

Radioprotective Effect of *Rosmarinus officinalis* L (Rosemary) Essential Oil on Apoptosis, Necrosis and Mitotic Death of Human Peripheral Lymphocytes (PBMCs)

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ABSTRACT

Background: Rosemary plant, with phenolic compounds, is known as an antioxidant herb and able to scavenge free radical agents in the biological environment; therefore, it is expected that the rosemary essential oil (R-EO) shows the radioprotective effect to protect individuals who are physically in contact with ionizing radiation.

Objective: This study aimed to assess the radioprotective effect of R-EO on human peripheral blood mononuclear cells (PBMCs).

Material and Methods: In this experimental study, the toxicity of the rosemary essential oil on PBMCs was assessed by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were irradiated at 0. 25 and 200 cGy using a 6 MV X-ray linear accelerator. The survival, apoptosis, necrosis, and survival enhancement factors of cells were analyzed by MTT and flow cytometry analyses with a non-toxic concentration of the rosemary essential oil (IC10).

Results: Irradiation of cells in the presence of R-EO caused a significant increase in cell survival compared with the control in both 25 and 200 cGy radiation doses. Also, the percentages of apoptosis and necrosis of cells showed a significant decrease compared with the control.

Conclusion: Rosemary essential oil as a natural and non-toxic compound could show favorable radioprotective effects in such a way that significantly increases the survival rate and decreases the percentage of apoptosis and necrosis of PBMCs.

Citation: Zhaeintan P, Nickfarjam A, Shams A, Abdollahi-Dehkordi S, Hamzian N. Radioprotective Effect of *Rosmarinus officinalis* L (Rosemary) Essential Oil on Apoptosis, Necrosis and Mitotic Death of Human Peripheral Lymphocytes (PBMCs). *J Biomed Phys Eng*. 2022;12(3):245-256. doi: 10.31661/jbpe.v0i0.2105-1333.

Keywords

Ionizing Radiation; Radioprotective Agent; Rosemary Plant; Apoptosis and Necrosis; Peripheral Lymphocyte

Introduction

In recent years, individuals such as staff in the nuclear energy industry, miners, radiologists, and diagnostic and therapeutic patients in medicine have been exposed to the potential risk of ionizing radiation. Ionizing radiation causes different damages in the biological system depending on the type, absorbed dose, exposure time, distance, and tissue sensitivity [1].

In the interaction of dispersed ionizing radiations (such as X-ray and gamma irradiation) with biological systems, especially water molecules,

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Received: 12 May 2021
Accepted: 20 July 2021

indirect effects in which some free radicals, as well as reactive oxygen species (ROS), are predominant. This reactive agent can further interact with vital macromolecules such as DNA and damage it [2].

The main mechanisms of radiation damage to the cells are apoptosis and cell death as a result of the break-in DNA; therefore, in the case of indirect ionizing radiation, compounds capable of scavenging free radicals could be considered as radioprotective agents [3]. Since 1949, compounds with a variety of structures and physiological functions have been examined as radioprotective or radiosensitive agents. However, the practical application of the majority of these synthetic agents has been limited in medicine due to undesirable side effects, such as their high toxicity at optimal protective doses [4-6]. Although synthetic thiol-containing compounds have the desired efficacy as radioprotectors, the toxicity of these compounds, such as hypotension, vomiting, nausea, hot flashes, somnolence, and hypocalcemia in optimal protective doses, have limited their use [7].

Therefore, the identification of non-toxic, effective, inexpensive, and acceptable compounds for prescription, has attracted the attention of researchers to natural compounds with less toxicity and greater effectiveness [8-10]. Radioprotection of plants also likely is due to its high levels of phenolic and flavonoid compounds, showing antioxidant activity [10, 11]. Antioxidants are natural or synthetic substances delaying the autoxidation mechanism by inhibiting the formation of free radicals or stopping the spread of free radicals. The most effective antioxidants are those that stop the free radical chain reaction and usually contain aromatic or phenolic rings [11].

Rosmarinus officinalis L. (rosemary) is a woody and evergreen herb and has a persistent aroma with a bitter and astringent taste that is native to the Mediterranean region and Asia and belongs to the *Rosmarinus* genus from the Lamiaceae family. The most significant me-

dicinal compounds of this plant include phenolic diterpenes and phenolic acids, such as carnosic acid, carnosol, rosmanol, ursolic acid, betulinic acid, and rosmarinic acid, showing antioxidant, anti-inflammatory, anti-viral, and anti-bacterial properties [12-14]. Due to the antioxidant properties of rosemary, radioprotective effects may be possessed; thus, in this study, the effect of radioprotection of R-EO on irradiated PBMCs by X-ray was investigated. For this purpose, the percentage of survival, apoptosis, and necrosis of PBMCs were analyzed.

Material and Methods

In this experimental study, RPMI-1640 culture medium, phosphate-buffered saline (PBS), and penicillin-streptomycin (penstrep) were purchased from Inoclon (Iran). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal bovine serum (FBS) was obtained from Sigma-Aldrich (USA). Ficoll and Lymphodex were purchased from Serena (Iran), apoptosis and necrosis kit (PI, anti-Annexin V-FITC) was obtained from IQ-product (Netherlands), and other solvents and chemical reagents were procured from Merck (Germany) without further purification.

Preparation of rosemary essential oil (R-EO)

Essential oil extraction from the rosemary plant was performed by the water distillation method (Hydro-distillation) using a Clevenger device (Zimax-Iran) [15]. To this aim, all parts of the plant were first ground and poured into a balloon containing distilled water and then connected to a Clevenger apparatus. After heating, the vapors containing the essential oil molecules were cooled in contact with the refrigerant tubes and placed as a lipid phase in the outlet of the device at the aqueous surface. The essential oil was slowly separated from the aqueous phase. Finally, to preserve the properties and prevent the oxidation process, the container containing essential oil was

sealed with parafilm and foil.

Fourier-transform infrared spectrophotometry of R-EO

In order to identify the type of functional groups of the essential oil of rosemary, a Fourier-transform infrared spectrophotometer device (FT-IR System 2000 Model, PerkinElmer, USA) was utilized. Since the sample was in a liquid form, a drop of the specimen was placed on potassium bromide powder and then formed into a thin film under high pressure.

Cell culture

In this method, blood samples were taken from four healthy female subjects (After receiving informed consent), in an age range of 25-35 years, without a history of smoking, radiotherapy, and genetic disorders. Blood samples were diluted 1:1 with PBS buffer and poured onto vials, and centrifuged at room temperature at 1800 rpm for 30 min. The lymphocyte layers were then gently separated and washed with PBS and then centrifuged at 2000 rpm for 5 min. Finally, the RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (Pen-Strep) was added to cells, and then the cells were transferred to a 5% CO₂ incubator (RS Biotech Galaxy-Australia) at 37 °C.

Toxicity assay

In order to evaluate the toxicity of rosemary essential oil, 10⁵ cells were seeded onto each well of a 96- well plate, and different concentrations of R-EO (0-600 µg / ml) were added to the cells (with four replications). After 96 h incubation (in a 5% CO₂ incubator), the cell survival percentage was measured by MTT assay. For this purpose, 20 µl of the MTT solution was added to each well of the 96-well plate, and after 4 h of incubation, the plates were centrifuged at 2000 rpm for 5 min. The supernatant was removed, and 100 µl Dimethyl Sulfoxide (DMSO) was added to each well.

Afterward, the plate was agitated for 10 min, and then the wells were read by an ELISA reader (Biotek Box 998-USA) at 570 and 630 wavelengths. Wells without R-EO were used as the control group, and finally, the IC₁₀ value of R-EO was determined as a non-toxic concentration to assess the radioprotective effect.

Analysis of radioprotective effect of R-EO

In order to analyze the radioprotection effect of R-EO, the cells were divided into two groups of control (no drug treatment) and treatment (treated with R-EO). In this method, 10⁵ cells were seeded in each well of a 96-well plate (with four replications). Then R-EO (at a concentration equivalent to IC₁₀) was added to the cell culture medium of the treatment group. After 24 h of incubation, both cell groups were irradiated at radiation doses of 0, 25, and 200 cGy using a 6 MV X-ray linear accelerator (for each dose, one plate). Finally, after 72 h of incubation, cell survival was determined by the MTT assay. The percentages of apoptosis and necrosis induced by radiation were also measured by the flow cytometry analysis.

Irradiation conditions

A 6 MV X-ray linear accelerator (Compact-Electa, England) was used to irradiate the cells at radiation doses of 0, 25, and 200 cGy. The monitor unit was calculated by three-dimensional treatment design software (Prowess Panther, Prowess Inc, Concord, CA) version 2.5. Irradiation to a plate containing cells was carried out by the Source to Axis Distance (SAD) method at a depth of 5 cm of solid phantoms and a field size of 20×20 cm². In order to optimize the electron equilibrium conditions, cells were cultured in the central wells of 96-well plates, and the side wells of the plates were filled with the culture media (without cells). Also, the wells were irradiated from the bottom of plates at 180° Gantry angle.

MTT assay

Following irradiation of PBMCs (at doses of 0, 25, and 200 cGy), the cells were incubated for 72 h. Next, the percentage of cell survival was measured by the MTT assay according to the protocol previously described in the section of "Toxicity assay of R-EO". Also, the survival enhancement factor (SEF) was defined as the ratio of cell survival in the presence of R-EO to the cell survival ratio in the absence of R-EO at each radiation dose to measure the radioprotective effect of R-EO.

Flow cytometry analysis

The flow cytometry analysis was used to assess the radioprotective effect of R-EO on the rate of apoptosis and necrosis of PBMCs induced by radiation. For this purpose, after irradiation of cells at radiation doses of 0, 25, and 200 cGy, cells were incubated for 72 h, poured into specific tubes for flow cytometry with 1 ml of added PBS buffer, and centrifuged at 1800 rpm for 5 min. The supernatant was then discarded, and after 20 s of shaking, 5 μ l of the Annexin-V antibody was added and incubated at 4 °C in a dark room for 20 min. Afterward,

0.5 ml of calcium buffer was added to cells and centrifuged at 1800 rpm for 5 min. Next, the supernatant was removed, and 5 μ l of PI dye was added. The cells were incubated at 4 °C in the dark for 10 min; then, 0.5 ml of PBS buffer was added to the cells. Finally, the optical absorbance was read by a flow cytometry apparatus (Partec-PASIII-Germany), and the obtained data were analyzed by the FlowJo software version 7.6 (BD-USA).

Statistical analysis

Data were analyzed by SPSS software version 16, using descriptive statistics (mean and standard deviation) and inferential statistics at a 95% confidence level (P -value \leq 0.05). The independent t-test was used to analyze the obtained values between the two groups of the study.

Results

Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of R-EO are depicted in Figure 1. The peaks of the aromatic rings (C-

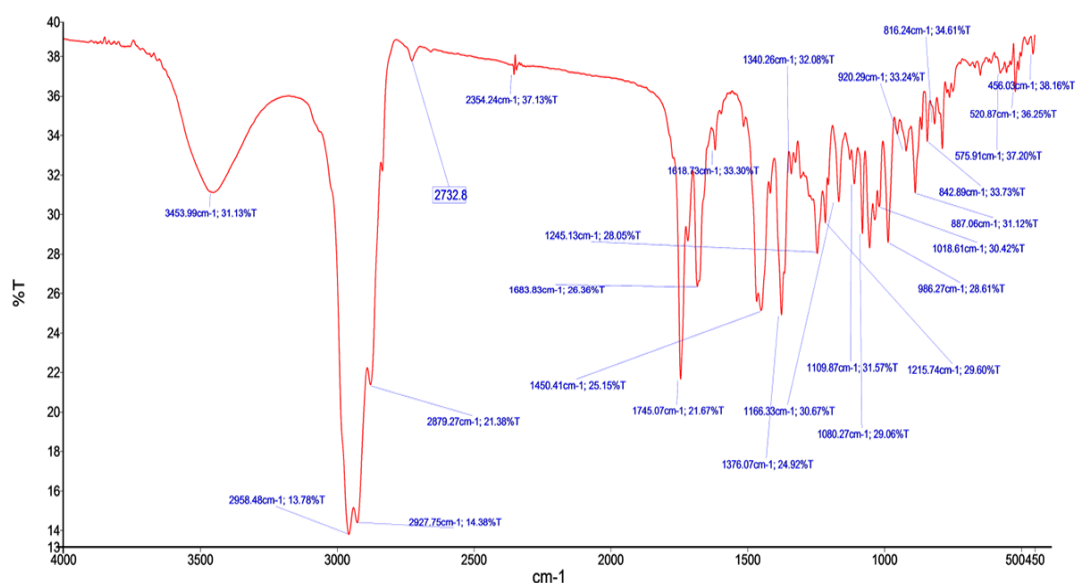


Figure 1: The fourier-transform infrared spectroscopy of Rosemary Essential Oil (R-EO); Peaks associated with aromatic rings (approx. 12900 cm^{-1}), hydroxyl groups (345/993 cm^{-1}), and carbonyl (1745-07 cm^{-1}) are visible

H) are well defined at 2958.48 cm^{-1} , 297.75 cm^{-1} , and 2879.27 cm^{-1} (approximately at 12900 cm^{-1}) that these aromatic rings are the main factors in creating antioxidant properties in plants. A peak at 3453.99 cm^{-1} belongs to the hydroxyl acid (O-H) group, observed in phenolic compounds and an absorption peak at $1500\text{-}1700\text{ cm}^{-1}$ corresponds to the C = C vibration bond, observed in α -pinene, camphene, and β -pinene. A peak at 1745.07 cm^{-1} is assigned to the carbonyl group (C = O), detected in the camphor structure [11].

The toxicity of the R-EO

As shown in Figure 2, the maximum survival rate is $20\text{ }\mu\text{g/ml}$ of rosemary essential oil, and then with an increase in the concentration of rosemary essential oil, the survival rate was decreased so that the IC₁₀ value of R-EO was obtained in a concentration equal to $150\text{ }\mu\text{g/ml}$.

Radioprotective effect of the R-EO

MTT assay

The survival percentage of PBMCs examined by MTT assay is shown in Figure 3. The percentages of cell survival in the presence

($150\text{ }\mu\text{g/ml}$) and absence of R-EO at a dose of 25 cGy , were 115.74 ± 23.46 and 76.00 ± 3.80 and at a dose of 200 cGy were 138.44 ± 7.22 and 66.24 ± 4.18 , respectively. The results of statistical analysis showed that at both radiation doses of 25 and 200 cGy , the difference in cell survival percentage was significantly ($P \leq 0.05$ & $P \leq 0.001$, respectively) increased compared with the control group (irradiation without R-EO).

The SEF values (the ratio of cell survival in the presence of R-EO to cell survival ratio in the absence of R-EO) at doses of 25 cGy and 200 cGy were reported to be 1.53 and 2.09 , respectively. The presence of R-EO significantly increases cell survival that is more obvious in survival rate and SEF at 200 cGy .

Flow cytometry analysis

The results of flow cytometry analysis are shown in Figure 4. The mean percentages of apoptosis in the presence ($150\text{ }\mu\text{g/ml}$) and the absence of R-EO at incubation time of 48 h after 25 cGy radiation doses were 0.99 ± 0.31 and 1.86 ± 0.31 , respectively, while at a radiation dose of 200 cGy such values were 1.18 ± 0.26 and 3.06 ± 0.66 , respectively.

At 72 h incubation, the mean percentages of

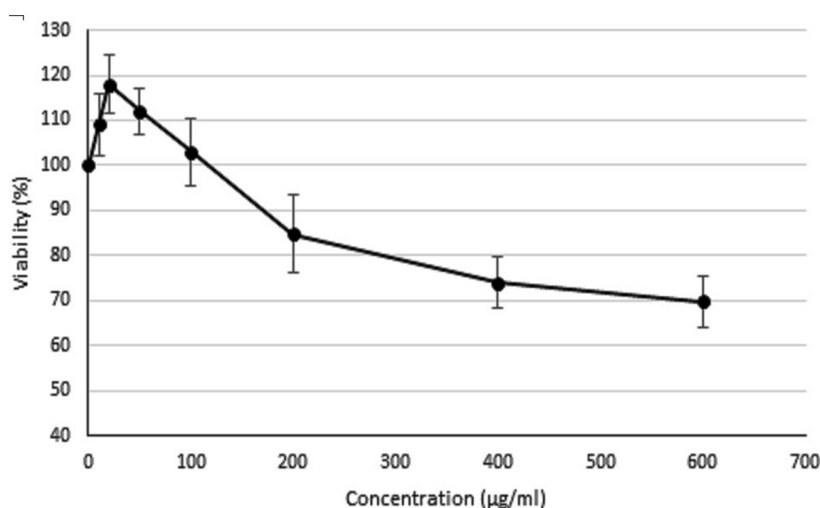


Figure 2: The survival rate of human peripheral blood lymphocytes in the presence of different concentrations of Rosemary Essential Oil (R-EO) ($0\text{-}600\text{ }\mu\text{g/ml}$) obtained from the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay with an incubation time of 96 h .

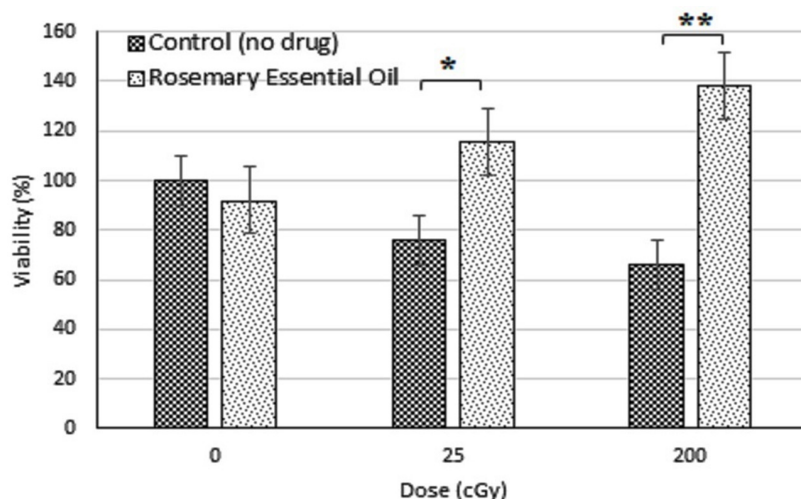


Figure 3: Survival percentage and statistical differences in human peripheral blood lymphocytes with 72 h incubation after irradiation using a 6 MV X-ray linear accelerator (at doses of 0, 25, and 200 cGy) in the presence of Rosemary Essential Oil (R-EO) at a concentration of 150 µg/ml (IC10 (Inhibitory Concentration which induce 10% toxicity)). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

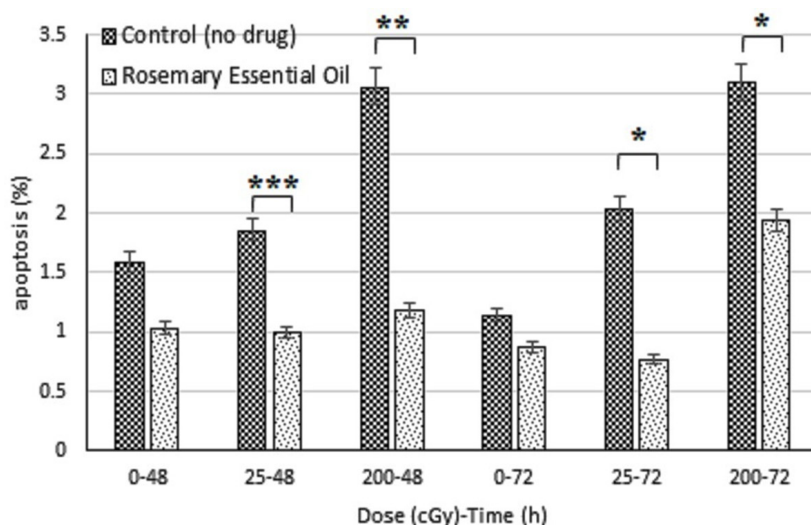


Figure 4: The mean percentage of apoptosis of human peripheral blood lymphocytes at 48 and 72 h irradiated with a 6 MV X-ray linear accelerator in the presence of Rosemary Essential Oil (R-EO) at a concentration of 150 µg/ml (IC10 (Inhibitory Concentration which induce 10% toxicity)) at various radiation doses (0, 25, and 200 cGy). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

apoptosis in the treatment and control groups at 25 cGy dose were 0.77 ± 0.56 and 2.04 ± 0.68 , respectively, while at the dose of 200 cGy, such values were 1.92 ± 0.52 , 3.10 ± 0.74 , respectively.

The flow cytometry results showed that at 48

h incubation, the mean percentage of apoptosis in irradiated PBMCs by both radiation doses of 25 cGy and 200 cGy was significantly lower in the treatment group compared with the control ($P \leq 0.001$ & $P \leq 0.05$, respectively). At 72 h incubation, the percentage of cell apop-

tosis in the treatment group was also less than the control ($P \leq 0.05$ at both radiation doses).

The mean percentages of necrosis at 48 and 72 h of incubation times are displayed in Figure 5. The mean percentages of necrosis in PBMCs, 48 h after irradiation by 25 cGy in the treatment and control groups were 8.52 ± 1.10 and 11.93 ± 1.57 , respectively, while such values at a dose of 200 cGy were 37.23 ± 2.63 and 42.65 ± 3.33 , respectively.

For 72 h incubation time, the mean percentages of necrosis in irradiated PBMCs by 25 cGy were 26.21 ± 6.64 and 28.72 ± 7.40 , respectively, while such values at 200 cGy radiation dose were 43.56 ± 2.02 and 47.30 ± 1.04 , respectively.

As shown in Figure 5, after 48 h incubation, the mean percentage of cell necrosis in the presence of R-EO also significantly decreased compared with the control group at both doses of 25 cGy and 200 cGy ($P \leq 0.01$ & $P \leq 0.05$, respectively). At 72 h incubation, this decrease was statistically significant only at a dose of 200 cGy ($P \leq 0.05$).

Figures 6 and 7 demonstrate an independent sample representing the results of different

treatment groups at a concentration of $150 \mu\text{g/ml}$ R-EO (IC10) at different radiation doses. The upper left quadrant, upper right quadrant, lower left quadrant, and lower right quadrant depict necrotic, late apoptotic, live cells, and early-apoptotic cells, respectively.

As observed in Figures 6 and 7 (B-F), the percentages of apoptosis and necrosis were significantly decreased in the treatment group compared with the control group. Also, the percentage of living cells (Q4) was higher in the treatment group compared with the control group.

Discussion

Due to the adverse effects of radiation, this study was performed to provide a plant extract to modulate injuries caused by ionizing radiation. The presence of a radioprotective agent in the body during radiation can reduce the negative effects of radiation, such as carcinogenicity and hereditary effects [7].

The damage of dispersing ionizing radiation (X-rays) in interaction with the biological system is often caused by producing chemically active agents such as free radicals [16].

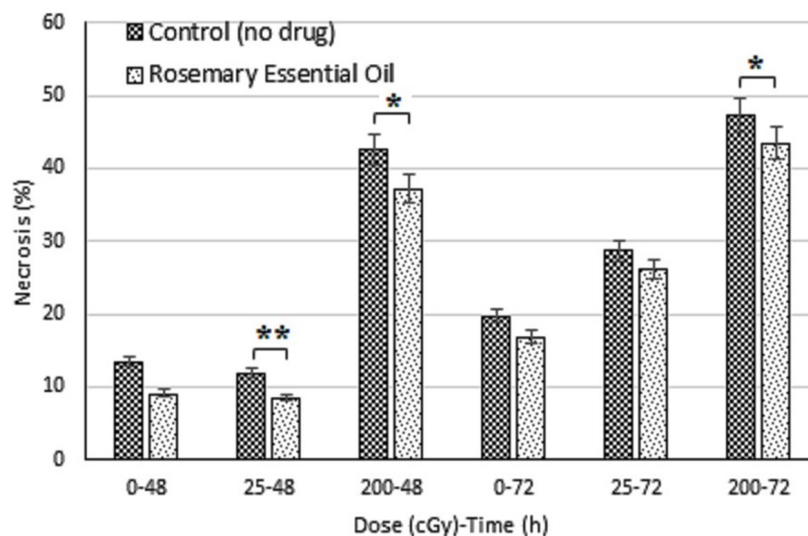


Figure 5: The mean percentages of necrosis in human peripheral blood lymphocytes at 48 and 72 h incubation irradiated with a 6 MV X-ray linear accelerator in the presence of Rosemary Essential Oil (R-EO) at a concentration of $150 \mu\text{g/ml}$ (IC10 (Inhibitory Concentration which induce 10% toxicity)) at different radiation doses (0, 25, and 200 cGy). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

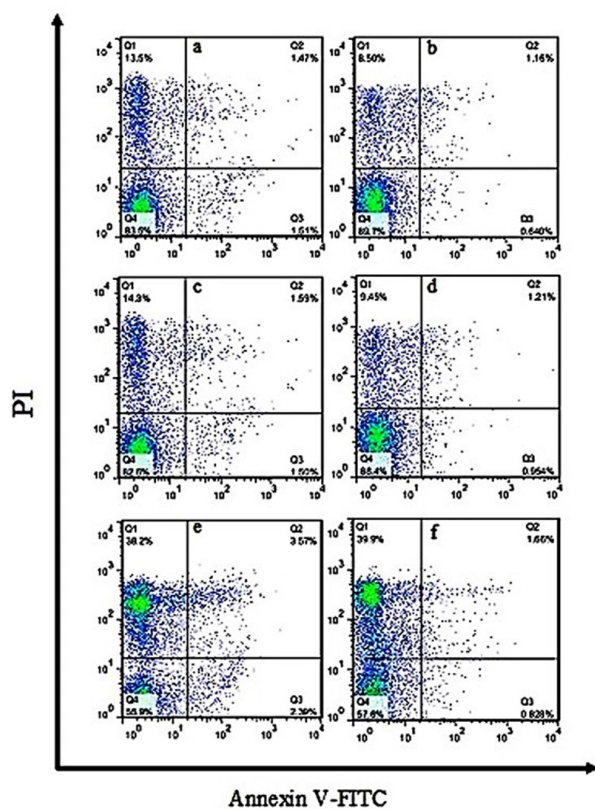


Figure 6: The flow cytometry analysis after 48 h incubation at a concentration of 150 µg/ml Rosemary Essential Oil (R-EO) at different radiation doses using a 6 MV X-ray linear accelerator. A: control group (no treatment) at zero radiation dose, B: treatment group (treated with R-EO) at zero radiation 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.

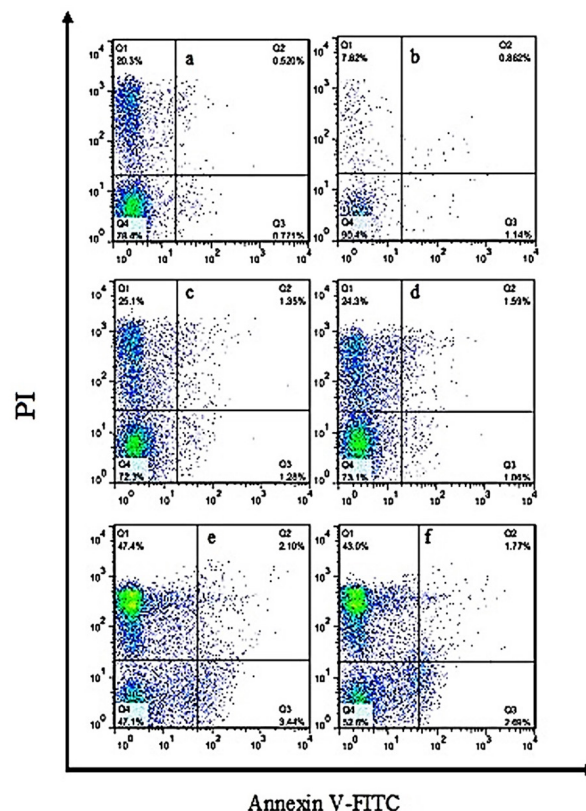


Figure 7: The flow cytometry analysis after 72 h incubation at a concentration of 150 µg/ml Rosemary Essential Oil (R-EO) at different radiation doses using a 6 MV X-ray linear accelerator. A: control group (no treatment) at zero radiation dose, B: treatment group (treated with R-EO) at zero radiation 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.

The prevailing view is that the consumption of natural substances containing antioxidants is capable of decreasing the risk of injuries through scavenging free radical agents in biological systems [10, 17]. The radioprotective effects of plants containing antioxidants and stimulants of the immune system have been extensively examined, and in some of them, the radioprotective effect has been reported [4, 10].

Rosemary contains compounds such as phe-

nolic diterpenes, caffeic, and rosemary acids, and flavonoids inhibiting lipid peroxidation and oxidative DNA damage and can scavenge free radicals [12]. Thus, R-EO was expected to exhibit the radioprotection effects against X-ray as a natural antioxidant and free radical scavenger.

In-vitro studies performed on the radioprotective effects of chemical agents demonstrated that peripheral blood lymphocytes are a suitable option for analyzing the effect

of ionizing radiation due to high radiosensitivity, ease of operation, and coordination at the G0 stage of the cell cycle. Lymphocytes are also considered a biological index of the average amount of exposure to radiation [7]. In this study, PBMCs were utilized to assess the radioprotective effect of rosemary essential oil. Since the radio-sensitivity of human peripheral blood lymphocytes does not follow Bergonie and Tribondeau law and is primarily due to the induction of apoptotic and necrotic death by ionizing radiation [18], reducing the percentage of apoptosis and necrosis as a marker of the radioprotection of R-EO was considered, and the flow cytometry analysis was used to measure such values. The MTT analysis was also applied to determine the survival of PBMCs.

Toxicity of R-EO

According to the toxicity results of R-EO, increased survival at low concentrations may be attributed to the antioxidant, anti-inflammatory, antiviral, and antibacterial properties of rosemary essential oil. As can be seen, even at high concentrations, R-EO has not shown significant toxicity. To measure the radioprotective effect of R-EO on human peripheral blood lymphocytes, a concentration of 150 µg/ml (IC10) was used as a non-toxic concentration for further analyses. Although the literature data suggest that polyphenols behave at higher concentrations as pro-oxidants and can induce apoptosis [19].

The 96-hour incubation time was chosen because it is in accordance with the selected incubation time in this study to measure apoptosis and cell necrosis using flow cytometry analysis (24 h before irradiation and 72 h after irradiation).

Radioprotective effect of R-EO

In order to evaluate the radioprotective effect of R-EO on PBMCs, mitotic, apoptotic, and necrotic deaths induced by ionizing radiation were analyzed by MTT assay and flow

cytometry analysis. cells were irradiated in the presence of R-EO (IC10) at two radiation doses of 25 and 200 cGy using a 6 MV X-ray linear accelerator.

A dose of 25 cGy was selected as a representative sub-lethal dose [20]. On the other hand, in the United States in 2007, the effective dose for adults in interventional, diagnostic radiology, nuclear medicine, and computed tomography tests was in ranges of 5-60 mSv, 0.01-8 mSv, 0.5-40 mSv, and 0.9-32 mSv, respectively [21]. Therefore, it can be expected that in developing countries, and especially in medical examinations, such as fluoroscopy and angiography, these dose ranges would be higher. Hence, it seems that the results of this study at a dose of 25 cGy can be generalized to doses received by personnel and diagnostic patients.

At the same time, the radiation dose of 25 cGy is out of the low dose range (0-150 mSv), and therefore the problems related to the accuracy and reproducibility of the data in this dose range (due to the effects of radio-adaptive response and radiation-induced bystander effects) is raised. A 200 cGy dose was also regarded as a boundary between lethal and sub-lethal doses [22, 23].

Flow cytometry analysis

According to Figures 4 and 5, the percentages of necrosis and apoptosis in the treatment group significantly decreased compared to the control group at both radiation doses.

Most of the antioxidant activity of rosemary is due to phenolic diterpenes, such as carnosic acid, carnosol, rosemanol, isoruzmanol, rosemial and epiruzmanol. These diterpenes act as interrupters in oxidative stress. Caffeine and rosemary acids are another important source of rosemary antioxidants associated with flavonoids [24]. In human peripheral blood lymphocytes, the percentages of apoptosis and necrosis increased following ionizing radiation [18]. Therefore, reducing the percentages of apoptosis and necrosis in PBMCs could be attributed to the increased radioprotective effect

of rosemary essential oil. Our findings indicated that R-EO increased survival and decreased the percentages of apoptosis and necrosis in irradiated PBMCs by X-ray, suggesting the capability of R-EO in the protection of irradiated lymphocytes by scavenging free radicals. As displayed in Figures 4 and 5, the percentages of apoptosis and necrosis in the treatment group at 48 h of incubation at both doses of 25 and 200 cGy were significantly lower than the control group; thus, the highest reduction was observed at 0.25 cGy. However, at a 72-hour incubation period, R-EO showed less radio-protection, leading to a short-term effect.

Conclusion

The results of this study showed that R-EO as a natural antioxidant can reduce the percentages of necrosis and induced apoptosis by ionizing radiation and also increases the survival of PBMCs, rendering a favorable radio-protection effect. This natural radioprotective substance could be used daily by all radiologists and staff who are exposed to ionizing X-rays, along with patients who underwent therapeutic and diagnostic procedures. Rosemary can be a good choice because of its different characteristics such as its pleasant odor that something is used aromatherapy and different useful effects on human cells.

Further studies are warranted to elucidate the mechanism underlying the radioprotective effect of rosemary essential oil. In future studies, the radiation protection effect of R-EO in a more radiation dose range should be investigated so that the results can be expressed in terms of the dose reduction factor (DRF), i.e the radiation protection effect of several radiation plants should be also investigated in the same way and the best sample should be selected to improve the kinetics of radioprotective agents with the help of nanocarriers.

Acknowledgment

This study was supported by a grant from Shahid Sadoughi University of Medical Sciences, Yazd,

Iran. This research was the subject of an MSc thesis of Parisa Zhaeintan and was approved by the ethical committee with a code number of IR.SBMU.MSP.REC.1399.236. The authors would like to gratefully thank the selfless contribution of Mr. M. Dehestani as a physicist of the radiation oncology center of Shahid Ramezanzade and Mr. H. Rahavi as a laboratory assistant in data collection.

Authors' Contribution

N. Hamzian designed and managed the study and A. Nickfarjam helped him. P. Zhaeintan obtained ethics approval and collected the data and wrote first version of manuscript. A. Shams designed and managed in vitro tests. S. Abdollahi-Dehkordi was involved in writing, editing and preparing the manuscript for submission. All authors contributed to the article and approved the submitted version.

Ethical Approval

The experimental procedures of the present research study were confirmed by the Ethics Committee of Shahid Sadoughi University of Medical Sciences in Yazd (IR.SSU.MEDICINE.REC.1397.129).

Funding

This work was supported by a grant from Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Conflict of Interest

None

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