

Article

Effect of Gamma Irradiation on Free Radicals and the Antioxidant Properties of Walnuts

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Abstract: The present work represented results from a comprehensive study of free radicals and the antioxidant properties of irradiated walnuts. The effects of gamma irradiation on free radical generation and their stability, as well as on the antioxidant activity in walnuts, were investigated by Electron Paramagnetic Resonance (EPR) spectroscopy, Oxygen Radical Absorbance Capacity (ORAC), and Hydroxyl Radical Antioxidant Capacity (HORAC) assays. Walnut samples were irradiated using ⁶⁰Co at two different doses: 10 and 25 kGy. As a marker for the identification of high-energy radiation treatment, characteristic cellulose radical signals were detected after irradiation and remained observable for over eight months. A significant increase in antioxidant activity was observed at higher irradiation doses, as measured by DPPH free radical scavenging activity, ORAC and HORAC assays.

Keywords: walnut; gamma irradiation; antioxidants; EPR; ORAC; HORAC

1. Introduction

Gamma irradiation is widely used for food sterilization, extending shelf life by reducing microbial and insect contamination, as well as preventing sprouting. It is a fast, efficient, secure, and absolutely safe method initially developed for food products in space missions, military use, and immune-compromised patients. Nowadays, it is increasingly applied to daily food products of the population and has been approved in many countries for preservation. The maximum allowable dose for the gamma irradiation of food is 10 kGy in the European Union, while in China, the USA, and Canada, the limit is 30 kGy for herbs and spices. Various analytical methods have been adopted to prove irradiation, required by global trade and consumer preferences. Among these, Electron Paramagnetic Resonance (EPR) spectroscopy is the leading technique, as it is included in three of the main European standards [1].

It is known that a single (central) signal is observed in the EPR spectra of all plant-based food products containing cellulose before irradiation. There are several suggestions about the origin of the paramagnetic species. According to EN 1787:2000 [2], after the irradiation with high energy radiation, a pair of lines (known as “satellite” signals), one on the left (at a lower magnetic field) and one on the right (at a higher magnetic field), appear in addition to the central signal. These signals are due to cellulose radicals formed by ionizing radiation. The distance between these signals is about 6 mT, and they are characteristic of radiation treatment. Standard EN 1787 is validated for pistachio nut shells, strawberries, and paprika and it deals with irradiated nuts.



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It is observed that hydrogen peroxide treatment (known as “bleaching”) of nuts can also induce free radicals, leading to the appearance of two weak lines at the same g-factors, as the satellite lines. However, for walnuts, the intensity ratio of these two signals is significantly higher in bleached samples irradiated with 12 kGy, whereas for those irradiated with 3 kGy, the difference is negligible. Therefore, at higher doses, this ratio can be used to distinguish between bleached and irradiated walnuts [3]. Interlaboratory comparisons of samples have confirmed that EPR spectroscopy can be used for routine official testing of irradiated walnuts on the Italian market. However, further investigations are needed to determine whether false positive results caused by bleached walnuts are a common issue [4].

Gamma-irradiated walnuts (shells and kernels) were tested for the identification of high-energy ionizing irradiation and dosimetry. The dependence of the signal intensity on microwave power, radiation dose, and time stability was analyzed. The results show that EPR is a reliable method for identifying and quantifying irradiation in walnuts using both shells and kernels [5]. A study by Raffi et al. [6], later confirmed by other researchers [7], suggests that the two satellite lines form part of a triplet signal induced by irradiation, while the central line corresponds to the same free radical present before irradiation but with an increased concentration. The single aliquot additive dose method is applied to evaluate the radiation dose in gamma-irradiated nuts, as well as to examine the time stability of the radiation-induced signal [8]. The earlier years of research were focused on identifying gamma sterilization of various food products, including nuts, using different analytical techniques based on European standards [9,10].

Walnuts are recognized as a superfood due to their high content of healthy fats and numerous health benefits. They contain several bioactive compounds, including polyunsaturated fatty acids, plant proteins, dietary fibers, sterols, folates, tannins, and polyphenols [11]. They are also an excellent source of unsaturated fatty acids [12]. On the other hand, walnuts exhibit high antioxidant activity, attributed to their phenolic compounds, which contribute to human health. These antioxidants prevent or delay the oxidation of fatty acids, thereby protecting the integrity of the lipid content of nuts [12]. Roasting treatment affects the phenolic content and antioxidant activity of nuts [13] including walnuts [14]. Previous studies have shown that gamma irradiation has little effect on the antioxidant activity of almonds [15]; whereas, in irradiated walnuts, antioxidant activity was found to increase when measured spectrophotometrically [16]. Different methods are used to assess antioxidant capacity. EPR spectroscopy utilizes the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Other methods as Oxygen Radical Absorbance Capacity (ORAC) and Hydroxyl Radical Antioxidant Capacity (HORAC) are fluorometric assays widely used for evaluating the antioxidant potential of foods, beverages, and supplements. An ORAC assay measures the peroxyl radical scavenging capacity of the sample, whereas HORAC is an indicator for its chelating ability and preventing role in hydroxyl radical formation. In previous studies, an increase in polyphenol content and antioxidant activity (measured by ORAC and HORAC methods) was observed in defatted almonds irradiated at 25 kGy [15].

The study aims to investigate the effect of gamma irradiation on free radicals in irradiated walnuts, their stability analyzed via EPR spectroscopy, and their antioxidant activity assessed using EPR, ORAC, and HORAC methods. Additionally, the polyphenol and flavonoid content of walnuts is examined due to its close association with the antioxidant properties. To the best of our knowledge, this is the first comprehensive study on the antioxidant capacity of walnuts, employing multiple analytical methods for assessment.

2. Materials and Methods

2.1. Materials

Walnuts were purchased from the local market. For the purposes of this study, it was essential that the samples had not been previously irradiated as prior gamma irradiation could affect the investigation. To ensure this, the walnuts were tested using EPR spectroscopy. The results confirmed that the walnuts obtained from the local market had not undergone irradiation and were therefore suitable for further analysis. Following this verification, the samples were subjected to subsequent analyses. Walnut shells (of equal weight for all samples) were used to study the stability of free radicals by EPR spectroscopy, while the defatted walnut powder was prepared for antioxidant activity investigations. The preparation process was as follows: accurately weighed portions of approximately 30 g of non-irradiated walnuts and walnuts irradiated at 10 kGy and 25 kGy were ground using a grinder and extracted in a Soxhlet apparatus for 8 h with hexane [17]. The extraction residue was then evaporated to dryness under a stream of nitrogen.

DPPH, high-purity chemical-grade Ethanol Absolute Alcohol 99.9%, and hexane (>99%) were purchased from Sigma Aldrich, Sofia, Bulgaria.

2.2. Sample Irradiation

Walnut samples were packed in polyethylene bags and they were irradiated with gamma rays from a ^{60}Co source (8200 Ci activity and 1.230 kGy/h dose rate) at the National Centre of Radiobiology and Radiation Protection, Sofia, Bulgaria. The irradiation was conducted at room temperature in the air using a semi-industrial “NIGU-7” radiation system. The equipment included four liters of a volume working chamber, which is rotated on its vertical axis during the irradiation. The delivered doses of 10 and 25 kGy were confirmed by Alanine dosimeters (Kodak BioMax, Wesertal, Germany), which were measured by an EPR spectrometer (E-scan Bruker, Bruker, Karlsruhe, Germany).

2.3. EPR Investigations

2.3.1. Preparation of Extracts

Extracts were prepared by adding 0.5 g of dried walnut residue (non-irradiated and irradiated with 10 and 25 kGy) to 7.5 mL absolute ethanol and 2.5 mL distilled water. The mixture was incubated for 24 h at room temperature without air exposure, then it was filtered through 150 mm filter paper, and the filtrate was used for further investigations. Fresh extracts were prepared before each experiment.

2.3.2. Determination of DPPH Free Radical Scavenging Activity (FRSA) by EPR

The stable free radical DPPH was used to estimate the effect of irradiation with different doses of gamma rays on the free radical scavenging activity (FRSA) of walnuts. The solutions were prepared as 1 mL of 0.002 M DPPH ethanol solution was mixed with varying amounts of the walnut extracts (0.03, 0.07, 0.1, 0.13, and 0.16 mL). After approximately 50 min of incubation, the EPR spectra were recorded.

During the measurement, the capillary tube was sealed and placed inside a standard EPR quartz tube, which was placed in the EPR cavity. Control samples containing 1 mL DPPH ethanol solution and the same amount of ethanol instead of the extract were used to check the dilution. The experiment was carried out at least three times for each sample to verify the reproducibility of the results.

The amount of scavenged DPPH radicals expressed in a percentage was calculated using the following:

$$\text{Scavenged DPPH radicals (\%)} = [(I_0 - I)/I_0] \times 100, \quad (1)$$

where I_0 was the intensity of the second DPPH peak of the control sample and I was the second peak intensity of the same EPR spectrum after the addition of the investigated extract.

In order to characterize and to compare the FRSA of walnut samples, the IC_{50} parameter was determined. The antioxidant abilities of the investigated sample were inversely proportional to the value of the IC_{50} .

2.3.3. Instrumentation

The EPR spectra were recorded at room temperature using a JEOL JES-FA 100 EPR spectrometer (JEOL, Tokyo, Japan) as the first derivative of the absorption signal. The spectrometer has a standard TE_{011} cylindrical resonator and it operates in X-band. The walnut shells were cut into small pieces suitable to insert in a quartz EPR tube and they were fixed in the cavity center. The EPR spectra were recorded under the following experimental conditions: modulation frequency 100 kHz, microwave power 0.4 mW, modulation amplitude 0.4 mT, sweep 15 mT, time constant 0.3 s, and sweep time 2 min.

2.4. Determination of the Total Polyphenol and Total Flavonoid Contents, ORAC, and HORAC

2.4.1. Extract Preparation

Approximately 2 g of powdered defatted walnuts were weighed accurately, transferred to an extraction tube, and mixed with 40 mL of 60% (v:v) acetone acidified with 0.5% formic acid. Samples were extracted for 1 h at room temperature using a magnetic stirrer (VELP Scientifica Srl, Usmate, Italy), centrifuged $6000 \times g$, 20 (MPW MED. Instruments, Warszawa, Poland) and supernatants were used for further investigations.

2.4.2. Determination of the Total Polyphenol and Total Flavonoid Contents

Total polyphenols were determined according to the method of Singleton and Rossi, 1965 [18] using gallic acid as a calibration standard. The results were expressed in mg of Gallic acid equivalents (GAE) per 100 g dry weight (DW) $\pm$ SD.

The total flavonoid content was determined according to Chang et al., 2002 [19], using $AlCl_3$ reagent and the calibration curve was built with quercetin dihydrate (10–200 mg/L) as a standard compound. The results are expressed as mg quercetin equivalents (QE) per 100 g DW $\pm$ SD.

2.4.3. Determination of Antioxidant Activity by ORAC and HORAC Assays

Oxygen Radical Absorbance Capacity and hydroxyl radical averting capacity were measured according to the methodology represented in the literature [20–22]. The results were expressed as micromole Trolox equivalents (μ mol TE) and micromole gallic acid equivalents (μ mol GAE) per gram of dry weight for ORAC and HORAC, respectively. The measurements were carried out on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

2.5. Statistical Analysis

Measurements for polyphenol and flavonoid content and antioxidant activity were carried out in triplicate. The results are represented as a mean value of three parallel determinations $\pm$ standard deviation and they were compared by a Students' *t*-test (MS Excel 2010 software). The values at $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Characteristics of EPR Spectra

Before irradiation, the EPR spectra of walnut shells displayed a single line signal with a *g*-factor of 2.0026 and a linewidth ΔH_{pp} of 0.7 mT (Figure 1a). This signal is

commonly observed in non-irradiated plant-based food products and it is attributed to semiquinone free radicals [23]. After gamma irradiation with 10 kGy and 25 kGy, a characteristic “cellulose-like” EPR spectrum was recorded (Figure 1b). It contained the irradiation-specific satellite lines from cellulose radical, spaced ~6 mT from each other (marked by arrows in Figure 1b), and increased intensity of the central line, which has a g factor 2.0055 and a linewidth ΔH_{pp} 0.8 mT. The left satellite line has $g = 2.0232$ and the right satellite 1.9853, and the linewidth is ΔH_{pp} 0.6 mT. The presence of these satellite signals is considered conclusive evidence of gamma irradiation, as defined by EU Standard EN 1787 [2]. This standard is based on interlaboratory tests using pistachio nut shells, paprika powder, and fresh strawberries. Additionally, the EPR spectrum of Mn^{2+} ions, naturally present in walnuts, was consistently observed (marked by asterisks in Figure 1). As is seen in Figure 1, the right satellite line overlaps the fourth line of Mn^{2+} ions. They can be used as a marker for the identification of treatment against which to compare the decrease in the radiation-generated signals. This is possible because the Mn spectrum is radiation and thermal insensitive. A detailed study [24] shows that the Mn spectrum can serve as a reference for radiation treatment when the specific satellite lines decrease with time or are completely absent. In the quoted study, significant changes in the ratio of the intensity of the central line to the intensity of the Mn signal of the irradiated samples before and after thermal treatment are observed. In non-irradiated samples after thermal treatment, a small change in the ratio was observed. However, in the present study, this method was not necessary, as the characteristic satellite lines are clearly visible. The quantity of the radiation-induced free radicals was similar between the 10 kGy and 25 kGy doses, with only a ~5% difference in the intensity of the central line. This suggests signal saturation at higher doses, likely due to radical recombination, as previously reported for almonds [15].

3.2. Kinetic Study

The stability of the central line and the satellite lines was monitored over 250 days after irradiation. The results indicate that all studied signals decrease exponentially over time, with the most significant decrease observed in the satellite lines of 25 kGy irradiated walnut shells within the first few days after irradiation (Figure 2). The central line intensity decreased by 73% for the samples irradiated with doses of 10 kGy and by 62% for 25 kGy irradiated samples after 250 days. The satellite lines decreased by ~67% and 70%, respectively, for 10 and 25 kGy doses. In contrast to almonds [15], where the central line intensity decreased by ~90%, the walnut samples decreased its intensity to a much lesser degree, which is visible against the Mn lines. As mentioned above, the quantity of free radicals was similar for both doses, and there was no significant difference in the central line intensity. However, the intensity of the satellite lines is higher for 25 kGy irradiated walnuts, confirming that at least two radiation-induced signals are present after treatment as for almonds [15]. The kinetic behavior of the satellite lines is similar for both doses, and the characteristic satellite signals remained detectable even after 8 months, confirming that irradiation with gamma rays walnuts can still be identified a long time after irradiation.

3.3. Total Polyphenol and Flavonoid Content

Table 1 represents the results for the total polyphenols and flavonoids content in the investigated samples. As is evident from the data, gamma irradiation at a higher dose has a pronounced effect on the phenolic composition of defatted walnuts, increasing the total polyphenols and flavonoid content by approximately 44% and 41%, respectively. Previous studies have also reported that gamma irradiation can enhance the concentration of phenolic compounds in plant matter [25]. However, this effect is quite complex and it depends on multiple factors including the plant matrix, the applied irradiation dose,

and the type and quantity of phenolic compounds present in the sample. In general, low to moderate irradiation doses have a less pronounced effect, as observed in the current study [26]. Several mechanisms may explain the increase in phenolic content following gamma irradiation. One of the most significant factors is the breakdown of high-molecular-weight polyphenols, such as tannins, into lower-molecular-weight phenolics, which are more bioavailable and exhibit enhanced antioxidant activity [27]. The observed increase in phenolic compounds was also associated with higher antioxidant activity, as measured by both assays used in this study (Table 1). Besides the higher content of polyphenols in the irradiated samples, the enhanced antioxidant activity may also be attributed to the cleavage of glycosidic bonds in flavonoid molecules, leading to the formation of sugar residues and aglycones. Aglycones typically exhibit higher antioxidant activity than their glycosylated counterparts [28].

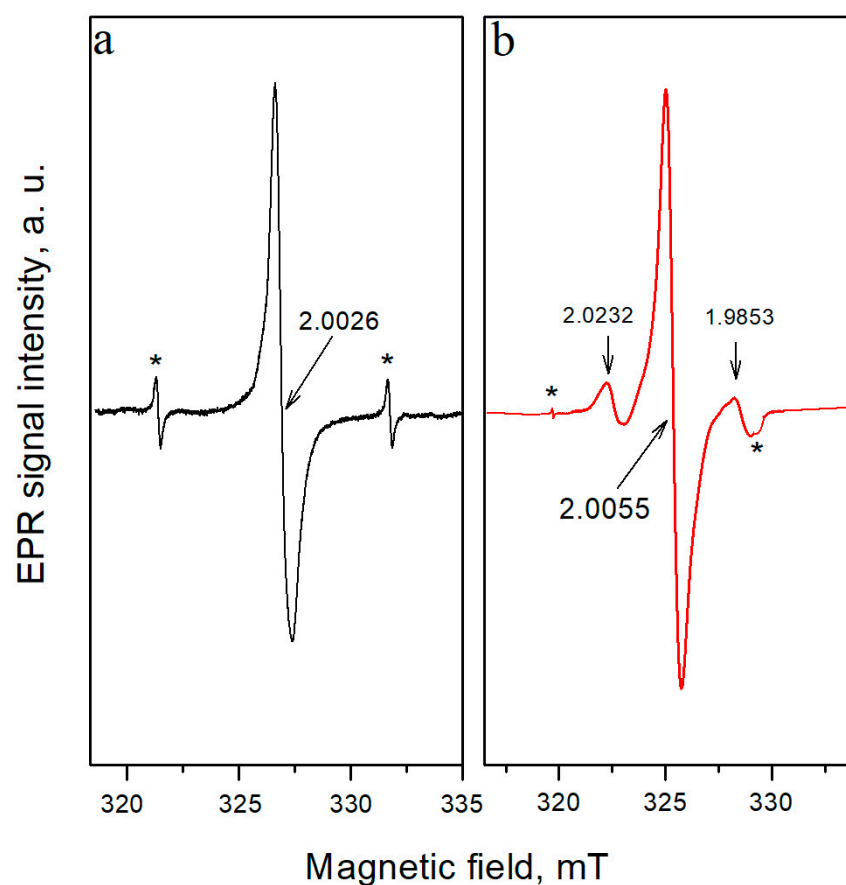


Figure 1. EPR spectra of walnuts: (a) before irradiation (The amplification used in order to record the EPR spectra of non-irradiated samples is 10 times higher than that for irradiated samples); (b) immediately after irradiation with 10 kGy; (*)—Mn signal.

Table 1. Polyphenol and flavonoid content and antioxidant activity of untreated (control) and γ -irradiated (10 kGy and 25 kGy) defatted walnuts.

	Total Polyphenols mg/100 g	Total Flavonoids mg/100 g	ORAC $\mu\text{mol TE/g}$	HORAC $\mu\text{mol GAE/g}$
Control	2915 $\pm$ 291	15.5 $\pm$ 0.7	235 $\pm$ 8	150 $\pm$ 7
10 kGy	2948 $\pm$ 19	15.4 $\pm$ 0.6	254 $\pm$ 9	167 $\pm$ 9
25 kGy	4210 * $\pm$ 12	26.0 * $\pm$ 1.1	320 * $\pm$ 6	178 * $\pm$ 8

* Results are listed as mean values $\pm$ standard deviations. Values marked by an asterisk are significantly different from the control one ($p < 0.05$).

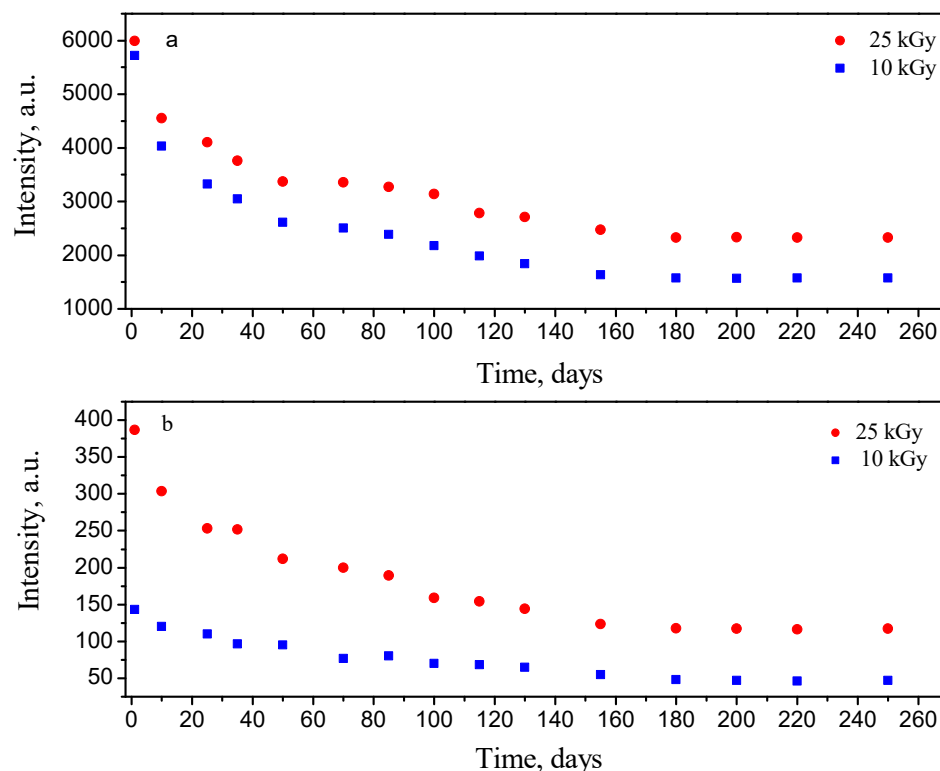


Figure 2. The time dependence of the EPR signal intensity of irradiated walnuts: (a) central line; (b) satellite lines.

3.4. Free Radical Scavenging Activity (FRSA) Assay by EPR

The relationship between DPPH scavenged radicals and the added walnut extract volume is shown in Figure 3. The results indicate that the ethanolic extract prepared from non-irradiated walnuts exhibited bioactivity for scavenging DPPH radicals. To characterize FRSA, the IC_{50} parameter was calculated. For the control sample, the IC_{50} value was 0.081. After the irradiation of defatted walnut samples by 10 kGy gamma rays, the IC_{50} value increased to 0.093, indicating a decrease in antiradical activity, as FRSA is inversely proportional to IC_{50} .

In contrast, an increase in free radical scavenging activity was observed in walnut samples irradiated with 25 kGy, with an IC_{50} value of 0.068. As previously mentioned, as a result of irradiation, free radicals are generated. The phenolic compounds present in walnuts, known for their antiradical properties, scavenge these radicals, and during the process, antioxidants are exhausted. This is the reason for the observed approximately 9% decrease in FRSA after walnut irradiation by a dose of 10 kGy. However, the recorded 13% increase in antiradical activity after 25 kGy walnuts irradiation is probably due to a significant increase in phenolic and flavonoid content (data are shown in Table 1). In this work, as in other reports [27], an excellent correlation was found between the total polyphenolic content and the antioxidant capacity of plant products. According to previous investigations [29], we observed different irradiation effects on the antioxidant capacity of walnut samples depending on the applied dose of gamma radiation.

Furthermore, some authors have indicated that plants contain considerable amounts of antioxidant phenolics in bound forms [30]. High-energy irradiation at 25 kGy generates more free radicals, which may break covalently bonded polymeric compounds into their free forms, thereby enhancing FRSA. Another possible explanation is the improved extractability of walnut phenolics after 25 kGy irradiation. Some nut phenolics may be associated with other plant constituents, such as proteins or carbohydrates. Thus, a certain

fraction of polyphenols is probably “unextractable” because of bonding with cell wall materials, mostly through hydrogen-bonding interactions. In walnuts, tannins are often found as protein complexes [31]. Probably, after gamma irradiation at 25 kGy, these hydrogen bonds in the complex may loosen and/or break, leading to the release of tannins or their weaker binding to proteins, thus making them more easily extractable.

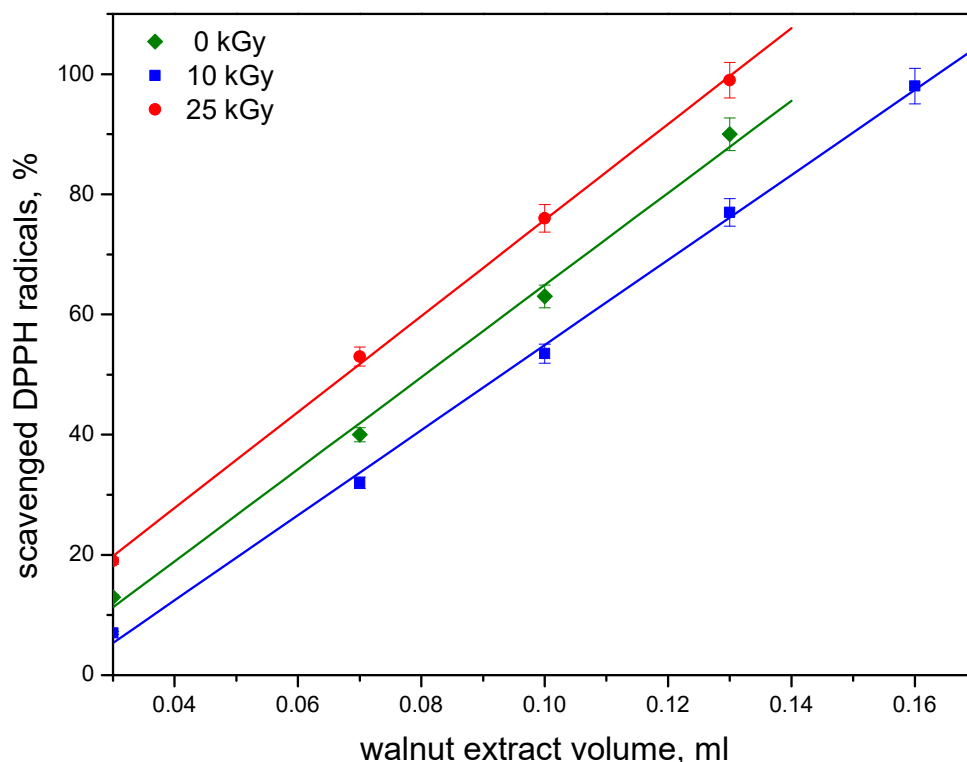


Figure 3. Dependence of scavenged DPPH radicals (%) on walnut extract volume (mL): non-irradiated (◆), irradiated by 10 kGy (■), and irradiated by 25 kGy (●). Error bars = SD, $n \geq 3$.

4. Conclusions

The current study presents a comprehensive analysis of irradiated gamma ray walnuts, focusing on free radical stability and antioxidant capacity. The findings contribute to a deeper understanding of the impact of gamma irradiation on the chemical composition and bioactivity of plant-based food products. EPR spectroscopy can confirm radiation-induced free radical formation for at least 8 months after irradiation. This long-term stability suggests that EPR spectroscopy can serve as a reliable tool for detecting irradiation treatment in walnuts and potentially other plant-based foodstuffs. A significant increase in polyphenol and flavonoid content was observed at 25 kGy, correlating with enhanced antioxidant activity, as evidenced by DPPH, ORAC, and HORAC assays. This indicates that gamma irradiation can alter the chemical structure of bioactive compounds, potentially increasing their accessibility and functionality. Higher irradiation doses improved FRSA, suggesting an increased availability of bioactive compounds. The dose-dependent effects of gamma irradiation demonstrate that while moderate doses (10 kGy) may lead to some antioxidant depletion due to free radical formation, higher doses (25 kGy) can enhance antioxidant potential by promoting the release of phenolic compounds. This suggests that controlled irradiation could be strategically used to optimize the health benefits of walnuts and other plant-based foods.

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of walnut defatted powder S.T.; statistical analysis—R.M.; writing—original draft preparation, K.A.; writing—review and editing, Y.K. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

EPR	Electron Paramagnetic Resonance
ORAC	Oxygen Radical Absorbance Capacity
HORAC	Hydroxyl Radical Antioxidant Capacity
DPPH	1,1-diphenyl-2-picrylhydrazyl
FRSA	Free radical scavenging activity
IC ₅₀	the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%
TEAC	Trolox equivalent antioxidant capacity
GAE	Gallic acid equivalents
SD	Standard deviation
QE	Quercetin equivalents

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