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ARTICLE

Gamma Irradiation Induced Enhancement in Isoflavones, Total Phenol, Anthocyanin and Antioxidant Properties of Varying Seed Coat Colored Soybean

Amit Kumar Dixit,[†] Deepti Bhatnagar,[†] Vineet Kumar,[‡] Anita Rani,[‡] J. G. Manjaya,[§] and Deepak Bhatnagar*,[†]

[†]School of Biochemistry, Devi Ahilya University, Khandwa Road, Indore - 452017, India, [‡]Directorate of Soybean Research, Khandwa Road, Indore-452017, India, and [§]Bhabha Atomic Research Center, Trombay, Mumbai, India

Three Indian soybean genotypes, namely, Kalitur, Hara soya and NRC37 with black, green and yellow colored seed coat respectively were gamma irradiated at a dose of 0.5, 2.0, and 5.0 kGy. The total isoflavones and total phenol content (TPC) in all the genotypes increased significantly at a dose of 0.5 and 2 kGy respectively. The anthocyanin content was high in Kalitur, while other genotypes showed no detectable amounts of it. The hydroxyl radical scavenging activity (HRSA), DPPH free radical scavenging activity (FRSA) and total antioxidant power (TAP) were highest in Kalitur with black seed coat color. However, maximum enhancement in antioxidant properties was found in NRC37 with yellow followed by Hara soya with green seed coat color at a dose of 0.5 and 2.0 kGy. It was also observed that the 3 soybean genotypes showed an increase in antioxidant constituents and antioxidative properties at lower doses of 0.5 and 2.0 kGy while, the antioxidant effects of soy seeds were either decreased or remained constant at a higher dose of 5.0 kGy. It is suggested that mild gamma irradiation enhanced the antioxidant constituents and, hence, antioxidant potential of soybean seeds.

KEYWORDS: Gamma irradiation; isoflavone; reducing power; DPPH; FRAP; reducing power

INTRODUCTION

Soybean is one of the most economical and nutritious foods, which may be of help to counter malnutrition and under nutrition in developing countries. Besides, it has a strong antioxidant role in minimizing the hazards and progression of various types of diseases such as cancer, diabetes, Alzheimer's disease, obesity, cardiovascular diseases, osteoporosis etc., associated with the generation of reactive oxygen species (ROS) (*1*−*4*). These health benefits of soy diet are mainly attributed to the phytochemicals and neutraceuticals present in soy food such as isoflavones, tocopherols, phytosterols, saponins and lunasin, which have been reported to be implicated in removal of ROS, chelation of metal ions and activation of antioxidant enzymes (*5*−*8*). The various adaptable food processing methods such as germination, soaking and fermentation have been shown to alter neutraceutical content in soybean foods (*9*). Gamma irradiation has been used to prevent fungal and bacterial infection of the seeds and foodstuffs. Gamma irradiation recommended for quarantine treatment of soybean has been reported to induce improvement in quality characters such as texture without production of off-flavor (*10*). It may be possible that gamma irradiation may also have some effects on the antioxidant aspects of soybean. However, this technology is not presently used to enhance the antioxidant properties in soybean. Therefore, in the present study, 3 Indian soybean genotypes with black, green and yellow seed coat color respectively were treated with gamma irradiation at 0.5, 2.0, and 5.0 kGy, in order to find the effects of irradiation on isoflavones, anthocyanin, total phenol content (TPC) and antioxidant properties such as hydroxyl radical scavenging activity (HRSA), free radical scavenging activity (FRSA) and total antioxidant power (TAP).

*Corresponding author. Tel: +91 9424072197. Fax: +91 731 2470372. E-mail: bhatnagarbio@yahoo.co.in.

MATERIALS AND METHODS

Kalitur, Hara soya and NRC37 (Ahilya 4) soybean genotypes with black, green and yellow seed coat color respectively were raised following standard agronomic practices in single row plots of 5 m long with 45 cm spacing between the rows in the experimental field of Directorate of Soybean Research (DSR), Indore, (22°N), Madhya Pradesh, India. Seeds of all three genotypes were harvested at their maturity and used for further experiments.

Gamma Irradiation. Fifty grams of seeds each of the soybean genotypes Kalitur, Hara soya and NRC37 with black, green and yellow colored seed coat respectively were exposed to doses of 0.5, 2.0, and 5.0 kGy gamma rays in a gamma cell GC 5000 with ^{60}Co source (dose rate 0.09072 kGy/min) installed at Bhabha Atomic Research Centre, Trombay, Mumbai, India. The seeds were irradiated for 5 min 51 s, 22 min 3 s, and 55 min 11 s to obtain irradiation doses of 0.5, 2.0, and 5.0 kGy, respectively. Dose rate was determined using a standard Fricke dosimetry (*11*). Calibration was done by keeping the dosimeter vials in the irradiation chamber at different positions. Packed seed samples without irradiation served as control. The treated seeds along with the parental control were used for the biochemical studies.

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Chemicals. All the chemicals and organic solvents used in this study were of analytical and HPLC grade. Acetonitrile (ACN), propyl gallate, 2-thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), daidzein, glycitein, genistein standards and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Chemical Co. St. Louis, MO.

Extraction of Antioxidants from Seeds. Irradiated seeds of different genotypes were kept in a hot air oven (50 °C) until they became moisture free as measured by moisturemeter. These dried seeds were ground using a metallic pestle and mortar and passed through 100 μm mesh sieve. Soy flour (1.0 g) was extracted with 15 mL of 70% aqueous acetone at 25 °C in the dark overnight (*12*). The mixture was centrifuged at 1000*g* for 10 min and stored at 4 °C in the dark for further analysis. The extraction and analysis were performed with four replicates.

Sample Preparation for Determination of Isoflavone Content. Finely ground soy flour (125 mg) was extracted in 80% ethanol (5 mL) and concentrated HCl (1 mL of 12 mol/L) mixture for 2 h in a boiling water bath. The method for extractions of isoflavones relies on the acid hydrolysis of 12 endogenous isoflavone isomers to their respective aglycon forms, i.e., daidzein, glycitein, and genistein (*13*). The suspension was centrifuged at 10000*g* for 10 min.

Conditions for HPLC. The supernatant obtained after centrifugation was passed through a syringe filter (Whatman 0.2 μm, 13 mm diameter) before loading into the HPLC system (Shimadzu LC10AT VP). The HPLC conditions for the quantification of isoflavones were as described by Kumar et al. (*14*). The retention time for daidzein, glycitein and genistein were 6.8, 7.4, and 10.8 min, respectively. Calibration curves were generated by injecting varying concentrations of standards of daidzein, glycitein, and genistein. The relative concentration of individual isoflavones in the sample was calculated by software CSW version 1.7 after superimposing the chromatogram of the sample on the standard curve. Individual isoflavone concentration was expressed as μg/g soy flour. Concentrations of aglycons were summed to calculate total isoflavone concentration.

Total Phenol Content of the Soy Extracts. The total phenol content of the soy extracts was determined using the Folin−Ciocalteau reagent (*15*). Briefly, a 50 μL aliquot of soy extract was added to 500 μL of Folin's reagent and 500 μL of 20% sodium carbonate. The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was read at 700 nm and the total phenols in the extracts were expressed as gallic acid equiv/g dry wt of soy flour, using a standard curve of a freshly prepared propyl gallate solution.

Determination of Anthocyanin Content. Anthocyanin quantification was performed by pH differential method of Giusti and Wrostld (*16*) with slight modification (*17*). Acetone extracts (0.1 mL) were diluted with 3.9 mL of 0.025 mol/L potassium chloride buffer (pH 1.0) and in another tube by the same volume with 0.4 mol/L sodium acetate buffer (pH 4.5). Samples were incubated for 15 min, and the absorbance of each dilution was measured at 513 and 700 nm in a spectrophotometer (Shimadzu, Japan). The corrected absorbance (A) of the diluted sample was calculated as follows:

$$A = (A_{513} - A_{700})_{\text{pH1.0}} - (A_{513} - A_{700})_{\text{pH4.5}}$$

The monomeric anthocyanin pigment concentration in the original sample was calculated as follows: (mg/mL) = ($A \times \text{MW} \times \text{DF}$)/($\varepsilon \times l$), where MW = molecular weight, i.e., 449.2, DF = dilution factor, ε = molar extinction coefficient, i.e., 26,900 for cyanidin-3-glucoside, and 1 = litre.

Inhibition of Deoxyribose Oxidation. The inhibition of deoxyribose oxidation was carried out as described by Halliwell et al. (*18*). This method was used to measure HRSA by studying the competition between deoxyribose and the test compounds for hydroxyl radical generated by Fe^{3+}−ascorbate−EDTA−H_2O_2 system (Fenton reaction). The hydroxyl radical attacked deoxyribose, and the extent of deoxyribose degradation was tested by the TBA method. The reaction mixture contained 50−200 μL of the acetone extracts containing 3.3, 6.6, 9.9, 13.2 mg of the soybean, deoxyribose (6 mmol/L), H_2O_2 (3 mmol/L), phosphate buffer (20 mmol/L, pH 7.4), $FeCl_3$ (400 μmol/L), EDTA (400 μmol/L) and ascorbic acid (400 μmol/L) in a total volume of 3 mL. The mixture was incubated at 37 °C for 1 h. One milliliter of 1% TBA and 1 mL of 2.8% trichloroacetic acid (TCA) were added to the mixture, which was then heated in a water bath at 90 °C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm. The % inhibition of deoxyribose oxidation was calculated using the following formula:

$$\text{inhibition (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance with the test samples.

Free Radical Scavenging Activity Using DPPH. The FRSA was measured using the method of Mellors and Tappel (*19*). To 3.0 mL of the ethanolic solution of DPPH (0.1 mmol/L) was added 0.1 mL of the extract. The decrease in DPPH absorption at 517 nm was measured after 10 min. BHT was taken as a positive control. The DPPH radical scavenging activity (%) was calculated using the following formula:

$$\text{radical scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 and A_1 were the absorbance of control and test sample respectively.

Total Antioxidant Power. The TAP using the ferric reducing antioxidant power (FRAP) was determined by the method of Benzie and Strain (*20*). Soy extract (0.1 mL) was mixed with 3 mL of FRAP reagent. The samples were incubated for 15 min at 37 °C, and the absorbance at 593 nm was recorded. The results were compared with a standard curve prepared with different concentrations of $FeSO_4 \cdot 7H_2O$. The change in absorbance was translated into a FRAP value in μM as follows:

$$\frac{\text{test sample OD 593 nm}}{\text{standard sample OD 593 nm}} \times \text{FRAP value standard } (\mu\text{M})$$

Statistical Analysis. All the data presented were arranged in completely randomized design, and values given were the means of 4 independent determinations with standard error. The statistical significance of the data was tested by using Tukey's test at $P < 0.05$ by SPSS evaluation version 14.

RESULTS

Isoflavone Content in Gamma Irradiated Soybean Genotypes. Kalitur genotype showed significant increase in genistein content, while daidzein and glycitein were not significantly altered at a radiation dose of 0.5 kGy as compared to control (**Figure 1a**). Glycitein in Hara soya genotype exhibited significantly ($P < 0.01$) higher increase as compared to daidzein and genistein at 0.5 kGy (Figure**1b**). Similarly, NRC37 genotype showed significant increase in genistein at a dose of 0.5 kGy (**Figure 1c**). The total isoflavone content was highest in NRC37 with yellow seed coat color followed by Kalitur and Hara soya genotypes (**Figure 1d**). Radiation at 0.5 kGy showed 12%, 38% and 17% increase in total isoflavone content of Kalitur, Hara soya and NRC37 respectively; however, the genotypes showed decrease in total isoflavone content at a higher radiation dose of 2.0 and 5.0 kGy.

Total Phenol Content. The TPC was found to be maximum in Kaliture with black seed coat color followed by NRC37 and Hara soya (**Figure 2**). Radiation at 0.5 kGy showed 17%, 14% and 3% increase in TPC of Kalitur, Hara soya and NRC37 respectively. The maximum enhancement of in TPC was found in Hara soya genotype (38%) with green seed coat followed by NRC37 (28%) and Kalitur (20%) at a radiation dose of 2.0 kGy. However, all 3 genotypes showed decrease in TPC content at a higher radiation dose of 5.0 kGy.

Anthocyanin Content in Soybean Genotypes. The anthocyanin was present in Kalitur genotype, which significantly increased at 2.0 and 5.0 kGy radiation doses. However, no detectable amount of anthocyanin was present in Hara soya and NRC37 genotypes (**Table 1**).

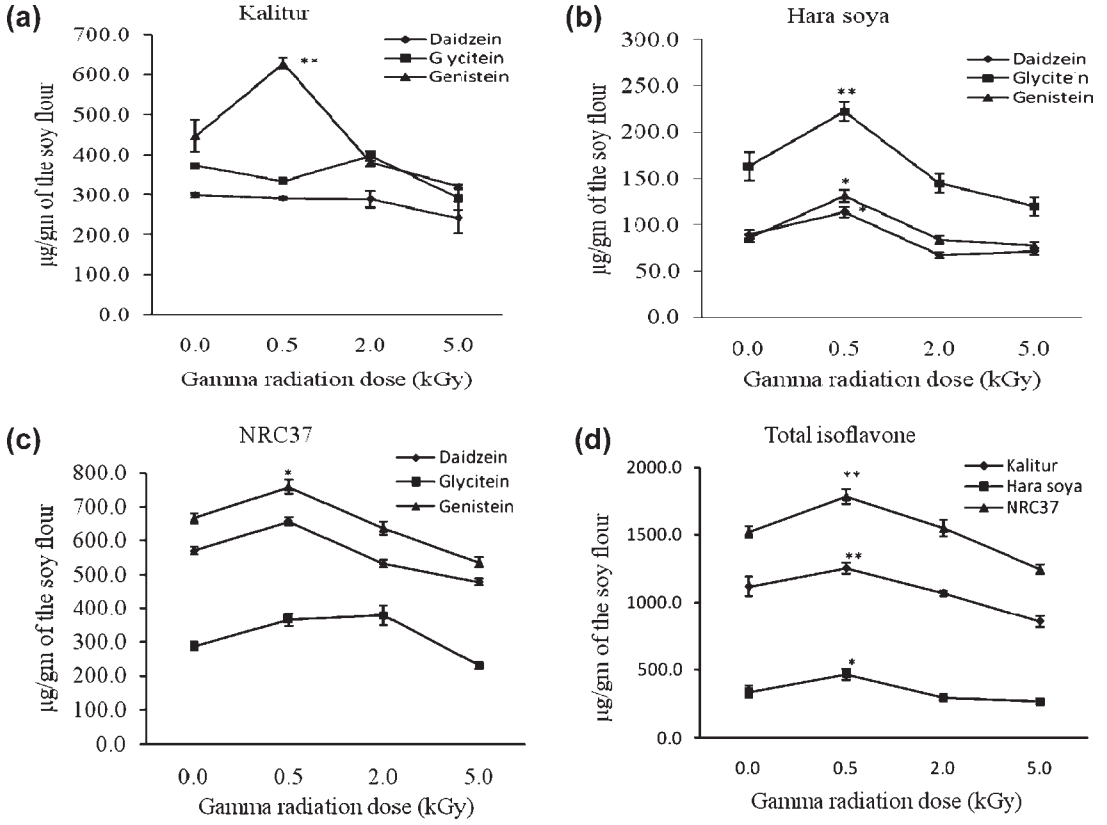

Figure 1. Isoflavone content in gamma irradiated soybean genotypes, i.e., Kalitur (**a**), Hara soya (**b**), NRC37 (**c**) and total isoflavone (**d**); *P* values as compared to control (*$P < 0.05$, **$P < 0.01$).

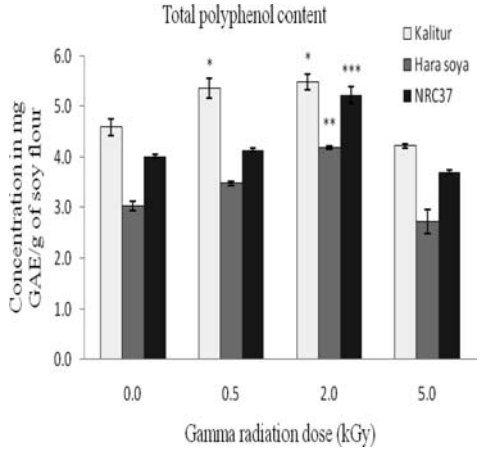

Figure 2. TPC in gamma irradiated soybean genotypes soybean. *P* values as compared to control (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Inhibition of Deoxyribose Oxidation. Increased % inhibition of deoxyribose oxidation was observed on increasing the amount of the samples at a particular dose of radiation (**Figure 3**). Kalitur, Hara soya and NRC37 showed 9%, 7% and 6% inhibition of deoxyribose with control sample, i.e., without gamma irradiation. Kalitur and Hara soya genotypes also showed nonsignificant increase in inhibition of the oxidation of deoxyribose at 0.5 kGy as compared to their unirradiated control. Maximum increase in inhibition of the oxidation of deoxyribose was observed with genotype Kalitur at a radiation dose of 2.0 kGy as compared to their unirradiated control. However, at 5.0 kGy radiation dose the inhibition of deoxyribose oxidation decreased in all 3 genotypes.

Table 1. Anthocyanin Content of Gamma Irradiated Soybean Flour[a]

radiation (kGy)	Kalitur (black)	Hara soya (green)	NRC37 (yellow)
0.0	0.81 ± 0.09	nd	nd
0.5	0.85 ± 0.07	nd	nd
2.0	1.06 ± 0.10 b	nd	nd
5.0	1.04 ± 0.03 b	nd	nd

[a] Anthocyanin content (mg/g of soy flour) in irradiated soybean was compared with control; letter b indicates $P < 0.01$; nd = not detected.

Free Radical Scavenging Activity Using DPPH. The FRSA as measured by reduction of DPPH was highest in Kalitur genotype with black seed coat color, among the 3 genotypes tested (**Table 2**). All 3 genotypes showed significant increase in FRSA at a radiation dose of 2.0 kGy; however, the maximum relative enhancement of 80% in FRSA was found in Hara soya followed by NRC37 (65%) and Kalitur (9%). The results also showed that FRSA decreased at 5.0 kGy in all 3 genotypes.

Total Antioxidant Power Using FRAP. Kalitur genotype with black seed coat color showed highest TAP as compared to Hara soya and NRC37 genotypes (**Table 2**). All 3 genotypes showed significant increase in FRAP at a radiation dose of 2.0 kGy, with maximum relative enhancement of 33% in FRAP in Kalitur followed by NRC37 (24%) and Kalitur (11%). The results also showed that FRSA decreased at 5.0 kGy in all 3 genotypes.

DISCUSSION

The radiation of soybean and other foodstuffs has been used to reduce postharvest food losses and to increase the shelf life. It has also helped to eliminate microorganisms responsible for the deterioration of foods (*10*). It was of interest to observe the effects of gamma irradiation on the antioxidant potential of Indian

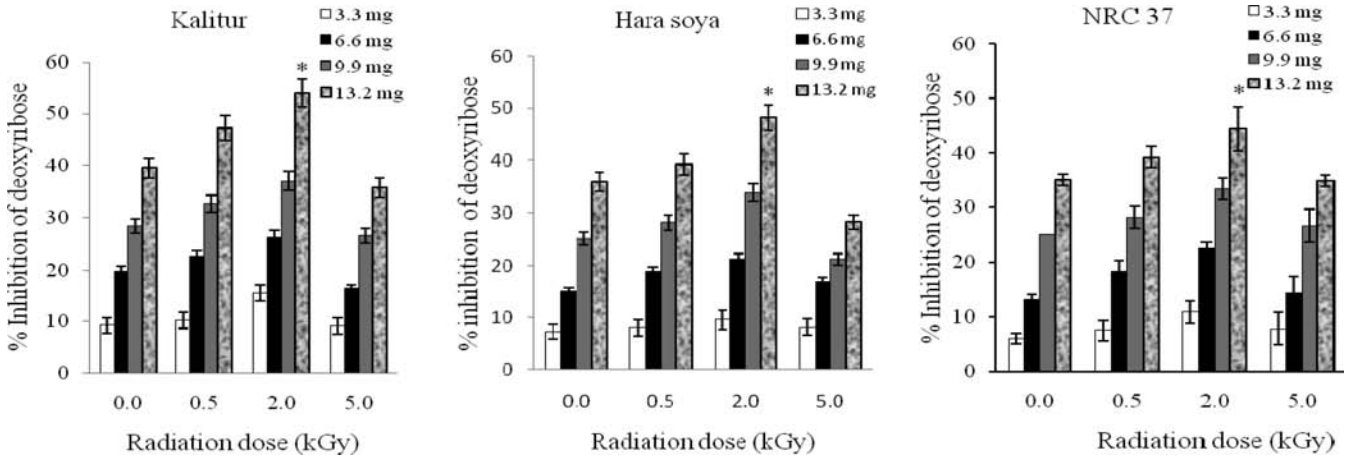

Figure 3. Inhibition of deoxyribose oxidation by gamma irradiated soybean genotypes. *P* values as compared to control (*$P < 0.05$).

Table 2. FRSA and TAP of Gamma Irradiated Soybean Genotypes[a]

	Kalitur (black)		Hara soya (green)		NRC37 (yellow)	
radiation (kGy)	FRSA[b]	TAP[c]	FRSA[b]	TAP[c]	FRSA[b]	TAP[c]
0.0	31.49 ± 1.21	51.06 ± 0.82	2.68 ± 0.13	9.19 ± 0.14	2.20 ± 0.04	7.43 ± 0.13
0.5	32.12 ± 1.12	62.33 ± 4.71 a	2.72 ± 0.04	9.40 ± 0.50	2.84 ± 0.03 a	7.84 ± 0.12
2.0	34.93 ± 0.38	67.97 ± 0.62 c	4.83 ± 0.34 c	10.67 ± 0.33 a	3.94 ± 0.12 c	9.19 ± 0.13 c
5.0	17.06 ± 0.72 c	43.96 ± 0.69	1.39 ± 0.13 b	9.13 ± 0.19	1.57 ± 0.14 c	6.67 ± 0.10 b

[a] Values in irrradiated soybean were compared with control. BHT as a positive control at 5 mM showed 55.6% DPPH radical scavenging activity; letter a indicates $P < 0.05$, b indicates $P < 0.01$, c indicates $P < 0.001$. [b] % of DPPH radical scavenging activity/mg of soy flour. [c] μmol/g of the soy flour.

soybean genotypes with black, green and yellow seed coat color. The antioxidant properties such as inhibition of deoxyribose oxidation, FRSA and TAP using FRAP were high in Kalitur as compared to Hara soya and NRC37. The hydroxyl radical scavenging activity as shown by inhibition of deoxyribose oxidation was high up to 2.0 kGy in soybean indicating that gamma irradiation increase antioxidant activity. Stajner et al. (*21*) have shown increase in antioxidant activity by FRAP method up to 1 kGy, which decreased with increasing dose of radiation up to 10 kGy. These authors have also shown increase in DPPH free radical scavenging activity, phenolic and tannin content of soybean seeds at a dose of 1 kGy.

The increased antioxidant activity of soybean genotypes at a low dose of gamma irradiation may be due to formation of free flavonoids, which have been shown to have greater antioxidant effects than glycosides. Variyar et al. (*22*) also suggested a radiation-induced breakdown of glycosides resulting in release of free isoflavones. Isoflavones are phenolic compounds, and increase in their concentration at a low dose of gamma irradiation is supported by the corresponding increase in the total phenolic content. Increased antioxidant activity of soybean genotypes at a low dose of gamma irradiation may be attributed to the increased levels of total phenolic content. Several studies have also shown an increase in phenolic compounds due to a spurt in the activities of key enzymes of the phenylpropanoid metabolic pathway at a low dose of gamma irradiation in other crop plants (*23*, *24*). Pendharkar and Nair (*23*) observed an increase in the phenolic compounds in potato tubers after exposure to gamma irradiation. Oufedjikh et al. (*24*) reported an increase in the total phenolic content due to an increase in phenylammonia lyase in Moroccan citrus fruit during storage, after exposure to a dose of 0.3 kGy. In addition, heating could also liberate low molecular weight antioxidant polyphenolic compounds, which are effective for potentiating the bioavailability of pharmacologically active natural products (*25*). Jeong et al. (*26*) also found that phenolic compounds with antioxidant activity could be released by heat treatment from citrus peels. In the present study, the energy of ionizing irradiation could bring the same effect of liberating phenolic components. Thus, in the gamma irradiated soybean genotypes, the increased antioxidant effects may be due to the release of free phenolic compounds including flavonoids. However, at a higher dose of gamma irradiation, a flux of free radicals is generated causing more deleterious effects and a decrease in antioxidant properties.

The results showed that NRC37 genotype with yellow seed coat color contains the highest amount of total isoflavone as compared to Kalitur and Hara soya with black and green seed coat, respectively. However, the DPPH and FRAP activity was low in NRC37 genotype. As isoflavones are weak antioxidants, it is possible that despite having high isoflavone content in NRC37, it may show weak FRSA and DPPH activity. On the other hand, Kalitur has moderate levels of isoflavone but has high polyphenols as well as anthocyanin content. In Kalitur with black seed coat, the anthocyanin content was high, while Hara soya and NRC37 with green and yellow seed coat respectively, showed no detectable anthocyanin content. Astadi et al. (*17*) showed high levels of phenolic and anthocyanin content and demonstrated considerable antioxidant activity in black soybean seed coat. Other authors have reported similar results showing high anthocyanin in black soybean with no detectable amounts of it in yellow genotypes (*27*). The recent studies on black soybean genotype have also reported higher total phenol, phenolic acid, anthocyanin, and carotenoid content (*28*). Free radical scavanging activity has also been shown to be higher in black genotypes as compared to yellow soybean genotype (*14*).

In conclusion, the results suggest that Kalitur with black seed coat color due to its high polyphenol and anthocyanin content showed higher HRSA, FRSA and TAP, whereas yellow and green soybean genotypes showed less of these properties. Gamma irradiation at 0.5 and 2.0 kGy increased, while a radiation dose of

5.0 kGy showed constant or reduced antioxidant properties in all 3 soybean genotypes.

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