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The Modulating Effect of Carumopticum on Human Peripheral Blood T-Cells Radiation Toxicity

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Herbs that possess immune modulating properties may be useful in reducing the risk of various radiation induced outcome in radiation therapy patients. The carumopticum extract has now been investigated for its effects on radiation induced apoptosis in blood cells. This study proposes a flowcytometry based method that uses fluorescein-annexin V binding FITC and propidium Iodide to enumerate viable, necrotic, and early and late apoptotic cells within specific subsets of a heterogeneous culture. Blood samples from healthy adult man with no mutagens materials contact history was collected in heparinized tubes. The peripheral blood T-cells that were isolated from blood samples cultured and irradiated while Carumopticum (Zenian in persian) extract was added to aquatic culture media just an hour before radiation treatment. So the irradiated cells underwent apoptosis and apoptotic cells were enumerated by annexin-V protocol and multicolor flow cytometry.

The results suggest that Carumopticum(ajowan) extract can potentiate the radiation induced apoptosis of PBLCs(73%).The results suggest that Carumopticum (ajowan) extract can enhance the radiation toxicity in blood T‑cells ($P < 0.05$).

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Introduction

Apoptosis or programmed interphase cell death is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissue. Tumor growth is regulated by the balance between cell proliferation and apoptosis. It has been found that most cancer chemotherapy drugs exert cytotoxic effects on malignant cells by inducing apoptosis [1-6].

Ionizing radiation causes damage to living or cancerous tissues through a series of molecular events, depending on the radiation energy [2-3]. The major radiation damage is due to the aqueous free radicals, generated by the action of radiation on water. These free radicals react with cellular macromolecules, such as DNA, and cause cell dysfunction, apoptosis and mortality. These reactions take place in tumor as well as normal cells when exposed to radiation. The radiation damage to a cell is potentiated or mitigated depending on several factors, such as the presence of oxygen and other molecules in the cellular milieu [2,4]. Oxidative damage to the cellular genetic material, i.e., DNA, plays a major role in mutagenesis and carcinogenesis [7-13]. Some herbs contains agents can modify or moderate effects of radiation due to the occurrence of natural antioxidants carrying out free radical scavengers and quenchers of singlet oxygen. [4-6, 14].The use of plants for medicinal purposes in traditional herbal medicine has been effective in the treatment of many diseases [8,9].Some herbal products have been used as anticancer agents, such as vincristine and vinblastine from Catharanthusroseus [4,15-18]. In spite of the extensive use of herbal therapies in all regions of the developing world, and developed countries there is insufficient scientific evidence to confirm their efficacy and safety. Thus, basic research aimed for clarifying the underlying mechanisms of any potential herbal effects are very important for the use of herbal medicine. Recently scientists have focused on the potential role of traditional medicinal herbs as alternative and complementary medications for cancer treatment. Even many investigations are undergoing on herbal drugs to replace them with conventional treatments of various types of cancer and other diseases [5, 19].

The activated immune effectors cells havebeen consistently found in the peripheral blood and, among these immune effectors, T cellshave been considered to be the most important because the regulation of T cell activation requires antigen specificity and a great amount of cytokines released in immune responses are from T cells. After development of autoimmune diseases, the failure to execute the appropriate apoptotic program may result in sustention of inflammatory process [20]. Research interest has been focused on various herbs that possess immune modulating properties that may be useful in reducing the risk of immune system diseases and cancers [20, 21]. Ajowan is an herb belonging to Apiaceae family and used in food and medicine. It is reported that the ripening seeds of ajowan contain 2-4% essential oil which is rich in mono terpenes like thymol and it is mainly used as an antiseptic agent as well as drug component in medicine. Ajowan oils are promising natural fungicides without any hazardous effects for [human and environment and can be used instead of harmful synthetic and chemical fungicides. The fruits of this plant can be used as an antispasmodic, antiflatulent, anti-rheumatic, diuretic and antimicrobial. Ajwain is an erect annual herb with striate stem originated in Iran and India [22, 14].In this research the effect of ajowan extract on radiation induced apoptosis of blood T- cells has studied.

Materials and methods

Materials

Ajowan extract were purchased from the local herbal market boiled, pasteurized, sterilized by dry autoclaving and cooled in sterilized manner, then and, the cooled decoction was filtered through a coarse sieve twice. Blood samples were taken from 16 healthy male adult donors. Following venopuncture Blood was diluted 1:1in PBS (Sigma-Aldrich GMBH Munich, Germany) and hold on Ficolle containing 10% fetal bovine serum. PBLCs were separated by Ficoll density gradient centrifugation using Ficoll-Paque Plus (Sigma-Aldrich GMBH Munich, Germany) [23, 24].

The extracted cells suspension was then divided into portions which were poured into 6 well (Spielsoth Korea) cell culture plates. After enough propagation of cells the plates of samples were irradiated in a tissue equivalent container for simulating realistic condition by Cobalt 60 radiotherapy unit (Theratron Phonix Canada) in Seyed Al-shohada hospital radiotherapy department in Isfahan (gamma photon energy 1.1 Mev) and subsequently incubated under standard culture conditions again (Figure 2). Irradiation was performed at a dose rate of 82.46cGy/min for dose 2Gy. For calibrating external dose of cobalt unit physical dosimetry was performed using a gas chamber dosimeter (Farmer 2570 Nuclear Enterprises, Zurich, Switzerland) as mentioned in IAEA Cytogenetic dosimetry technical report [23]. Ajowan extract added to culture media just one hour before samples irradiation.

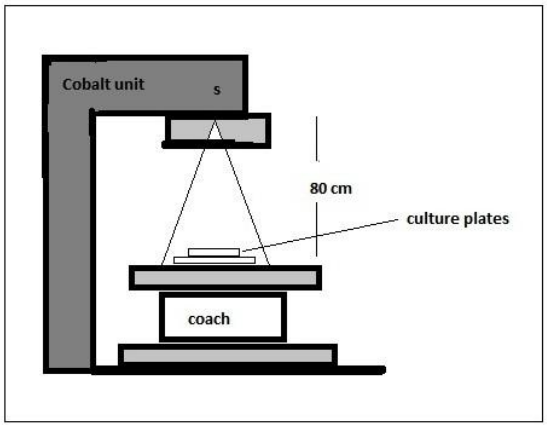

Fig. 1. Geometry of cultured cell samples irradiation.

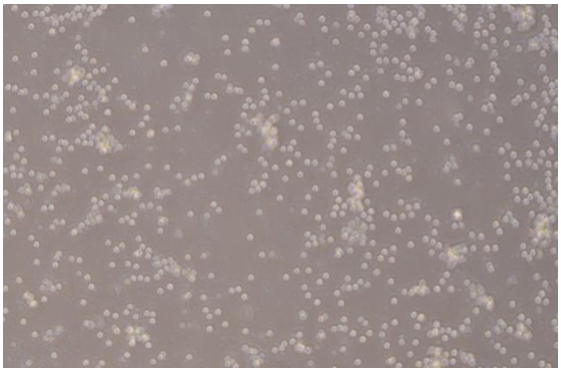

Fig. 2. The cells were incubated at 37°C in a 5% CO_2.

After 24 hours incubation the plates content were centrifuged at 1500rpm (300g) for 5min at room temperature for isolating T-cells. Isolated T-cells in pellet form resuspended in approximately 200µl of the remaining solution. The cells were subsequently measured by flowcytometry in approaches that has been recommended by elow kit supplier and articles [24, 25]. Forward and side light scattering and stain-induced fluorescence at two different wavelengths (530 nm, green and 640 nm, red) were simultaneously measured from each cell. The forward scatter (FSC) signal is proportional to the cell size, whereas the side scatter (SSC) signal is proportional to cellular granularity (intracellular superstructure). Using these two parameters it was possible to discriminate the three major types of leucocytes and gate the lymphocytes for acquisition (Fig. 3B). Staining the cells with FITC (green fluorescence) identifies cell type and PI quantifies cellular DNA content. Apoptotic lymphocytes were defined as those cells staining positively for their Annexin-FITC, and displaying reduced DNA content and cell size. Data from each leucocytes sample were acquired immediately after preparation. Acquisition was performed by a Becton-Dickinson FACS can flow cytometer using its Cell Quest software. Four parameter acquisitions permitted discrimination of different subpopulations of leucocytes. Using forward scatter versus side scatter two parameter dot plots, three subpopulations of leucocytes (monocytes, granulocytes and lymphocytes) as well as the cell debris could be distinguished and the lymphocytes selected (Fig. 3B,3D). Data for 10000 cells were acquired in about 2–3 min. Apoptotic lymphocytes must be included in the cells selected during data acquisition. The apoptotic cells form a discrete population separate from the normal lymphocytes and can be distinguished by their smaller size (FSC) and slightly larger granularity (SSC). However, non-appropriate lymphocytes and debris must first be excluded. A two-dimensional dot plot of antibody immune fluorescence versus DNA content was used for quality control. Figure 3B shows how this population was identified. The population displaying high cellular DNA content and high FITC positivity contains the normal, FITC-positive

cells, the population with high cellular DNA content but low Annexine-FITC positivity contains other lymphocytes. Below these two clusters two further populations are observed, both with reduced DNA content; these are the apoptotic cells. The population with the lowest DNA values and low FITC positivity represents the debris. The FITC-positive apoptotic cells were selected, and the two-dimensional FSC versus SSC dot plot showed the presence of these apoptotic cells within the selection gate [26, 27, 28].

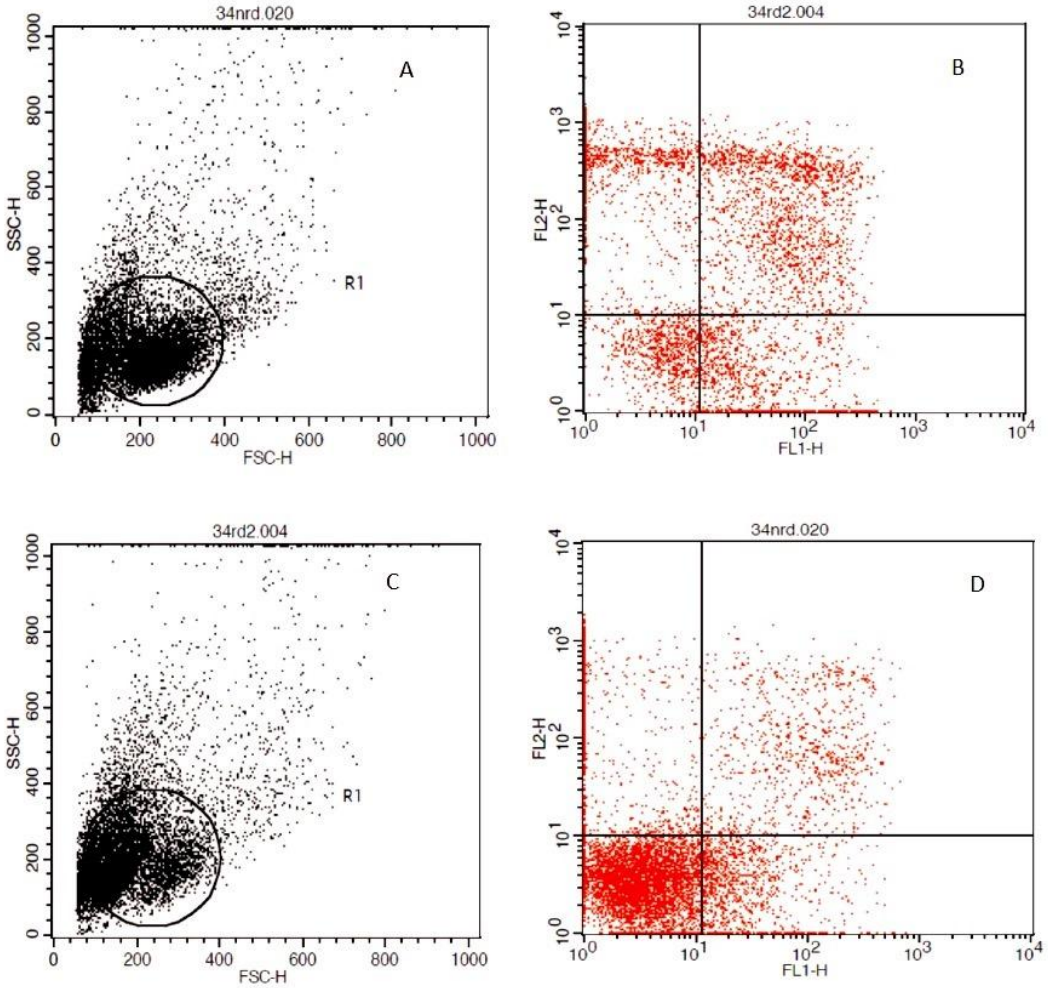

Fig. 3. A) Zone gating for appropriate non irradiated cells. B) Two parameter dot plot Pi/FITC for counting apoptotic non irradiated cells. C) Zone Gating for appropriate irradiated cells. D) Two parameter dot plot Pi/FITC for counting apoptotic irradiated cells. Difference of two counts (B, D) is net radiation induced apoptosis

Results and discussion

Radiation-induced apoptosis in T-lymphocytes was measured at next day after irradiation. The volunteer had no radiotherapy history and continuous chemical or physical mutagen agents contacts.

Annexin-v Fluoresceine Iso Thio Cyanate (FITC) conjugated assay was used for determining apoptosis in t-cell lymphocytes by flowcytometry. The samples were analyzed in five batches. For every sample doses of 0, 10, 20, 30 40µg /ml of Ajowan extract was added in different wells of culture plate. The Ajowan extract were added to culture plate an hour before radiation treatment.

Wells of culture plate with 0 doses of drug used as controls and a plate with 0 doses of drug without irradiation used as blank. Every culture plate was irradiated with 2Gy dose of cobalt gamma radiation. The well content then poured into 15ml centrifuge tube and prepared for measurement of the cells apoptosis count with annexin and flowcytometry assay. The averaged results and their STDE presented in table (1) and showed graphically figure (5). In every time of measurement the results confirmed by microscopic speculation.

Table 1. Result of assessed apoptosis after 2Gy dose of irradiation for different dose of Ajowan extract.

Dose(µg/ml)	0	10	20	30	40
1	12	21	22	28	42
2	9	20	22	32	38
3	9	18	20	31	42
4	13	20	18	27	38
5	12	22	17	28	43
6	13	19	18	31	39
7	12	18	21	27	37
8	11	19	19	29	42
9	10	19	21	32	41
10	9	19	21	27	37
11	9	23	21	30	38
12	9	23	17	34	43
13	13	24	23	33	37
14	12	21	17	29	36
15	8	19	18	28	44
16	11	19	18	30	44
Average	10.75	20.2	19.5625	29.75	40.0625

Discussion

Ajowan products contains different important components such as carbohydrates, glucosides, saponins and phenolic compounds (carvacrol), volatile oils (thymol), terpiene, paracymene and betapinene, protein, fat, fiber, and minerals including calcium, phosphorus, iron, and nicotinic acid (niacin) [29].

There is a lot of scientific evidence for the therapeutic effects of this plant in gastrointestinal disorders, such as reflux, cramps, abdominal tumors, abdominal pain, and Helicobacter pylori, as well as in eye infection disorders, its beneficial effects have been demonstrated [30]. Reported beneficial effects of ajowan seeds also include carminative, antiseptic, amoebiasis expectorant, antimicrobial, antiparasitic, antiplatelet-aggregatory and antilithiasis. Positive results in treating common cold and acute pharyngitis also was abtained [30].

Valiollahi1 et al concluded that the use of ajowan had almost similar beneficial effects on body performance, cholesterol profile and antibody titre in broiler chicks [31].

Therapeutic uses of ajowan seeds also include carminative, antiseptic, amoebiasis expectorant, antimicrobial, antiparasitic, antiplatelet-aggregatory and antilithiasis as well as treating common cold and acute pharyngitis [30]. Abortifacient, galactogogic, and diuretic activities have been observed for this plant [32, 33].

There is also anticarcinogenic potential evidence for ajowan [34]. It has been shown that this plant has also foetotoxicity, abortion potential, and galactogogue properties [35]. In previous studies, different pharmacological effects were shown for ajowan. In addition, the plant has been used widely in traditional medicine. Therefore, for evaluating of other aspect of pharmacological effects of ajowan and its constituents on radiation apoptosis and related outcomes the present study was proposed.

To study if apoptosis rate relates to age of samples age and apoptosis rate correlation for 2Gy of gamma radiations were examined. Correlation coefficient was less than 0.2 for all of all doses inserted. This means no relation exist between age and apoptosis of samples. The volunteers age distribution has presented graphically in Figure 4.

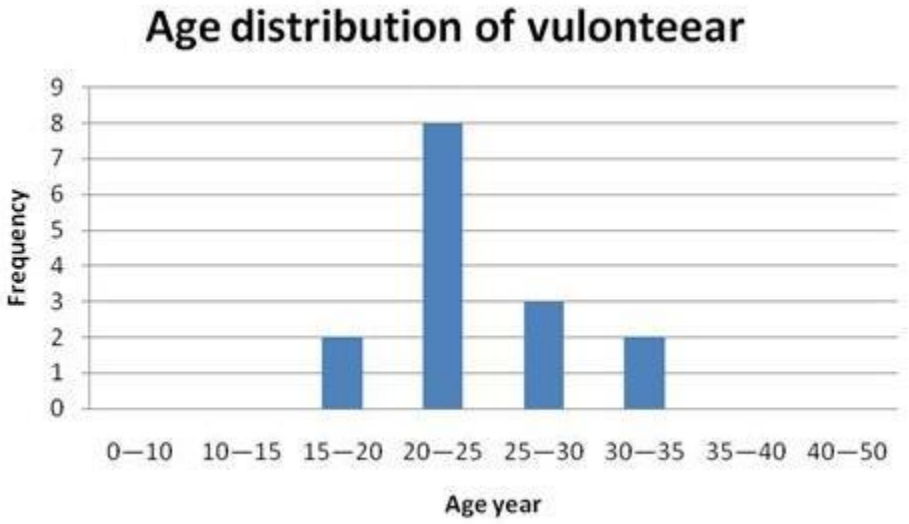

Fig.4. Age distribution of sampled volunteer.

Cells treated with 0, 10, 20, 30, 40 µg/ml of Ajowan extract before irradiation. There was a significant increase (<75 %) in cell radiation induced apoptosis when cells were treated with Ajowan extract. The T-Test assay indicated significant difference between cell culture with and without extract ($p<0.04$). Therefore ajowan extract significantly enhanced radiation induced apoptosis. Increasing ajowan extract concentrations from 10µg/ml not appeared to cause more apoptosis changes, indicating that apoptosis in the tried concentrations occurred in a non concentration-dependent fashion. Some sign of media clearance same as antibiotic effect was seen but not confirmed with later experiments.

Annexin V binding is a well-established marker for apoptosis and a potentially attractive biomarker for identifying radio toxicity patients in radiotherapy programs and radiation accident victims [22]. Annexcin V binding to peripheral blood lymphocytes increases as samples stored before analysis, so increase the apoptosis results. It observed that time delay after irradiation and before flowcytometry positively affects on the result of annexin assay. It suggests that first-order Radiation induced apoptosis strongly changes for different storage conditions specially relay time of storage in binding buffer. This time dependent apoptosis result in blurring the difference between results and leads to a significant decline in the stability and reproducibility for annexin v as a diagnostic test for radiation toxicity. In this regards this result should be evaluated with some different methods to confirm this finding for next steps of reasoning and usage. Forever this assay indicated that Ajowan extract enhance radiation induced apoptosis. But it should be examined that this effect can help apoptosis of tumor cells or decrease radiosensitive outcomes such as skin effects. This need to examine effect of some drug doses on tumor cell radiation induced apoptosis.

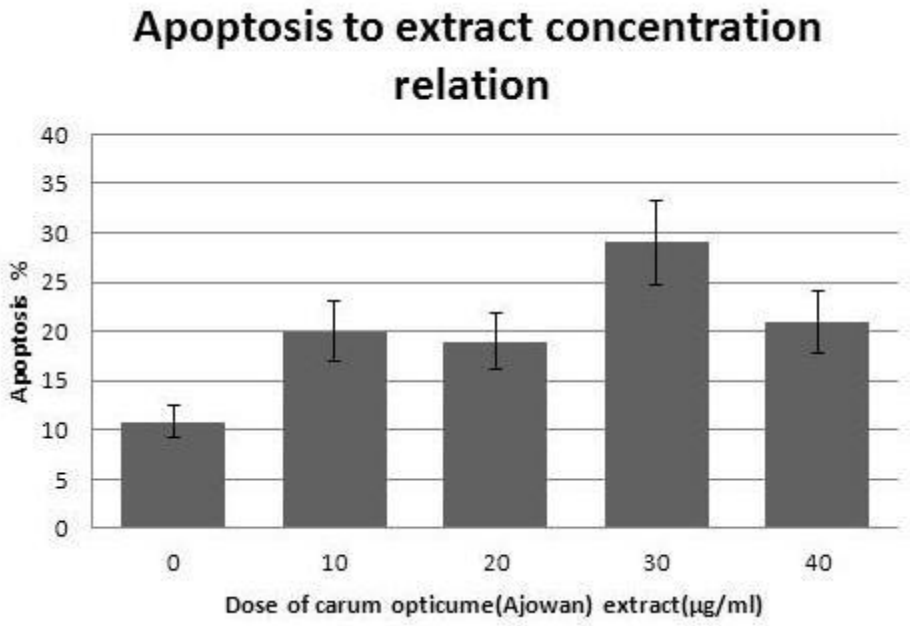

Fig. 5. Relation of apoptosis to carumopticume extract concentration.

Conclusion

Although the results of this study are very significant, but this study require other studies to confirm and complete findings. For validation of these results required to ensure reproducibility of these finding using a second, independent cohort. This would also allow integration of other potential confounders, such as radiation dose and apoptosis of cells at Ajowan extract added media relations that not included in the current study. Ultimately such an assay could be tested for its utility by measuring lymphocyte sensitivity in extract added media before radiation treatment decisions and by documenting whether the results had an impact on cellar apoptosis management and, more importantly, a reduction or increase in long-term toxicity.

Acknowledgment

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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