluorescein isothiocyanate- and propidium iodide (PI)-labeled annexin-V to assess apoptosis in blood T-cells after irradiation in both presence and absence of fenugreek extract. T-cells peripheral blood lymphocyte isolated from blood samples of healthy individuals with no irradiated job background. The media of cultured cells was irradiated 1-h after the fenugreek extract was added. The number of apoptotic cells was assessed by annexin-V protocol and multicolor flowcytometry. An obvious variation in apoptotic cells number was observed in presence of fenugreek extract (>80%). The results suggest that fenugreek extract can potentiate the radiation induced apoptosis or radiation toxicity in blood T-cells ($P < 0.05$).

Key words: *Apoptosis, fenugreek, flowcytometry, Herbal drug, lymphocyte, T-cells*

INTRODUCTION

Apoptosis or programed cell death is a normal physiological process to eliminate damaged or unwanted cells. This ability can compensate the number of cells, enabling healthy tissues to maintain homeostasis.[1-3] Dysfunction in this process is in favor of cell proliferation in cancerous tissue, where the disregulation results in cancer onset or progression.

Most approaches in cancer treatment employ cytotoxic methods induce apoptosis on malignant cells.[4,5] Ionizing radiation causes damages to normal or cancerous tissues through a series of similar molecular events. Eighty percentage of the human tissues is water, so the major damages of radiation are because of the free radicals of water. These free radicals interact with cellular macromolecules such as DNA and cause cell dysfunction and mortality.[6-8] It takes place in tumor as well as normal cells. Intensity of the damages depends on several factors such as the presence of oxygen, sulfhydryl compounds, enzymes, and some herbal component.[9-12] In spite of the extensive use of herbal therapies, there is insufficient scientific evidence proving their efficacy and safety. Thus, the basic researches aimed for elucidating the mechanisms underlying every tentative herbal effect. The results are very important if herbal medication as be considered an alternative or complementary medication to treat the diseases such as cancers.[13-15] Many studies are investigating the capability of herbal drugs to substitute for some of natural anticancer agents such as vincristine and vinblastine from *Catharanthus roseus*, taxol, and docetaxel from *Taxus brevifolia* to replace them with conventional treatments of various types of cancer and other acute diseases.[16-22] The regulation of T-cell activation requires antigen specificity and a great amount of cytokines in immune responses are released from T-cells.[23] Because of these properties and some other T-cells have been considered as the most important cell of the immune system.[11] Researchers are interested in various herbs that possess immune modulating properties expecting that they can reduce the risk of various immune systems diseases and cancers.[10] Fenugreek (*Trigonella foenum*) which is called "Shanbalile" in Persian is cultivated all over Iran and other countries in the world is used as herbal medicine in many parts of the world.[12,24,25] Hence, this study intends to investigate the effects of fenugreek extract on radiation toxicity by evaluating radiation induced

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apoptosis of peripheral blood T-cells and measure the ability of the extract to induce or suppress apoptosis in blood cells when one radiotherapy session dose of radiation exerted to primary *in vitro* cultured T-cells and fenugreek extract exist in cellar milieu. This study demonstrated that fenugreek extract exhibits radiation induced apoptosis enhancing activity toward T-cells, and T-cells are more susceptible to cytotoxic effects of radiation in the presence of the extract.[14]

MATERIALS AND METHODS

The study was carried out with all medical ethics consideration. All experiments were performed on fresh heparinized human blood taken from 15 healthy adults who were aware of the study and freely consent to incorporate.

Cell culture and irradiation were done by standard preparation strategies previously described in International Atomic Energy Agency Technical Publication.[26] All blood samples were diluted with a ratio of 1:1 in phosphate buffer saline (PBS) (Sigma–Aldrich GMBH Munich, Germany) and peripheral blood lymphocytes (PBLCs) were separated by density gradient centrifugation using Ficoll-Paque Plus (Sigma–Aldrich GMBH Munich, Germany).[27] The isolated cells were added to Roswell Park Memorial Institute Medium 1640 culture media that was enriched by fetal bovine serum, glutamine, antibiotics, and phytohematoglutinin [Figure 1]. The cultured cells were incubated at 37°C in a 5% CO_2 atmosphere.

Fenugreek extract was purchased from the local herbal market boiled and sterilized by dry autoclaving and cooled down preparing to use in culture media before irradiation.

The enough reproduction of cells for the irradiation took place after 10 days.

At this time, every sample cells suspension was split into five portions in a 6-well culture plate in order to add the extract in five different concentrations to each one.

Irradiation was performed at a dose rate of 82.46 cGy/min for 2 Gy dose of gamma ray of cobalt unit (Theratron Phoenix, Ottawa, Canada) in the Radiotherapy Department of Seyed Al-shohada Hospital in Isfahan [Figure 2], while samples were on a tissue-equivalent plate. A gas chamber dosimeter (Farmer 2570 Nuclear Enterprises, Zurich, Switzerland) and standard protocol was used for physical dosimetry.[26]

After 24 h incubation, the cell suspensions were centrifuged at 1500 rpm for 5 min at room temperature. The cell pellet was resuspended in approximately 200 µl of the remaining solution. Four milliliter of Becton–Dickinson (BD) buffer solution diluted with a ratio of 1:10 in double-distilled water was added to the suspensions, and left for 15 min at room temperature. The cells were then centrifuged at 1500 rpm for 5 min, the supernatant was removed, and fluorescein isothiocyanate (FITC) -conjugated annexin-V (BD, Basel, Switzerland) was added to the suspensions. Following the final round of centrifugation and aspiration, the cells were resuspended in 0.5 ml of phosphate buffer (BD) containing 5 µl of propidium iodide (PI) stock (1 mg/ml in PBS) and incubated at room temperature for 5 min in order to stain the DNA. Annexin-V protocol was done as kit producer recommended (BD Pharmingen™, www.eBioscience.com, eBioscience, Inc. 10255 Science Center Drive San Diego, CA 92121 USA).[28] The cells were subsequently measured by flowcytometry. Forward and side light scattering and stain-induced fluorescence were simultaneously measured at two different wavelengths (530 nm, green and 640 nm, red). The forward scatter signal is proportional to the cell size, whereas the side scatter signal is proportional to cellular granularity (intracellular superstructure). Using these four parameters it was possible to discriminate the three main types of leucocytes and gate the lymphocytes to acquire the more information [Figure 3a-d].[27]

A two-dimensional dot plot of annexin-FITC fluorescence on the basis of DNA content was used for quality control.

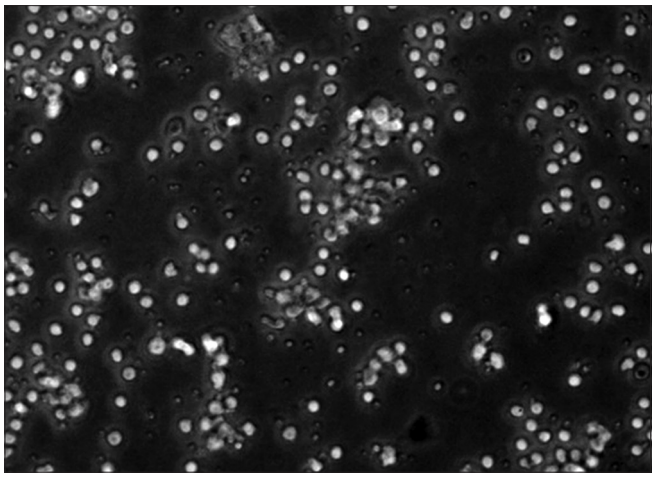

Figure 1: Extracted lymphocyte under microscope vision

Figure 2: Irradiation of samples on a standard protocol

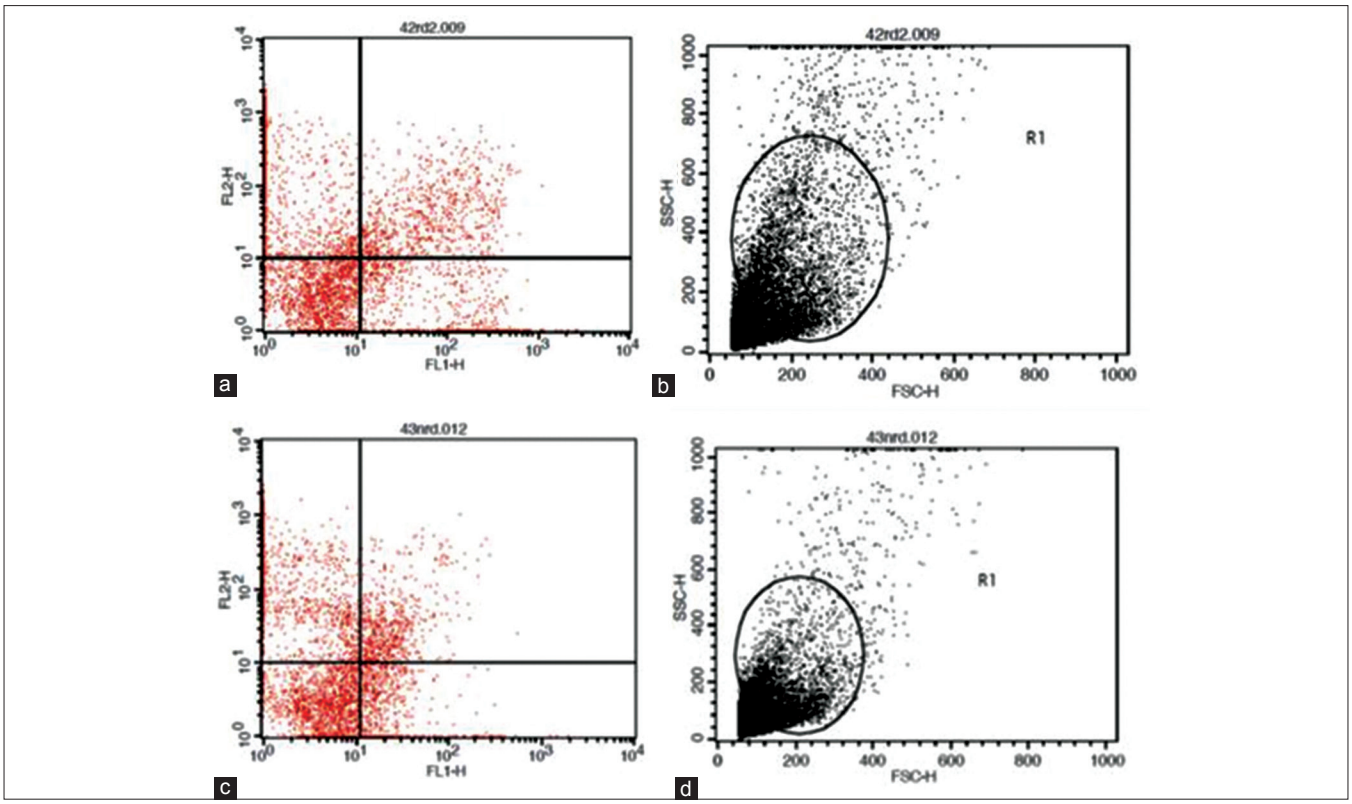

Figure 3: (a) Two parameter dot plot for irradiated cells. (b) Gated zone for appropriate irradiated cells. (c) Two parameter dot for unirradiated cells (d) Gated zone for appropriate irradiated stained cells

RESULTS

The range of the ages and its distribution of fifteen healthy blood donors, without radiotherapy history and continuous toxic chemical or physical agents contact were presented graphically in Figure 4. As it's obvious, the distribution follows a normal distribution model

FITC conjugated assay was used for determining apoptosis in T-cell lymphocytes by flowcytometry. Figure 3 shows how this population was identified. The population displaying high cellular DNA content and high FITC positivity contains the normal, FITC-positive cells, and the population with high cellular DNA content, but low Annexin-FITC positivity contains other lymphocytes. Below these two clusters the two further populations were observed, both with reduced DNA content; these are the apoptotic cells and the population with the lowest DNA values and low FITC positivity represents the debris [Figure 3b].[28,29] The identification of apoptotic cells in this method is depend on DNA content, so the population with reduced DNA content and FITC positivity were selected to gate for more information. Before main measurement, the background apoptosis without fenugreek extract in culture media were measured. The measured apoptosis of cells without irradiation and with 2 Gy dose of radiation presented in Table 1.

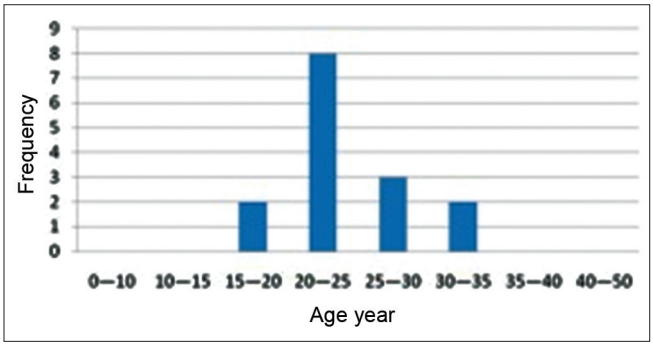

Figure 4: Age distribution of sampled volunteer

Some wells of cell suspension without any drug used as control. The results confirmed by microscopic speculation in every period of assessment. The results presented in Table 2.

DISCUSSION

Fenugreek has been used not only for food and condiment but also for medicinal purposes for ages. The study results suggests that radiation-induced apoptosis of cell is potentiated by fenugreek herbal component in the cellular milieu,[9-12] and T-cells are more susceptible to the cytotoxic effects of radiation in presence of the extract. However, some confounder such as age, radiation dose, and pure toxic

effect of the extract on cells exist that should be discussed. Net apoptotic effect of fenugreek extract in cited references are discussed.[16-21]

To study relation of apoptosis rate to age of subjects' age and apoptosis rate correlation for 2 Gy of gamma radiations was examined. Correlation coefficient was <0.2 for all of all doses exerted. Therefore, it suggests no relation between age and radiation induced apoptosis exist.

Cells were treated with 10, 20, 30, 40 μg/ml of fenugreek extract before irradiation.

There was a significant increase (>80%) in radiation induced apoptosis when cells were treated with fenugreek extract. The *t*-test assay indicated significant difference between radiation-induced apoptosis in cell culture with fenugreek extract treatment and radiation-induced apoptosis in cell culture without fenugreek extract treatment ($P < 0.03$). Therefore, it can be resulted fenugreek extract significantly enhances radiation induced apoptosis [Figure 5]. Increasing fenugreek extract concentrations from 10 μg/ml not appeared to cause more apoptosis increase, indicating that apoptosis in the these concentrations occurred in a nonconcentration-dependent approach or variation occurred in very smaller amount of extract that suggests this effect should be surveyed at more accurate experiment. Some sign of media clearance same as antibiotic and anti-fungic effect was seen under microscopic speculations but not confirmed with later experiments.

Table 1: Background apoptosis without fenugreek extract

Radiation dose	0 Gy	2 Gy
1	6	14
2	7	9
3	6	8
4	8	10
5	4	9
6	3	10
7	4	14
8	6	13
9	0	14
10	3	9
11	5	11
12	4	10
13	6	7
14	6	13
15	4	12
Average	4.81	10.875

Table 2: Apoptosis percentage after adding four dose of extract to culture media and irradiation

Dose of extract	10	20	30	40
1	23	28	20	25
2	22	22	16	24
3	24	23	15	20
4	25	26	19	22
5	16	24	15	25
6	19	22	17	20
7	18	29	20	25
8	17	24	16	20
9	25	21	14	19
10	26	26	18	23
11	18	23	17	20
12	19	29	15	23
13	18	24	18	24
14	21	27	19	21
15	17	28	16	19
Average	20.5	25.125	17	22
SD	3.26	3.72	2.41	3.42

SD – Standard deviation

Annexin-V binding is a well-established marker for apoptosis and a potentially attractive biomarker for identifying radio toxicity in radiotherapy subjects and radiation accident victims.[3,4] Binding of annexin-V to PBLCs increases as samples stored before analysis, so increase the apoptosis counted results.[4] Besides, it observed that time delay after irradiation and before flowcytometry positively effects on the result of annexin assay.

It suggests that measured apoptosis strongly changes for different storage and holding conditions specially relay time of storage in binding buffer. This time relation of apoptosis yields blurring the difference between results and leads to a significant decrease in the stability of this assay as a diagnostic test for measuring radiation-induced apoptosis. In this regards, it is suggested that the results should be evaluated with some different methods to confirm these findings for next steps of conclusion and usage.[30,31] Several authors have studied the effects of fenugreek on living systems. The Zargar study provided the evidence indicating the therapeutic effect of the extract prepared from the dried seeds of fenugreek on an animal model of hepatotoxicity and on cell proliferation and has implications in finding a treatment for liver cirrhosis by a natural herbal drug with

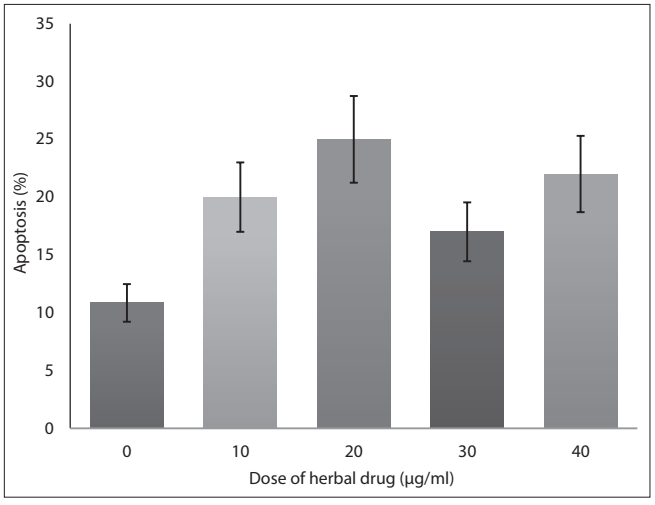

Figure 5: Relation of apoptosis to dose of fenugreek extract concentration

no side effects.[32] Gopal *et al*. study has been focused on the anti-leukemic properties of fenugreek, as a herb with proven anti-diabetic, antitumor, and immune-stimulating functions. They tested five fenugreek varieties of seed extracts on B-cells. Their study results indicated that there is a good degree of potentiality of anti-leukemic activity of all five fenugreek germplasms. This report showing fenugreek as an apoptosis inducer on B-cells, hold a great promise in the future treatment of leukemia.[33] The effect of extracts of fenugreek seeds on cell proliferation of breast cancer cells. MCF-7 was studied by MTT assay at a concentration range of 20–320 μg/ml by Sreeja *et al*. They resulted that extracts of fenugreek seeds stimulated the proliferation of MCF-7 cells.[34]

Xue *et al*. study showed that fenugreek extract can lower kidney/body weight ratio, blood glucose, blood lipid levels, and improve humorological properties in experimental diabetic rats following repeated treatment for 6 weeks.[35] On the basis of Raju *et al*. findings, the fenugreek constituent seems to have potential novel colon cancer preventive effects.[36]

Rosioru *et al*. papers' reported an investigation of hepatoprotective action of fenugreek seed flour in ethanol intoxicated rats. Good effects were obtained especially for WBC count and enzymatic activities, and specially with the higher fenugreek dose.[37] Alizadeh *et al*. treated the cell line KG-1 with various concentrations of fenugreek seeds extract with various durations and evaluated cellular enumeration, viability test, staining and light microscopy, and apoptosis induction. Their 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 seed extract did not change the apoptosis count and morphology of normal lymphocytes. Applying herbal medicines could be an effective and safe treatment for leukemia. This is the first study that suggests significant chemotherapeutic effects of fenugreek seeds against these cell lines.[38]

CONCLUSIONS

This study demonstrated that fenugreek extract exhibits apoptosis enhancing activity and T-cells are more susceptible to the cytotoxic effects of radiation in presence of the extract. The results of this study are considerable and they can be so practical that deserve more other studies to complete findings and confirm for applying the fenugreek extract in cancer treatment or other therapies.

To validate these results it's required to ensure reliability of these findings using a second, independent cohort. This would also allow elimination of potential unwanted confounders, such as apoptosis rate of cells at fenugreek extract media and radiation dose-dependence that's not included in the current study. Ultimately, the assay of measuring lymphocytes sensitivity in fenugreek extract should be done before any decision in radiation treatment and the impact of extract on cellular apoptosis or even more important, the nature of possible long-term toxicity of that should be documented. If these finding be confirmed by second independent assay with enough reliability both *in vitro* and *in vivo* experiments, the extract can be used as a radio sensitizer or modifier for practical applications such as transplantation, whole body irradiation, or as an agent for reducing side effects in patients who are sensitive to radiation.

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