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Consequences of Gamma Irradiation on Triphala's Phytochemical Compositions, Microbial Burden and Antioxidant Properties

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Abstract

Triphala, a renowned polyherbal blend comprising three fruits, *Phyllanthus emblica* Linn., *Terminalia chebula* Retz., and *Terminalia bellirica* (Gaertn.) Roxb., in equal proportions, holds a rich historical lineage in both Ayurvedic and Thai traditional medicine. Triphala has been reputed as the indigenous medicine in the Thailand National List of Essential Drugs: List of Herbal Medicinal Products which has been widely used as a natural remedy for relieving cough and phlegm. However, the inherent challenge of microbial contamination in herbal remedies necessitates effective interventions. Gamma (γ) irradiation emerges as a pivotal method to mitigate the microbial burden in medicinal plants, albeit with potential repercussions on their chemical composition and biological properties. This study investigated the impact of γ-irradiation doses of 5, 10, and 25 kGy exposed to Triphala and the microbial contamination along with antioxidant activity, total phenolic content (TPC), gallic acid (GA), and chebulagic acid (CA) contents. The TPC and antioxidant activity of non-irradiated and irradiated Triphala were determined by using the Folin-Ciocalteu method and DPPH assay, while the GA and CA contents were quantified by HPLC analysis. The results demonstrated the efficacy of γ radiation doses (5-25 kGy) in diminishing microbial loads without significantly altering TPC or DPPH scavenging activity. Intriguingly, irradiation at 5 and 10 kGy, resulted in a notable increase in GA contents and CA contents ($p < 0.05$). Thus, the γ-irradiation emerges as a promising avenue for preserving Triphala quality and antioxidant properties amidst microbial contamination challenges.

Keywords**:** *Gamma (γ) radiation; irradiation effect; Triphala; microbial burden; phenolic compounds; antioxidant*

1. Introduction

Triphala is an Ayurvedic herbal formula preparation that contains three fruits, namely, *Phyllanthus emblica* Linn., *Terminalia chebula* Retz., and *Terminalia bellirica* (Gaertn) Roxb., in proportional amounts (Belapurkar et al., 2014; Peterson et al., 2017). Triphala was initially documented in the Ayurvedic traditional Charaka Samhita and has been employed in the treatment of several aliments for millennia (Mahdihassan, 1978). Triphala has been widely utilized and prescribed in both Ayurvedic and Thai traditional medicine. According to the Thailand National List of Essential Medicines (List of Herbal Medicinal Products), Triphala is effective in relieving cough and phlegm (National Essential Drug List Committee, 2023)

Triphala has been substantiated by recent clinical investigations to possess a diverse range of biological activities, such as antioxidative, antibacterial, laxative and immunomodulatory properties.

It has demonstrated efficacy in treating constipation, gingivitis, osteoarthritis, glaucoma, and several other illnesses or diseases (Mukherjee et al., 2006; Gupta et al., 2010; Baliga et al., 2012; Kalaiselvan, & Rasool, 2015; Baratakke et al., 2017). Triphala has been illustrated to have a significant amount of phenolic molecules as indicated by phytochemical tests (Avula et al., 2013). Gallic acid and chebulagic acid are the primary components responsible for the bioactivities of Triphala (Pawar et al., 2009; Russell et al., 2011; Lu, & Basu, 2015).

Herbals are susceptible to infestation by microbes and pests like insects and/or rodents during processes of preparation and storage. This diminishes their longevity and, in exceptional cases, induces severe illness, particularly when the herbs are mixed with pathogenic bacteria like *Salmonella* and *Staphylococcus aureus*. Gamma (γ) irradiation is chosen over alternative decontamination procedures due to its ability to completely eradicate bacteria without leaving behind any chemical residues, ensuring safety and offering ecological benefits (Calucci et al., 2003; Khattak et al., 2008). γ-irradiation is also efficient, rapid, and simple because it can be performed at room temperature. Several investigations have examined the consequences of γ-irradiation on the phenolic substances and antioxidant activity in nutritious foods and herbal products. According to Kumar et al. (2010), certain studies indicate that γ-irradiation has advantageous effects, whereas other findings imply negligible or even harmful consequences (Kumar et al., 2010). The quality of herbal compounds, including total phenolic content (TPC) and antioxidant activity, remained unchanged when exposed to γ-irradiation concentrations ranging from 0.25–30 kGy (Koseki et al., 2002; Calucci et al., 2003; Thongphasuk, & Thongphasuk, 2012; Thongphasuk, & Thongphasuk, 2013; Ito et al., 2016; Pereira et al., 2017). Moreover, numerous investigations have demonstrated that exposing herbs to γ-irradiation considerably increases the total phenolic compounds (Pereira et al., 2017; Hadiati et al., 2021).

2. Objectives

This proposed study investigated the impact of gamma (γ) irradiation on Triphala, determining total microbial contamination, total phenolic content (TPC), DPPH free radical scavenging, and bioactive contents as gallic acid and chebulagic acid. The Folin-Ciocalteu method and DPPH assay were used to determine the TPC and antioxidant activity of non-irradiated and irradiated Triphala. HPLC quantification was used to analyze the gallic acid and chebulagic acid content.

3. Materials and methods

3.1 Chemicals

Sodium carbonate was supplied by Ajax Finechem (Australia). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and gallic acid were purchased from Sigma-Aldrich (Germany). Folin-Ciocalteu reagent was obtained from Merck (Germany). Chebulagic acid was procured from Chengdu Biopurify Phytochemicals Ltd. (China). The HPLC grades acetonitrile and methanol were acquired from QReC (New Zealand).

3.2 Gamma irradiation

The desiccated fruits of *Phyllanthus emblica* Linn., *Terminalia chebula* Retz., and *Terminalia bellirica* (Gaertn.) Roxb. were acquired at a nearby herbal store in Nonthaburi Province, Thailand. Mr. Nirun Vipunngeun, a botanical taxonomist and instructor at the Department of Pharmacognosy, College of Pharmacy, Rangsit University, verified the authenticity of the herbal samples. The Thailand Institute of Nuclear Technology (Public Organization), conducted gamma irradiations at their facility in Ongkharak district, Nakhon nayok province, Thailand. The irradiations were performed using a gamma irradiator source (^{60}Co gamma irradiator, Paul Stephen consultancy LTD, England) with a dose rate of 2.84 kGy/hr. The sample was contained in a carrier box with a diameter of 30 cm and a height of 15 cm, which was surrounded by biological shielding measuring 1.88 meters. Triphala underwent γ-irradiation at doses of 5, 10, and 25 kGy. The samples that were not exposed to radiation (0 kGy) were employed as control samples.

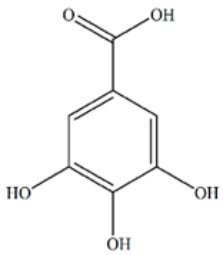
3.3 Sample preparation

The samples, both irradiated and non-irradiated, weighing 0.5 g each, were extracted using 50 mL of hot water at a temperature of 100°C. The samples were allowed to stand for a duration of 10 minutes before the extraction process. The supernatants were passed through filter paper No.1 (with a pore size of 11 µm, diameter of 110 mm, manufacturer by Whatman, England). The filtered solutions were stored at –20°C until they were analyzed.

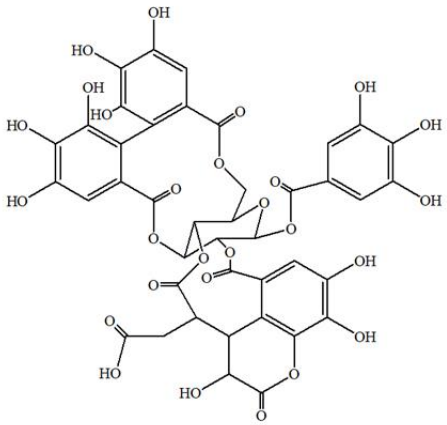
3.4 Microbial analysis

The purpose of the study was to assess the total number of live bacterial (CFU/g) and the total number of yeast and mold (CFU/g) presented in both the non-irradiated and irradiated Triphala samples. Total plate count was performed following the method of FDA-BAM chapter 3 (Food and Drug Administration, 2001) while total yeast and mold counts were investigated using FDA-BAM chapter 18 (Food and Drug Administration, 2001). *Staphylococcus aureus* (per 1 g) was examined using Association of Official Analytical Chemists (AOAC) (2003).11 (Petrifilm) guideline and *Salmonella* spp. (per 25 g) was examined using FDA-BAM chapter 5 (Food and Drug Administration, 2008). *Escherichia coli* (per 1 g) was examined under AOAC, 2005.03 (SimPlate) guideline while *Clostridium* spp. (per 10 g) was determined following USP40 chapter 62 (2019).

(a) Triphala composed of *Phyllanthus emblica* Linn., *Terminalia chebula* Retz., and *Terminalia bellirica* (Gaertn.) Roxb.



(b) gallic acid



(c) chebulagic acid

Figure 1 Three fruits of Triphala (a) as *Phyllanthus emblica* Linn., *Terminalia chebula* Retz., and *Terminalia bellirica* (Gaertn.) Roxb. which enriches of gallic acid (b) and chebulagic acid (c)

3.5 Determination of total phenolic content (TPC)

The TPC was detected utilising the Folin-Ciocalteu assay (Brighente et al., 2007). The samples (n=3) were combined with 0.2 N of Folin–Ciocalteu and mixed extensively in a microplate. The mixture solution was left undisturbed at a temperature of 25°C for a period of 5 minutes prior to the addition of 7.5% Na_2CO_3. Subsequently, the combination was left undisturbed for 120 minutes and its absorbance was determined at a wavelength of 760 nm, relative to a control sample. The spectrophotometric analysis was conducted using a Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories (UK) Ltd). The TPC was established using a calibration curve based on gallic acid. The results were expressed as mg gallic acid equivalents (mg GAE) per 1 g of dry weight of Triphala.

3.6 Free radical-scavenging assay with DPPH

The activity of DPPH in scavenging radicals was assessed using a modified version of the technique proposed by Cavin et al. (1998) and Thongphasuk, & Limsitthichaikoon (2023). The assessment of free radical scavenging activity in terms of percentage (%) of inhibition was conducted using spectrophotometry. One hundred microliters of samples that had been filtered were combined with 100 µl of methanol solution containing DPPH radicals (0.1 mM) and allowed to stand for 30 minutes. Subsequently, the absorbance of the mixture was measured at a wavelength of 517 nm, and the % inhibition was calculated.

3.7 Determination of gallic acid and chebulagic acid content by HPLC

The quantification of gallic acid (GA) and chebulagic acid (CA) concentrations was performed using high-performance liquid chromatography (HPLC) according to Monton et al. (2020). The quantification of GA and CA concentrations was conducted using an HPLC apparatus (Agilent 1260 infinite, Agilent, USA). The ACE C18-PFP column, measuring 250 × 4.6 mm with an internal diameter of 5 µm, was used at a controlled temperature of 25°C. The mobile phase consisted of a mixture of acetonitrile (ACN) and a 1% solution of acetic acid solution (AA). The procedure commenced by first maintaining a concentration of 5% ACN for a duration of 1 minute. Subsequently, the concentration was elevated to 10% ACN within a span of 3 minutes, followed by a further increase to 15% ACN over a period of 8 minutes. The concentration was then raised to 35% ACN in 20 minutes, and thereafter to 50% ACN in 3 minutes. Finally, the concentration was reduced to 5% ACN within 1 minute and held steady for 4 minutes. The rate at which the mobile phase flowed was 1 mL per minute. The injected volume was 10 µL. The calibration of the photodiode array detector was performed at a wavelength of 270 nm.

3.8 Statistical analysis

The analysis was conducted three times in order to ensure accuracy and reliability (n=3). The continuous variable data was given as averages with standard deviations (SD). To investigate differences between or among the groups participating in the experiment, the student t-tests and analysis of variance (ANOVA) were carried out utilizing the SPSS software (version 13, SPSS Inc, Chicago, IL, U.S.A.). Statistical significance is characterized as $p < 0.05$.

4. Results and discussion

4.1 Effects of gamma irradiation on microbial decontamination

The results showed that Triphala exposed to γ-irradiated significantly observed the deduction of microbial loads in the total aerobic count, total yeast count, and *Staphylococcus aureus* as shown in Table 1. Triphala before and after radiation treatments revealed that the γ-irradiation significantly reduced the contamination of aerobic count fungi and *Staphylococcus aureus*. No instances of *Salmonella* spp., *Escherichia coli*, or *Clostridium* spp. were found.

Thai Herbal Pharmacopoeia (2021) (Bureau of Drug and Narcotic, Department of Medical Science, 2021) established guidelines for the permissible levels of microbial contamination in herbals and medicinal plants. Prior to γ-irradiation, our findings on microbiological contamination exceeded the permitted limits. However, following the radiation treatment, the microbial levels were found to be within the acceptance range.

Table 1 Effects of gamma irradiation on Triphala microbial decontamination

	Gamma irradiation			
	0 kGy	**5 kGy**	**10 kGy**	**25 kGy**
Total aerobic count (CFU/g)	7.40 x 10^2	< 10 EAPC	< 10 EAPC	< 10 EAPC
Total fungi (CFU/g)	7.4 0x 10^3	< 10 EAPC	< 10 EAPC	< 10 EAPC
Staphylococcus aureus (per 1 g)	1.00 x 10^2	< 10	< 10	< 10
Salmonella spp. (per 25 g)	Not found	Not found	Not found	Not found
Escherichia coli (per 1 g)	< 10	< 10	< 10	< 10
Clostridium spp. (per 10 g)	Not found	Not found	Not found	Not found

*EAPC represents the estimated aerobic plate counts.

4.2 Effects of gamma irradiation on total phenolic content (TPC)

The results of the TPC expressed as mg equivalents of gallic acid/g dry weight of Triphala were 46.91±0.67 mg GAE/g of non-irradiation (0 kGy) and 48.05±0.75, 45.85±0.83 and 47.16±0.08 mg GAE/g exposed to 5, 10 and 25 kGy γ-irradiation, respectively, as shown in Figure 2. There were no significant changes ($p > 0.05$) in the TPC after being exposed to 5-25 kGy γ-irradiation compared to the non-exposed Triphala which is similar to that of other herbals that have been treated with γ-irradiation (Hadiati et al., 2021; Suryadi, & Mun'im, 2021). Our study found that the γ-irradiation had no effect on phenolic compounds. However, other research had found that γ-irradiation can increase, decrease, or maintain TPC levels (Amiri et al., 2021; Hadiati et al., 2021; Heidarieh et al., 2021; Ahmed, & Hassan, 2023).

4.3 Impact of gamma irradiation on the ability to neutralize DPPH free radicals

The antioxidant properties of Triphala were assessed using DPPH methods, both in the presence and absence of γ-irradiation. The free radical-scavenging activity was evaluated as percentage (%) of inhibition. The inhibition percentages were 90.15±3.11% of non-irradiation samples (0 kGy), and 93.82±1.77%, 95.43±2.49% and 94.64±2.05% for samples treated to 5, 10, and 25 kGy γ-irradiation, respectively, as shown in Figure 2. The scavenging activity of both the control and radiation-processed samples at 5, 10 and 25 kGy did not show any significant changes ($p > 0.05$). This indicates that γ-irradiation did not have an impact on the free radical-scavenging ability of Triphala, as measured by DPPH test.

The effects of γ-irradiation on bioactive components, including antioxidant and volatile compounds, depend on various factors such as the radiation level, dose rate, composition of raw materials, etc. (Khattak et al., 2008; Thongphasuk et al., 2014). Our investigation concluded that the γ-irradiation had no effect on the capacity of Triphala to eliminate free radical, which is consistent with other research done on botanical and plant materials (Harrison, & Were, 2007; Hadiati et al., 2021; Ahmed, & Hassan, 2023).

4.4 Effect of gamma irradiation on gallic acid and chebulagic acid content determined by HPLC

Gallic acid (GA) and chebulagic acid (CA) are the main components in Triphala. After exposure to γ-irradiation, the results exhibited as percentages (%) of GA content were 0.9735 ±0.0006% of non-irradiation (0 kGy) and 1.0842 ±0.0018, 1.1351±0.0025 and 0.9246±0.0183 % after exposure to 5, 10 and 25 kGy γ-irradiation, respectively, as shown in Figure 3. The percentage (%) of CA content was 0.6079±0.0041% of non-irradiation (0 kGy) and 0.7443±0.0051, 0.8626 ±0.0103 and 0.6117±0.0174 % exposed to 5, 10 and 25 kGy γ-irradiation, respectively. The GA and CA contents detected by HPLC analysis detected a significant ($p < 0.05$) increase in both GA and CA contents when exposed to 5 and 10 kGy irradiation whereas at the irradiation dose of 25 kGy exposure did not significantly affect the GA and CA contents compared to the non-irradiation.

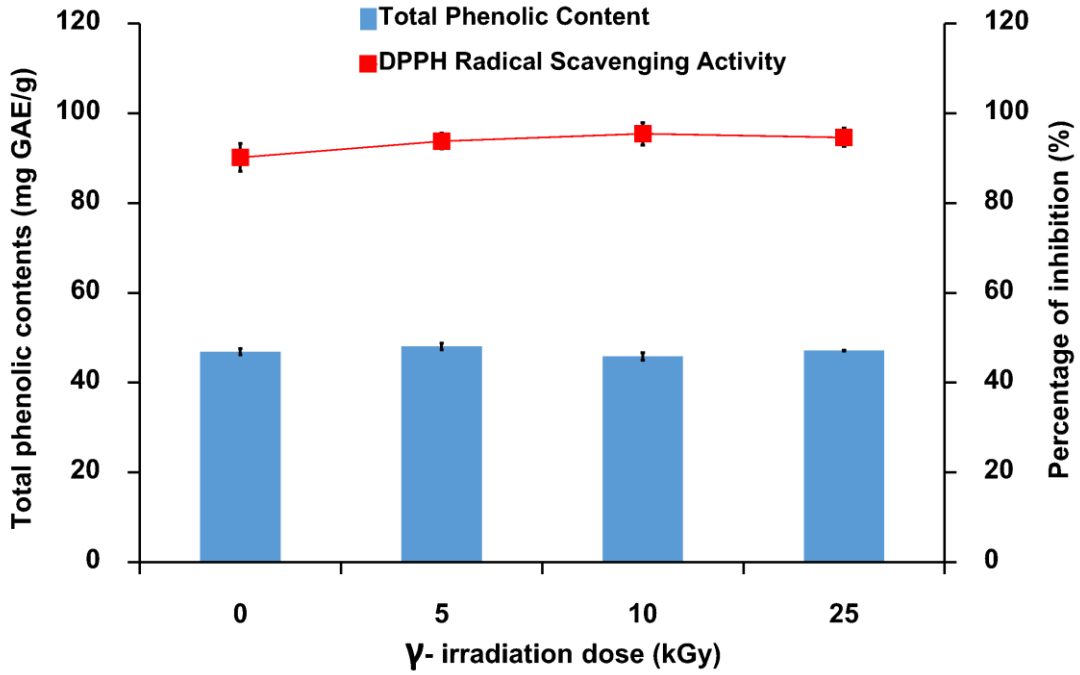


Figure 2 Total phenolic content (TPC) and antioxidant by DPPH free radical scavenging of Triphala exposed to γ-irradiation dose at 5-25 kGy compared with Triphala non exposed to γ-irradiation (0 kGy)

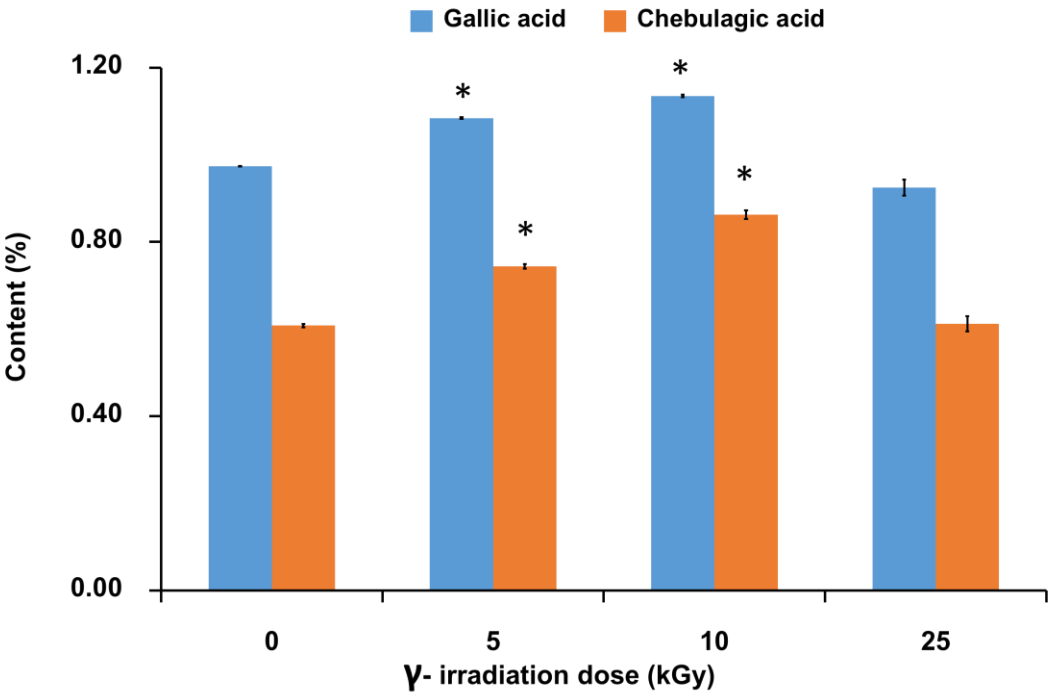


Figure 3 Gallic acid and chebulagic acid content determined by HPLC assay. The data are presented as the mean ± standard deviation (SD, n = 3). Asterisks indicate statistically significant changes as compared to non γ-irradiation (0 kGy) ($p < 0.05$)

Triphala after being exposed to γ-irradiation at doses of 5 and 10 kGy showed evident increases in percentage of GA and CA content, which may relate to the study of Harrison, & Were (2007), that γ-irradiation may trigger the breakdown of phenolic substances from glycosidic parts and deterioration of greater phenolic molecules into smaller compounds like GA and CA (Harrison, & Were, 2007; Hadiati et al., 2021).

Fresh and dried herbals are often contaminated by fungal, yeast, microbial, and insect pests throughout the collection and storage procedure. γ-irradiation has been chosen over other decontamination procedures because to its effectiveness in eliminating germs without leaving any chemical residues, thereby ensuring both safety and ecologically acceptability (Calucci et al., 2003; Khattak et al., 2008; Hadiati et al., 2021). Several studies have examined the impact of γ-irradiation on phenolic components and the antioxidant activity of foods and medicinal plants (Amiri et al., 2021; Heidarieh et al., 2021; Ahmed, & Hassan, 2023).

Recent studies used γ-irradiation for preventing microbial contamination without altering the TPC, antioxidant and bioactive compounds (Amiri et al., 2021; Heidarieh et al., 2021; Ahmed, & Hassan, 2023) which suggests that the application of γ-irradiation has been effective and valuable in the herbal product industry (Calucci et al., 2003; Khattak et al., 2008). Conversely, another study reported a reduction of TPC in several plants. For example, the study of Koseki et al. (2002) revealed a substantial reduction in TPC in dried rosemary following irradiation at doses from 10 and 30 kGy, compared to controls. Irradiation at a dosage of 10 KGy on *Aloysia citrodora* Paláu had a similar outcome (Pereira et al., 2017). The effects of γ-irradiation on TPC may vary depending on the plant type and parts, phenolic composition and chemical structure, experimental condition, solvent and procedure for extraction, storage conditions and irradiation levels (Harrison, & Were, 2007; Khattak, & Simpson, 2010).

Gallic acid and chebulagic acid are widely recognized for their diverse biological activities, which encompass not only antioxidant properties but also anti-atherogenic, anti-cancer, antidiabetic, anti-inflammatory, anti-fibrotic, antiulcer, antiviral, and hepatoprotective effects (Yang, et al., 2013; Shanmuganathan, & Angayarkanni, 2018; Dhingra et al., 2022). Consequently, the augmentation of GA and CA derivatives of Triphala exhibits a multitude of pharmacological effects, thereby necessitating more investigations.

Our results showed evidently that γ-irradiation specifically reduces the microbial burden, while having no impact on total phenolic content (TPC) and free radical scavenging plus increasing the bioactive compounds, such as GA and CA content. Thus, γ-irradiation is recommended over other decontamination methods for Triphala treatment due to its ability to kill microorganisms without destroying the bioactive components.

5. Conclusion

Triphala, a traditional Ayurvedic and herbal medicine, is known for its natural remedy for cough and phlegm. However, microbial contamination poses a challenge. Gamma-irradiation, a method to mitigate microbial burden, has been investigated. The study found that irradiation at doses of 5, 10 and 25 kGy effectively reduced microbial loads without significantly altering the TPC and DPPH radical-scavenging activities. However, irradiation at 5 and 10 kGy increased GA and CA contents, suggesting γ-irradiation as a promising method for reducing microbial contamination and preserving Triphala's quality.

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