Radioprotective Effect of Resveratrol, Crocin, and Their Combination on Cytogenetic Alterations in Human Lymphocytes

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ABSTRACT

Background: High-dose radiation altering the genetic material in patients' bone marrow cells can lead to hematopoietic radiation syndrome. Accordingly, the presence of radiation protections agents is critical to preventing these adverse effects.

Objective: This study aimed to evaluate the radioprotection of the exclusive or combination effect of resveratrol and crocin extracts at various concentrations on irradiated human lymphocytes.

Material and Methods: In this experimental study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to evaluate the cell viability in pre-treatment with resveratrol, crocin, or a combination of both, using a concentration range of 5 to 4800 µM / ml in 24 h. The chromosomal aberration test was employed to determine the aberration frequency in 48 h. This study was performed on human peripheral blood lymphocytes treated with 2 Gy radiation and reliability of measurements performed by the triplicate repeat.

Results: MTT results showed that the groups treated with either resveratrol or crocin at concentrations of 5 to 4800 µM had no significant reduction in cell viability. The cytogenetic analysis of irradiated lymphocytes with 2 Gy X-rays revealed a reduction in the frequency of dicentric chromosomes in all treated groups in contrast with the control group. The most significant reduction occurred in those treated with a single agent at the concentration of 100 µM and a combined drug at the concentration of 50 µM.

Conclusion: The combination of resveratrol and crocin is considered a potential radioprotector and prophylactic for patients before radiation therapy.

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Keywords

Radiation Protection; Resveratrol; Crocin; DNA Damage; Human Lymphocyte; Chromosome Aberrations

Introduction

It is well-known that high-dose irradiation altering the genetic material in bone marrow cells of patients can lead to hematopoietic irradiation syndrome. Therefore, radioprotector agents are necessary to prevent these adverse effects. Radioprotection of bone marrow, the main site for blood cell production, is an important factor in the survival and well-being of patients after exposure. Free radical and deoxyribonucleic acid (DNA) damage induced by radiation are the underlying

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causes of chromosomal aberrations (CAs) that alter normal cells to tumor cells. Irradiation of the whole body with a dose of 2 Gy and more increases CAs in bone marrow cells that impair the hematopoietic system [1-3]. Radiation is often delivered in fractions with a radiation dose of 2 Gy per session [4, 5] to avoid damage to the bone marrow.

Ideal radioprotectors should mitigate tissue injury caused by free radicals induced by radiation and should not produce toxicity along with their effect [6]. Various cellular growth factors, including sargramostim, filgrastim, and pegfilgrastim are administered after exposure to aid the recovery of radiation-induced bone marrow failure [7].

Synthetic radioprotectors, such as palifermin and amifostine are used to decrease the incidence and duration of oral mucositis caused by radiotherapy for head and neck cancers. However, none of these agents are approved by the United States Food and Drug Administration (FDA) in preventing hematopoietic diseases with significant adverse effects [8]. Currently, no natural protective drug exists with FDA approval [9]. Natural radioprotectors are vital especially due to their impact as natural polyphenols on healthy cells (anti-apoptotic) and sensitivity to tumor cells (pro-apoptotic) [10].

Resveratrol is a natural polyphenol substance in cis/trans isoforms [11] with antioxidant and anticancer properties and radiation protection effects [12] and also reduces the incidence of oxidative cardiovascular diseases, such as heart attack and atherosclerosis by reducing low-density lipoprotein (LDL) levels, platelet aggregation and adhesion, and DNA oxidative damage [13]. Besides, resveratrol can scavenge free radicals induced by radiation, chemicals, and H_2O_2 that damage DNA molecules on chromosomes [14]. Its radioprotective effect is mainly due to intracellular antioxidants, such as glutathione and superoxide dismutase [15]; however, its polyphenol properties are influenced by various concentrations and cell types [16].

Crocin is also a natural substance from the carotenoid family derived from saffron that possesses anticancer properties through altering the apoptotic gene expression and the potential of reducing the doses of chemotherapeutic agents in cancer patients [17]. Moreover, crocin also inhibits the growth of cancer cells with the least detrimental effects on healthy cells [18]. Because of its antioxidant properties, crocin inhibits the chemical alteration of irradiated cells and reduces oxidative stress significantly [19]. Recent studies have shown that crocin alone can reduce the chemical damage caused by light radiation in animal retinal cells [20-22] and play an essential role in cell division and cytotoxic function of T lymphocytes in childhood leukemia. Additionally, some studies have indicated that crocin can significantly inhibit cell division in tumors [23-25]. A study published in 2019 showed that using a combination of crocin and resveratrol extracts of different concentrations can protect human retinal pigment epithelial (hRPE) cells from damage caused by light radiation [26]. The current study aimed to evaluate the radioprotective concentration-dependent effects of resveratrol as a polyphenol and crocin as a carotenoid on irradiated healthy human lymphocytes.

Material and Methods

Reagents

In this experimental study, resveratrol and crocin were obtained from Sigma Aldrich, USA. Ethanol (95%) and Dimethyl sulfoxide (DMSO) were used as a solvent for resveratrol and crocin, respectively. Resveratrol was stored at -20 °C and crocin at 2-8 °C in dark conditions; the drugs were made in various concentrations of 5, 50, 100, 200, 400, and 800 µM for cytogenetic analysis and additional concentrations, such as 1600, 2400, 3200, 4000, and 4800 µM for the MTT test. RPMI1640, FBS, and PHA (Merck Company) were used for blood culture. Methanol and

acetic acid (purchased from Merck) were used as fixative agents, and Gimisa (Sigma Aldrich) was applied d for staining chromosomes.

Irradiation Conditions

Blood samples were taken with the individuals' consent and kept in heparinized tubes (to prevent blood clotting) and pretreated with different concentrations of resveratrol, crocin, or both at 37 °C for 1h before irradiation. Various treated groups were firstly placed in a 6-well culture plate and exposed to 2 Gy X-ray radiation at a 6 MV using Elekta Compact linear accelerator (at a dose rate of 50 monitor units per minute). All radiation procedures were performed in Asia Hospital in Tehran, Iran. Irradiated samples were immediately transferred to the laboratory, cultured in an appropriate medium, and incubated for 48 h. The International Atomic Energy Agency (IAEA) protocols were observed in all stages [27].

Culture Conditions

For each treatment group, 0.5 ml of whole blood was added to 4.5 ml of Roswell Park Memorial Institute medium (RPMI) culture medium along with 20 µl of resveratrol, crocin, or a combination of both in equal proportions as a radioprotector in different concentrations (5 to 800 µM). Each 6-well culture plate was irradiated following the addition of phytohaemagglutinin (PHA), fetal bovine serum (FBS), and penicillin-streptomycin. Plates were incubated at 37 °C for 48 h and transferred into tubes. A total of 50 µl of colcemid was added to each culture tube 2 h before harvest to arrest the cells in the metaphase phase. The cells were harvested and their nuclei were removed and fixed with a stabilizing solution (ethanol and acetic acid with a 1: 3 ratio).

Cytogenetic analysis

A total of 3 to 5 slides were prepared from each cell group by lysing the nuclei to obtain chromosomes. Slides with cellular chromosomes were dried, aged for 2 to 3 days, and then stained with Giemsa to perform the karyotyping [20]. One hundred metaphases were analyzed for each group using light microscopy. Furthermore, CAs are counted as the dicentric, ring, acentric, chromatid exchanges, and gaps. Other aberrations, such as chromatid breaks (Ctb), translocations, and inversions were recorded only by providing visible.

MTT assay

The MTT assay was performed to determine the potential cytotoxic effects of each agent. The assay works with water-soluble yellow tetrazolium substance regenerated by a living cell system of mitochondria, converted to water-insoluble purple formazan cells' proliferation, and the interfering cytotoxic properties were investigated indirectly [28]. First, lymphocyte cells were isolated and counted using Ficoll-Hypaque; 10,000 cells were secondly suspended in 96-well culture plates. The samples were prepared in triplicate for each group, followed by pretreatment with resveratrol, crocin, or a combination of both extracts at different concentrations ranging from 0 to 4800 µM/ml. The culture plates with the treated cells were incubated at 37 °C with 5% CO_2 for 24 h. A total the 50 µl of the MTT solution was added to each well 24 h after incubation, and plates were then transferred to the incubator for an additional 4 h. The culture medium containing MTT solution was removed, and the remaining precipitate was exposed to 200 µl of DMSO with 25 µl of Sorensen's buffer incubated at 37 °C for 30 min. Finally, the culture plates were read at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Statistical analysis

The data and diagram were prepared and analyzed using the SPSS (version 26) and PRISM software (version 8), respectively. In addition, the Kolmogorov-Smirnov test, Student's t-test, and Poisson distribution of the obtained data were conducted.

Ethical consideration

The Ethics Committee of Iran University of Medical Sciences approved the protocol of the study, and the informed consent of the volunteers that agreed to participate was obtained in this study as well.

Results

Measurement of cell viability by MTT method

The MTT was conducted for each pretreated group with resveratrol, crocin, or a combination of both (Figure 1).

Examination of different concentrations of resveratrol (from 5 to 4800 µM) showed no significant reduction in the viability of healthy cells. On the other hand, concentrations of resveratrol increased cell viability compared to the control group, which was significant at concentrations of 4000 and 4800 µM (P<0.05, and P<0.001). However, the examination of different concentrations of crocin (5 to 4800 µM) showed no significant reduction in the level of cell viability, it shows increased viability at 200 µM concentration compared to the control group (P<0.05). The group treated with both resveratrol and crocin concentrations of 5 to 4800 µM also had no significant decline in cell viability. However, an increase in cell viability compared to the control group was observed at two concentrations of 400 and 4800 µM (P<0.01).

Cytogenetic results in treatment groups after 2 Gy X-ray irradiation

The cytogenetic analysis of irradiated blood lymphocytes in the presence of different extract concentrations is shown in Table 1.

The frequency of dicentric chromosomes decreased in all treated groups compared to the control group, with the most significant reduction in both resveratrol and crocin groups at a concentration of 100 µM (P<0.05). In the group with the combined drugs, dicentric aberration decreases significantly at a concentration of 50 µM (P<0.05). Accordingly, the level of protective criteria for each treated group was measured by the following formula in equation 1 [8]:

$$Y = 100 - \frac{\text{dicentric frequency in treatment groups} \times 100}{\text{dicentric frequency in the control group}} \quad (1$$

Where Y is protective criteria (%), and

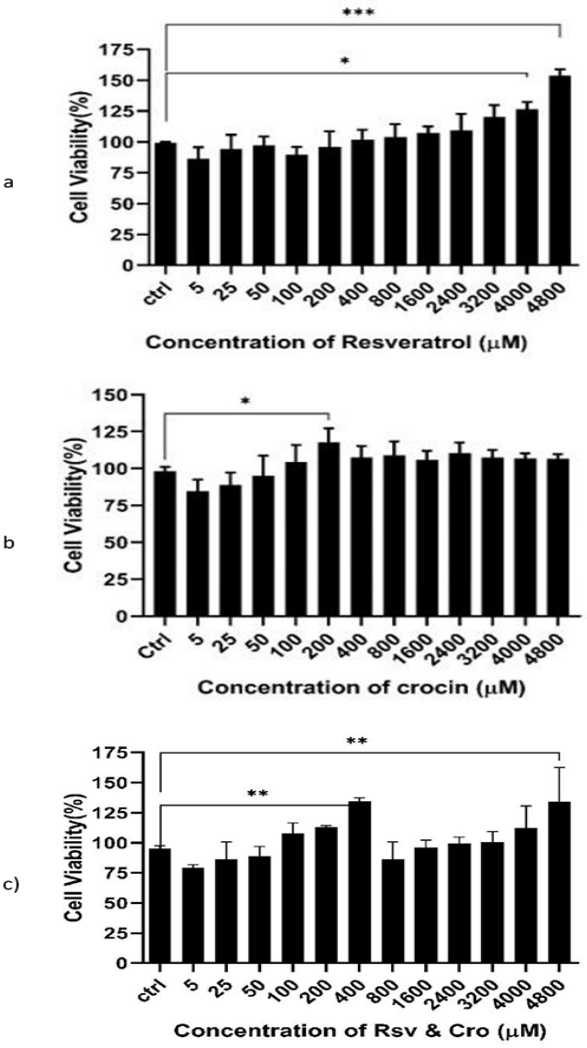

Figure 1: The survival rate of normal blood lymphocytes, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and treated by (a) resveratrol, (b) crocin, and (c) combination of both. *P value>0.05 **P value>0.01 ***P value>0.001

Table 1: Chromosome aberrations in human lymphocytes exposed to 2 Gy of X-ray radiation in different conditions: without extracts (control group) and pretreated with various concentrations of resveratrol, crocin, and combination

Chromosomal aberration	N	Dic	$Y_{dic} \pm SE$	R	Ace	[a]Other chromosome aberration			Total aberrant cell
							Ctb	gap	others
0 (with 2Gy)	100	30	0.30±0.054	4	36	2	0	2	46
Resveratrol (µM)									
5	100	24	0.24±0.048	0	16	6	0	0	30
50	100	14	*0.14±0.037	2	12	0	0	2	24
100	100	10	*0.10±0.031	2	12	0	0	0	18
200	100	12	*0.12±0.034	2	9	4	0	4	22
400	100	18	*0.18±0.042	2	34	6	0	2	26
800	100	26	0.26±0.051	0	18	10	0	0	44
Crocin (µM)									
5	100	16	*0.16±0.040	0	22	4	0	0	36
50	100	18	*0.18±0.042	4	28	6	0	0	30
100	100	14	*0.14±0.038	4	12	0	0	0	20
200	100	16	*0.16±0.040	4	10	0	0	0	22
400	100	20	0.20±0.044	2	24	2	0	0	36
800	100	16	*0.16±0.040	2	34	2	0	0	22
Combination (µM)									
5+5	100	28	0.28±0.052	2	13	4	0	0	34
50+50	100	8	*0.08±0.028	4	9	4	0	0	16
100+100	100	10	*0.10±0.031	2	15	6	0	2	18
200+200	100	12	*0.12±0.035	2	16	2	0	0	26
400+400	100	16	*0.16±0.040	2	26	4	0	0	32
800+800	100	12	*0.12±0.035	2	16	2	0	0	24

N: Number of analyzed cells, Dic: Dicentrics, $Y_{Dic} \pm SE$: Frequencies of dicentrics ± standard errors, R: Rings, Ace: Acentric fragments, Ctb: Chromatid breaks

a: Others included translocations and inversions

*: The different frequency of dicentric chromosomes with statistically significant ($P<0.05$)

dicentric frequency is the number of dicentric chromosomes.

Based on the above formula, the maximum level of radioprotection in the resveratrol group occurred at around 66% with 100 µM concentration and the minimum with 13% in 800 µM concentration. In the crocin group, the maximum protection, with 53%, was related to 100 µM concentration and the minimum belonged to the concentration of 400 µM with a rate of 33%. Moreover, the highest protective effect in the group treated with the combined drugs was detected at a concentration of 50 µM with 73%, and the least (6%) at the concentration of 5 µM. The changes in the frequency of dicentric aberration and abnormal metaphases, resulting from different drug concentrations separately and cooperatively are shown in Figures 2 and 3. Decreased dicentric frequency was observed in the separate groups of cells treated with resveratrol or crocin at a concentration of 100 µM and in the

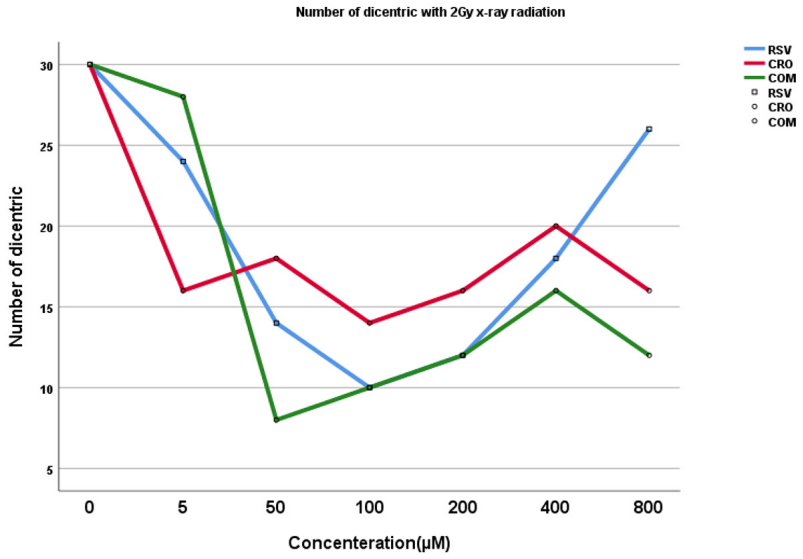

Figure 2: The effect of various concentrations of resveratrol, crocin, and their combination on the frequency of dicentric chromosomes in lymphocytes irradiated at 2 Gy X-ray.

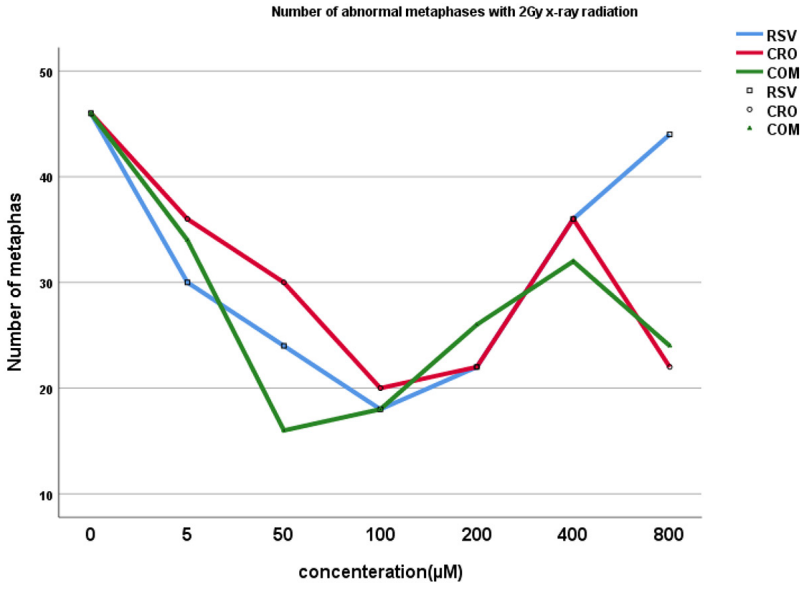

Figure 3: The effect of various concentrations of resveratrol, crocin, and their combination on the frequency of abnormal metaphase in lymphocytes irradiated at 2 Gy X-ray.

combined group at a concentration of 50 µM.

Cytogenetic results in treatment groups without X-ray irradiation

Table 2 shows the results of cytogenetic analysis of treated groups at various concentrations without irradiation. Neither the dicentric nor ring chromosomes were generated in the pretreated group. The majority of CAs were acentric chromosomes in the treatment groups, and the frequency of acentric chromosomes was proportional to the number

Table 2: Chromosomal aberrations in cultures pretreated with resveratrol, crocin, and combined state non-exposed of X-ray irradiation

Chromosomal aberration	N	Dic	R	Ace	Ctb	Gap	Other	Total aberrant cell
0 (Non-Expose)	100	0	0	1	0	0	0	4
Resveratrol (µM)								
5	100	0	0	2	0	0	0	3
50	100	0	0	2	0	0	0	3
100	100	0	0	1	0	0	0	2
200	100	0	0	3	0	0	2	2
400	100	0	0	2	1	0	1	4
800	100	0	0	6	1	0	0	6
Crocin (µM)								
5	100	0	0	3	0	0	1	6
50	100	0	0	2	1	0	0	4
100	100	0	0	1	1	0	3	0
200	100	0	0	0	0	0	0	2
400	100	0	0	4	0	0	0	6
800	100	0	0	1	0	0	0	2
Combination (µM)								
5+5	100	0	0	1	0	0	0	3
50+50	100	0	0	0	0	0	0	2
100+100	100	0	0	2	0	0	0	4
200+200	100	0	0	2	0	0	1	3
400+400	100	0	0	2	0	0	0	5
800+800	100	0	0	0	0	0	0	2

N: Number of analyzed cells, Dic: Dicentrics, R: Rings, Ace: Acentric fragments, Ctb: Chromatid breaks

of abnormal metaphases. The highest abnormal metaphases and acentric chromosomes were detected at the highest concentration in the resveratrol group (800 µM), and the highest level of CAs was considered at the crocin concentration of 400 µM. A lower CA was observed in combined groups (resveratrol and crocin) in contrast to groups with a single drug.

Discussion

In the present study, two natural compounds (resveratrol and crocin) with antioxidant and radioprotective properties were used with appropriate compositions and concentrations to protect against irradiation-induced genetic malformations [29, 30]. An ideal radioprotection should have no or very low toxicity for normal cells, a wide range of effective concentrations for administration, and well-defined protection for the cellular genome against irradiation biohazards [31].

The MTT test was used to determine the toxicity by measuring the cell viability at various concentrations, from 5 to 4800 µM for each extract. Resveratrol did not cause any significant reduction in cell viability; however, a significant increase in cell viability was detected at two concentrations of 4000 and 4800 µM compared to the control group (P<0.05, and P<0.001). Based on these results, resveratrol acts as a potent stimulant of cell proliferation

with no negative effects on healthy cells. In agreement with these results, a study by Hosseinmehr et al. also showed that resveratrol could increase the growth and proliferation of healthy cells [32]. The crocin extract did not cause any reduction in cell survival at the prepared concentrations, indicating no toxicity to cells. However, at a crocin concentration of 200 µM, a significant increase in survival was detected (P<0.05). Another study also showed that crocin inhibits the growth of cancer cells while it does not harm the growth and viability of normal cells [33]. The combination of resveratrol and crocin extracts at various concentrations did not cause a significant decrease in cell viability, whereas concentrations of 400 and 4800 µM showed increased viability compared to the control group (P<0.05). According to the present results, all the pretreated concentrations of extracts in combination did not harm cell growth and in turn, provided a better radioprotection. Another study showed that using both resveratrol and crocin has no toxic effect on the viability of normal cells and can even increase its rate [26].

Chromosome analysis of pretreated cells with selected concentrations of resveratrol without radiation showed a small number of structural aberrations, such as acentric and chromatid breaks in healthy lymphocytes. Several CAs, such as gap, acentric, and chromatid breaks were also observed in the presence of a different concentration of crocin extract without irradiation. However, the number of CAs in the non-irradiation group pretreated with combined extracts was very low and negligible. The extracts in the combined state can seemly neutralize their negative effects on the chromosome. In agreement with these results, a study by Sebastia et al. showed the presence of small cytogenetic effects (acentric and gaps) of resveratrol on healthy blood cells without radiation [8, 14]. The leading cause of structural aberration in non-irradiated chromosomes pretreated with resveratrol extract could be a specific activity known as topoisomerase toxicity. The toxicity of topoisomerase results from its ability to increase new homologous and non-homologous compounds in normal cells [34]. The study by Hoshyar et al. showed that with increasing the crocin concentration, cell division in the HepG2 cell line decreases providing an increasing the rate of apoptosis and cell death [35].

In the irradiated group treated with either resveratrol or crocin at different concentrations, the CAs reduction occurred along with specific structural abnormalities, including dicentric chromosomes. The resveratrol, at a concentration of 100 µM, showed the greatest effect on reducing CAs (including dicentric and abnormal metaphases) occurring at the rate of 66%; however, the resveratrol had the least effect of 13% at concentrations of 800 µM. The highest (53%) and lowest (33%) levels of radioprotection of crocin were detected at a concentration of 100 µM and 400 µM, respectively. Cells treated with both radioprotectors in the combined state with a lower concentration (50 µM) showed a significant reduction in dicentric chromosomes (P<0.05) in comparison to a higher concentration of each agent alone. Therefore, 100 µM of radioprotectors crocin and resveratrol in the combined state not only reduced the CAs but also increased protection against structural aberration induced by radiations even at a lower concentration. The lowest radioprotection was observed in 5 µM at 6%, and the highest was in 50 µM at 73%.

According to the results of the present study, resveratrol had a biphasic effect [36], in which the highest and lowest radioprotection was observed at a low concentration (100 µM) and a high concentration (800 µM), respectively. Evidence maintains that polyphenolic compounds can exert these protective properties [8, 34]. Reduced CAs by resveratrol-dependent free radical scavenging oxidative damage were associated with hydroxyl groups in the resveratrol structure [37]. Other studies have

concluded that crocin protection against DNA damage was due to antioxidant, anti-inflammatory, and protective properties against free radicals [34, 38]. The findings of the present study indicate that the group treated with the combined drug at 50 µM concentration had the most significant protection by reducing the number of CAs.

In the concentration of 800 µM, the resveratrol-treated group had the least radioprotection; however, cells treated with crocin or a combination of both had a better protective response at this concentration. The improved response in the combined state may be due to the moderation of polyphenolic properties of resveratrol by crocin [1, 39, 40] and also the reduction of irradiation-induced free radicals, leading to a decrease CAs in treated cells as well. Various evidence indicates that polyphenolic compounds have hormesis or a biphasic response curve in which positive and negative effects are shown at low and high concentrations, respectively [34]. An in-vitro study by Stefan et al. (2019) was reported that the combination of resveratrol and crocin provided more protection against DNA damage in the cell by increasing intracellular glutathione and reducing oxidative stress [17].

Conclusion

The results of the present study confirmed that combining the two extracts produced a more substantial protective effect on healthy cells by reducing free radicals and CAs. Therefore, the results of this study can be useful in radiation therapy before starting the treatment process. In this study, a wide range of different drug concentrations were prepared to assess their effects that the combination of the two extracts at lower concentrations demonstrated a greater prophylactic effect against DNA damage than at higher concentrations.

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Authors' Contribution

SA. Moosavi and A. Neshasteh-riz designed the study. Sh. Faraji performed the experiments, and S. Mayahi analyzed the experimental findings. S. Cheraghi supervised the work. All authors read and approved the final version of the manuscript.

Ethical Approval

This study was approved by the Iran University of Medical Sciences (Ethics code: IR.IUMS.REC.1397.1093).

Informed consent

Blood samples were taken from volunteers with a complete description of the plan and informed consent.

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Conflict of Interest

None

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