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Effect of Gamma Irradiation on Total Phenolic Contents and Antioxidant Activities of Methanolic Extract of Java Plum Seeds

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Abstract

The synthetic antioxidant butylated hydroxyanisole (BHA) has been widely added to lipids and food products as a preservative due to inhibiting lipid oxidation. However, concerns have arisen about the potential health impacts of this synthetic antioxidant on consumers. Therefore, there is a need for an alternative substitute for BHA that is safe for human health. The methanolic extract of Java Plum seeds (MEJS) contains a diverse group of phenolic compounds and has the potential to serve as a natural antioxidant. The antioxidant must remain stable during food processing, such as irradiation. This research aimed to determine the effect of γ-irradiation on the total phenolic content and antioxidant activity of MEJS. When γ-irradiation was applied to MEJS at doses up to 12.5 kGy, there was a slight decrease in total phenolic and tannin content but a significant increase in total flavonoid content. This increase in total flavonoids led to a rise in both free radical scavenging and reducing power activities.

Keywords: antioxidant; java plum; seed extract; γ-irradiation

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Introduction

Synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been widely added to lipids and food products as preservatives, effectively inhibiting lipid oxidation (1;2). However, synthetic antioxidants have raised concerns about potential health impacts on consumers. The U.S. Department of Health and Human Services released the 14th Report on Carcinogens (RoC), listing BHA as a food additive classified as 'reasonably anticipated to be a human carcinogen' (3). As a result, there is a need for alternative substitutes to BHA that ensure safety for human health.

The extract from Java Plum seeds (JS) contains a diverse group of phenolic compounds such as (+)-catechin, quercetin, (+)-epicatechin, rutin, gallic acid, ellagic acid, kaempferol, and the galloylglucose group (4;5). Meanwhile, the methanolic extract of Java Plum seed (MEJS) was found to be abundant in rutin and (+)-catechin (6). Many of the Java plum phenolic compounds mentioned above have been reported to possess potent antioxidant activity (7;8;5;9;10;11;12).

Plant phenolic compounds' antioxidant effects have been studied to prevent lipid oxidation over the last few decades (13;2). Previous research has shown that MEJS exhibits potent reducing agents, radical scavenging, and hydrogen donor properties (5;6). Additionally, it has been noted that MEJS possesses natural antioxidant potential (5;14;10). When used as a food additive, antioxidants must remain stable during food processing, particularly thermal processes such

as irradiation (15). Gamma irradiation has been highly influential in inhibiting the growth of undesirable microbes (16;17). However, it simultaneously causes a decrease in total phenolic content (TPC) and DPPH radical scavenging activity in the extracts after irradiation (16). Furthermore, Song et al. (18) reported that γ-irradiation increased the extraction yield and TPC rather than maintaining the DPPH radical scavenging and antimicrobial activities. Nevertheless, the influence of γ-irradiation on the antioxidant activity and TPC of MEJS has not been studied yet. Therefore, the present study aims to determine the effect of γ-irradiation doses (0-12.5 kGy) on the total phenolic content and antioxidant activity of MEJS.

Material and Methods

Materials

Java Plum (*S. cumini* Linn.) seeds were ground to a powder (60 mesh) with 10% water content. Chemicals used included methanol (>99.5% purity, Merck), gallic acid, tannic acid hydrate (Sigma-Aldrich, Belgium), (+)-catechin (Sigma Chemical Co., St. Louis, USA), hydrochloric acid (HCl), ferrous chloride (FeCl2), ferric chloride (FeCl3), ammonium thiocyanate, potassium ferricyanide (III), trichloroacetic acid (TCA), phosphotungstic acid, 1,1-*diphenyl-2-picrylhydrazyl radical* (DPPH) (Sigma-Aldrich Chemical Co.), Whatman filter paper (Whatman International, Ltd., England), Folin-Ciocalteu reagent, and phosphate buffer. All reagents and chemicals were of analytical grade. The equipment used included an analytical balance (Shimadzu AUW 120, Shimadzu, Kyoto, Japan), a rotary vacuum evaporator (IKA-RV10 Basic), a freeze dryer (Virtis SP Scientific Sentry 2.0), an oven, a vortex mixer (Velp Scientifica Europe), a water-bath shaker (Julabo SW 22), a UV-Visible spectrophotometer (UV1601 Shimadzu, Japan), and a Cobalt-60 Irradiator (Ob-Servo Ignis, Izotop Hungaria).

Sample preparation

The Java Plum fruits (Genthong variety) were harvested in October 2018 during the optimal season. Local farmers in Semarang Regency, Central Java, Indonesia, picked the fruits at their optimum maturity stage. After harvesting, the fruits were stored in cold storage at -18°C until their use in May 2019. The Java Plum seeds were obtained by separating them from the pulp. The seeds were then cut into pieces using a sharp knife and dried using a cabinet dryer at 55±5°C. Once dried, the seeds' kernels were extracted and separated from the husk and seed coats. The kernels were milled using a cutting mill and then sieved to obtain Java Plum seed powder (60 mesh) with less than 10% water content. This Java Plum seed powder (JSP) was airtight packaged and stored in a dark, dry room until its use in the subsequent steps.

Proximate assay

In brief, the JSP was subjected to proximate analysis, which included the determination of water content using the gravimetric method of analysis as outlined by AOAC (19), protein content assessed through Kjeldahl extraction methods as per AOAC (19), lipid content determined using Soxhlet extraction methods following AOAC (19) guidelines, estimation of crude fiber content using the standard procedure outlined in AOAC (19), analysis of ash content on a dry matter basis using AOAC (19) analysis methods, and calculation of total carbohydrates *by difference* according to AOAC (19).

JSP extraction

The extraction of JSP was conducted following a modified method as described by Rohadi *et al.* (6). Briefly, approximately 30 g of JSP was extracted using a 50% (v/v) aqueous methanol solution at a materials-to-solvent ratio of 1:10. The mixture was macerated on a water bath shaker at 40 ± 1°C for 6 hours with agitation at 100 rpm. Subsequently, the mixture was filtered using Whatman filter paper, and the resulting filtrate was collected in a 1000-milliliter Erlenmeyer flask. The residue underwent two additional extractions using the same methods. The collected extracts were concentrated using a rotary vacuum evaporator (IKA-RV 10 Basic, Germany) to obtain the methanol-free extract. This extract was then freeze-dried (Virtis SP Scientific Sentry 2.0) to remove residual solvent. The resulting methanolic extracts of Java Plum seeds (MEJS) were stored in a refrigerator for further analysis. This extraction procedure was repeated three times.

Irradiation of MEJS

The MEJS was irradiated at doses of 2.5, 5.0, 7.5, 10, and 12.5 kGy using a Cobalt-60 irradiator (Institute of Isotopes Co. Ltd) with an activity of 12 kCi, operating at a dose rate of 3.93 kGy/h at room temperature. The irradiated

extracts were then stored at 4ºC for further analysis.

Total phenolic assay

The total phenolic compounds were determined using the modified Folin–Ciocalteu method (20). In brief, 0.5 mg of MEJS was diluted in 1 mL of methanol, and 0.5 mL of Folin-Ciocalteu reagent (1:1) was added, followed by homogenization. After 8 minutes, 4.5 mL of 2% sodium carbonate (Na_2CO_3) solution was added and thoroughly mixed. Subsequently, the mixture was stored in a dark room at room temperature. The reaction resulted in the development of color, and the absorbance at λ = 765 nm was measured after 60 minutes using a UV-1601 Shimadzu spectrophotometer (Japan) against the blank. The same procedure was repeated using a standard solution of gallic acid. The results were expressed as grams of gallic acid equivalent (GAE) per 100 grams of MEJS.

Total Flavonoid assay

The total flavonoid compounds were determined using the method described by Ebrahimzadeh *et al.* (20). In brief, 0.5 mg of MEJS was diluted in 1.5 mL of methanol, followed by adding 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was then homogenized and incubated at room temperature for 30 minutes. Absorbance was measured at 415 nm using a spectrophotometer (UV-1601 Shimadzu, Japan). The total flavonoid content was calculated as (+)-catechin equivalent (CE) using a calibration curve described previously.

Total Tannin assay

The total tannin content was determined using the method outlined by Palici *et al.* (21). Briefly, 2 mL of MEJS (0.01 – 0.001%) was mixed with 1 mL of phosphotungstic acid and 17 mL of 50% sodium bicarbonate (Na_2CO_3). The mixture was then incubated for 2 minutes before measuring its absorbance. Absorbance was recorded at λ = 750 nm using a spectrophotometer (UV-1601 Shimadzu, Japan). The total tannin content was calculated as tannic acid equivalent (TAE) using a calibration curve described previously.

DPPH radical scavenging activity assay

The DPPH radical scavenging activity was assessed using the method outlined by Vasi and Austin (8). In brief, 0.5 mL of MEJS solution at various concentrations (25, 50, 100, 200, and 400 ppm) was mixed with 0.5 mL of DPPH solution (100 µM) and then incubated at room temperature (37±2°C) for 15 minutes. The absorbance was measured at λ = 517 nm. The reaction was subjected to absorbance measurement again at λ = 517 nm. Radical scavenging activity (RSA) was expressed as the percentage of inhibition of the DPPH free radical and calculated as follows:

Inhibition RSA-DPPH (%)

$$= 1 - [\frac{Abs.sample}{Abs.control}] \text{ x } 100 \text{ \% } \ldots\ldots\ldots\ldots 1$$

Reducing Power assay

The reducing power activity was assessed using the method described by Vasi and Austin (8). Briefly, 2.5 mL of MEJS solution at various concentrations (25, 50, 100, 200, and 400 ppm) was combined with 2.5 mL of phosphate buffer (0.2 M, pH = 6.6) and 2.5 mL of 1% potassium ferricyanide solution. The mixture was incubated at 50°C in a water bath for 20 minutes. The reaction was then terminated by adding 1 mL of 10% trichloroacetic acid (TCA) to each reaction. Afterward, the solution was centrifuged (3000 rpm/10 min). The upper layer of the mixture (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% iron (III) chloride solution, and the absorbance was measured at λ = 700 nm. A higher absorbance indicates a higher total reducing power.

Statistical Analysis

All experiments in this study were conducted in triplicate, and the results were expressed as mean ± SD. The data were analyzed using one-way analysis of variance (ANOVA) with the statistical package for the social sciences (SPSS) for Windows, version 22.0 (SPSS Inc., Chicago, IL). Statistical significance was declared at a significance level of $p < 0.05$.

Results and Discussion

The primary characteristic parameters of JSP revealed the following chemical contents: water content 9.1 ± 0.1%, lipid 0.25 ± 0.04%, protein 3.89 ± 0.02%, crude fiber 2.14 ± 0.07%, ash 1.07 ± 0.01%, and carbohydrate (by difference) 85.73 ± 0.1%. Previous research indicated that Java plum seeds constituted solid waste (20.65%) of fresh fruits. JSP was found to be rich in dietary fiber, with sucrose (0.17%), fructose (2.78%), glucose (2.24%), and serving as a source of minerals including potassium (8813 ppm), magnesium (2162 ppm), iron (137 ppm), and sodium (115 ppm), along with trace

minerals like calcium, phosphorus, and copper (10). The yield of JSP extraction was determined to be 12.84 ± 0.8%, slightly lower than the reported 13.89 ± 0.5% (10). These differences may arise due to variations in sample freshness and particle size. Vasi and Austin reported a yield extraction of Java plum seed with 50% ethanol at 12.96% (8).

Phenolic compounds

The phenolic compounds of MEJS before irradiation were quantified using three methods: total phenolic content, flavonoid content, and total tannin content. The total phenolics content was determined as grams of gallic acid equivalent per 100 grams of dry extract, and this was derived from a calibration curve equation: $Y_1 = 7.145X - 0.034$, with an R^2 value of 0.986. The total flavonoid content was calculated as grams of (+)-catechin equivalent per 100 grams of dry extract, referencing a calibration curve equation: $Y_2 = 0.0012X - 0.0038$, with an R^2 value of 0.998. The total tannin content was expressed as grams of tannic acid equivalent per 100 grams of dry extract, and this was determined based on a calibration curve equation: $Y_3 = 0.84X + 0.031$, with an R^2 value of 0.996. These values are provided in Table 1.

Table 1. The phenolic compounds of MEJS

Solvent	Phenolic compounds		
	Total phenolic (g-GAE/ 100 g)	Total flavonoid (g-CE/ 100 g)	Total tannin (g-TAE/ 100 g)
Methanol:water (1:1)	34.73 ±0.3	7.12±0.17	29.81 ±0.15

As presented in Table 1, the MEJS contains the following phenolic compounds: total phenolic content of 34.73 ± 0.3 (g-GAE/100 dry extracts), flavonoid content of 7.12 ± 0.17 (g-CE/100 dry extracts), and total tannin content of 29.81 ± 0.15 (g-TAE/100 dry extracts). Notably, differences exist between the results of phenolic compound content in MEJS and those from previous studies (10). These discrepancies may arise from variations in factors such as maceration temperature, particle size, and sample freshness. Additionally, using (+)-catechin as a standard in the total flavonoid assay for samples is crucial. Consequently, the MEJS contains more (+)-catechin than quercetin (6). Many publications have reported using (+)-catechin as the standard in total flavonoid assays (22;23). Vayuparp and Laksanalamal reported a yield of 14.86 ± 0.03% and a total phenolic content of 32.86 ± 0.04% (g-GAE/100 g dry extract) from grape seed extraction using 50% ethanol and maceration (50°C/6 hrs, 1:10) (1).

Phenolic Compound and Antioxidant Activity Assay

Table 2 below shows the effect of various irradiation doses on the total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and in vitro antioxidant activity immediately (0 days) after irradiation.

Table 2. Effect of γ-irradiation on phenolic compounds and in vitro antioxidant activity of MEJS

Irradiation Dose (kGy)	Phenolic compounds			In vitro Antioxidant Activity*	
	TPC (g-GAE/100g-DE)	TFC (g-CE/100 g-DE)	TTC (g-TAE/100 g-DE)	RSA-DPPH (%)	FRAP (OD)
0	34.73 ± 0.30^{a}	7.11 ± 0.10^{e}	29.77 ± 0.16^{a}	71.1 ± 0.14^{b}	1.89 ± 0.007^{f}
2.5	33.76 ± 0.08^{b}	7.33 ± 0.17^{e}	29.37 ± 0.55^{a}	89.82 ± 0.1^{a}	1.93 ± 0.002^{e}
5	28.59 ± 0.08$_{c}$	7.95 ± 0.16^{d}	29.48 ± 0.11^{a}	90.67 ± 0.1^{a}	1.99 ± 0.001^{d}
7.5	29.70 ± 0.04^{c}	9.16 ± 0.20^{c}	29.21 ± 0.14^{a}	90.99 ± 0.1^{a}	2.12 ± 0.003^{c}
10	29.36 ± 0.67^{c}	9.95 ± 0.22^{b}	29.00 ± 0.14^{a}	91.20 ± 0.1^{a}	2.45 ± 0.003^{b}
12.5	29.69 ± 0.04^{c}	10.43 ± 0.10^{a}	28.66 ± 0.10^{a}	91.41 ± 0.1^{a}	2.48 ± 0.003^{a}

The mean in the same column with different alphabetical letters is significantly different (p<0.05).
*Antioxidant activity was measured at 400 ppm extract.

The effect of γ-irradiation on phenolic compounds and in vitro antioxidant activity of the samples revealed intriguing phenomena. Gamma irradiation did not yield the same effect on phenolic compound assays and antioxidant activity. The TFC and average in vitro

antioxidant activities, specifically RSA-DPPH and FRAP, of the control (0 kGy) samples, when compared to the irradiated (at 2.5 kGy) samples, increased by 3.1%, 26.3%, and 2.1%, respectively. However, in the TPC and TTC assays, there was a decrease of 2.8% and 1.3% (although not statistically significant). Subsequently, γ-irradiation at higher dose levels (5-12.5 kGy) led to a decrease in the TPC and TTC assays (not significantly, $p > 0.05$) by 12% and 2.4%, respectively. Conversely, it resulted in an increase in radical scavenging activity by 1.8% (not significantly), while the total reduction increased significantly by 28.55% ($p < 0.05$) (Table 2).

Mali *et al.* (17) reported that the decrease in the TPC and TTC assays could be attributed to the degradation of tannins present in MEJS powder, which have a higher molecular weight, leading to the release of simple phenolic compounds such as quercetin (+)-catechin, and tannic acid. Gamma irradiation at the initial step (2.5 kGy) may break down the tannin complexes, facilitating the release of these simple active compounds, which could contribute to the increase in the total phenolic content (TPC) assay. This perspective aligns with the opinions raised by Kumari *et al.* (24) and Mali *et al.* (17). However, continued irradiation at higher doses (5-12.5 kGy) led to a decrease in the TPC and TTC assays and an increase in the TFC assay. This increase in the TFC assay was then followed by an enhancement in antioxidant activity (Table 2). The augmented antioxidant activity after irradiation may be attributed to the degradation of tannins present in MEJS, resulting in the release of free simple bioactive compounds such as rutin, quercetin, and (+)-catechin.

DPPH radical scavenging activity assay

DPPH free radical scavenging activity, expressed as (% inhibition), was used to assess the in vitro antioxidant assay of MEJS samples (both irradiated and control). The effect of γ-irradiation on the in vitro antioxidant activity of the samples is depicted in Figure 1 below. The results showed that increasing irradiation doses (0-12.5 kGy) applied to the samples (25-400 ppm) enhanced their free radical scavenging activity. At 25 ppm, the scavenging activities of MEJS against the DPPH radical were 3.66%, 10.93%, 18.39%, 21.91%, 21.91%, and 23.08% for 0 kGy (control), 2.5 kGy, 5.0 kGy, 7.5 kGy, 10.0 kGy, and 12.5 kGy irradiation doses, respectively (Fig. 1). Meanwhile, at the higher concentration (400 ppm), the scavenging activities of the samples were 71.1%, 89.82%, 90.67%, 90.99%, 91.2%, and 91.41% for control (0 kGy), 2.5 kGy, 5.0 kGy, 7.5 kGy, 10.5 kGy, and 12.5 kGy irradiation doses, respectively. There were significant differences in the effect of γ-irradiation dose on the scavenging activity of the samples ($p < 0.05$). This finding is consistent with the research results conducted by previous researchers (17;25;26). Tseng and Mau stated that the free radical scavenging activity of cold water extract from *Ganoderma tsugae* increases with the concentration of the extract (26).

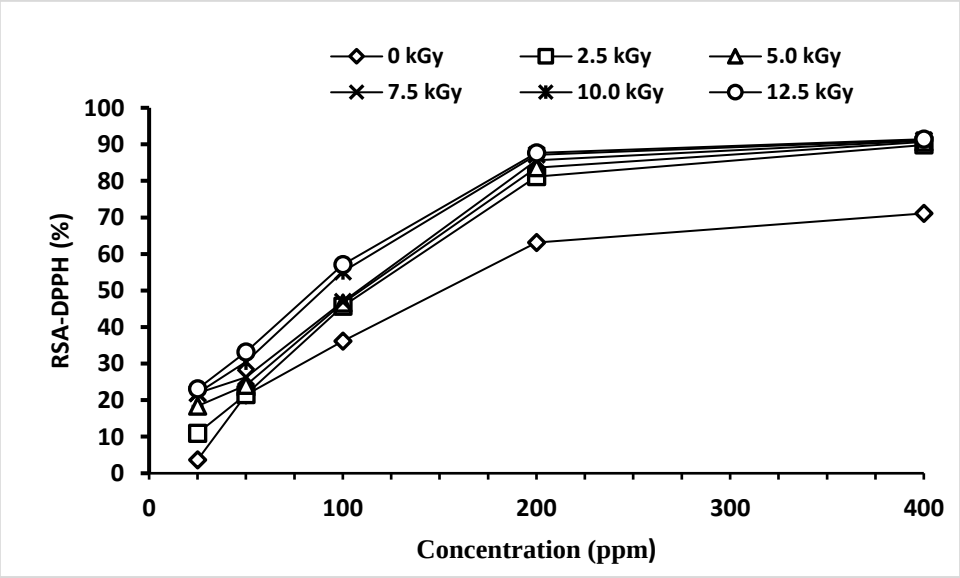

Figure 1. Free radical scavenging activity of MEJS (25-400 ppm) in various γ-irradiation doses. There is a significant effect of γ-irradiation dose against antioxidant activity ($p<0.05$). Each value is expressed as mean ± sd, n=3.

However, this contrasts with the findings of Naveed *et al.* (27), who reported that γ-irradiation tends to decrease the antioxidant activity of various methanolic extracts of selected herbs. Additionally, Gumus *et al.* (16) noted that the DPPH radical scavenging activity of the methanolic extract of four plant spices from Turkey decreased after irradiation. γ-Irradiation has been observed to have no impact on the antioxidant activity status, instead maintaining it, as reported by Song *et al.* (18). Previous research on the effect of irradiation on foods and its influence on phenolic and antioxidant activity has indicated that γ-irradiation does not significantly affect changes in phenolic compounds (28;27;29).

Reducing Power assay

Ferric reducing antioxidant power, expressed as absorbance value (OD), was used to assess the in vitro antioxidant assay of MEJS samples (irradiated and controlled). The effect of γ-irradiation on the in vitro antioxidant activity of the samples is depicted in Figure 2 below. In general, the results showed that increasing the dose of irradiation (0-12.5 kGy) applied to the samples (25-400 ppm) led to an increase in their ferric-reducing antioxidant power. At 25 ppm, the ferric reducing power values were 0.18, 0.21, 0.212, 0.221, 0.223, and 0.28 for 0 kGy (control), 2.5 kGy, 5.0 kGy, 7.5 kGy, 10.0 kGy, and 12.5 kGy irradiation doses, respectively (Fig. 2).

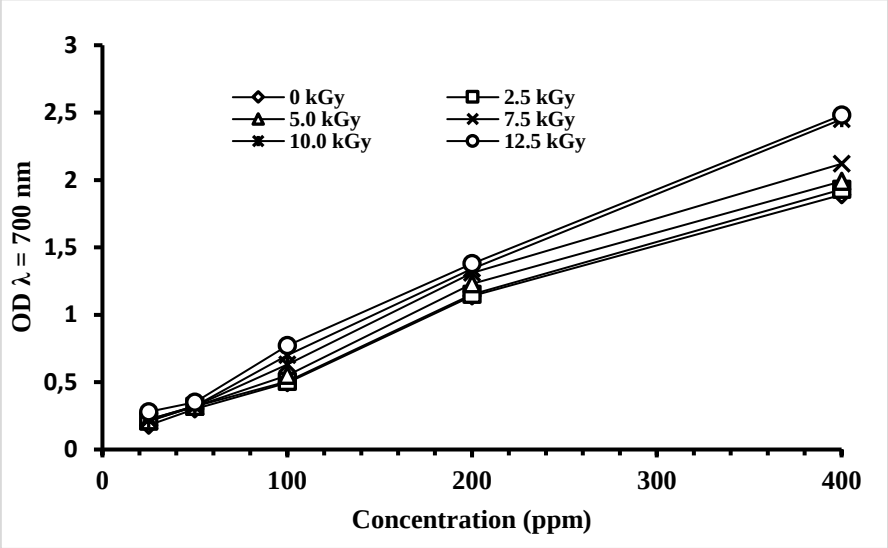

Figure 2. Reducing power of MEJS (25-400 ppm) in various γ-irradiation doses. A significant effect of γ-irradiation dose against antioxidant activity (p<0.05). Each value is expressed as mean ± sd, n=3.

Meanwhile, at the higher concentration (400 ppm), the reducing power of the extract was 1.89, 1.93, 1.99, 2.12, 2.45, and 2.48 for 0 kGy (the control), 2.5 kGy, 5.0 kGy, 7.5 kGy, 10.5 kGy, and 12.5 kGy irradiation doses, respectively. There were significant differences in the effect of γ-irradiation dose on the ferric-reducing power of the samples (p < 0.05). This finding is consistent with the research results conducted by previous researchers (26;25). Tseng and Mau stated that the total reduction properties of the cold water extract from *Ganoderma tsugae* increase in line with the increasing concentration of the extract (26).

However, this contrasts with the findings of Naveed *et al.* (27), who reported that γ-irradiation tends to decrease the antioxidant activity of various methanolic extracts of selected herbs. γ-Irradiation has not impacted the antioxidant activity status. Instead, it has maintained it (18). Some of the previous research that explored the effect of irradiation on foods against phenolic compounds and antioxidant activity revealed that γ-irradiation did not significantly correlate with changes in phenolic compounds (27;29). The effect of irradiation applied to foods could decrease certain identified phenolic compounds, while conversely, it could increase them, depending on the applied dose (30).

EC_{50} value in antioxidant properties

The antioxidant properties were typically expressed as EC_{50} value (mg-extract per liter) for comparison. Efficacy in antioxidant properties is inversely correlated with the EC_{50} value. The EC_{50} values of the irradiated MEJS were 200 ppm, 156 ppm, 142 ppm, 133 ppm, 116 ppm, and 105 ppm for the

irradiation doses of 0 kGy (control), 2.5 kGy, 5.0 kGy, 7.5 kGy, 10 kGy, and 12.5 kGy, respectively. Regarding antioxidant efficacy, irradiated MEJS with doses of 10 kGy and 12.5 kGy were much more effective, as evidenced by their lower EC_{50} values compared to the others. The enhanced antioxidant efficacy after irradiation may be attributed to the degradation of tannins present in MEJS, resulting in the release of free simple bioactive compounds like rutin, quercetin, and (+)-catechin. The effect of irradiation applied to foods may lead to a decrease in some identified phenolic compounds, while on the other hand, it could increase others depending on the applied dose. However, irradiation generally demonstrated a slight decrease in some of the identified phenolic compounds (30). This study observed a positive correlation between the increase in total flavonoids and the free radical antioxidant activity. Meanwhile, the correlation between total flavonoids and the EC_{50} value is described by the linear regression equation $y = -24.99x + 361.6$, $R^2 = 0.728$.

Conclusion

Gamma irradiation applied to MEJS at doses of up to 12.5 kGy should result in a slight decrease in the total phenolic content and tannins, simultaneously leading to a significant increase in the total flavonoid content. This increase in total flavonoids enhances free radical scavenging and reduces power activities. The improved antioxidant activity observed after irradiation can be attributed to the degradation of glycoside compounds and tannins present in MEJS, resulting in the release of free simple bioactive substances such as rutin, quercetin, and (+)-catechin, which strongly contributes to the antioxidant activities. γ-Irradiation might convert antioxidant components from glycoside form to aglycone form. Gamma irradiation can be applied to Java plum seed extract up to a dose of 12.5 kGy without significantly reducing its antioxidant activity.

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Conflict of Interest

The authors listed below certify that they have no affiliations or involvements with any organizations or entities with financial interests, such as educational grants, related to the subject matter or materials discussed in this article.

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