Radioprotective effect of nanoniosome loaded by Mentha Pulegium essential oil on human peripheral blood mononuclear cells exposed to ionizing radiation

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Research Article

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Abstract

Introduction

: Despite the applications and advantages of ionizing radiation; there are many radiation risks to biological systems that are necessary to be reduced as much as possible. The present study aimed to assess the radioprotective effect of nanoniosomes loaded by *Mentha Pulegium* essential oil (MPEO-N) as a natural antioxidant on human peripheral blood mononuclear cells (PBMCs).

Materials and methods

MPEO-N nanoparticles were prepared by the lipid thin-film hydration method, and its physicochemical characteristics were analyzed. PBMCs were then irradiated with X-ray using a 6 MV linear accelerator at radiation doses of 25 and 200 cGy in the presence of non-toxic concentrations of MPEO-N nanoparticles (IC10). After 48 and 72 h of incubation, the radioprotective effect was investigated by measuring survival, apoptosis, and necrosis of PBMCs, using MTT assay and flow cytometry analysis.

Results

The hydrodynamic diameter and zeta potential of nanoniosomes were 106.0 nm and − 15.2 mV, respectively. The mean survival percentage of PBMCs showed a significant increase only at a radiation dose of 200 cGy compared with the control group. The percentages of apoptosis and necrosis of cells in the presence of MPEO-N nanoparticles at both radiation doses and incubation periods (48 and 72 hours) demonstrated a significant reduction compared with the control.

Conclusion

MPEO-N nanoparticles as a natural antioxidant, exhibited a favorable radioprotective effect by a significant reduction in the percentage of apoptosis and necrosis of irradiated PBMCs.

1. Introduction

Numerous epidemiological studies indicate carcinogenic risks of ionizing radiation. The occupational and medical radiation exposure, although not in the moderate and high radiation dose ranges, can pose significant radiation risks to staff and especially to diagnostic patients [1]. X-ray, which is most commonly used in medicine and industry, have a low linear energy transfer rate and therefore their indirect interaction is predominant, due to which biological macromolecules are mainly damaged by interaction with water radiolysis products, such as reactive oxygen species (ROS) [2] An effective way to counteract the effects of ROS is the use of antioxidant compounds to delay or prevent the oxidation of biological macromolecules by different mechanisms [3]. In some studies, antioxidant compounds have

been shown to exert radioprotective effects [4]. Although many studies have indicated significant benefits of radioprotectors, these compounds are not typically prescribed as a dietary supplement or with food to radiation staff or to patients before or after medical imaging [5]. Natural compounds have most of the required criteria to be applied as radioprotective agents. They employ several mechanisms to exert their radioprotective effects on living organisms [3]. For example, they eliminate the destructive effect of oxidant agents by donating electrons to peroxyl or hydroxyl radicals and, in this process, might be converted into free radicals with less deleterious effects [6]. Studies show that some plant-derived natural antioxidant compounds are more effective than synthetic ones and have lower toxicity. Therefore, there is an increasing desire to study and use plant-derived natural antioxidants in medicine [7, 8].

Mentha Pulegium belongs to the Lamiaceae family and contains natural antioxidant compounds such as flavonoids, alkaloids, and polyphenols. *Mentha Pulegium*, along with its essential oil, has been known as a scavenger of free radicals [7]. Despite the benefits of natural antioxidants, there are limitations to using these valuable substances. These limitations include low solubility, low shelf-life, uncontrolled release, and instability in digestion and absorption in the gastrointestinal tract. Hence, to overcome such challenges, nowadays, the use of nanocarriers has been developed [9, 10]. Studies have shown that loading of natural antioxidants on nanocarriers results in their controlled release into cell membranes and improves cell uptake, preserving their compounds from premature degradation, enhancement of biodegradability, and improvement of drug retention time in the blood circulation [10]. Nanoniosomes with a flexible structure have been accepted by numerous researchers as carriers and are suitable for loading hydrophilic and hydrophobic compounds. Nanoniosomes are surfactants-based non-ionic vesicles that are structurally similar to liposomes but have some advantages, such as lower manufacturing costs and greater stability than liposomes. At the same time, they are biocompatible and biodegradable and cause less toxicity due to their non-ionic nature [9].

The aim of this study was to assess the radioprotective effect of Nanoniosomes-loaded *Mentha Pulegium* Essential Oil (MPEO-N) on human peripheral blood mononuclear cells (PBMCs) as a radiosensitive cell line. For this purpose, the percentage of survival, apoptosis, and necrosis of irradiated PBMCs was evaluated in the presence of nanoparticles using MTT assay and flow cytometry analyses.

2. Materials And Methods

2.1 Materials

Cholesterol was purchased from Sigma-Aldrich (USA). Tween-60 and polyethylene glycol (PEG) were obtained from Ludwigshafen (Germany). RPMI1640 culture medium, phosphate-buffered saline (PBS), and penicillin-streptomycin (Pen-strep) were obtained from Inoclon (Iran). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (USA). Ficol was purchased from Serena (Iran). Apoptosis and Necrosis Kits (PI, anti-Annexin V-FITC) were obtained from IQ-product (Netherland). Other solvents and chemical reagents were procured from Merck (Germany) without further purifications.

2.2 Extraction of MPEO

The preparation of MPEO was performed by the steam distillation method (using the Clevenger apparatus (Simax-Iran). In brief, 100 g of the leave of the *Mentha Pulegium* were powdered in a mortar and then mixed with 500 ml of distilled water in the balloon of the device. Then, the essential oil was separated for 4 h and subsequently transferred to a microtube covered with aluminum foil until use.

2.3 Preparation of formulations

The lipid thin-film hydration method was applied to prepare MPEO-N nanoparticles [9]. To this aim, 180.0 mg of Tween 60 and 22.8 mg of cholesterol were dissolved in chloroform and transferred to a round-bottom balloon, and adjusted to a volume of 20 ml with chloroform. Next, 2.0 mg of MPEO was dissolved in 2 ml of methanol, and the resulting mixture was added to the balloon. Afterward, the balloon contents were homogenized, and the solvent was removed by a rotary device (Heidolph- Germany) for 30 minutes (150 rpm, 37°C). Then, in order to hydrate the lipid thin-film, 10 ml of PBS was added to the balloon and agitated at 45°C for 30 minutes. The product was obtained as single-layer and multilayer nanoparticles. Then, 11.2 mg of mPEG was added to the formulation and stored in the dark at 25°C to cover the surface of vesicles. After that, to reduce the size and create small unilamellar vesicles, a sonicator probe device (Ultrasonic, Iran) was used for 30 minutes (15 seconds on, 10 seconds off), and the product was filtered using a 0.2 µm filter. Finally, the product was transferred into a dialysis bag and placed on a heater (Heidolph, Germany) containing 150 times the sample volume of PBS to separate the unloaded MPEO. The nanoparticles were stored at 4°C and kept away from light for subsequent analyses. MPEO-free nanoniosomes were prepared in a similar method, except that MPEO was not used in the oil phase.

2.4 Physicochemical characterization of MPEO-N nanoparticles

2.4.1 Morphology and hydrodynamic diameter

The morphology of MPEO-N nanoparticles was analyzed using atomic force microscopy (AFM) (Hitachi-Japan) and scanning electron microscope (SEM) (NOVA NanoSEM, USA). The hydrodynamic diameter, zeta potential, and polydispersity index (PDI) of nanoparticles were also determined using a Zetasizer instrument (HORIBA, Japan) by the dynamic light scattering (DLS) method.

2.4.2 Encapsulation efficiency (EE %) and loading capacity (LC %)

To calculate the EE % and LC%, first the maximum absorbance wavelength of MPEO was obtained by UV-Vis spectrophotometry (Bio Tek, USA) in a wavelength range between 200 to 600 nm. For this purpose, the diluted concentration of MPEO in methanol was analyzed in an absorbance range of 1, and methanol was used as a blank. Then, 1 ml of the MPEO-N nanoparticle suspension (equivalent to 1 g of formulation) was dissolved in 1 ml of isopropanol, and the amount of MPEO in the solvent was determined by a UV-Vis spectrophotometer at the wavelength of maximum absorbance of MPEO (using

isopropyl as blank and corresponding calibration curve). Finally, the LC and EE values of MPEO-N nanoparticles were calculated based on the following equations [11]:

$$\mathrm{LC}(\%) = \frac{\mathrm{drugweightinnanoparticle}}{\mathrm{theweightofnanoparticle}} \times 100 \; \mathrm{EE}(\%) = \frac{\mathrm{Residualdruginthenanoparticle}}{\mathrm{Initialfeedingamountofdrug}} \times 100$$

2.5 MPEO-N release curve

In order to measure the release rate of MPEO from nanoniosomes, 1 ml of the formulation was poured into a dialysis bag and stirred in 10 ml of PBS buffer under body conditions (pH 7.4, 37°C). At regular intervals of 30 minutes up to 72 hours, 1 ml of the buffer in the container was replaced by 1 ml of a fresh buffer, and its absorption at the maximum absorption wavelength of MPEO (300 nm) was read by UV-Vis spectrophotometer (using PBS as blank and related standard curve). Finally, the amount of released MPEO from nanoniosomes was calculated at each time and the release curve.

2.6 Cell culture

For cell culture, in brief, after obtaining written consent (according to the Helsinki Declaration), blood samples were obtained using prefilled heparin syringes from five volunteer males, in the age range of 20–30 years old, without a history of radiotherapy, systemic diseases, and smoking. Next, human peripheral blood lymphocytes were isolated by means of Ficoll density gradient centrifugation. Lymphocytes were washed twice with PBS and centrifuged at 1500 rpm at room temperature for 5 minutes each time. After the removal of the supernatant, RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic (pen-strep) was added to the cells. The cells were then counted using trypan blue. Then, 10^5 cells were seeded onto a 96-well plate and incubated in a %5 CO_2 incubator at 37°C.

2.7 Toxicity assay

The toxicity of MPEO and MPEO-N nanoparticles assessed by the MTT assay. Briefly, the cells were seeded in a 96-well cell culture plate (at a density of 1×10^5 cells/well) and equivalent concentrations of 30–480 µg/ml of MPEO-N nanoparticles from different formulations were added to the wells with five replications for each concentration,. After 96 h of incubation, 20 µl of the MTT solution (5 mg/ml in PBS) was added to each well. After 4 h of incubation, the plate was centrifuged at 1800 rpm at 4°C for 5 minutes. Afterward, the supernatant was discarded, and 100 µl of DMSO was added to each well. After 10 minutes of shaking, the optical absorbance of the wells was read by an ELISA reader (Biotek, USA) at a wavelength of 570 nm vs. 630 nm (as blank). Finally, the cell survival percentage in different groups and the IC10 of each formulation were calculated. Wells filled with PBMCs without nanoparticles were considered as a control group.

2.8 Investigation of radioprotective effect

2.8.1 Irradiation conditions and treatment groups

Before irradiation, the cells were divided into 3 main groups of control (without drug), MPEO treatment group, and MPEO-N treatment group. Cells were irradiated in different groups with radiation doses of 0,

25, and 200 cGy using a 6MV linear accelerator X-ray (Compact-Electa, England). Irradiation was performed according to the Source to axis distance (SAD) technique at a depth of 5 cm of the tissue-equivalent solid phantom at a 180 ° Gantry angle with a field size of 20×20 cm^2. The monitor units were calculated by Prowess Panther treatment planning system (TPS) version 5.2 (Prowess, Inc., CA, USA), according to the attenuation coefficient of the plates. In order to promote the dosimetry conditions, central plate wells were used for cell culture, while the marginal wells were filled with culture medium.

2.8.2 Determination of cell survival

In order to evaluation of radioprotective effect of formulations on the survival of irradiated PBMCs, the cells were seeded in 96-well cell culture plate (at a density of 1×10^5 cells/well*)*. After 24 h of incubation, concentrations of different formulations equivalent to IC10 of MPEO-N nanoparticles were added to the wells in different treatment groups, and after 24 h of incubation, irradiation was performed as previously described. Finally, the percentage of cell survival was measured at 48 and 72 h of incubation by the MTT assay (as described in toxicity assay section). To quantify the radioprotective effect, the survival enhancement factor (SEF) was defined in each radiation dose and calculated as the survival in the presence of drug to survival in the absence of drug.

2.8.3 Determination of apoptosis and necrosis

To investigate the radioprotective effect of the formulations on the percentage of apoptosis and necrosis of the PBMCs, as previously described, irradiation was performed in different groups. At two different incubation times of 48 and 72 hours, cells were poured into tube specified for flow cytometry analysis. Then 1 ml of PBS was added to the cells and after stirring, the tubes were centrifuged at 1800 rpm for 5 minutes. The supernatant was then removed and the tube was centrifuged again with 0.5 ml PBS. Then, the supernatant was discarded, and 4 µl of Annexin-V was added to the tube. After30 minutes of incubation of samples at 4°C and in the dark, 0.5 ml of PBS was added to tube, and after a few seconds of shaking, samples were centrifuged at 1800 rpm for 5 minutes. Following centrifugation, the supernatant was separated. Then, 4 µl of PI was added to the cells and centrifuged at 1800 rpm for 5 minutes. The supernatant was removed, and the cells were incubated at 4°C for 10 minutes in the dark. Finally, after brief agitation of samples, 0.5 ml of PBS was added to samples, and the percentages of apoptotic and necrotic cells were analyzed by flow cytometry (Partech, Germany). The obtained data were analyzed by FlowJo software version 7.6 (BD, USA).

2.9 Statistical analysis

Data were analyzed by GraphPad Prism software version 9 using descriptive statistics (mean and standard deviation) and inferential statistics at a 95% confidence level (*P*-value < 0.050). Since the data were normally distributed, the difference between groups was analyzed by one-way analysis of variance (one-way ANOVA).

3. Results

3.1 Physicochemical properties

The morphology of MPEO-N was assessed by AFM and SEM. As shown in Fig. 1, nanoparticles are spherical and distributed separately.

According to the results of DLS analysis, the mean hydrodynamic diameter and PDI of MPEO-N nanoparticles were 106.0 nm and 0.20, respectively. The zeta potential of MPEO-nanoparticles was − 15.2 mV.

The EE % and LC % of MPEO-N nanoparticles were 44.37 and 3.40%, respectively.

3.2 Release curve

The release curve of MPEO-N nanoparticles in PBS (pH = 7.4) is shown in Fig. 2. After 24 hours, the release percentages of *Mentha Pulegium* from MPEO-N nanoparticles at 37 and 42 ºC, were 52.21% and 60.98%, respectively.

3.3 Toxicity of formulations

The toxicity of formulations on PBMCs was assessed after 96 h of incubation using the MTT assay. As shown in Fig. 2, the concentrations of MPEO and MPEO-N nanoparticles at the peak of the survival curve were 20 and 40 µg/ml, respectively. Survival was then gradually reduced so that the IC10 value of MPEO was reported to be 80 µg/ml, while the IC10 value of MPEO-N nanoparticles was approximately equal to 170 µg/ml.

3.4 Radioprotective Effect on survival of PBMCs

The survival of PBMCs was assessed using the MTT assay. Figure 3 depicts the survival percentage of PBMCs in different groups at two incubation times of 48 and 72 hours. The concentration of all formulations was considered to be equivalent to the IC10 value of MPEO-N nanoparticles (170 µg/ml). The mean survival percentage of PBMCs at a dose of 200 cGy and at two incubation times of 48 and 72 h was 78.80 ± 7.56 and 81.49 ± 8.53, respectively, which showed a significant increase compared to the control group ($P < 0.05$ and $P < 0.01$, respectively).

At 25 cGy, despite the increase in survival percentage compared to the control group, such an increase was not statistically significant. The percentage of cell survival in the MPEO treatment group also increased compared to the control in both radiation doses and both incubation times; however, such increment was not statistically significant.

The maximum SEF values for MPEO-N nanoparticles were obtained at a radiation dose of 200 cGy reported to be 1.16 and 1.26, at 48 and 72 h of incubation, respectively.

3.5 Rafioprotective effect on apoptosis and necrosis of PBMCs

The mean percentage of apoptosis and necrosis of irradiated PBMCs in the presence of different formulations at the concentrations equivalent to the IC10 value of MPEO-N nanoparticles (170 µg/ml) was determined by flow cytometry. As shown in Fig. 4, the mean percentage of apoptosis in all treatment groups showed a significant decrease compared with the control group at both radiation doses of 25 and 200 cGy and both incubation times of 48 and 72 h ($P < 0.05$).

The percentage of apoptosis in the MPEO-N treatment group at a radiation dose of 200 cGy at 48 and 72 h of incubation was 6.57 ± 0.99 and 4.56 ± 1.24, respectively, which compared to the control group (10.17 + 2.50 and 10.78 + 2.17, respectively) showed a significant decrease ($P \leq 0.001$ for both). Also, the reduction in the apoptosis percentage was significantly higher at a dose of 200cGy than a dose of 25cGy (at both incubation periods).

In the MPEO treatment group compared to the control group, the highest decrease in the percentage of apoptosis in PBMCs was related to a dose of 200 cGy and 72 h incubation time, so that the percentage of apoptosis has significantly decreased from 10.16 ± 2.51 to 7.20 ± 1.40 ($P < 0.01$).

The percentage of apoptosis in the MPEO-N group showed a greater decrease compared to MPEO group. Such a difference was statistically significant ($P < 0.05$) except for the treatment group with a radiation dose of 25 cGy at 72 h of incubation.

As shown in Fig. 5, the percentage of necrosis in the MPEO-N treatment group in both radiation doses and both incubation times showed a significant decrease compared to the control group. The highest decrease ($P < 0.001$) was observed at a radiation dose of 200 cGy with an incubation time of 72 h for the MPEO-N treatment group (26.30 ± 4.10) compared to the control group (38.76 ± 4.15). However, the reduction in the necrosis percentage of the MPEO treatment group compared to the control group was not statistically significant in both incubation times and both radiation doses ($P \geq 0.05$).

In Fig. 6, the gating of the population of PBMCs after 72 h 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).

4. Discussion

4.1 Study parameters

Due to the widespread use of ionizing radiation in various areas of human life, the target population in this study was people who were exposed to occupational radiation and medical radiation. The application of ionizing radiation in medicine has been increasing in recent decades [6, 5]. According to

NCRP Report No. 160, approximately half of radiation exposure in the United States is due to medical exposure, which according to reports of ANSCEAR in 2008[12], is estimated at 42% worldwide and appears to be increasing every year. In diagnostic imaging, although the dose of each examination has been reduced due to improved technology and protocols, as well as the increased awareness of radiation staff, the annual effective dose has been elevated owing to the increasing number of medical examinations [5]. According to reports of UNSCEAR and NCRP, the dispersed radiations such as X-ray and gamma rays play a major role in medical exposure [13].

It has now been shown that the interaction of dispersed ionizing radiation such as X-rays with the biological systems leads to the production of free radicals and reactive oxygen species (ROS) [14]. On the other hand, the ability of antioxidants to scavenge free radical has been confirmed in numerous studies [15]. Hence, due to the antioxidant nature of MPEO, as a result of possessing phenolic and phenoloid compounds [7], it would be expected that this essential oil can be an efficient radioprotector. The low toxicity of MPEO in comparison with synthetic compounds, as well as the possible removal of its limitations by loading on nanoniosomes, gives us hope for a desirable radioprotective agent.

In this study, PBMCs were chosen to investigate the radioprotctive effect of MPEO-N. PBMCs are considered an example of natural non-proliferating tissue cells which are at the G0 phase. These types of cells are obtained using minimally invasive methods, and a large number of cells can be collected serially in a short time [16]. In addition to the above properties, due to the availability and high radio-sensitivity, they have always been considered in radiation protection studies [17]. Despite Bergonié and Tribondeau's law, which states the relationship between proliferation and radio-sensitivity [18], PBMCs have high radiation sensitivity, due to radiation-induced apoptosis [19] Therefore, in the present study, the radioprotective effect of nanoniosome loaded by *Mentha Pulegium* essential oil was investigated by measuring apoptotic, necrotic, and mitotic death in PBMCs by MTT assay and flow cytometry analyses.

For this aim, the cells were irradiated at two radiation doses of 25 and 200 cGy. A radiation dose of 25cGy was considered as approximately representative of the range of radiation dose to individuals [20] in common applications of X-rays (especially in diagnostic imaging), in such a way that it is out of the low dose range (which is highly debatable in studies due to bystander effect, adaptive radiation response, etc. [21]. The 200cGy radiation dose has also been considered as the boundary between lethal dose and sub-lethal dose in radiation protection studies [22].

4.2 Characteristic of MPEO-N nanoparticles

The physicochemical properties of nanoniosomes have a remarkable influence on their behavior and systemic activities [21]. The size of nanoniosomes is one of the main characteristics that directly affect cell uptake. Nanocarriers less than 150 nm can cross through the capillary endothelium of the liver [23, 24]. Nanoniosomes prepared by the thin-film hydration method are larger than those prepared by the ether-injection method; also, increasing the amount of cholesterol increases the size of the vesicles [25, 26]. In this study, Tween 60 was used, and the average hydrodynamic diameter of MPEO-N nanoparticles was 106.0 nm, which in comparison with studies with the same preparation methods, seems to be in an

appropriate range for in-vitro and in-vivo treatments [25, 23, 27]. The PDI of nanoparticles was also less than 0.3, implying the acceptable particle-size distribution [28].

Studies show that nanoparticles with a zeta potential range between − 41.7 to -58.4 mV have appropriate electrostatic stability [23]. However, due to the fact that excessive addition of charged molecules can disrupt the mechanism of nanoniosome formation [28], the zeta potential of MPEO-N in this study (-15.2 mV) seems to inhibit aggregation and is sufficient to create stability and this was observed in practice by keeping nanoparticles in the body's simulated environment for a long time [29].

The encapsulation efficiency and loading capacity of MPEO-N nanoparticles were 44.37% and 3.4%, respectively. These two quantities generally depend on various parameters, such as the type of compound loaded in nanoniosomes, the method of preparation, the length of the non-ionic alkyl surfactant chain, and the ratio of surfactant to cholesterol. Studies have shown that the use of Tween60 in the production of nanoniosomes increases the loading of target compounds compared with other surfactants and also causes further stability [25, 26].

4.3 *Mentha Pulegium* release rate

The release curve (Fig. 2) of MPEO-N nanoparticles showed that the release rate of *Mentha Pulegium* from nanoniosomes is continuous and controlled so that a 50% release (T1/2) occurred during 24 h under the normal body condition (37°C, pH = 7.4). The longer alkyl surfactant chain is able to lower the drug release rate, reduce drug leakage, and enhance the stability of nanoniosomes [25, 30, 27]. At 42°C, the release rate was carried out with a steeper slope (T1/2= ⁓7 hours), and after 10 hours, an almost constant trend was reached. It is clear that the increase in temperature had a direct effect on the release rate. According to previous studies, PEGylation of nanoniosomes also improves the hydrophilicity of their molecular surface and thus prevents their recognition and elimination by phagocytic systems, leading to the increased stability and half-life of PEGylated nanoniosomes [30].

4.4 Toxicity of formulations

In order to assess the radioprotective effect of MPEO-N nanoparticles on PBMCs, a non-toxic concentration of nanoparticles was used [31], which in this study was considered IC10. To evaluate the toxicity of different formulations, MPEO-N equivalent concentrations of each formulation with 96 h of incubation were used, which was proportional to the incubation time used to determine the radioprotective effect of formulations by MTT assay and flow cytometry analyses. As the results showed, MPEO and MPEO-N nanoparticle did not show toxicity up to a concentration of 170 µg/ml.

4.5 Radioprotective effect of MPEO-N

4.5.1 MTT assay

As illustrated in Fig. 3, the results showed that despite an increase in the survival of PBMCs in all treatment groups compared with the control, such an increase in a radiation dose of 25 cGy at both incubation times was not statistically significant. Only at a radiation dose of 200 cGy and for the MPEO-N

treatment group, the survival of PBMCs was significantly higher than the control, as the highest increase was observed at 72 h of incubation ($P < 0.01$). Notably, the increase in the survival of PBMCs in MPEO-N treatment group was not statistically significant compared to MPEO treatment group. In radiation protection studies, to quantify the radioprotective effect of an agent, the well-known quantity of DRF is introduced, which is defined at a radiation dose that results in 50% survival (D50) [32]. Since the radioprotective effect of MPEO-loaded nanoniosomes was examined at two radiation doses of 25 and 200cGy, the quantity of survival enhancement factor (SEF) [33] was defined which is the ratio of cell survival in the presence and absence of radioprotector in each radiation dose [34]. According to this definition, the maximum SEF value was reported to be 1.29, related to the MPEO-N treatment group, at a dose of 200 cGy in 72 h of incubation.

To explain the reasons for these results, it has previously been stated that the main mechanism underlying radiation damage in PBMCs is apoptosis, and in fact, PBMCs are considered resistant to radiation-induced mitotic death due to their differentiation and non- proliferation [19, 18]. Consequently, the lack of the optimal radioprotective effect of MPEO-N in the increase of the survival of PBMCs may be related to this issue.

4.5.2 Flow cytometry analysis

According to the Fig. 5, the results of the flow cytometry analysis showed that the percentages of apoptosis and necrosis of PBMCs are increased in response to irradiation which is consistent with previous studies [16, 5]. Of note, such an increase was moderated in the presence of MPEO and MPEO-N. In the MPEO-N treatment group, the percentage of apoptosis and necrosis of irradiated PBMCs was significantly reduced in both radiation doses and both incubation times compared to the control group ($P < 0.01$ for apoptosis and $P < 0.05$ for necrosis). Such a decrease in the MPEO group was significant only for the percentage of apoptosis ($P < 0.05$, compared to control).

Comparing the radioprotective effect of MPEO-N and MPEO, the results showed that the reduction of apoptosis percentage of PBMCs in MPEO-N treatment group was more than MPEO treatment group, but this difference was significant only in 200 cGy radiation dose ($P < 0.05$). Also, the percentage of necrosis in PBMCs showed a significant decrease only in the MPEO-N treatment group compared to the control, and this clearly indicates that the MPEO-N is more effective than the MPEO on the radioprotective effect.

Among the natural compounds, useful studies have been performed on the radioprotective effect of curcumin on PBMCs [35, 36], the results of which may be generalized to MPEO. In a study carried out on PBMCs irradiated with a radiation dose of 2 Gy, it was shown that dendrosomal nanoformulation of curcumin, by modulating the NF-κB and Nrf-2 pathways, affects the expression of genes whose products are involved in cell cycle regulation, DNA damage detection, and apoptosis, thereby increasing cell survival [36].

Amifostine is the first FDA-approved radioprotector to reduce the incidence of moderate to severe xerostomia after radiation therapy of head and neck cancer [37]. Despite the favorable radioprotective

effect, its use is usually discontinued in 15–20% of patients due to severe side effects, such as hypotension, fatigue, and drowsiness. Also, the short half-life of this drug in patients diminishes its effectiveness [38]. Another group of radioprotectors discussed in the last two decades, which have been studied extensively, are Fullerenol nanoparticles or other water-soluble derivatives of Fullerenol (C60). However, there is much controversy about their toxicity, and most of the effectiveness of these agents is limited to ionizing radiations with low LET [39].

Although numerous studies have been conducted from the past to the present to analyze the radioprotective effects of chemical and natural compounds, it appears that we are still far from introducing an ideal radioprotector agent with versatile clinical applications [40]. One of the major challenges in the development of radioprotectors is the lack of a comprehensive system to biologically examination of these compounds [41]. The radioprotective effect of a radioprotector candidate depends on various parameters, the most important of which are the radiation dose, cell line, and the mechanism to study. These variables make it difficult to compare the radioprotective effect of different mediators. Hence, it seems that a single system for measuring the radioprotective effect is necessary to select a radioprotector with the optimal performance for additional analyses.

5. Conclusion

The present study evaluated for the first time the radioprotective effect of nanoniosomes-loaded *Mentha Pulegium* essential oil. MPEO-N nanoparticles show their optimal radioprotective effect by reducing the percentage of apoptosis and necrosis of PBMCs. Due to the unique properties of MPEO-loaded nanoniosome, such as low toxicity, biocompatibility, biodegradability, and controlled release; it can be a useful candidate for further studies aimed to developing functional food products or daily supplements for radiation staff, as well as patients who are exposed to low LET radiation. Further studies are needed to better elucidate the mechanism of radioprotective effect of MPEO-N. In this regard, the evaluation of other cell lines that are sensitive to radiation-induced mitotic death would be recommended.

Declarations

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Compliance with Ethical Standards

This research was conducted with the code 6743 in Shahid Sadoughi University of Medical Sciences as a master's thesis of Moslem Najmi-Nejad and was approved by the Iran National Committee for Ethics in Biomedical Research with the code IR.SSU.MEDICINE.REC.1398.326. The authors declare no conflict of interest.

Availability of Data and Materials

The datasets generated during current study are available from the corresponding author on reasonable request.

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Figures

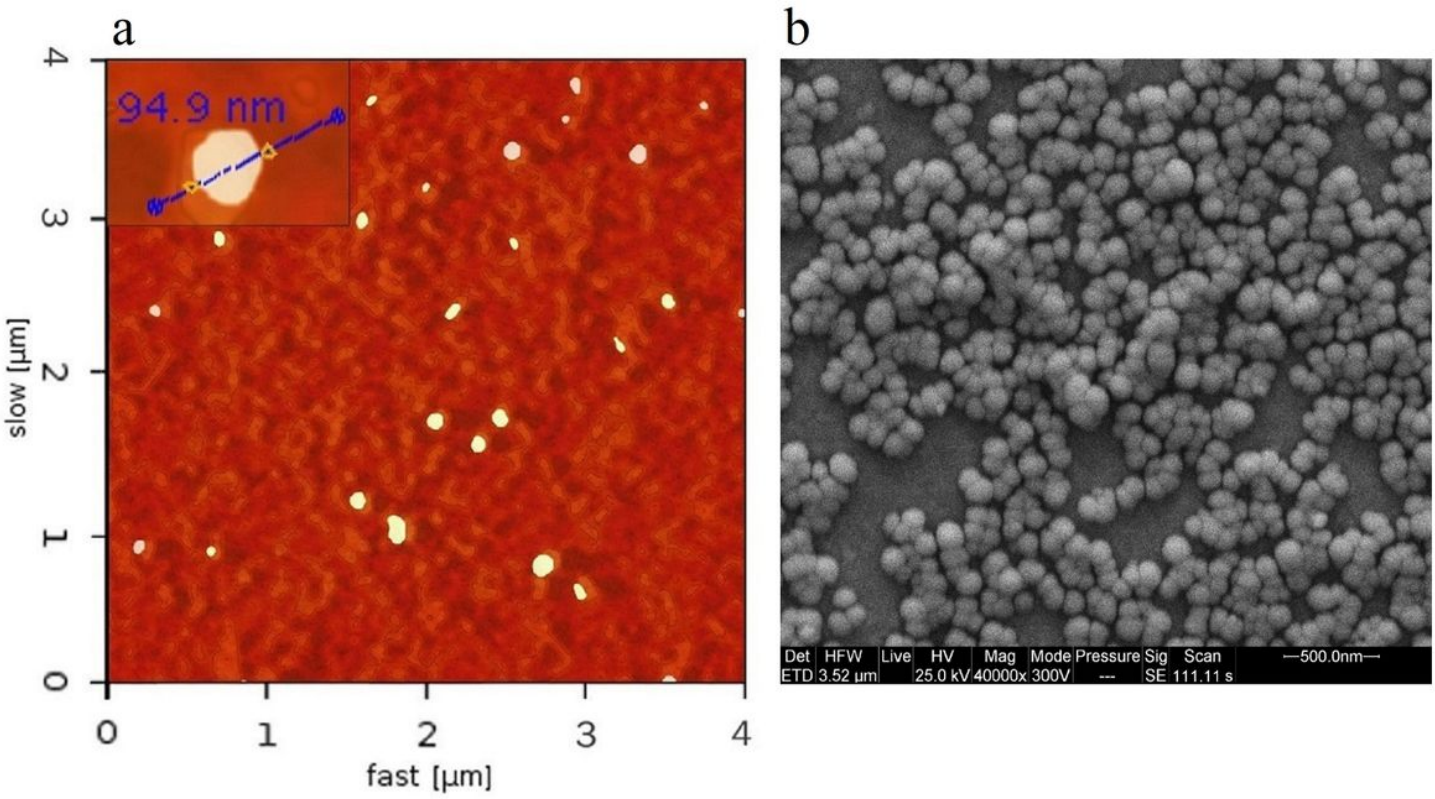

Figure 1

a: Atomic force microscope (AFM) image of MPEO-N nanoparticles, b: Scanning electron microscope (SEM) image of MPEO-N nanoparticles.

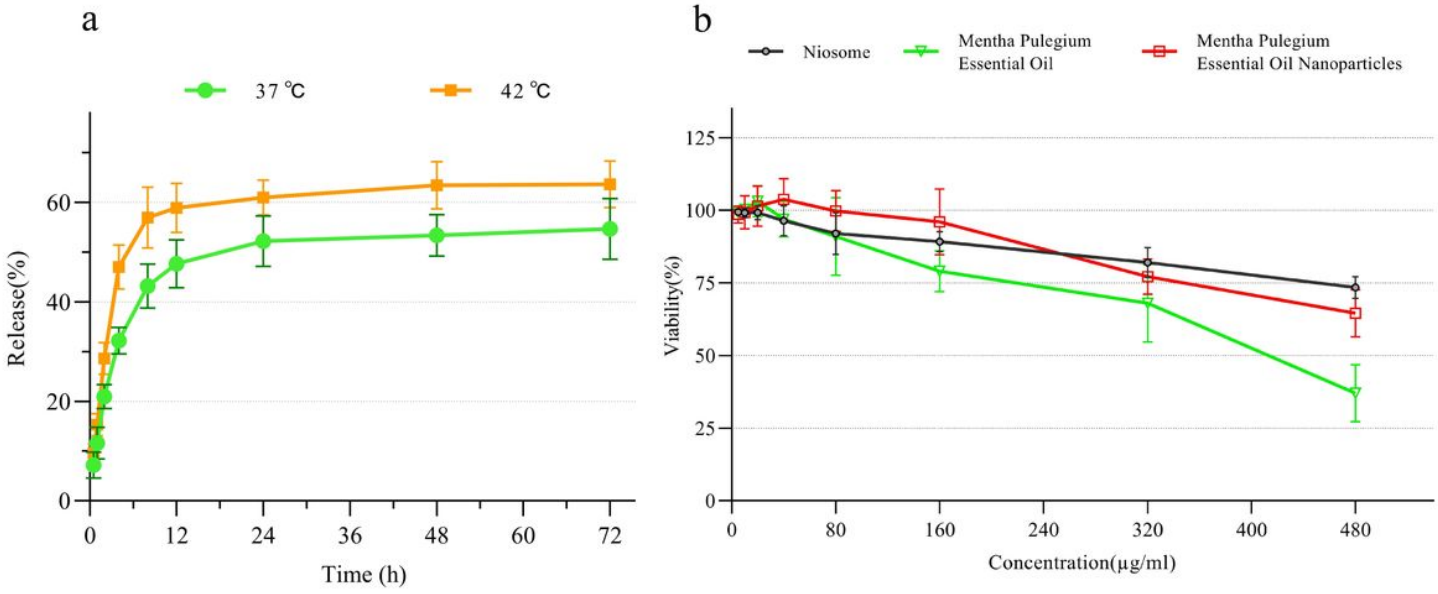

Figure 2

a: Release curve of nanoniosome-loaded *Mentha Pulegium* essential oil at 37 and 42 °C. b: MTT assay- Toxicity of different formulations on PBMCs after 96 h of incubation.

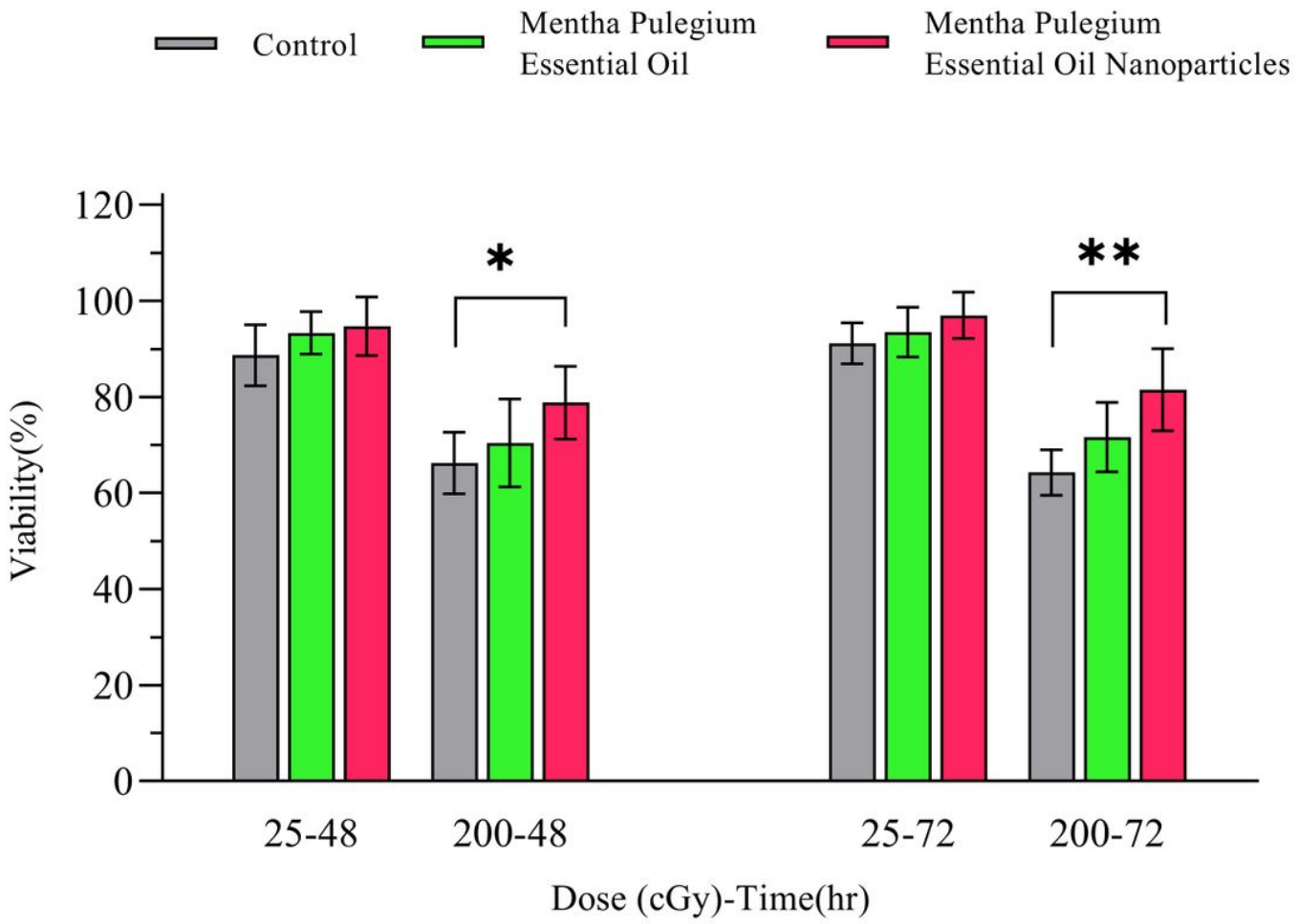

Figure 3

MTT assay- Survival percentage of irradiated PBMCs with different radiation doses of X-ray in the presence of different formulations with concentrations equivalent to the IC10 of nanoniosome-loaded *Mentha Pulegium* essential oil (MPEO-N) at 48 and 72 h of incubation. *P< 0.05, ** P<0.01.

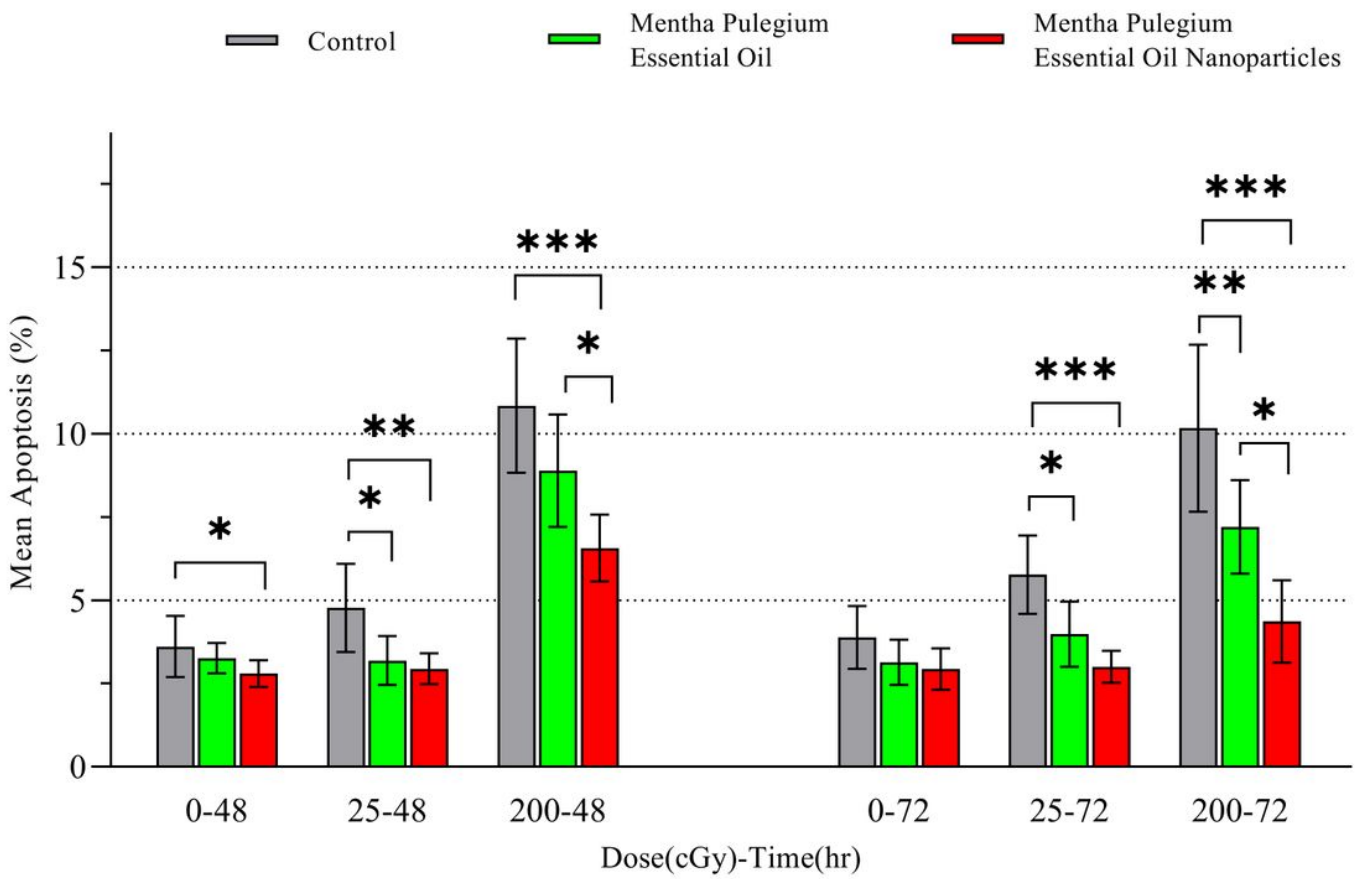

Figure 4

Flow cytometry analysis- The mean percentage of apoptosis in irradiated PBMCs at radiation doses of 0, 25, and 200 cGy in various treatment groups with concentrations equivalent to IC10 of nanoniosome-loaded *Mentha Pulegium* essential oil (MPEO-N) (170 µg/ml) at 48 and 72 h of incubation. **P*< 0.05, ** *P*<0.01, *** *P*<0.001.

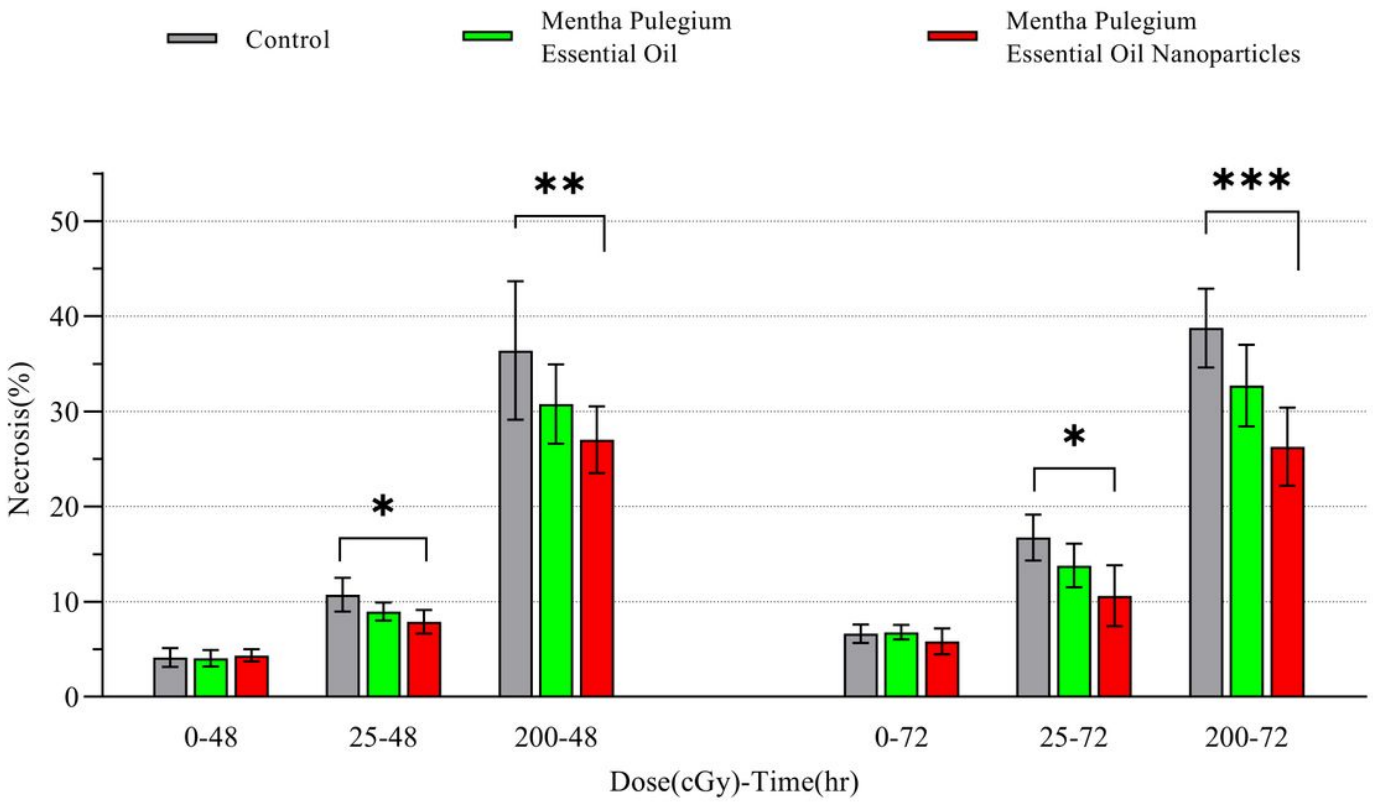

Figure 5

Flow cytometry analysis- The mean percentage of necrosis in irradiated PBMCs at radiation doses of 0, 25, and 200 cGy in various treatment groups at a concentration equivalent to IC10 of nanoniosome-loaded *Mentha Pulegium* essential oil (MPEO-N) (170 µg/ml) at 48 and 72 h of incubation. *P< 0.05, ** P<0.01, *** P<0.001.

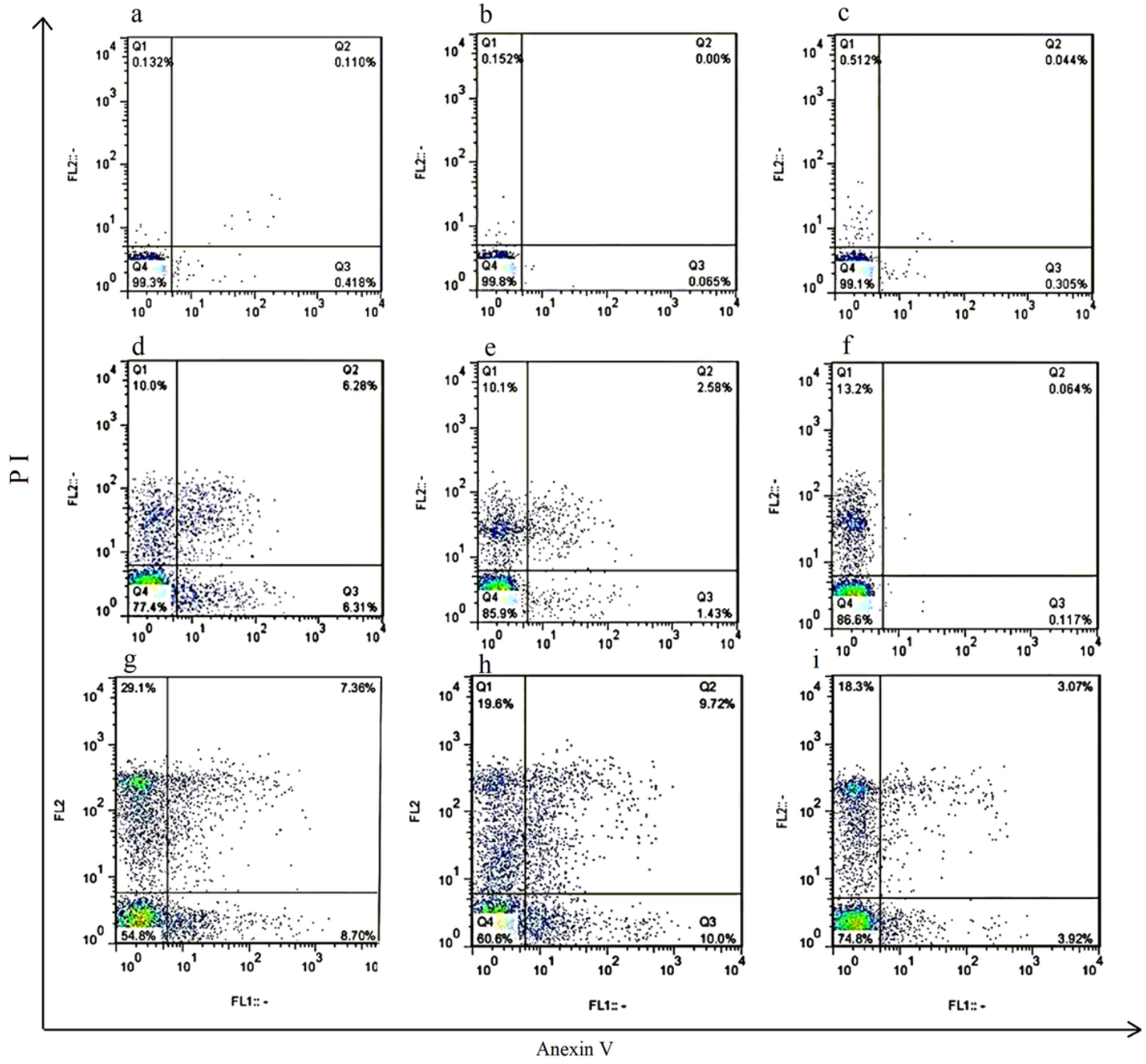

Figure 6

Flow cytometry analyses on irradiated PBMCs after 72 h of incubation with an equivalent concentration of IC10 of nanoniosome-loaded *Mentha Pulegium* essential oil (MPEO-N). In each graph, the lower and left quadrants represent the percentage of living cells, and the lower and right quadrants represent the apoptotic cells. a: control group (untreated) with zero radiation dose, b: treatment group with *Mentha Pulegium* essential oil (MPEO) with zero radiation dose, c: MPEO-N nanoparticles treatment group with zero radiation dose, d: Control group with 25cGy radiation dose, e: MPEO treatment group with 25cGy radiation dose, f: MPEO-N nanoparticles treatment group with 25cGy radiation dose, g: Control group with

200cGy radiation dose, h: MPEO treatment group with 200cGy radiation dose, i: MPEO-N nanoparticles treatment group with 200cGy radiation dose.