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Radiation Treatment Enhanced the Free Form of Phenolic Acid and Flavonoids in Leafy Greens and therefore Bioactivity in Terms of Antimutagenicity

H. D. Khade[1, 2], Sachin N. Hajare[1] and Satyendra Gautam*[1, 2]
[1]Food Technology Division, Bhabha Atomic Research Centre, Mumbai-400085, India.
[2]Homi Bhabha National Institute, Anushakti Nagar, Mumbai - 400094 India.

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***Corresponding Author Email:** sgautam@barc.gov.in

Abstract

In the current study, radiation treatment (gamma as well as electron beam of 2 kGy) was used to hygienize leafy greens spinach (cv. Semi-Savoy), mint (cv. menthol mint) and coriander (cv. Co-2), which were rich in microbial load including presumptive *Salmonella* and coliforms. Effect of radiation treatment on major phenolics such as kaempferol (spinach), quercetin (coriander) and rosmarinic acid (mint) contents was studied as phenolics are known to possess functional properties through TLC and HPLC analyses. Kaempferol, quercetin and rosmarinic acid (Gallic Acid Equivalent) content in fresh spinach, coriander and mint leaves was found to be 538 µg/100g, 14 mg/100g, and 59 mg/100g (fresh weight) respectively. There was significant increase ($p \leq 0.05$) in their contents after irradiation which further enhanced during storage at 4-6 ^{o}C for 15 days. Among the radiation sources, electron beam was found to be more effective than gamma rays presumably due to higher dose rate. Thus, radiation treatment besides helping in achieving hygienization, does value addition to leafy greens in terms of its functionality, which was validated in terms of antimutagenicity potential in *E. coli* MG 1655 (wild type) cells using rifampicin resistance forward mutation detection assay.

Practical Application

The radiation treatment was found to enhance phenolic content of the leafy vegetables. Phenolics are known to possess functional attributes such as antimutagenic activity. Thus, the treatment is found to play dual role i.e. hygienization of the leafy vegetables as well as improvement in the functional property.

Short title: Radiation Processing of Leafy Greens led to Nutritional Enrichment

Keywords

Electron beam /Gamma; kaempferol; quercetin; rosmarinic acid; leafy greens.

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1. INTRDUCTION

Leafy greens such as spinach, coriander and mint are known to have microbial load as reported in our earlier study (unpublished data) as well as from other laboratories worldwide [1]. To address the concern, radiation treatment was optimized where 2 kGy of gamma as well as electron beam radiation (Food Safety and Standard Authority of India (FSSAI) approved dose) were found to reduce the microbial load to safe limits and also the presumptive coliforms to below detectable level. Coliforms are often used as an indicator of pathogenic contamination [2]. Moreover, the shelf life of these commodities was extended up to 15 days at chilled temperature of 4-6 ^{o}C (unpublished data).

Fresh leafy vegetables are known to be the rich source of vitamins, minerals and phenolics having antioxidant, anti-mutagenic and immunomodulatory properties [3]. These phyto-chemicals present in vegetables which help to improve health by preventing free radical formation [4]. Radiation processing has been reported to impart benefits such as food hygeinization and preservation, still its uses are less explored at commercial scale [5].There is always a concern in consumer's mind when such products are subjected to radiation processing for hygienization, how these phytoconstituents get affected and therefore their bioactivities [6].

Present study evaluate significant effect of radiation processing (both gamma and electron beam) on the major phenolic/ flavonoids present in these vegetables namely kaempferol (in spinach), quercetin (in coriander) [7] and rosmarinic acid (in mint) [8], in terms of their amount and analysis of their antimutagenic potential [9].

2. MATERIAL AND METHODS

2.1 Chemicals

The standard chemicals like quercetin, kaempferol and gallic acid were procured from Sigma Chemical Co. and the HPLC-grade solvents such as methanol (HPLC grade), acetic acid was from Merck. Milli-Q water (Millipore) were used for preparation of samples as well as HPLC solvents.

2.2 Procurement, minimal processing and radiation processing of leafy greens

Experimental samples for the present study were obtained from Nashik region of Maharashtra state namely coriander (cv. 'Co-2'), spinach (cv. 'Semi-Savoy') and mint (cv. 'menthol mint'). Roots of the plants were cut, and the shoots were thoroughly cleaned first with potable water for 5 minutes, and then treated with sodium hypochlorite (200 ppm) for the same time (5 min) for sanitation. Later these samples were air dried for 30 min and were packed (150 g) in tray with wet bed and covered with PVC film.

Gamma irradiation at the dose of 2 kGy (D_{min}) was carried out at the institute (Dose rate, 26 Gy/min) and the proper dose delivery was ascertained by Cerric – Cerous dosimetry system as per the standard protocol [10]. Ratio of dose uniformity was 1.4. At electron beam facility radiation processing was carried out (D_{min} 2 kGy), (max dose rate 10kGy per sec). BF3 (boron trifluoride) dosimeters were used to ensure 2 kGy dose delivery [11].

2.3 Extraction of flavonoids from spinach and coriander

For extraction of flavonoids 40 g sample of spinach and coriander leaves were finely grounded in liquid nitrogen. Extraction of the total powder obtained was carried out in 80 % methanol (100ml) for three times and supernatant was collected after centrifuging at 10000 g for 10 minutes.

Supernatant was concentrated in a flash evaporator (BUCHI Make) till all the traces of methanol were removed. The aqueous portion was then extracted with hexane (100 ml) three times. Subsequently, aqueous fraction was further extracted with ethyl acetate (100 ml) twice. Ethyl acetate phases were pooled together, washed thrice with water (100 ml) and the aqueous phase was treated with 1 % HCl and incubated at 70 ^{o}C for 1h to hydrolyze the glycoside bound flavonoids. Flavonoids extracted in ethyl acetate were concentrated in methanol to make 1 % solution and used for detection and quantification.

2.4 Detection and quantification of flavonoids by TLC followed by High Pressure Liquid Chromatography

2.4.1 Standard

10 mg kaempferol, quercetin and gallic acid were weighted accurately and dissolved in methanol (1 ml) in to separate volumetric flask. Working standard solutions (10 to 100 µg/ml) prepared by using original stock of standard, methanol was used as diluents. All solutions were stored at 4 °C till analysis which was performed within 2 days of sample preparation.

2.4.2 Identification of flavonoids by TLC analysis

Commercially prepared TLC plates (Merck Life Sciences Pvt. Ltd., Mumbai) were used for analysis of flavonoids. Samples (10 µl) were spotted on to the TLC plate (20 cm X 20 cm) and the spots were air dried. The TLC was developed in a solvent system consisting of toluene: ethyl formate: formic acid, (in the ratio 5:4:1) and spots were visualized by exposing the TLC plates to iodine. Standard of quercetin (2µg)

and kaempferol (2µg) were spotted on plate to compare and identify the flavonoids in samples.

2.4.3 Quantification of flavonoids (quercetin and kaempferol) using HPLC

HPLC (DIONEX make) equipped with C18 analytical column and a double-wavelength UV detector set at 350 nm was used in this study. Before the analysis, the HPLC column was pre-treated with HPLC grade methanol. 2% acetic acid as solvent-A and absolute methanol solvent –B was used as mobile phase. Flow rate was maintained 1.2ml/min and gradient 0-100 % B in 30 min [12]. Standard curves were constructed using quercetin (0.01 to 0.05 mg/ml) and kaempferol (0.001 to 0.003 mg/ml) and the concentration of the quercetin and kaempferol in vegetable samples was calculated accordingly.

2.4.4 Extraction of phenolic acid

About 0.5 g of fresh mint (after minimal processing as detailed above) sample was finely ground using liquid nitrogen and extracted in 80 % methanol for 10 min. Later, the suspension was centrifuged at 10000 rpm 10 min at 20 ^{o}C. The supernatant filtered through Whatman No. 1 paper and concentrated filtrate was used for HPLC analysis.

2.4.5 Phenolic acid analysis by HPLC

HPLC (DIONEX make) equipped with C18 analytical column and a double-wavelength UV detector set at 330 nm was used in this study. Before the analysis, the HPLC column was pre-treated with methanol (HPLC grade). Solvent A was 0.1 % Orthophosphoric acid in water, solvent B was 0.1 % Orthophosphoric acid in methanol (v/v). Gradient flow was 0 - 10 min, gradient from 40 to 50 % B; 10-15 min, gradient from 50–60 % B; 60 % B was maintained for 25 min and 1 ml/min was flow rate. Flow rate was set at 1 ml/min. The chromatographic peaks of phenolic acid were confirmed by comparing their retention time in the above said solvent system as reported earlier [13]. Standard curves were constructed using gallic acid in the concentration range of 0.005 to 0.020 mg/ml and the phenolic content in these samples was expressed as equivalent to gallic acid.

2.5 Antimutagenic activity of spinach and coriander extract

Antimutagenicity assay was performed by method mentioned by [14]. Overnight grown culture of *E. coli* (MG1655) was diluted in 25 ml Luria broth and incubated at 37 ^{o}C at 150 rpm for 3h. (mid log phase). Later, the culture (*E. coli* (Mg1655) was kept on ice up to 15 min and centrifuged (7,000 X g for 15 min). Pellet washed in 10 ml luria broth (two times) and cells were resuspended in luria broth (25 ml). The cells were then incubated separately with spinach and coriander extract (1%) for 15 min. An aliquot (2 ml) of cell suspension was incubated at 37^{o}C on a rotary shaker (150 rpm) for 45 min with EMS (133 mM). This concentration of EMS has been optimized as an effective concentration for the assay in earlier studies [15].

In control sample, vegetable extract was replaced with sterile distilled water. EMS is a directly acting DNA alkylating agent mutagen and thus induces mutagenesis [16]. After EMS treatment cells were centrifuged and washed with Luria broth (2 ml) twice and suspended in 2 ml of Luria broth. From this 50 µl cells inoculated in fresh Luria broth and further incubated overnight at 37 ^{o}C on shaker (150 rpm). Next day proper dilution of culture was spread plated on Luria agar plates containing rifampicin (100 µg/ml) and also plain Luria agar plates and incubated at 37 ^{o}C for 24 h. Total viable count (TVC) was calculated from Luria agar plates while Rif^{R} mutants were estimated by taking the count from Luria agar plates with rifampicin. Mutation frequency was calculated by the ratio of number of Rif^{R} mutants per millilitre to the Total Viable Cells (TVC) per millilitre of culture. Cell suspension incubated without mutagen gave spontaneous mutation frequency.

2.6 Statistic analysis

Altogether three independent sets of experiments were performed, and each set was performed in triplicate. The data was analysed using the Origin software version v7.5714 (B714, OriginLab., Northhampton, MA), and means and standard deviations were calculated by taking all the data points in the study.

3. RESULTS AND DISCUSSION

Leafy vegetables are rich source of health protective phytoconstituents including phenolics and flavanoids [17]. Along with their nutritive value, they are equally vulnerable to microbial contaminations including pathogens primarily from the soil and contaminated irrigation water. Microbiological quality as well as shelf life of leafy greens such as spinach, coriander and mint has been increased by sanitizing the leafy greens with approved sanitizers followed by air drying, packing and radiation processing (unpublished data). Many a time's consumers are concerned about effect of radiation treatment on nutritional content of food. These leafy greens are the not primary source of nutrients for calorific gain else they have been reported to be rich in phytoconstituents such as phenolics and flavonoids known to have health protective potential [18].

In one study Nuutila et al. (2002), worked on spinach and found that kaempferol content in the range of 4.4 g/kg and 0.5 g/kg in dry weight and fresh weight,

respectively [19]. Effect of radiation processing on the phenolics and flavonoid content in these vegetables has been studied by many researchers. Pinela et al. (2016), have shown that 5 kGy dose preserved the antioxidant activity, total flavonoids, MUFA, tocopherols and phenolics in *Tuberaria lignosa* sample [20]. Edimecheva et al. (2005), have shown that O-glycoside bond was cleaved due to radiolysis in solution of lactose, maltose and cellobiose [21]. Therefore, in this study too major phytoconstituents of these leafy vegetables were evaluated in radiation treated and stored samples and compared with fresh control samples.

3.1 Kaempferol content in spinach enhanced upon radiation treatment

The major flavonoid in spinach was found to be kaempferol as confirmed by TLC analysis (Table 1, Fig. 1A and 1B and Fig. 2). In fresh control sample of spinach leaves kaempferol content was found to be 538 µg/100 g fresh weights. Sample irradiated with gamma and electron beam (2 kGy) resulted in enhanced kaempferol content up to 881 and 950 µg/100g fresh weight respectively on day one (Fig. 3A-D). Further upon storage at 4 °C for 15 days kaempferol content was found to be enhanced up to 1072 µg/100g fresh weight in gamma and 1204 µg/100g fresh weight in EB treated samples (Table 1). Thus, the results showed that after radiation processing and storage at above conditions there was increase in kaempferol content of spinach leaves.

Table1.Content of major phytochemicals in leafy greens as analyzed by HPLC

Sr. No	Sample type	Control (Day 1)	Gamma Radiation Treated Sample (Day 1)	Electron Beam Treated Sample (Day 1)	Gamma Radiation Treated Sample (Day 15)	Electron Beam Treated Sample (Day 15)
1	**Kaempferol content in spinach (µg/100g)**	538±7.21^{a}	881±9.53^{b}	950±10.06^{c}	1072±13.11^{d}	1204±11.55^{e}
2	**Quercetin content in coriander leaves (mg/100g)**	13.85±0.97^{f}	17.42±0.47^{g}	18.67±0.57^{h}	20.14±0.35^{i}	22.45±0.54^{j}
3	**Rosmarinic acid conc. in mint leaves GAE mg/100g**	59±2.2^{k}	77±4.3^{l}	127±5.4^{m}	175±10.6^{n}	184±12.3^{lo}

Different letters across rows indicate significant difference [p ≤0.05] within sample means as analysed by ANOVA (one-way).

Other than radiation, intense pulsed light (IPL) is also being used for surface decontamination. This treatment was shown to enhance total polyphenolic content of spinach in the range of 5–10% treated with IPL 20 kJ/m^2 and 32–34% were treated with 40 kJ/m^2 [22]. Study carried out by other researchers have shown that, in fresh spinach leaves polyphenols were 270 mg / kg tannic acid equivalent and 390 mg / kg as catechin equivalents, and in these kaempferol was 30 mg/ kg [23]. Earlier studies Hussain et al. (2016), have shown that upon irradiation ferric reducing power of spinach increased from 4.1% to 42.8% and OH* scavenging increased from 1.5% to 2.4% [24]. Thus, enhancement of kaempferol content in spinach is significant. Antimutagenic potential varied from vegetable varieties and no correlation found with antioxidant activity [25]. The role of kaempferol in health protection has been studied by many researchers. They have shown that kaempferol is present in wide varieties of plant species and possesses significant anti-inflammatory properties. Kaempferol acts as both a chemo-preventive and chemotherapeutic agent. It also acts to prevent various disorders especially neoplastic induction. Kashyap, D. et al. (2017), have shown that kaempferol acts on a wide range of extracellular and intracellular targets involved in the pathway of cell signalling [26]. It regulates the cancer growth progression processes like apoptosis, cell division and metastasis.

3.2 Quercetin content in coriander too enhanced upon radiation treatment

Quercetin was found to be a major phenolic compound in coriander as analyzed by TLC. Quercetin content in fresh control sample of coriander was found to be 13.85 mg/100g. Samples irradiated with gamma and electron beam (2 kGy) resulted in enhanced quercetin content up to 17.42 mg/100 g and 18.67 mg/100 g, respectively on day one (Fig. 1A and 1B; Fig. 2; Fig. 4A-D). Further quercetin content was found to be increased to20.14 mg/100 g and 22.45 mg/100 g in gamma and EB

treated samples, respectively, which were stored at 4 °C for 15 days (Fig. 4A-D). It is further observed that, EB treated sample showed higher content of quercetin upon irradiation and storage compared to gamma treated samples. This may be attributed to the higher dose rate of EB, resulting in production of free flavonoids from bound ones. Nambiar et al. (2010), have confirmed by chromatography that, coriander leaves possess quercetin and kaempferol as major flavonoids. In earlier reports too, irradiation of coriander resulted in microbial decontamination and improvement in keeping quality at 8–10 °C [27, 28]. Here author too found enhancement in extractability of carotenes and chlorophylls due to irradiation. Quercetin has been reported as a major flavonoid in vegetables like *Celosia argentea, Alternanther asessilis* and *Cardiospermum helicacabum* and in the same study authors have shown the presence of total phenolics in the range of 3.89 to 8.55 mg Gallic Acid Equivalent (GAE)/g (dry weight), and flavonoid in the range of 9.0 to 38 mg/g (dry weight) quercetin equivalent [29]. As per USDA database (2007) quercetin content in fresh coriander leaves has been reported to be 52.9 mg/100 g (fresh weight) [30]. It indicates that there is significant variety-based variation in the phytoconstituents of leafy greens [31]. The effect of radiation on the content of quercetin in onion (*Allium cepa* L.) has been reported earlier. Radiation dose of ~10 kGy enhanced the yield of quercetin from 36 to 153 µg /ml. It was also shown that optimum gamma irradiation dose of 10 kGy is sufficient to release soluble phenols by breaking the physical and chemical bonds [32]. Here onion perhaps had been used as a model system to study the effect of high dose irradiation. However, the gamma radiation dose required for sprout inhibition of onion and potato is less than 1 kGy (FSSAI, USFDA). Study carried out by Patel et al., has shown that, quercetin possesses pharmacological role in cardiovascular disease, such as ischemia-reperfusion injury, cardio toxicity, endothelial cell dysfunction, heart failure and hypertension [33]. Beside it has also been found to be relevant in case of oxidative stress and atherosclerosis. In systemic circulation, presence of quercetin has significance due to this health protecting property [34].

Fig.1. TLC analysis of flavonoids extracted from coriander, A (Un-hydrolysed), B (Hydrolysed): spot 1 (10 µl), spot 2 (30 µl), and spot 3 (Standrad quercetin 10 µl).

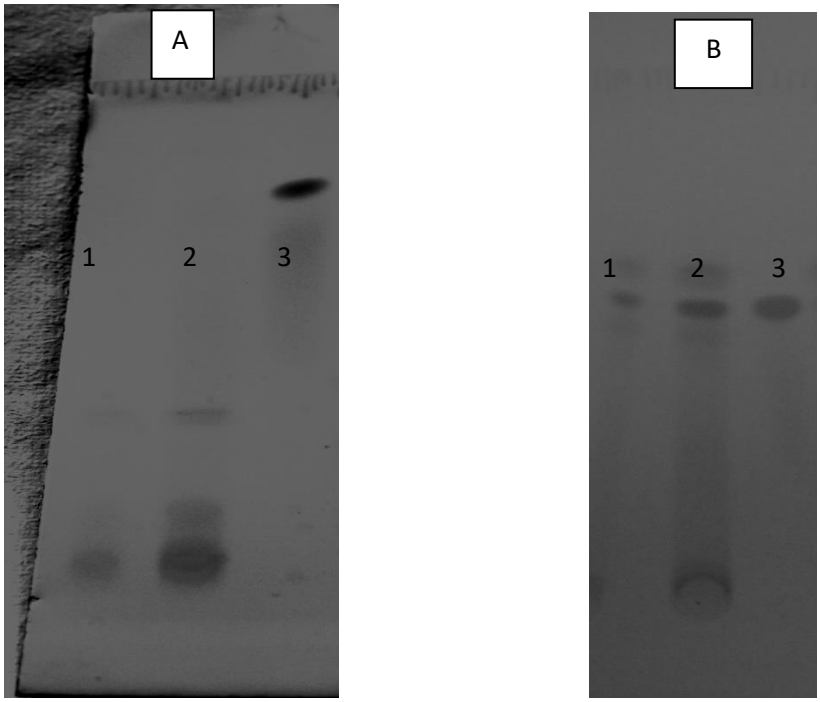


Fig.2. TLC analysis of flavonoids extracted from radiation treated coriander and spinach with respective controls and standard: spot 1: Standard quercetin; spot 2. Standard kaempferol; spot 3. coriander control sample; spot 4. coriander 2 kGy gamma radiation treated acid and hydrolysed sample; spot 5. coriander 2 kGy EB treated and acid hydrolysed sample; spot 6. spinach control sample; spot 7. spinach 2 kGy gamma treated and acid hydrolysed sample; spot 8. spinach 2 kGy EB treated and acid hydrolysed sample; spot 9. spinach unhydrolyzed control sample; spot 10. spinach unhydrolyzed 2kGy gamma treated sample; 11. spinach unhydrolyzed 2 kGy EB treated sample.

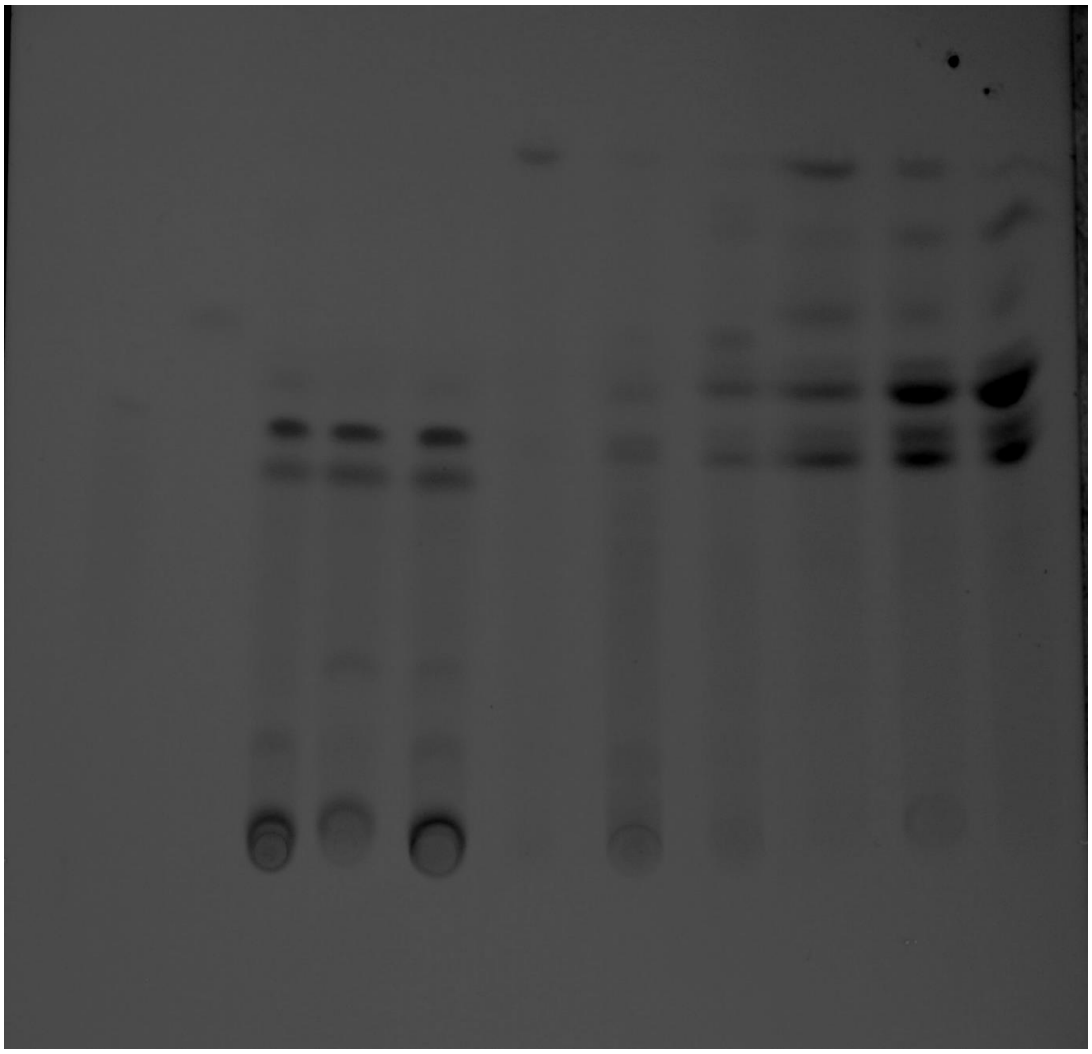


Fig.3. HPLC analysis of flavonoids extracted from spinach, A: Kaempferol, B: spinach control acid hydrolysed sample; C: spinach 2 kGy gamma treated acid hydrolysed sample; D: spinach 2 kGy EB treated acid hydrolysed sample.

Fig. 3A

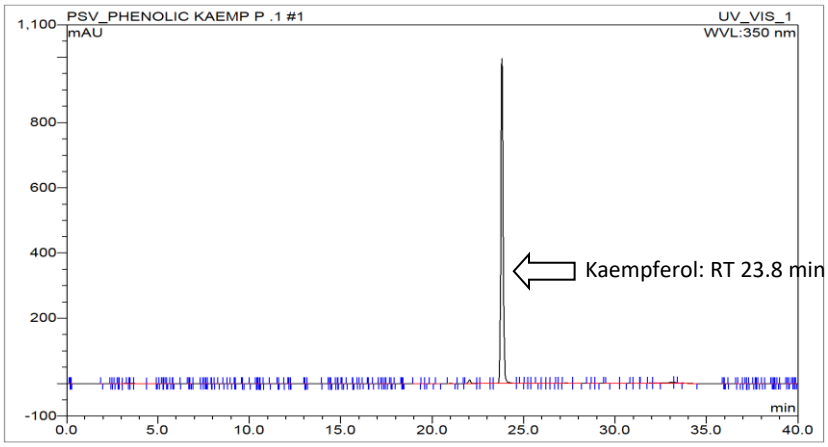


Fig. 3B

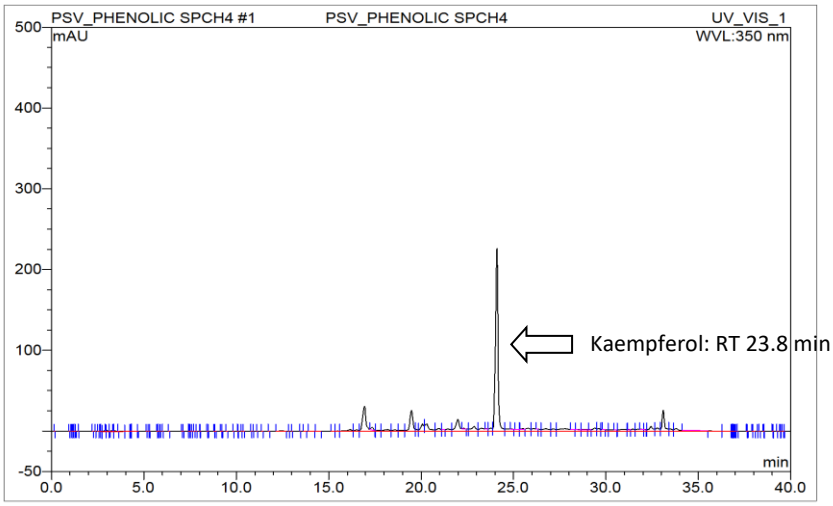


Fig. 3C.

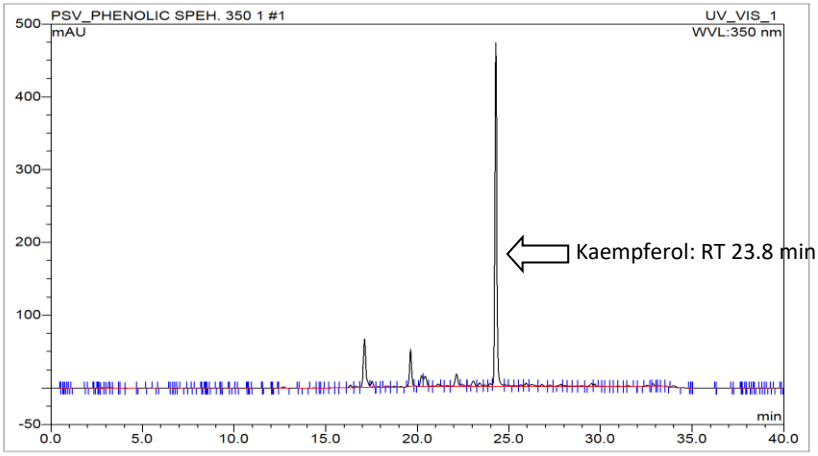


Fig. 3D.

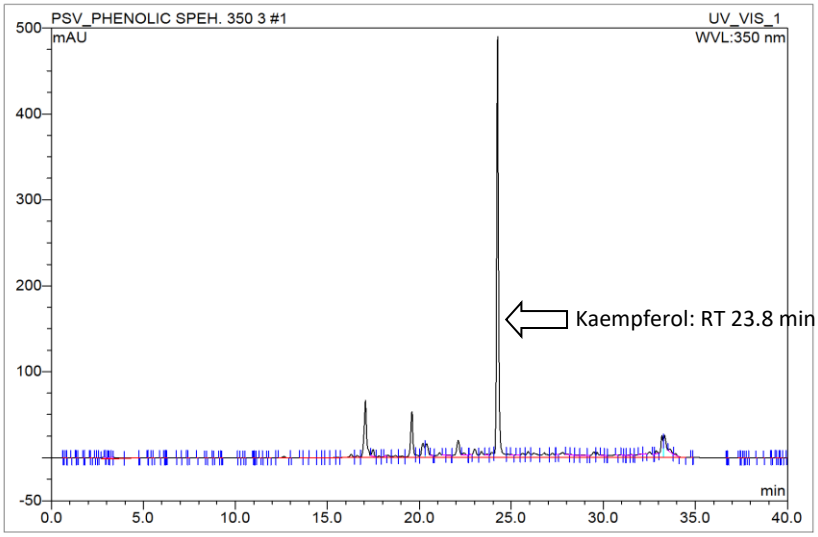


Fig.4. HPLC analysis of flavonoids extracted from coriander, A: Quericitin, B: coriander control acid hydrolysed sample; C: coriander 2 kGy gamma treated acid hydrolysed sample; D: coriander 2 kGy EB treated acid hydrolysed sample.

Fig. 4A

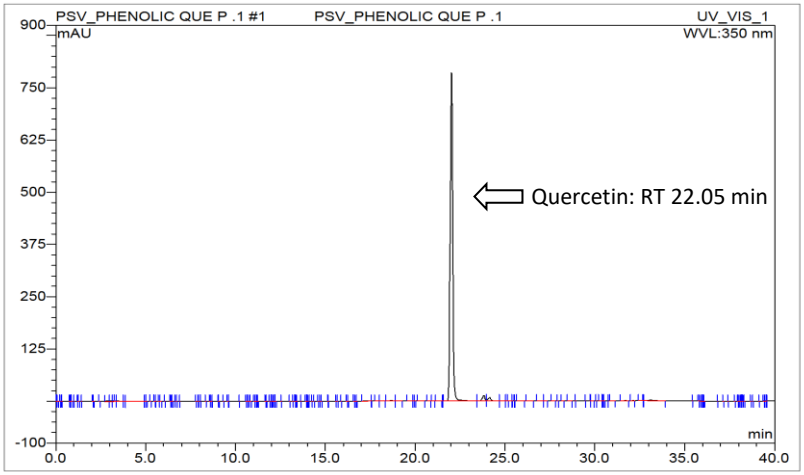


Fig. 4B

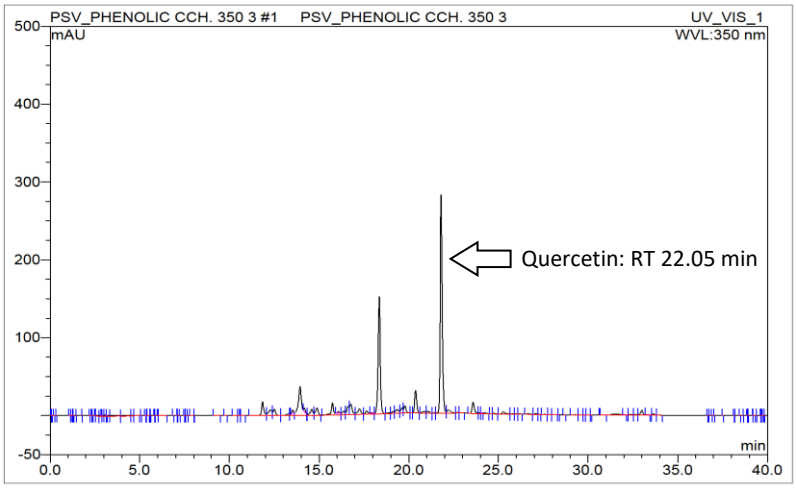


Fig. 4C

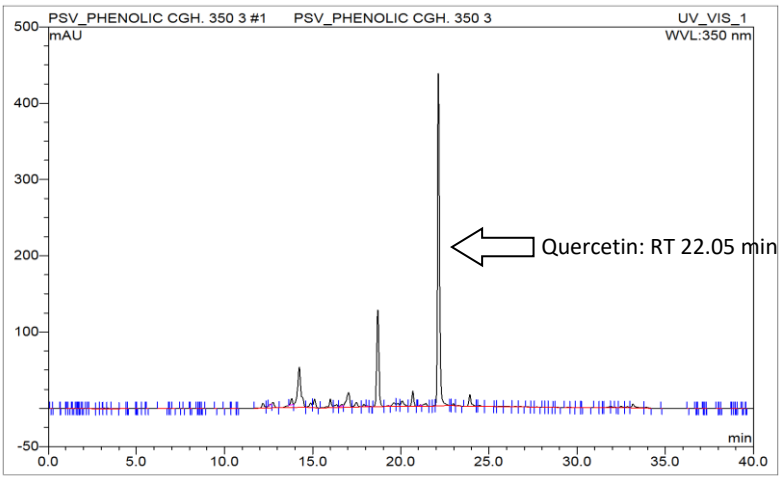


Fig. 4D

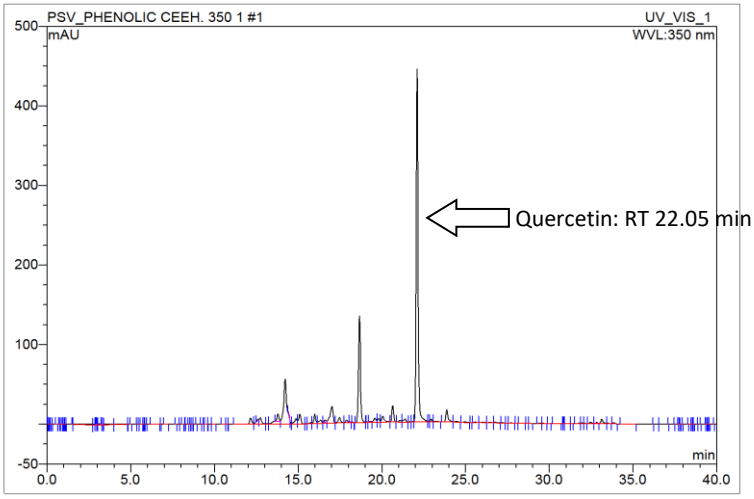


Fig.5. Rif^R Mutation frequency: A. Coriander and B. Spinach. Different letters across bar indicate that the means for control and irradiated samples were significantly different (P ≤ 0.05) when analysed with ANOVA (one-way).

Fig. 5A

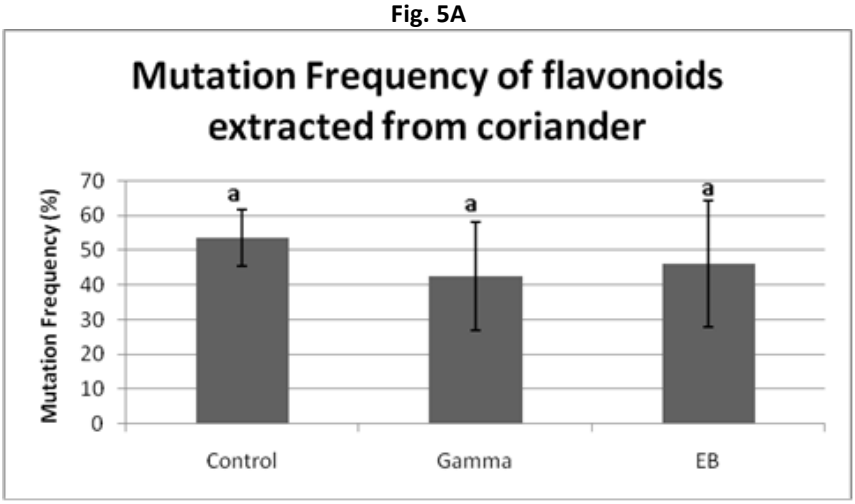
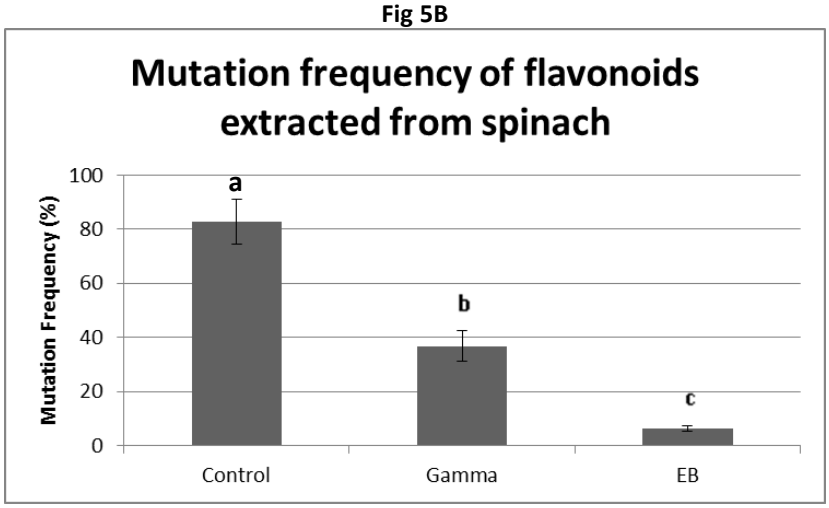


Fig 5B



3.3 Rosmarinic acid content in mint increased upon irradiation

Rosmarinic acid is found to be the major phenolic and present in mint (*Mentha piperitae)*. Rosmarinic acid content in fresh control sample of mint was found to be 59 mg/100 g (fresh weight). Samples irradiated with gamma and electron beam (2 kGy) resulted in enhanced rosmarinic acid content up to 77 mg per 100g of fresh weight in gamma irradiated samples and 175 mg per 100 g of fresh weight in electron beam treated samples on day one. Rosmarinic acid content in 15 days stored (4 ^{o}C) samples was found to be 127 mg/100g (fresh weight) in gamma and 184 mg/100 g (fresh weight) in EB treated samples. In earlier studies Trigo et al. (2009), showed that 0.5 kGy of gamma radiation could reduce bio-burdan while maintaining organoleptic attributes and functional qualities [35]. Thus, evidences support the concept that radiation can hygienize the leafy vegetables as well as enhance the health protective property by increasing the phytoconstituent's content in many cases. Mirondo & Barringer (2016) too showed rosmarinic acid as a major phenolic in fresh mint leaves [36]. Content of rosmarinic acid was also studied by Daniella et al. (2015), they reported the rosmarinic acid content in herbs like *Rosmarinus officinalis L.* and *Origanum vulgare L.* as 1.33 mg/g and 124 mg/g (dry weight), respectively [37]. Study carried out by Milica et al., (2015) has shown that *Salvia verbenaca* L. contains ~94 µg/mg of rosmarinic acid [38]. In another study, hydrolysis of crude mint extract resulted in increase in free rosmarinic acid from 12 mg/g to 17 mg/g (air dried leaves) in *Mentha piperitae* [39]. Study has shown that in leafy vegetable like iceberg lettuce gamma irradiation in the range of 0.5 to 2 kGy, and storage period up to 8 days at 8 ^{o}C resulted in enhanced phenolic content and antioxidant capacity [40]. Phenolics have been found to have significant role in enzyme inhibitory activity which was responsible for control of skin disorders, Alzheimer's disease and Diabetes mellitus [41]. Rosmarinic acid has been reported to play role in stimulated activation of the mTOR/S6K pathway based on hepatocyte proliferation [42]. Impaired liver function was found to be improved by rosmarinic acid, which helped in liver regeneration too [43]. Thus, increase in phenolic acid content upon radiation processing imparts the prophylactic advantages to the people consuming irradiated leafy greens.

3.4 Antimutagenic potential of spinach and coriander too increased upon radiation processing

Antimutagenic activity was monitored by observing change in rifampicin sensitive phenotype to rifampicin resistance phenotype due to induced mutation in *E. coli* MG1655 [44] (wild type) cells [45, 46]. *E. coli* cells which are not exposed to the mutagen do not show growth on rifampicin containing media because rifampicin act on DNA dependent RNA polymerase. *E coli* cells develop resistance to rifampicin by mutations in the *rpoB* gene that reduces affinity of rifampicin to RNA polymerase [47]. This assay is robust as compared to AMES mutagenicity assay as it shows very low level of spontaneous mutation frequency. Moreover, it is very sensitive, specific and simple [48].

The antimutagenic potential of control, gamma irradiated, and electron beam irradiated spinach and coriander was evaluated by EMS induced Rif^{R} antimutagenesis assay. The flavonoids extracted from coriander showed about 46% reduction in mutagenicity. Gamma irradiation and electron beam irradiated samples showed about 57 and 54% reduction in the mutagenicity. There was slight

increase in the mutagenicity in both gamma as well as electron beam irradiated coriander samples. However, these variations were not found to be significantly different. In case of spinach the extracted flavonoids in unirradiated samples showed reduction (of about 17%) in mutagenicity (Fig. 5A and B). Gamma radiation treatment showed significant increase in antimutagenicity by 63% as compared to control while electron beam irradiation showed highest antimutagenic activity (93%) as compared to control (Fig. 5A and B). This can be attributed to the enhanced extractability of flavonoids due to radiation treatment in spinach and also the nature of bioactivities. Various bioactive compounds work through different molecular mechanism of action and free flavonoids seem to possess high antimutagenic potential as seen in case of spinach. Antimutagenicity was also found in anthocyanins and various polyphenols extracted from tea and rose (*Rosa centifolia*) petals [49].

4. CONCLUSION

In leafy vegetables like spinach, coriander and mint, kaempferol, quercetin and rosmarinic acid, respectively were found to be the major bioactive compound. Their contents were found to be significantly increased upon radiation processing by both gamma and electron beam radiation (2 kGy). It was also observed that, in case of electron beam treated samples enhancement in their contents was more as compared to gamma treated samples. Thus, the radiation processing which is known to ensure microbial safety of leafy greens and thus extend their shelf life also has potential to enhance the free bioactive contents. This helped in enhancing their bioactivity in terms of antimutagenic potential and thus enhanced prophylactic value(s).

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