Effects of Ionizing Radiation on Human Peripheral Blood Mononuclear Cells (PBMCs) in the Presence of *Mentha-Pulegium* Essential Oil: A Study on the Radioprotective Effect

Nima Hamzian[1], Abolfazl Nickfarjam[1], Ali Shams[2], Fateme Haghiralsadat[3], Moslem Najmi-Nezhad[4,5]*

[1]PhD, Department of Medical Physics, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[2]PhD, Department of Immunology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[3]PhD, Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[4]MSc, Department of Medical Physics, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[5]MSc, Department of Biochemistry and Biophysics, School of Paramedicine, Iranshahr University of Medical Sciences, Iranshahr, Iran

ABSTRACT

Background: The Mentha-Pulegium essential oil (MP-EO) contains different antioxidant compounds and reduces the indirect effects of dispersed ionizing radiation on biological systems.

Objective: The current study aimed to assess a possible radio-protective effect of MP-EO on peripheral blood mononuclear cells (PBMCs).

Material and Methods: In this experimental study, MP-EO was firstly prepared and PBMCs were then irradiated in various groups with doses of 25 and 200 cGy of X-rays in the presence of *IC10* of MP-EO. After incubation times of 48h and 72h, the survival, apoptosis, and necrosis percentages of PBMCs were determined by MTT assay and flow cytometry analyses; the radio-protective effect of MP-EO was examined.

Results: In the presence of 80 µg/ml (*IC10*) MP-EO, the mean survival percentage of irradiated PBMCs by radiation doses of 25 and 200 cGy was significantly increased after 48h of incubation compared with the control. At 72h of incubation, the mean survival percentage of irradiated PBMCs was significantly increased only at 25 cGy. The percentage of apoptosis and necrosis of PBMCs was significantly reduced in the presence of the MP-EO at both incubation times and radiation doses; therefore, the highest reduction was at 200 cGy and 48h incubation compared to the control.

Conclusion: MP-EO as a natural, non-toxic, and cost-effective compound can exhibit a favorable *in-vitro* radio-protective effect by increasing the survival and decreasing the percentage of apoptosis and necrosis of irradiated PBMCs.

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Keywords

Lymphocytes; Mentha Pulegium Essential Oil; Radiation, Ionizing; Radioprotective; Apoptosis; Necrosis

*Corresponding author:
Moslem Najmi-Nezhad
Department of Medical Physics, School of medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
E-mail: moslem.najminezhad@gmail.com

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Introduction

The risk of exposure to ionizing radiation, as an important tool in the diagnosis and therapy, is high in medicine and can also cause serious side effects to living organisms; developed techniques have increased the number of X-ray examinations in the diagnosis and treatment of patients. Accordingly, public awareness of the harmful effects of ionizing radiation leads to further public concerns [1, 2]. Interna-

tional Commission on Radiological Protection (ICRP) established a guideline for physicians and radiographers about the principles of radioprotection due to numerous changes, which are also confirmed by the ALARA principle (As Low As Reasonably Achievable), over the past three decades; minimizing the radiation exposure can be reasonably achievable [3, 4]. Exposure to ionizing radiation can directly cause different types of Deoxyribonucleic acid (DNA) damage, such as a double-strand break, the most severe. Ionizing radiation can also cause damage by indirect effects, such as the production of reactive oxygen species (ROS). In dispersed ionizing radiation (low Linear Energy Transfer [LET]), such as X-ray and gamma rays, the damage due to reactive oxygen species is dominant compared to the direct damage to DNA [5]. X-ray and gamma rays are most used in medical diagnosis and treatment and play a major role in the effective annual dose due to natural and man-made radiation sources [6, 7]. Generation of free radicals and ROS by the interaction of X-rays with the biological systems can lead to an imbalance between intracellular antioxidants; ROS results in oxidative stress damage to macromolecules, such as DNA, Ribonucleic Acid, and proteins [8].

Antioxidants are compounds known as scavengers and neutralizers of free radicals, capable of inhibiting the oxidation process [9]. Therefore, antioxidants can reduce the harmful effects of radiation caused by ROS production. Plant-derived antioxidant compounds are considered due to their lower toxicity than synthetic antioxidants [10]. Mentha pulegium is a fragrant plant of the Lamiaceae family with different species in various parts of the world, especially in Iran [11].

Mentha-pulegium contains a variety of phenolic, flavonoid, and terpenoid compounds that have exhibited antioxidant activity [12, 13]; therefore, Mentha-pulegium essential oil can possess a favorable radio-protective impact on cells and living organisms by reducing the detrimental effects of radiation. Before animal testing using the animal laws of the United States Food and Drug Administration, an essential step identifies and selects a biomarker that can represent an objective and measurable feature to develop the potential targets of radiation exposure. One of these potential targets is peripheral blood mononuclear cells (PBMCs) [14], which can be a desirable radiation biomarker due to their high sensitivity, ease of access with minimal invasion, and circulation throughout the body.

The present study evaluated the radio-protective effect of Mentha-Pulegium essential oil (MP-EO) on PBMCs. Therefore, apoptosis, necrosis, and mitotic death of irradiated cells were examined in the presence of MP-EO.

Material and Methods

In this experimental study, Phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640, and Penicillin-Streptomycin (Pen-Strep) were obtained from Inoclon (Iran). A total of 3-(4, 5 Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Aldrich (Munich, Germany); Fetal bovine serum (FBS) was procured from GIBCO (USA). The apoptosis-necrosis kit (PI, Anti-Annexin V-FITC) and Lymphodex were obtained from IQ products (Netherlands) and Serana (Iran), respectively. Dimethyl sulfoxide (DMSO) and Heparin were procured from BioIDEA (Iran) and Caspian (Iran), respectively. Other solvents and chemical reagents were procured from Merck (Germany).

Preparation of MP-EO

The MP-EO was prepared by water distillation (hydro-distillation method) [15] using a Clevenger apparatus. A total of 100 gr of the plant leaves and flowers were completely powdered and mixed in a balloon with 500 ml of water and then connected to a Clevenger device. After 4h, MP-EO was separated and stored in a microtube covered with aluminum

foil. Mentha-Pulegium essential oil is a concentrated hydrophobic liquid, containing volatile chemical compounds from the Mentha-Pulegium plant that evaporates easily at room temperature.

Culture of PBMCs

First, heparinized blood samples were drawn from 5 healthy men with no history of systemic diseases, smoking, and radiotherapy, in an age range of 20 to 30 years. The blood was then diluted in the same proportion with PBS and transferred to Falcon tubes containing Ficoll. The tubes were then centrifuged (Universal-Iran) at 1800 rpm for 20 min; the lymphocytes were isolated and PBS was added. Next, the samples were centrifuged again at 1500 rpm at 25 °C for 5min. The supernatant was discarded, and the culture medium (RPMI), supplemented with 10% FBS, 1% antibiotic was added. Subsequently, the cells were incubated in a 5% CO_2 incubator at 37 °C.

Assessment of MP-EO toxicity

The toxicity of MP-EO on PBMCs was assessed by the MTT assay. For this purpose, 10^5 cells were seeded onto each well of a 96-well plate, performed for both experimental and control groups. After 24h of incubation, serial concentrations of MP-EO (5-320 µg/ml) were added to the cell culture medium of the experimental groups with 5 replications. After 96h of incubation (37 °C, 5% CO_2), cell survival was measured by the MTT assay in both control and different experimental groups. Accordingly, 5 mg of tetrazolium powder was added to 1 ml of PBS and after agitation by vortex-shaker (IKA-Germany), the resulting solution was filtered under sterile conditions (0.2 µm). Afterward, 20 µl of the tetrazolium solution was added to each well; after 4h of incubation (37 °C, 5% CO_2), the plate containing the cells was centrifuged at 4 °C for 5 min. The supernatant was then removed, and 100 µl of DMSO was added to each well; after about 5min, the optical density of wells was read by an enzyme-linked immunosorbent assay at a wavelength of 570 nm and a reference wavelength of 630 nm. Finally, the percentage of cell survival in experimental groups in comparison with the control group according was calculated according to the below formula:

$$\text{Survival percentage} = \frac{OD_{\text{treated}} - OD_{blank}}{OD_{\text{control}} - OD_{blank}} \times 100 \quad (1)$$

where OD_{treated} and OD_{control} are the optical density of treatment and control groups, respectively.

Finally, the *IC10* value was recorded as the concentration without obvious toxicity of MP-EO to measure its radioprotection.

Analysis of the radioprotective effect of MP-EO on PBMCs

PBMCs were exposed to different doses of linear accelerator X-rays in the presence and absence of MP-EO to analyze the radioprotection effect of MP-EO. The survival, apoptosis, and necrosis of PBMCs in the presence of MP-EO were then measured using MTT assay and flow cytometry analysis. Additionally, a total of 10^5 cells were seeded onto each well of a 96-well plate, and after 24h of incubation, equivalent to a concentration of 80 µg/ml of MP-EO (*IC10*) was added to the cell culture medium. After 24h of incubation (37 °C, 5% CO_2), plates containing different treatment groups were irradiated with various doses of 0. 25, and 200 cGy ionizing radiation. Cells that were not treated with MP-EO were considered the control group. Finally, the percentage of survival, apoptosis, and necrosis of cells in the two incubation periods (48 and 72h) after irradiation were measured using MTT and flow cytometry techniques.

Irradiation protocol

PBMCs were irradiated with a 6 MV linear accelerator (Electa Compact -England) at radiation doses of 25, and 200 cGy, based on the Source to Axis Distance (SAD) technique at a depth of 5 cm of the solid phantom and a Gantry angle of 180° in a field size of 20×20

cm^2. Computing the radiation monitor unit in predefined doses, considering the attenuation coefficient and the dimensions of the plate, was analyzed using Prowess Panther 3D conformal treatment planning system version 2.5. Central plate wells were used for cell culture as much as possible to better perform the dosimetry conditions, and the culture medium was poured into the marginal empty wells.

MTT assay

A total of 10^5 cells were seeded onto each well of a 96-well plate, and a concentration of 80 µg/ml of MP-EO (*IC10*) was added to the culture medium of the cells to measure the radioprotective effect of MP-EO on the survival rate of irradiated PBMCs. After 24h of incubation (37 °C, 5% CO_2), plates in different treatment groups were irradiated with various doses of X-rays (25, and 200 cGy). Cells that were not treated with the MP-EO were regarded as the control group. Finally, the percentage of cell survival was measured by the MTT assay (according to previous), and the survival enhancement factor (SEF) was calculated as the ratio of cell survival in the presence and absence of MP-EO at different radiation doses.

Flow cytometry analysis

The radioprotective effect of MP-EO on the percentage of apoptosis and necrosis of irradiated PBMCs was determined by the flow cytometry (Partec-Germany) analysis. Similar to the protocols used for the MTT assay, 10^5 cells were poured into each well of a 96-well plate, and a concentration of 80 µg/ml of MP-EO (*IC10*) was added to the cells; after 24h of incubation, irradiation was performed at doses of 25, and 200 cGy. The percentage of apoptosis and necrosis of cells in the two incubation periods of 48 and 72h after irradiation was obtained by flow cytometry. The cells belonging to each treatment group were transferred into tubes specific for the flow cytometry analysis, and 1 ml of PBS was added to the cells. After shaking, the tubes were centrifuged at 1800 rpm for 5 min. Next, the supernatant was slowly discarded, and 5 µl of Annexin V was added to each 100 µl of the cell suspension. Afterward, tubes containing the cells were shaken for 20s and incubated at 4 °C for 20 min in the dark. At this time, the calcium buffer of the Annexin V-PI kit was diluted up to 20 times with deionized water. Moreover, 1 ml of calcium buffer was added to tubes, shaken for 5s, and centrifuged at1800 rpm for 5min as well. The supernatant was removed, and 5 µl of propidium iodide was added to the cells, agitated then for 10s, and then incubated at 4 °C for 10 min. Finally, 500 µl of PBS was added to the cells, and the cell population was counted by flow cytometry.

Statistical analysis

The obtained data were analyzed using GraphPad Prism Software (version 9) through descriptive statistics (mean and standard deviation) and inferential statistics at a 95% confidence level (P-value ≤ 0.05). The independent t-test was used to compare the difference between the experimental and control groups.

Results

Toxicity of MP-EO

Figure 1 depicts the survival of human PBMCs in the presence of serial concentrations of MP-EO. Cell survival is maximal at a concentration of 20 µg/ml, and the *IC10* value of MP-EO was obtained at a concentration equivalent to 80 µg/ml. Additionally, the percentage of cell survival at the highest concentration of the graph finally reached about 60%.

Effect of MP-EO on Survival of irradiated PBMCs

Figure 2 displays the mean survival percentage of irradiated PBMCs (at radiation doses of 25 and 200 cGy) in the absence and the presence of 80 µg/ml of MP-EO (equivalent to *IC10*) at two incubation times of 48 and 72h. The survival rate of PBMCs at a dose of 25

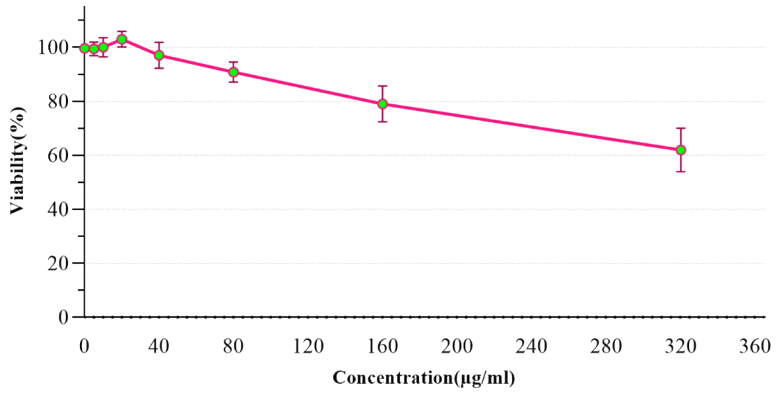

Figure 1: The survival percentage of Peripheral blood mononuclear cells (PBMCs) in the presence of different concentrations of Mentha Pulegium Essential Oil (MP-EO) after 96 hours of incubation (Colorimetric assay for assessing cell metabolic activity (MTT assay)).

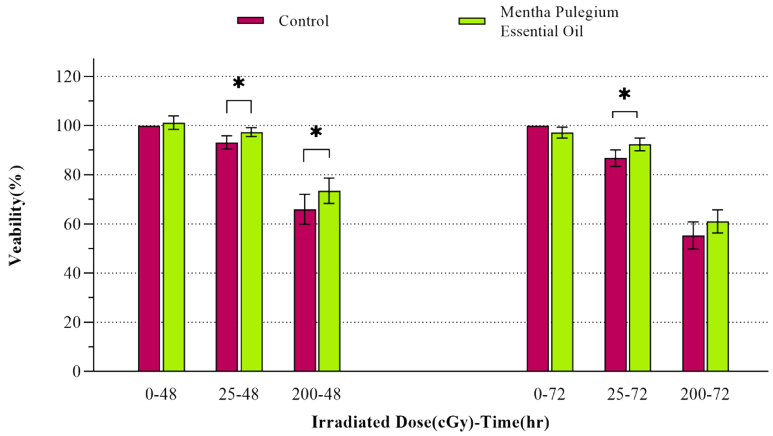

Figure 2: The Colorimetric assay for assessing cell metabolic activity (MTT assay)-Mean survival percentage of irradiated Peripheral blood mononuclear cells (PBMCs) at radiation doses of 0, 25, and 200 cGy in the presence and absence of Mentha Pulegium Essential Oil (MP-EO) at 48 and 72 hours of incubation periods, * $P \leq 0.05$.

cGy at both 48 and 72h of incubation periods was significantly increased compared with the control group ($P \leq 0.05$). The survival percentage of PBMCs at a radiation dose of 200 cGy, only in 72h of incubation was increased significantly compared to the control ($P \leq 0.05$). At a radiation dose of 25 cGy, the SEF values of MP-EO at 48 and 72h of incubation periods were reported 1.04 and 1.06, respectively. At a dose of 200 cGy, the SEF values at 48 and 72h of incubation periods were reported 1.11 and 1.14, respectively.

Effect of MP-EO on Apoptosis and necrosis of irradiated PBMCs

The mean percentage of apoptosis in irradiated PBMCs in the presence of 80 µg/ml of MP-EO (*IC10*) was obtained in different groups by the flow cytometry analysis (Figure 3). At 48h of incubation, the mean percentage values of apoptosis of PBMCs in the presence of MP-EO at radiation doses of 25 and 200 cGy were reported 3.40±0.60 and 6.78±0.95, respectively, with a significant decrease ($P \leq 0.01$ for both radiation doses) in comparison with

the control group (5.62±1.43 and 9.04±1.45, respectively). At 72h of incubation, the mean percentage values of apoptosis of PBMCs at 25 and 200 cGy radiation doses were reported 3.17±0.68 and 6.27±1.02, respectively, showing a significant reduction compared to the control group (4.26±0.89 and 7.87±1.31) at both radiation doses ($P \leq 0.05$).

The flow cytometry results of mean percentages of necrosis in irradiated PBMCs in different groups were illustrated in Figure 4. At 48h of incubation, the mean percentage values of necrosis of PBMCs in the presence of *IC10* of MP-EO at radiation doses

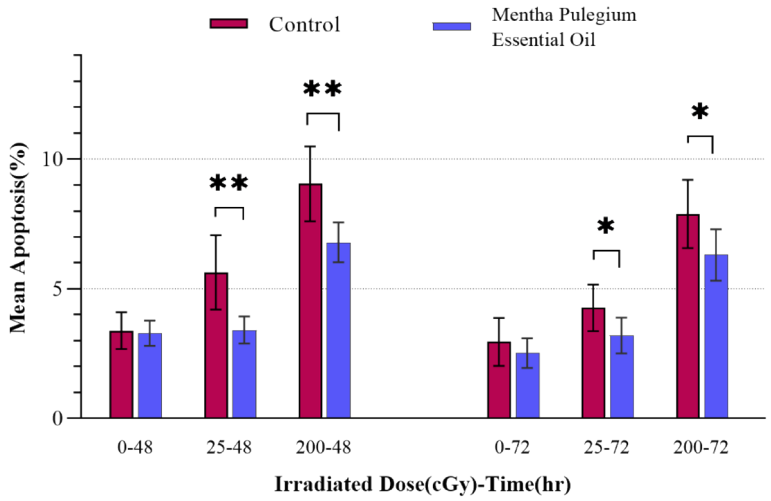

Figure 3: The flow cytometry analysis- The mean percentages of apoptosis of irradiated Peripheral blood mononuclear cells (PBMCs) by radiation doses of 0, 25, and 200 cGy, in the presence and absence of Mentha Pulegium Essential Oil (MP-EO) at two incubation periods of 48 and 72 hours, *$P \leq 0.05$ and **$P \leq 0.01$.

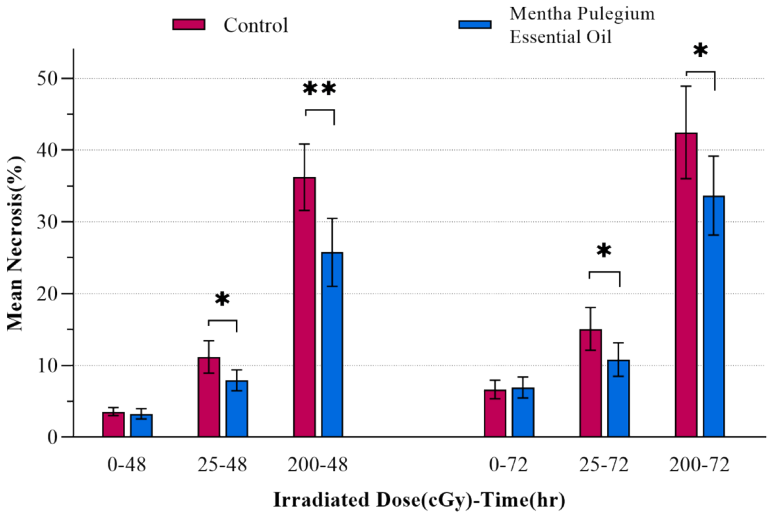

Figure 4: The flow cytometry analysis- The mean percentages of necrosis of irradiated Peripheral blood mononuclear cells (PBMCs) at radiation doses of 0, 25, and 200 cGy, in the presence and absence of Mentha Pulegium Essential Oil (MP-EO) at two incubation periods of 48 and 72 hours, * $P \leq 0.05$ and ** $P \leq 0.01$.

of 25 and 200 cGy were reported 7.92±1.44 and 25.74±4.73, respectively with a significant reduction ($P \leq 0.05$ and $P \leq 0.01$, respectively) in comparison with the control group (11.18±2.25 and 36.23±4.62, respectively). At 72h of incubation, the values at 25 and 200 cGy radiation doses were reported 10.74±2.27 and 33.67±5.49, respectively, showing a significant reduction compared with the control group (42.50±6.47 and 15.08±2.96) at both radiation doses ($P \leq 0.05$).

In Figures 5 and 6, the gating of the population of PBMCs after 48 and 72h of incubation in different groups is shown as a pseudocolor graph obtained from the flow cytometry analysis. Necrotic cells are located in the first quadrant (Q1), late apoptosis in the second quadrant (Q2), apoptosis in the third quadrant (Q3), and living cells in the fourth quadrant (Q4).

In columns of both Figures 5 and 6, apopto-

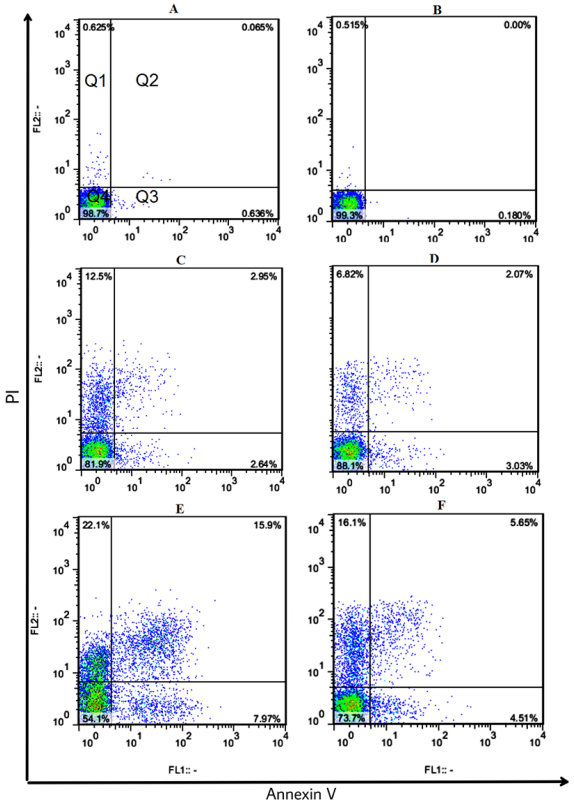

Figure 5: The flow cytometry analysis after 48 hours of incubation at a concentration of 170 µg/ml Mentha Pulegium Essential Oil (MP-EO) at different radiation doses using a 6 MV X-ray linear accelerator. A: control group (no treatment) at zero radiation dose, B: treatment group (treated with MP-EO) at zero radiation dose, C: control group at 25 cGy radiation dose, D: treatment group at 25 cGy radiation dose, E: Control group at 200 cGy radiation dose, F: Treatment group at 200 cGy radiation dose.

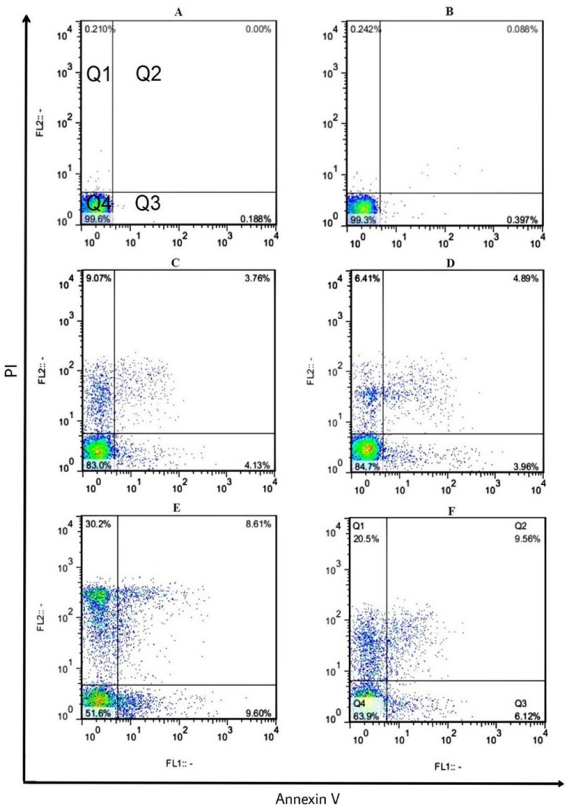

Figure 6: The flow cytometry analysis after 72 hours of incubation at a concentration of 170 µg/ml Mentha Pulegium Essential Oil (MP-EO) at different radiation doses using a 6 MV X-ray linear accelerator. A: control group (no treatment) at zero radiation dose, B: treatment group (treated with MP-EO) at zero radiation dose, C: control group at 25 cGy radiation dose, D: treatment group at 25 cGy radiation dose, E: Control group at 200 cGy radiation dose, F: Treatment group at 200 cGy radiation dose.

sis and necrosis increased from top to bottom with increasing radiation dose. In addition, right-sided graphs (treatment groups with MP-EO) showed greater survival than left-sided graphs (untreated groups).

Discussion

The aim of this study was to evaluate the radioprotective effect of a natural antioxidant on protecting radiation staff in radiation centers and patients exposed to radiation for therapeutic and diagnostic purposes. Dispersed X-ray is widely used, especially in medical diagnosis and treatment, and plays a major role in the effective annual dose from natural and man-made radiation sources [7]. The main mechanism of the interaction of these radiations with the biological system is through the generation of free radicals and ROSes [5, 16]. Attenuation or neutralization of the deleterious effects of ROS is possible by compounds called antioxidants. In recent years, increasing awareness of the toxic nature of some synthetic compounds has led to greater efforts to identify antioxidants of plant origin and low toxicity [17-19]. Mentha-pulegium has powerful antioxidant properties as a result of possessing a variety of natural compounds, such as flavonoids and phenols [12, 20].

The antioxidant activity of Mentha-pulegium by measuring the free radical agent, such as 2,2-diphenyl-1-picryl-hydrazyl-hydrate, Ferric Reducing Antioxidant Power Assay, and other assays is reported [12, 13]; however, discrepancies in their results are significant due to different extraction methods and changes in the type as well as the amount of chemical compounds. In this study, the positive effects of this plant on protecting cells against oxidative damage caused by ionizing radiation were expected due to the favorable antioxidant properties of Mentha-Pulegium [12]. Therefore, the radioprotective effect of MP-EO on PBMCs was evaluated that PBMCs are broadly used in the analysis of radioprotection studies due to their availability and radiosensitivity. Additionally, the results obtained from measuring the toxicity of MP-EO on PBMCs showed that the essential oil has low toxicity on normal human peripheral blood lymphocytes even at high doses. However, based on the study conducted by Shirazi et al. Mentha-Pulegium has toxicity on several cancer cell lines, such as ovarian, lung, cervical cancer cells, and Hela cells [21].

Unlike Bergoni and Tribondeau's law [22], showing the relationship between proliferation and radio-sensitivity, PBMCs have high radiosensitivity due to ionizing radiation apoptosis [23, 24]. Therefore, apoptotic as well as necrotic death and analyzing the mitotic death were measured to assess the radioprotective effect of MP-EO.

PBMCs were irradiated in the presence of MP-EO at two doses of 25 and 200 cGy. A dose of 25 cGy was selected as the low dose. At the low doses range (0.1-10 cGy), the data are variable due to the existence of the bystander effect and radiation adaptive response [25, 26]; therefore, a higher dose of 25 cGy was applied there. The radiation dose of 200 cGy is also commonly used in radioprotection studies as a boundary between lethal and sublethal doses [27].

Radioprotective effect of MP-EO on PBMCs viability

In the present study, the quantity of SEF at each radiation dose was defined and used (as the ratio of cell survival in the presence and absence of MP-EO at each radiation dose). This quantity corresponds to the well-known quantity of Dose Reduction Factor (DRF), which is defined at D50 [28, 29].

The results of the MTT assay on survival rates of PBMCs showed that the presence of MP-EO in the cell culture medium before irradiation significantly increases cell survival compared with the control group; however, the value of SEF at the highest dose was 1.14. Therefore, an adequate radioprotection effect was not observed in comparison with other ra-

dioprotection agents in other studies [30, 31], due to the mechanism of ionizing radiation effect on lymphocytes, in which the induction of apoptosis and necrosis predominates, while lymphocytes are considered resistant to mitotic death induced by ionizing radiation [22].

Radioprotective effect of MP-EO on PBMCs apoptosis and necrosis

Flow cytometry indicated that apoptosis and necrosis are increased following irradiation of cells, which is consistent with other studies conducted in radioprotection fields [32-34]. The results of flow cytometry showed that in the treatment group of MP-EO, in both radiation doses of 25 and 200 cGy and both incubation periods of 48h and 72h, apoptosis and necrosis rates of PBMCs were significantly reduced compared to the control group that was greater for apoptosis during the 48-hour incubation period ($P \leq 0.05$).

Necrosis is usually considered as the final fate of cells, so it can be expected at a 72-hour incubation period, a group of apoptotic cells is categorized into delayed apoptotic and necrotic cells [35]. Unlike apoptosis, necrosis is an uncontrolled cell death due to stress and external damage, leading to the rupture of cell membranes and the shedding of organelles as well as cell contents [35].

The investigation of radioprotectors in various *in-vitro* studies is influenced by several factors, such as the type of radiation, radiation dose, cell line, mechanism, and the quantity examined. The lack of a unified system in this area leads to difficult comparison and evaluation of a radioprotectant. However, studies have been conducted on radioprotective effect of natural or chemical compounds on PBMCs by measuring apoptosis [32-34, 36]. The difference in radiation dose and other parameters, or the lack of the same quantities leads to difficult comparisons. Further, Fardid et al. examined the radioprotection effect of hesperidin on peripheral blood lymphocytes in rats at 2 and 8 cGy doses by analyzing apoptosis and necrosis, without any significant reduction in apoptosis and necrosis in a dose of 2 cGy [37]. In a study conducted on cell survival by Menkovic et al. the radioprotective effect of Mangiferin extract was shown on peripheral blood lymphocytes in low doses [38].

Based on our results (Figures 3 and 4), in both radiation doses and incubation periods, the percentage of necrosis in treatment groups of MP-EO was significantly decreased compared with the control group. Reducing the percentage of radiation-induced apoptosis and necrosis of PBMCs in the presence of MP-EO shows the radio-protective role of this essential oil against low LET ionizing radiation.

Conclusion

MP-EO as a natural antioxidant shows a remarkable radioprotective effect on irradiated PBMCs with X-ray that is shown more by reducing the apoptosis and necrosis of the cells, possibly thereby scavenging free radicals.

Due to the natural compound, low toxicity, and cost-effectiveness of Mentha-pulegium essential oil, after complementary studies, it could be daily used as a radioprotector by radiation staff and patients exposed to radiation. Further studies are suggested for improving its kinetics to increase the efficiency of Mentha-pulegium essential oil.

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Authors' Contribution

N. Hamzian designed and managed the study and edited the manuscript and A. Nickfarjam helped him. A. Shams managed to perform in vitro tests. F. Haghiralsadat assisted in extract the essential oil. M. Najmi-Nezhad obtained ethics approval, collected and analyzed the data and wrote first version of the manuscript. All the authors read, modified, and approved the final version of

the manuscript.

Ethical Approval

The experimental procedures of the present research study were confirmed by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.MEDICINE.REC.1398.326).

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Conflict of Interest

None

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