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Comparative study of experimental approaches to increase the availability of phytochemicals using gamma radiation

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Abstract. In recent years, there has been significant interest in the reduction of health risks due to diseases caused by oxidative stress. Many plants contain compounds that mitigate such decay, and there are many studies that attempt to increase the availability of these phytochemicals using gamma radiation. This paper reviews 14 such studies, in attempt to shed light on the overarching elements of the employed experimental designs. Most studies of gamma irradiated plants evaluated the total phenolic and flavonoid content, as well as the antioxidant capacity of plant extracts, while only a few evaluated the antimicrobial activity and the vitamin or mineral content. The assays used to quantify this data were rather similar however, the dosimetry and extraction methods varied greatly, according to the plant which was being studied. Among the reviewed papers, only a few found that the irradiation of plant material decreased or had no effect on the assayed parameters. The majority of studies showed significant increases, at various doses, among the assayed data.

1. Introduction

Fruits and vegetables confer health benefits that have in part been shown to derive from their polyphenol contents, with research in the past decade being focused on flavonoids due to their biological effects. Phenolic compounds contribute to the antioxidant activities in plants, and are classified as hydroxylated compounds of cinnamic and benzoic acids [2]. Antioxidants are bioactive compounds that inhibit the harmful effects of free radicals. There are several illnesses in which antioxidants can have a mitigating role, such as pulmonary diseases, hypercholesterolemia, obesity, allergies [1].

High levels on phytochemicals such as amino acids, vitamins, antioxidants, minerals are associated with a risk reduction capability for degenerative diseases such as diabetes, cancer, hypertension, osteoporosis and cardiovascular diseases, due to the connection of these illnesses to high levels of free radicals [15].

There is a growing number of studies regarding the influence of irradiation processes on the compounds responsible for antioxidant activity. A number of research articles that used plant material as an experimental model showed that antioxidant properties were maintained or enhanced by gamma rays. This type of irradiation has been shown to up regulated the activity of the enzyme phenylalanine ammonia-lyase, responsible for the synthesis of polyphenolic acids [2].



The energetic processes of gamma radiation and oxidation are able to break down polyphenols into soluble, low molecular weight phenols, thus increasing the number of compounds with antioxidant properties. However, radiation treatment has a specific effect on phytochemicals and the level of antioxidants that will be particular to the applied dose and the sensibility of the phytochemicals in each plant type [1].

The effect of irradiation comes about by way of two mechanisms, the indirect and direct. Through the indirect mechanism, water undergoes radiolysis with the generation of hydroxyl and hydroperoxide radicals, as well as hydrated electrons.

These free radicals can break the glycosidic bonds of procyanidin polymers found in plants, with the formation of procyanidin monomers, leading to an increase in the total phenolic and flavonoid content of irradiated plants [2].

The aim of this study was to evaluate the result of different experimental approaches and to discuss the results of published research papers in the field of gamma irradiation induced changes on harvested plant material.

The plants were largely edible fruits or therapeutic herb material, with most studies focusing on changes in the phenolic and flavonoid content as well as in antioxidant activity. Some authors designed their experiments to also find the minimum dose required to sterilize the assayed material, a necessary procedure in industrial food irradiation. Only a few studies choose to assay changes in vitamin and mineral elements, changes in the antimicrobial or antitumor activity of irradiated plants.

2. Correlation between plant species and employed experimental design

The majority of evaluated experimental designs irradiated dry plant material (bark [7,8,9], leaves or branches [3,4,9,12], fruit [2,5,11,14], peels or hulls [10,13], pomace flour [1]), in some cases milled to a powder [1,4,10,13,14]. Only in two instances did the authors irradiate fresh plant material [2,6], with a high-water content, and in only one of these the plants were unrefrigerated [2].

Thus, the working hypothesis behind these designs was that gamma radiation interacts with the compounds found in the dry plant material, leading to a higher extraction rate. None of the evaluated research papers set up experiments in which living plants were subjected to gamma rays, and aimed to record changes in plant metabolites as a consequence.

All the surveyed papers focused on the impact of gamma rays on the extractability, quantity and chemical properties of compounds found in dead plants.

Extraction methods differed according to the chosen biological model (plant species and parts) and the types of assays which followed.

Table 1. Dosimetry, types of assay and extraction methods for irradiated plants.

	Dose / Intervals	Dose Rate	Irradiator Type	Biological Material	Assay Type	Extraction Method
[1]	0, 1, 2 kGy dose at times of 0, 3, 6 and 9 months	0.87 kGy/h	Gammacell Excell 220—MDS Nordion	<i>Malus pumila</i> (pomace flour)	Phenolic content and antioxidant activity	Phenolic compounds were extracted from mashed apple flour according to the methodology described by Alberti et al. (2014) 40 g of date fruits were blended for 3 min. Samples were macerated for 10 h in 120 ml methanol:water (80:20, v/v) at room temperature and filtered. The alcohol is then removed under vacuum at 40°C and evaporated until dry
[2]	0, 0.5, 1, 2.5 kGy	Unspecified	Gammacell Excell 220—MDS Nordion	<i>Phoenix dactylifera</i> (fresh fruits)	Phenolic content and antioxidant activity	

	Dose / Intervals	Dose Rate	Irradiator Type	Biological Material	Assay Type	Extraction Method
[3]	0, 1, 10 kGy	2.57 kGy/h and 1.91 kGy/h, respectively	Co-60 experimental chamber Precisa 22	<i>Ginkgo biloba</i> (dry leaves)	Phenolic content	using a rotary evaporator. Methanol/water extracts and infusions were prepared accordingly to the methodology described by Barros et al. (2013)
[4]	0, 3, 5, 7, 10 kGy	5.0 kGy/h	Multipurpose Co-60 Gamma Ray Facility	<i>Ilex paraguariensis</i> (dried leaves and branches, milled)	Phenolic content	Extracts were carried out according to a method adapted from Mazzafera (1997)
[5]	0, 0.5, 1, 1.5, 2 kGy	1.7 kGy/h	SLL 515 batch irradiator	<i>Pleurotus ostreatus</i> (dried mushrooms)	Phenolic and flavonoid content, and antioxidant activity	Mushroom extracts were prepared using a protocol by Stankovic (2011)
[6]	0, 0.5, 1 kGy	Unspecified	Multipurpose Cobalt-60 Irradiator Source	<i>Daucus carota sativus</i> (fresh, refrigerated)	Phenolic content	Phenolic compounds were extracted, according to Surjadinata and Cisneros-Zevallos (2012)
[7]	0, 5, 10, 15, 20 kGy	10.04 kGy/h	GammacellEx cell 220—MDS Nordion	<i>Spondias lutea</i> L. (dry bark)	Phenolic content	The extract was obtained from the bark of the hog plum tree by maceration for 72 hours and consecutively extracted with ethanol/water 70%. The extracts were subsequently filtered and evaporated.
[8]	0, 1, 10 kGy	2.57 kGy/h and 1.91 kGy/h, respectively	Co-60 experimental chamber Precisa 22	<i>Cochlospermum mangolensis</i> W. (dry bark)	Phenolic and flavonoid content, antioxidant activity, lipophilic and hydrophilic compounds, anti-hepatocellular carcinoma activities.	Methanol/water extracts and infusions were prepared accordingly to the methodology described by Barros et al. (2013)
[9]	0, 5, 7.5, 10 kGy	10.040 kGy/h	GammacellEx cell 220—MDS Nordion	<i>Anacardium occidentale</i> (bark and leaves)	Phenolic content, tannins, antioxidant, antimicrobial activity	A. occidentale extracts were obtained from leaves and bark, macerated for 72 h in 70% ethanol. Afterwards extracts were filtered and evaporated until dry.
[10]	0, 5, 10, 15, 25 kGy at times of 0, 30, 60 and 90 days	1.93 kGy/h	Gamma Chamber-900	<i>Punica granatum</i> L. (peel powder)	Phenolic content, antioxidant, microbiological	Solvent extraction studies were carried out using modified method as described by Chirinos et al. (2007)
[11]	0, 0.5, 1, 1.5, 2 kGy at times of 0 and 12 months	1.7 kGy/h	SLL 515 batch irradiator	<i>Pleurotus ostreatus</i> (dried mushrooms)	Vitamins and mineral elements	Extracts were obtained using a mortar and pestle, with 50 ml cold acetone. The filtered extracts were divided using 20 ml petroleum spirit in separating funnel. The solvent was dried by passing it

	Dose / Intervals	Dose Rate	Irradiator Type	Biological Material	Assay Type	Extraction Method
[12]	0, 1, 5, 10 kGy	1.7 kGy/h	Co-60 experimental chamber Precisa 22	<i>Aloysiaticrodora</i> (dry leaves)	Phenolic content, mycotoxin analysis	through anhydrous sodium sulfate placed on cotton wool. The total volume was recorded. Samples were extracted by maceration following the procedure of Pereira et al. (2015); Ochratoxin A extraction was done using the procedure of Zhao et al. (2014) For extraction of the antioxidant content, 1 g of every irradiated sample was added to 20 ml of methanol and stirred for 30 min. The yielded extracts were filtered using filter paper and kept at 4 °C.
[13]	0, 2, 6, 10 kGy	0.8 kGy/h	PX-30 Gamma Cell Facility	<i>Amygdalus communis</i> L. (hull powder)	Phenolic and flavonoid content, antioxidant activity	
[14]	0, 0.5, 1, 2.5, 5 kGy	13.068 kG/h	Gammacell Excell 220—MDS Nordion	<i>Ziziphusjuzub a var. vulgaris</i> (dried fruits, milled)	Phenolic and flavonoid content, water soluble vitamins, anthocianins, total acidity (TA), total soluble solids (TSSs)	Solvent extraction studies were performed using a modified method adapted from Chew et al. (2011) and Chirinos et al. (2007)

All of the surveyed articles used irradiators with a Cobalt 60 source, although they differed in design and function – most were experimental chamber type machines, while other were industrial multipurpose gamma ray facilities [4,6].

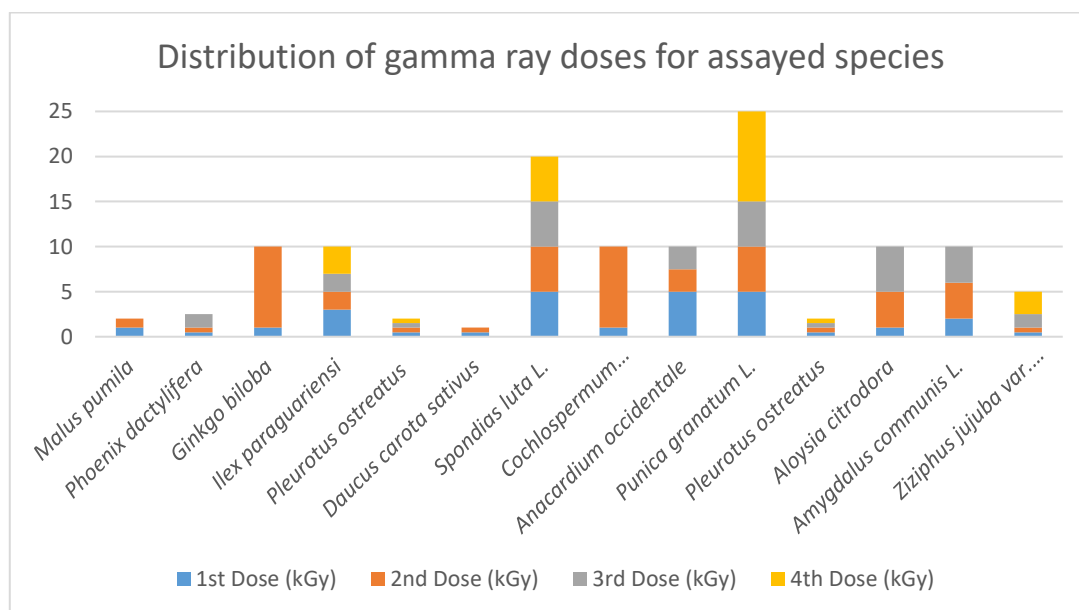


Figure 1. Dose distribution according to plant species.

All experimental designs used at least two irradiation thresholds, with some articles reporting three [2,9,12,13], and with a maximum of four levels being employed [4,5,7,10,11,14]. Dose rates ranged from 0.8 kGy/h [13] to 13.068 kGy/h [14], and the chosen doses at which to irradiate the plant material varied greatly, from 0.5 kGy [2,5,6,11,14] to the maximum dose of 25 kGy [10].

In order to understand how the gamma dose was given, a distinction must be made between chronic and acute irradiation. Chronic irradiation represents a prolonged exposure to a radioactive source, at a low dose, whereas acute irradiation occurs in a single higher dose. In the case of the surveyed experimental designs, all authors used an acute irradiation method.

Doses and dose rates varied greatly, and were given according to knowledge of the individual resistance of plant species to penetrating gamma radiation and to the different chemical compounds that they contained.

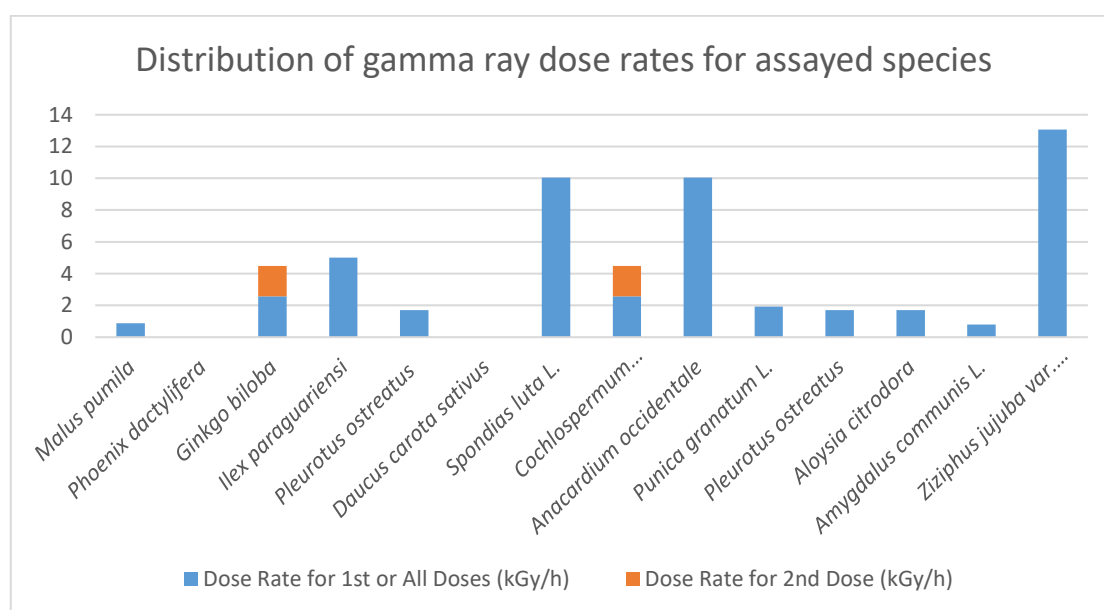


Figure 2. Dose rate distribution according to plant species.

3. Experimental data for assayed bioactive compounds from irradiated plants

Malus pumila irradiation experiments using pomace flour showed that the values for phenolic compounds and antioxidant activity (FRAP) of the samples with the 1 kGy dose at time 0 were greater ($p < 0.05$) than the samples treated with doses of 0 and 2 kGy. Also, the levels of hydroxycinnamic acids in the samples increased proportionally with time for the irradiated samples. The inhibition of DPPH for the 0 kGy sample at time 9 was reported at 30.68% while the 1 kGy dose at time 0 was 31.66% of reduction. The sample with the 1 kGy dose had the highest antioxidant potential, with the most total phenolic compounds [1].

Phoenix dactylifera irradiation experiments using fresh date palms demonstrated significant ($p < 0.01$) increases in the total phenolic compounds of irradiated as compared to control samples at the 2.5 kGy dose. Furthermore, there were no significant changes in TPC and antioxidant activity by reducing-power data at doses of 0.5 and 1 kGy compared to the control. However, a significant increase in the antioxidant activity of Mazafati dates at doses of 2.5 kGy was reported [2].

Ginkgo biloba irradiation experiments using dry leaves quantified the samples for TPC and also found that flavonoids were the main constituents, with two kaempferol derivatives being the predominant compounds found. Most abundant was kaempferol-3-O-dirhamnosylglucoside, found in all the prepared infusions and in the methanol: water extract irradiated at 1 kGy, while kaempferol-3-O-rutinoside was predominant in the control and in the sample irradiated at 10 kGy [3].

Ilex paraguariensis irradiation experiments using milled dry leaves and branches determined that there were constant levels of phenolic compounds in mate samples with increase in radiation dose. Furthermore, a slight increase was found in the quantity of phenols at the 3 and 7 kGy doses compared with the control. This increase in phenols was attributed to their release from glycosidic compounds and decay of larger phenols due to gamma radiation [4].

Pleurotus ostreatus irradiation experiments using dried mushrooms showed a significant ($p < 0,05$) increase in the phenols extracted from samples irradiated with a 0,5 kGy dose, while the 2 kGy dose recorded the least with comparison to the control. Flavonoid content was greatest for the 0,5 kGy dose and least for the 2 kGy. The DPPH scavenging activity was consistent with the total phenolic and flavonoid [5].

Daucus carota sativus irradiation experiments using refrigerated baby carrots determined that non-irradiated samples had TPC levels of 330 μg eq. gallic acid/g, while samples which received a 0,5 and 1 kGy dose had mean values of $308 \pm 8,3$ and $266 \pm 10,6$ μg eq. gallic acid/g, respectively. Thus, the 0,5 kGy dose showed a reduction of 10% compared with the control, while samples with a 1 kGy dose had a 20% loss in TPC [6].

Spondias lutea L. irradiation experiments using dry bark demonstrated that TPC of the samples showed no significant changes for doses of 5, 10, 15, and 20 kGy, with respective μEAG values of $6,25 (\pm 0,2)$; $6,70 (\pm 0,5)$; $6,25 (\pm 0,5)$; $6,85 (\pm 0,4)$ compared to the control $6,45 (\pm 0,4)$. The results showed that the employed doses of gamma radiation did not statistically influence, the bark polyphenol content percentage [7].

Cochlospermum angolensis Welw. irradiation experiments using dry bark showed that gamma irradiation did not significantly affect the nutritional value of the borututu samples. However, the highest energetic contribution, organic acids, total sugars, total tocopherols, and PUFA contents were found with the sample irradiated at 10 kGy. This same sample also had the highest TPC and TFC and the highest antioxidant activity [8].

Anacardium occidentale irradiation experiments using bark and leaves determined that the levels of total tannins and phenols in the bark samples had not been influenced statistically by the chosen irradiation doses. However, the leaf material was statistically ($p < 0,05$) influenced by radiation, with TPC between $3,13 \pm 0,04$ (0 kGy) and $3,50 \pm 0,08$ (10 kGy) and TTC between $2,47 \pm 0,06$ (0 kGy) and $2,93 \pm 0,04$ (10 kGy). The antimicrobial effects of gamma irradiated bark and leaves was also increased [9].

Punica granatum L. irradiation experiments using peel powder determined that at day 0 there was a significant increase in TPC and antioxidant activity of the samples irradiated at 10 kGy with regard to the control ($16,80 \pm 0,15$ g GE/100 g DW and $16,10 \pm 0,21$ g GE/100 g DW respectively). At day 60 after irradiation, there was a statistically significant increase ($p < 0,05$) in TPC and TEAC values of the samples, however at day 90 there was a decrease in both. The 5 kGy and above doses were shown to completely remove the microbial load [10].

Pleurotus ostreatus irradiation experiments using dried mushrooms found that vitamin A and C concentration immediately post irradiation in the samples were similar to those after 12 months of storage. It was determined that low gamma doses had significant ($p < 0,05$) effect on vitamin A and ascorbic acid content. Storage time and gamma radiation had no significant ($p > 0,05$) effect on the vitamin D content of the mushrooms. The TTS did not change after 1 year of storage. Sodium and magnesium content showed significant differences ($p < 0,05$) with increasing dosages [11].

Aloysia citrodora irradiation experiments using dry leaves showed that among the phenolic compounds found in the samples, three out of eleven identified phenols displayed a significant increase in content ($p < 0,05$). Five of the remaining eight showed a decrease, with two compounds showing a significant decrease ($p < 0,05$). However, at the highest dose of 10 kGy only one compound significantly increased while four others decreased [12].

Amygdalus communis L. experiments using hull powder compared irradiated samples stored for five years to freshly irradiate bones. The effect of gamma irradiation lowered ($p < 0,05$) the total phenolic content of the fresh extracts compared with the control. Stored samples showed an increase in

TPC by about 21% at a dose of 10 kGy in regard to the control, however the 2 and 6 kGy doses moderately decreased the TPC of the stored extracts. The TFC value of the fresh samples was decreased by 41% at the 2 kGy dose, but the 6 and 10 kGy doses decreased the TFC by 16% compared to the control [13].

Ziziphus jujuba var. *vulgaris* experiments using dried and milled fruits demonstrated that the TSS values of irradiated jujube samples remained unaffected at the studied doses, and that the level of total acidity had a significant ($p \leq 0,05$) increase at doses higher than 2,5 kGy. Doses up to 2,5 kGy showed a significant increase in the TPC and total monomeric anthocyanin content, but both values decreased after the 5 kGy dose. The results indicate that doses below 2,5 kGy can be used to improve the availability of phytochemicals in the jujube fruit [14].

4. Experimental design for chronic irradiation using a multipurpose Co-60 gamma ray facility

In order to study the effect of ionizing radiation, produced by a high intensity gamma ray Co⁶⁰ source, on plant metabolites or on the microbiological footprint of different materials, we present an experimental design suitable for an industrial irradiation facility, but not for a gamma chamber type device, with which to screen through a large number of doses for a correlation with a desired effect.

In pursuit of the optimum amount of radiation (that stimulates the production of a given metabolite, has a certain effect on chemical compounds that occur in plants, reduces microorganism count to a desired degree), samples can be exposed in a field of increasing radiation intensity and thus receive different doses.

In this design, plant metabolites or microorganisms are then quantified using different methods, either biochemical - according to the nature of the targeted compound, or microbiological. Once determined, the optimum dose can then be given in a single exposure, enabling quick transfer of the technological procedure to industrial applications.

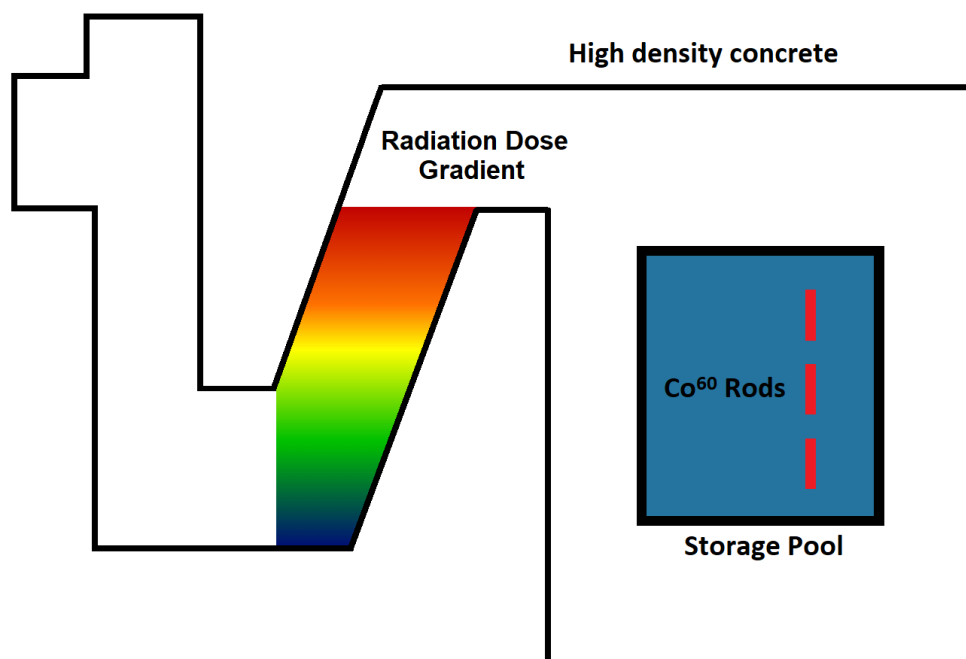


Figure 3. Layout of a typical Co⁶⁰ multipurpose irradiator, with a radiation dose gradient.

5. Conclusions

The overall experimental approach of the evaluated studies relied on a Co⁶⁰ gamma ray source, with most experimental designs using a gamma chamber type machine, to irradiate at different doses

various plant material. These doses were very different, and related to the particular physiology of the chosen plant part and to the degree of processing that had previously taken place.

Most studies of gamma irradiated plants evaluated the total phenolic and flavonoid content, as well as the antioxidant capacity of plant extracts, while only a few evaluated the antimicrobial activity and the vitamin or mineral content. The assays used to quantify this data were rather similar however, the dosimetry and extraction methods varied greatly, according to the plant which was being studied. Among the reviewed papers, only a few found that the irradiation of plant material decreased or had no effect on the assayed parameters. The majority of studies showed significant increases, at various doses, among the assayed data.

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