Research Article

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Assessment of the impact of γ-irradiation on the piperine content and microbial quality of black pepper

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Abstract: The major bioactive component of black pepper (*Piper nigrum*) is piperine which has demonstrated beneficial therapeutic properties. The purpose of this research was to investigate the effects of different irradiation doses on the content of piperine in black pepper. Samples were irradiated with ^{60}Co γ-rays (at absorbed doses of 0.5, 1, 3, 5, 7, 10, and 12 kGy). Thin-layer chromatography (TLC) and UV–Vis spectrophotometry methods were used for measuring the piperine content in the samples. TLC was performed using three mobile phases (1. toluene:ethyl acetate, 7:3 v/v; 2. acetone:*n*-hexane, 6:4 v/v; 3. toluene:methanol, 8.5:1.5 v/v) and the retention factor (R_f) value for piperine was equal to 0.66, 0.94, and 0.67, respectively. The content of piperine in γ-irradiated samples of black pepper was found to be between 0.04 and 1.05% w/w from the spectrophotometry analyses. Irradiation slightly decreased the piperine content of black pepper. It was found that piperine crude yield from black pepper was from 1.10 (the unirradiated sample) to 1.69, 1.07, 0.60, 0.90, 0.30, 1.20, 0.80% for irradiated samples, respectively. Microbiological analyses were performed with standard plate count method, which resulted in a decreasing number of the total cell count of microbial cells with increasing the radiation dose. Treatment with irradiation reduced the population of bacteria by 4 logs.

Keywords: γ-irradiation, piperine, black pepper, TLC, UV–Vis spectrophotometry, microbial quality

1 Introduction

Throughout history and even in modern times, the fruits of *Piper nigrum* (black pepper) have found extensive utilization both as a common kitchen spice and in diverse traditional medicinal practices. The significant therapeutic characteristics of *P. nigrum* fruits are largely attributed to the presence of piperine, a piperidine alkaloid, which constitutes approximately 2–7.4% of the fruit's composition [1,2].

The concentration of piperine in plants of the Piperaceae family may be influenced by the climatic conditions or the geographical location of their growth [3]. Black peppercorns, which are the desiccated whole berries of the pepper vine, are the preferred form for traditional trading. Piperine serves as the primary alkaloid accountable for the spiciness, while the volatile (essential) oil contributes to the aroma and taste [4]. Pharmacological and clinical investigations have demonstrated that piperine exhibits central nervous system depressant, antipyretic, analgesic, anti-inflammatory [5], antioxidant [6], and hepatoprotective [7] properties. Notably, piperine is recognized as a bioenhancer [8]. When referring to piperine as a bioenhancer, it is typically in relation to the bioavailability of other compounds that are ingested, such as nutrients and phytochemicals. Specifically, piperine has demonstrated the ability to augment the bioavailability of substances such as curcumin, resveratrol, and catechins. These are all examples of specific compounds found in

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certain foods or supplements, and piperine's ability to increase their bioavailability can potentially lead to greater health benefits. Studies have indicated that piperine can elevate the bioavailability of various drugs, with enhancements ranging from 30 to 200% [8,9]. Piperine is a promising component in terms of its use in medicine for various purposes such as treating lymphedema [10], inhibiting the spread of prostate cancer and breast cancer [11,12], as well as many health benefits of piperine, especially against chronic diseases [13]. Therefore, it is important that during the processing of food products, which are consumed by the general population, the piperine content does not decrease and its quality and safety are maintained due to its importance to the human health.

The allowable absorbed doses for food irradiation are typically established in accordance with regulatory guidelines, which outline the specific ionizing radiation sources, irradiation conditions, and doses applicable to particular food types and objectives. Approved irradiation doses range from 0.05 kGy, employed to prevent sprouting in white potatoes, to 30 kGy, utilized for the sterilization of herbs and spices [14].

Various research studies validate that extended boiling and cooking under pressure contribute to the degradation of piperine, while inadequate storage conditions exposed to light promote the formation of isopiperine [15]. Furthermore, steam treatment demonstrated a substantial decrease in the piperine content of black pepper [28]. Regarding the microwave treatment, it represents a secure and appropriate method for the decontamination of black pepper, minimizing the loss of flavor compounds to a greater extent compared to the recommended doses of gamma irradiation [16].

However, there is a limited body of research investigating the specific effects of ionizing radiation on the piperine content, and the available studies are deficient.

Black pepper is often a source of contamination with fungi and bacteria [17] and when added to food it can cause food spoilage. The addition of contaminated spices to raw or minimally processed foods poses a substantial hazard, leading to the potential development of severe foodborne illnesses. Ground black pepper has been found to be contaminated by pathogenic bacteria, including *Salmonella* sp. [18]. However, while thermal processing can effectively kill most bacteria, there are some heat-resistant bacteria, such as *Salmonella*, that may survive this process [19]. Additionally, thermal processing may not be enough to completely eliminate bacterial spores or viruses that can cause foodborne illnesses. Spices are often stored for long periods of time, resulting in an elevated likelihood of contamination by bacteria, fungi, and other microorganisms. Irradiating spices can help to ensure their safety by reducing the risk of contamination and extending their shelf life. Various methods of decontamination, such as ethylene oxide fumigation [20] and steam treatment [21], are used for disinfecting spices. However, both methods are associated with undesirable effects. Due to the carcinogenic nature [22] of ethylene oxide, the safety regulations governing its use have progressively become more stringent. On the other hand, the utilization of steam on spices is connected with adverse effects such as the deterioration of color, reduction in volatile oil content, and an elevation in moisture levels within the spices, ultimately resulting in a diminished shelf life [23].

Ionizing irradiation represents an alternative approach that proficiently manages foodborne pathogens and can protect public against different foodborne diseases [24]. Employing irradiation as a substitute for the utilization of toxic and carcinogenic substances like ethylene oxide and methyl bromide allows for the elimination of microbiological contamination, offering a less detrimental impact on spices compared to heat sterilization [25].

The objective of this study was to assess the effectiveness of ionizing radiation in sterilizing black pepper with the aim of enhancing its quality, while also investigating the alterations in its chemical properties, particularly the impact of ionizing radiation on the piperine content. The impact of ionizing radiation led to a reduction in the piperine content, and in terms of the microbiological aspect, we have similar findings in the literature, and a decrease in the number of microorganisms is also observed. Numerous publications have examined the chemical composition of essential oils from irradiated spices at different doses, but till now very few articles have been devoted to evaluation of the effect of ionizing irradiation in the piperine content in black pepper taking in consideration its contribution to human health.

2 Materials and methods

2.1 Sample collection

Black pepper, in form of grains was obtained as a gift from the food processing factory "Vitaminka A.D." from Prilep (North Macedonia). Piperine standard was isolated from the fruits of *Pepper nigrum.* Methanol was from Fluka (Switzerland), sodium hydroxide (99.8%) was supplied from Alkaloid A.D. (North Macedonia), while toluene (99.8%), ethyl acetate (99.8%), acetone (99.8%), and *n*-hexane (99.8%) were from Merck (Germany). *Sabouraud* 2% glucose agar and nutritive agar were supplied from Merck (Germany). All

reagents were with analytical purity. Piperine determination was conducted utilizing a thin-layer chromatography (TLC) technique, followed by UV–Vis spectrophotometric analysis. The absorbance was recorded using the spectrophotometer DLAB SPUV – 1100 (PRC). Cuvettes used for spectrophotometry were quartz, type Q130010. For TLC analysis, a Merck pre-coated silica gel plate measuring 5 × 10 cm (60F254, 200 µm) was employed, and subsequent visualization of the plates was performed at 254 nm using a UV lamp manufactured by Analytikjena (Germany).

2.2 Irradiation of the samples

The irradiation process was performed using a cobalt-60 gamma irradiator, specifically the tote box CEA model from Saclay, France. The irradiation facility is located at the Vinča Institute for Nuclear Sciences in Serbia. The samples were subjected to irradiation at various doses, including 0.5, 1, 3, 5, 7, 10, and 12 kGy, with a radiation speed of 9.1 kGy/h. The irradiation process was conducted at room temperature for a duration of 46 min.

2.3 Extraction and isolation

The extraction of piperine was done with the employment of the Soxhlet method. Each black pepper sample (10 ± 0.0001 g) was previously finely grounded and weighed on analytical balance (VWR LA 124, Austria) with 0.1 mg accuracy. The samples were placed in filter bags and extracted with 100 mL methanol in Soxhlet extractor for period of 4 h at 70°C. After extraction, all samples were placed in the distillation apparatus. Right before finishing with the distillation process, alkaline methanolic solution was added to the remaining extract in the bottom flask. The alkaline methanolic solution was prepared by dissolving 6 g NaOH in 50 mL methanol in a volumetric flask. The mixture (the extract + solution [NaOH + methanol]) was filtered and 40 mL of distilled water was added to the filtrate and allowed to stand for 18 h in the refrigerator at 4°C, where the precipitation of piperine crystals was slowly obtained. The obtained crystals were filtered again through a membrane filter using Büchner funnel and transferred in small dark bottles and were stored for further analysis. This procedure was repeated with all the samples. The steps of extraction and isolation of piperine are presented in Figure 1.

2.4 Determination of piperine in black pepper using spectrophotometry

2.4.1 Calibration curve of piperine

Standard stock solution was prepared by dissolving 10 mg of piperine in 100 mL of methanol. From the stock solution, standard working solutions of piperine were prepared in

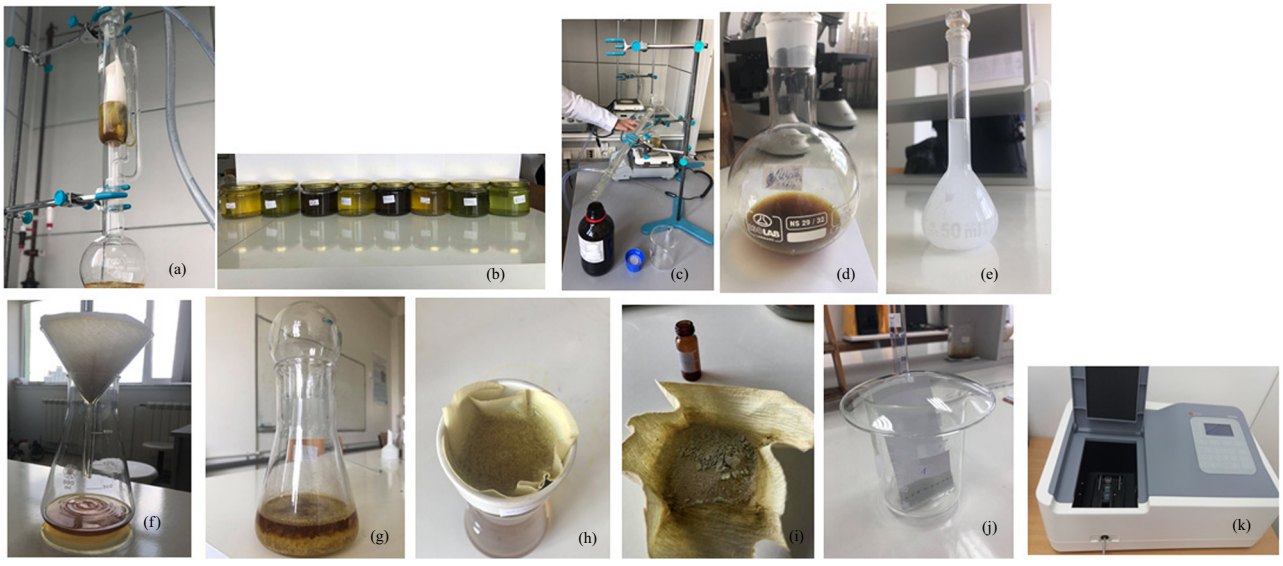

Figure 1: Steps of the extraction, isolation, and analyzing of piperine in black pepper samples. Extraction of piperine with Soxhlet method (a), obtained extracts (b), distillation of the extracts (c), residue left after distillation (distilled extract) (d), the alkaline methanolic solution (e), filtration of the mixture (the distilled extract + solution [NaOH + methanol]) (f), water added to the filtrate (g), filtration of the final solution (h), isolated piperine sample (i), developing a TLC plate (j), and spectrophotometry (k).

the concentration range of 2–20 µg/mL in 100 mL volumetric flasks using methanol as the solvent. The absorbance of these solutions was measured using a UV–Vis Spectrophotometer (DLAB SPUV – 1100, PRC) at a wavelength of 342 nm. A calibration curve was then constructed by plotting the absorbance values against the corresponding concentrations (Figure 2).

The method was validated taking into consideration linearity and range, precision and accuracy, reproducibility, limit of detection (LOD), limit of quantification (LOQ), and robustness according to the recommendations written by International Conference on Harmonization (ICH) guidelines [26].

Calibration curve exhibited exceptional linearity (R^2 > 0.99). The intra-day and inter-day precision and accuracy of the method was calculated with the analysis of the repeatability of the values of the working standard solutions of piperine in methanol. The percentage of recoveries was analyzed of the obtained piperine in irradiated black pepper. The average values of the standard deviation of response and slope of the calibration curve were utilized to calculate the LOD and LOQ. The LOD was determined using the equation LOD = 3.3σ/S, and the LOQ was calculated using the equation LOQ = 10σ/S, where σ represents the standard deviation of the response and S represents the slope of the calibration curve. The robustness of the method was done by changing the wavelength ±3 nm.

2.4.2 Sample measurement

The piperine crystals obtained from the irradiated and control samples were dissolved in methanol in a 100 mL volumetric flask and the sample solutions were measured at a wavelength of 342 nm with methanol serving as a blank sample. The maximum absorption obtained from sample piperine solution (unirradiated control sample) was 2.01 µg/mL.

2.4.3 Determination of the crude yield of piperine

The crude yield of piperine was determined by measuring the masses of the amount of piperine obtained by extraction and isolation and the initial amount of black pepper.

The crude yield of black pepper (%) was calculated by the following formula:

$$\text{Crude yield of piperine (\%)} = \frac{\text{The amount of piperine obtained } (g)}{\text{The amount of black pepper originally used } (g)} \times 100\%. \quad (1)$$

2.5 TLC

The analysis of piperine was conducted utilizing TLC. To prepare the samples, a single grain was dissolved in 0.5 mL of methanol for all the three phases. For the analysis, a comparative study was conducted using three different mobile phase solvent systems. The first system consisted of a mixture of toluene and ethyl acetate (7:3 v/v), the second system consisted of a mixture of acetone and *n*-hexane (6:4 v/v), and the third system consisted of a mixture of toluene and methanol (8.5:1.5 v/v). The distance that a compound travels on the TLC plate is expressed as the R_f

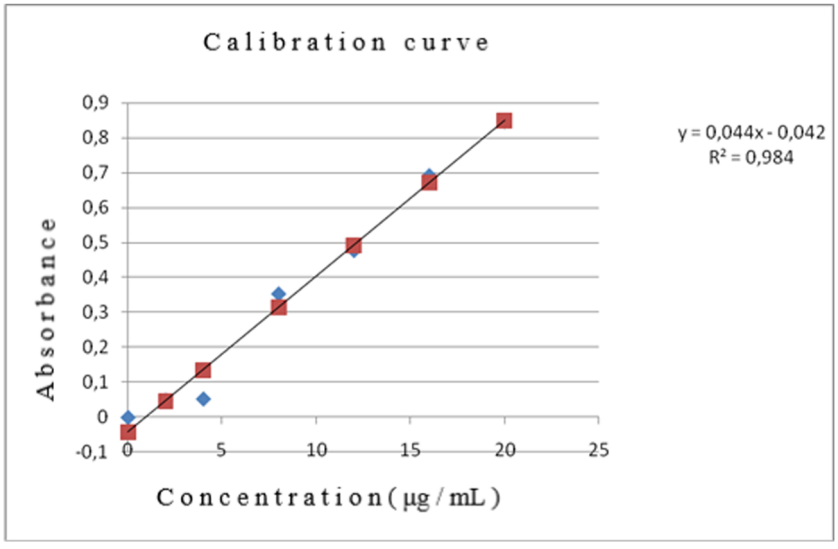

Figure 2: Calibration curve of piperine.

value, which is the ratio of distances from the starting line to the compound and to the solvent front.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}. \quad (2)$$

2.6 Determination of the microbiological quality of black pepper

The impact of irradiation on the overall cell population of the spice samples was examined using standard plate counts, which were expressed as the number of colony-forming units per gram of the sample (cfu/g). To conduct the analysis, 5 g of the samples were diluted in 45 mL of water [24,27]. Further dilutions were made until reaching a $1{:}10^5$ dilution, at which point 1 mL of the last two dilutions was inoculated onto Petri dishes containing 10 mL of *Sabouraud* 2% glucose agar for quantifying yeasts and molds, and nutritive agar for enumerating total aerobic mesophilic bacteria. The culture media were subjected to sterilization at a temperature of 121°C for a duration of 15 min. Following sterilization, the nutritive agar plates were placed in an incubator set at a temperature of 35°C, while the *Sabouraud* 2% glucose agar plates were incubated at a temperature of 30°C. Both types of agar plates were incubated under aerobic conditions for a period of 5 days. All analysis has been performed in triplicates.

3 Results and discussion

3.1 Effect of irradiation doses on the content of piperine according to the spectrophotometry analysis

The absorbance characteristics indicated that experimental data for piperine follows Beer Lambert's law within the concentration range 2–20 µg/mL at 342 nm with the coefficient of determination of 0.99 and calibration equation $Y = 0.0451X - 0.0455$. The linearity range is within the given interval. The % w/w content of piperine in the samples preparations of black pepper was found to be between 0.04 and 1.05 for piperine γ-irradiated samples (Table 1) in comparison to the unirradiated control sample (2.01%).

The results showed that with the increase dose of the irradiation, the piperine content was changed in the way of decreasing the piperine content in the samples. The piperine content of black pepper was slightly reduced as a result of irradiation. These outcomes align with the findings of Arifan et al. [27] and Waje et al. [28], which demonstrated a significant decrease in the piperine content of black pepper following irradiation. The obtained values for LOD and the LOQ were 2.6248 and 7.9540 µg/mL, respectively. Results of the robustness test for ±3 nm showed non-significant alteration of the absorption of the sample and standard piperine in methanol.

The method of least square regression analysis was used to calculate linear equation at 342 nm. The results of repeatability test show that the precision was over a short interval of time, as well as during inter-day assessment. The proposed value of 2% relative standard deviation for intra- and inter-day is presented in Table 2.

The statistical data processing involved the utilization of least square regression analysis and the ANOVA test. The validation of the linear regression was conducted through the ANOVA test, where the calculated F value was found to be higher than the critical F value of 5.7003 (4.6001).

3.2 Impact of irradiation doses on the crude yield of piperine

The impacts of various irradiation doses on the yield of piperine are shown in Figure 3. The effects of irradiation doses on the crude extracted yield were studied by varying

Table 1: Linearity of piperine in methanol and content in black pepper

Standard	Concentration (µg/mL)	Absorbance[1] ± SD	RSD (%)	Piperine irradiated sample (kGy)	Piperine content in the sample (% w/w)
St1	0.0	0.0001 ± 0.0001	0.02	0.5	0.85
St2	2.0	0.0493 ± 0.0006	1.17	1	0.04
St3	4.0	0.0530 ± 0.0005	1.08	3	1.05
St4	8.0	0.3530 ± 0.0006	0.16	5	0.32
St5	12.0	0.4770 ± 0.0001	0.21	7	0.99
St6	16.0	0.6910 ± 0.0006	0.08	10	0.33
St7	20.0	0.8455 ± 0.0005	0.06	12	0.31

[1]Mean ± standard deviation (n = 3); SD: standard deviation; RSD: relative standard deviation.

Table 2: Results of precision

Concentration (μg/mL)	Intra-day (n = 3)[1]		Inter-day (n = 3)[2]	
	Experimental concentration ± SD (μg g/mL)	RSD (%)	Experimental concentration ± SD (μg g/mL)	RSD (%)
2.0	1.9999 ± 0.0006	1.63	1.9998 ± 0.0005	1.59
4.0	4.0011 ± 0.0005	0.42	3.9991 ± 0.0004	1.91
8.0	7.9999 ± 0.0004	0.97	8.0001 ± 0.0001	0.54
12.0	12.0001 ± 0.0003	1.31	11.9990 ± 0.0001	1.27
16.0	15.9999 ± 0.0004	1.07	16.0001 ± 0.0005	1.83
20.0	20.0001 ± 0.0003	0.44	19.9999 ± 0.0004	1.75

[1]Mean ± standard deviation (n = 3); [2]mean ± standard deviation (n = 9); SD: standard deviation; RSD: relative standard deviation.

at different does from 0.5 to 12 kGy. Comparing with the unirradiated control sample there was a slightly small increase in the piperine yield as irradiation dose increased to a value of 1 kGy in black pepper. This can be related to its capacity of absorbing energy to break of the cell wall of the pepper matrix [29,30]. Beyond these, quantity of crude yield extracted decreased with a corresponding increase in irradiation doses shown in Figure 3. The decrease which occurred upon irradiation could be due to that γ-irradiation may induce the formation of free radicals which can cause degradation, resulting in reduced amounts of piperine. There were no remarkable alterations observed in the composition of piperine when exposed to radiation doses of 5 and 10 kGy, which have been scientifically established as the maximum safe dose in terms of toxicity and nutrition [31]. Regardless, with the increase of radiation doses to 12 kGy, a minor decline in the piperine content was detected.

3.3 Separation and identification of piperine through TLC in fluorescence mode

TLC analysis of the compound was carried out on silica gel plates coated in advance, utilizing three different mobile phases, namely: mobile phase A: toluene:ethyl acetate (7:3, v/v), mobile phase B: acetone:*n*-hexane (6:4, v/v), and mobile phase C: toluene:methanol (8.5:1.5, v/v). Figure 4 reveals the developed TLC plates.

The TLC method was introduced as a control method and in the same time as an orthogonal method in order to access the specificity test for the determination of piperine in samples. The choice of the mobile phase was based on the behavior of the substance in various mobile phases. The spots indicated the piperine presence in the sample comparing the determined values of the retention factor with the standard piperine sample. The spots in Figure 4 are in the order with the increasing utilized irradiated

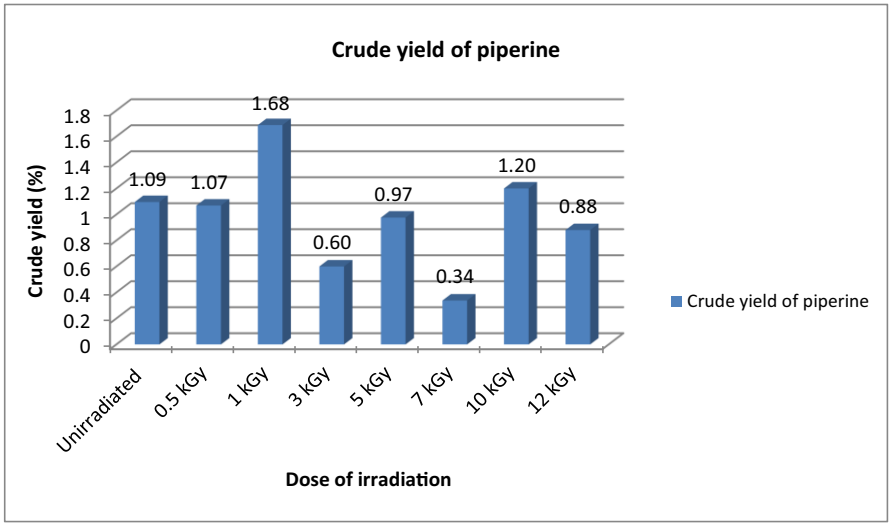

Figure 3: Effects of different irradiation doses on the extracted crude yield of piperine in black pepper.

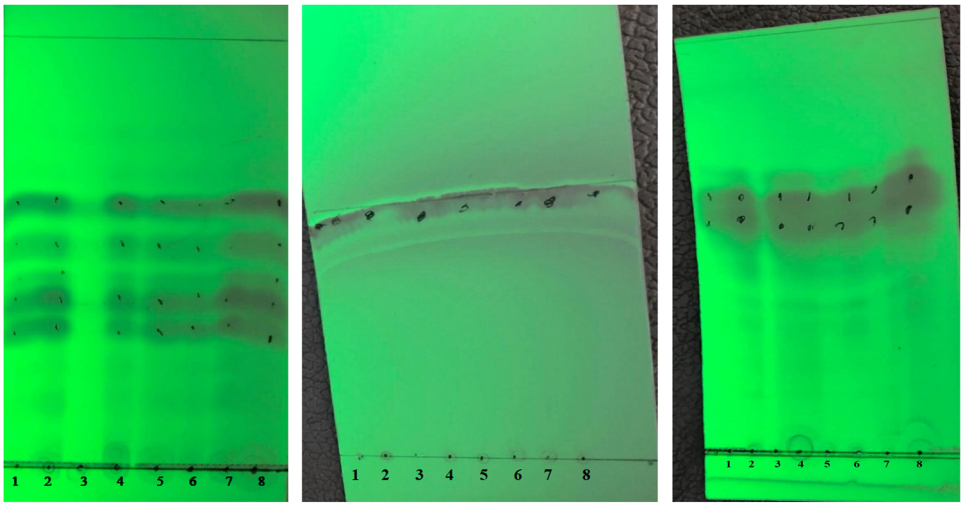

Figure 4: Chromatogram of piperine. Mobile phase (a), toluene:ethyl acetate (7:3, v/v); mobile phase (b), acetone:*n*-hexane (6:4 v/v); mobile phase (c), toluene:methanol (8.5:1.5 v/v). Lane 1, control unirradiated sample; lanes 2–8 irradiated samples (0.5, 1, 3, 5, 7, 10, and 12 kGy).

doses from 0.5 to 12 kGy, starting with the control sample which was not irradiated. The R_f value for piperine using mobile phase A was found to be 0.666, with the use of the mobile phase B the value of R_f was 0.948, while with the use of the mobile phase C the R_f was 0.670. The best separation (Figure 4a) was achieved with the use of the mobile phase (A) toluene:ethyl acetate (7:3, v/v) and the R_f value for piperine was found to be 0.666 which corresponded to that of piperine [32–34].

3.4 Irradiation effect on the microbiological analysis

The impact of ionizing irradiation at varying doses (0.5–12 kGy) on the microbial sterilization of black pepper was investigated. To assess the efficiency of irradiation as a technique for preserving food, a microbiological method (agar plate total cell count) [24,27] was employed to determine the total count of aerobic mesophilic bacteria and fungi/yeasts. The culture plates (containing nutritive broth agar and *Sabouraud* glucose broth) were incubated with diluted samples from irradiated and unirradiated black pepper, ranging from dilutions of 10^2–10^5 times.

Figure 5 displays the results of enumerating colonies from the black pepper sample, both unirradiated and irradiated, on the two different types of inoculation media used. The figure clearly demonstrates that the colony-forming units of total mesophilic bacteria decrease with an increase in the irradiation dose, as was expected. The initial population of the total aerobic mesophilic bacteria in the untreated black pepper was relatively high (105 cfu/g),

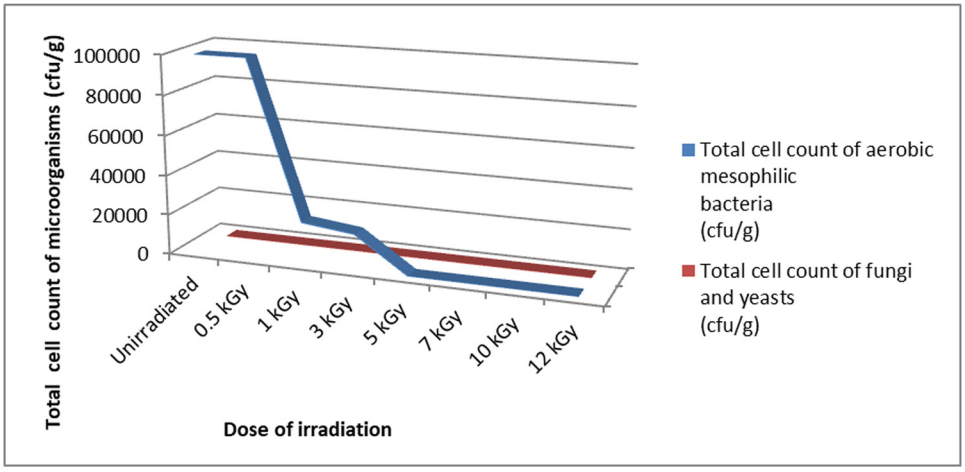

Figure 5: Total number of microbial cells (cfu/g) in black pepper unirradiated (control) and irradiated with different doses of irradiation.

but irradiation treatment resulted in a reduction of 4 logs. Irradiated black pepper with 0.5, 1, 3 kGy showed 1×10^5, 21×10^3, 18×10^3 cfu/g bacterial contamination, respectively. No bacterial growth was observed on the nutritive growth media when black pepper, irradiated with 5 kGy, was inoculated. It was found out that a dose of 5 kGy is sufficient for the reduction of total bacterial contamination. In a similar study by Sádecká et al., it was likewise demonstrated that the total microbial count of 10^6 colonies in the untreated black pepper sample decreased upon irradiation with a dose of 5 kGy [31].

Based on the findings depicted in Figure 5, it is evident that both the irradiated and unirradiated samples of black pepper exhibited no signs of fungal and yeast contamination. This outcome can be attributed to the presence of essential oils in black pepper, which are recognized for their potent antimicrobial properties [24]. Pepper oil has been shown to possess inhibitory effects on fungal growth, and as a result, black pepper can be regarded as an unfavorable medium for fungal proliferation [34]. Waje et al. conducted a similar investigation which revealed that the counts of yeasts and molds decreased to non-detectable levels following storage [28]. The counts of the untreated peppers decreased as well after the storage duration. Therefore these results can be explained due to the temporary storage of black pepper and showed that storage could further enhance the microbial quality of treated peppers since the injured microbial cells, as a result of irradiation, were unable to repair and proliferate over time [28].

After culturing the black pepper spice samples on nutritive agar plates, the microorganisms were initially identified. As a result, the bacterial strains preliminarily thought to be present in the unirradiated black pepper samples were confirmed to be *Bacillus subtilis.* According to the results, it is noticed that there is a reduction of total number of aerobic mesophilic bacteria at radiation doses of 0.5–3 kGy. However doses up to 3 kGy are too low to be used for sterilization of black pepper. The most optimal dose for sterilization is 5 kGy, as expected.

4 Conclusion

Although spices, herbs, and dried vegetable seasonings are subjected to ionizing radiation treatment to eliminate microbial contamination, it is important to note that this process can potentially modify the chemical composition depending on the dosage of radiation employed.

From the results obtained from this research, we have concluded that ionizing radiation is very effective, safe, useful, and successful in terms of microbial quality, namely in controlling the microbial contamination that can be effectively used to improve the safety of our food supply. In this research it is evident that although a dose of 10 kGy is allowed for the disinfection and sterilization of spices, from the obtained results we conclude that the radiation dose of 5 kGy is sufficient enough to eliminate microorganisms from black pepper. Results show that this radiation dose has minimal effect on the piperine content in black pepper.

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Conflict of interest: The authors of this manuscript have no conflicts of interest to declare.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on rational request.

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