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Mutagenic influence on biochemical and phytochemical composition of Senna (*Cassia angustifolia* Vahl): A comparative study of gamma irradiation and EMS

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Abstract

This study investigates the biochemical and phytochemical responses of Senna (*Cassia angustifolia* Vahl) seeds to mutagenic treatments using gamma irradiation and ethyl methane sulfonate (EMS). Various biochemical parameters, including chlorophyll, protein, carbohydrate, proline, phenolics, flavonoids, sennosides, and antioxidant enzyme activities (SOD, CAT and POD), were analysed at different doses. The results showed that moderate doses (40 Gy gamma irradiation and 2.0% EMS) significantly enhanced chlorophyll (2.30 mg/g FW), protein (17.3 mg/g FW) and carbohydrate (54.7 mg/g DW) content, indicating a stimulatory effect on biosynthetic pathways. Proline content increased consistently with higher doses, peaking at 5.35 µg/g FW (40 Gy) and 5.05 µg/g FW (2.0% EMS), highlighting its role in oxidative stress mitigation. The highest sennoside accumulation was observed at 40 Gy and 2.0% EMS, supporting their role in medicinal efficacy. Tannin levels increased up to 2.0% EMS and 40 Gy, followed by a decline beyond these thresholds. Antioxidant enzyme activities showed significant increases, with SOD (3.85 U/mg protein), CAT (6.12 U/mg protein) and POD (4.75 U/mg protein) peaking at moderate doses before declining at higher stress levels. However, at higher mutagenic doses (beyond 50 Gy gamma irradiation and 2.5% EMS), metabolic imbalances led to reduced biochemical activity, possibly due to oxidative damage and enzymatic inhibition. These findings suggest that controlled mutagenic treatments can improve Senna's phytochemical composition and pharmacological value. Future research should explore molecular mechanisms and optimize mutagenic approaches for sustainable medicinal plant improvement, with implications for pharmaceutical and agricultural applications.

1. Introduction

Senna (*Cassia angustifolia* Vahl) is a well-known medicinal plant belonging to the Fabaceae family, widely recognized for its therapeutic benefits. It is cultivated in various tropical and subtropical regions for its leaves and pods, which are rich in bioactive compounds. These compounds, including sennosides, flavonoids, glycosides and anthraquinones, contribute to its strong laxative, antimicrobial, anti-inflammatory, and antioxidant properties (Ali *et al*., 2018; Singh and Kaur, 2020). Given its pharmaceutical significance, there is a growing need to enhance its yield, quality, and medicinal value through scientific interventions (Figure 1). Mutation breeding has emerged as a key technique in improving medicinal plants by inducing genetic variations (Bhat *et al*., 2017). Among the widely used mutagens, gamma radiation (a physical mutagen) and ethyl methane sulfonate (EMS, a chemical mutagen) have shown considerable promise. These mutagens alter the DNA structure, leading to changes in plant morphology, growth patterns, and biochemical profiles (Khan *et al*., 2019). In Senna, the application of mutagenesis can be an effective approach to enhance plant productivity, improve phytochemical content and strengthen its pharmacological properties (Gupta *et al*., 2021). Gamma radiation is a form of ionizing radiation that affects cellular structures and induces genetic modifications (Hussain *et al*., 2020). The extent of mutation is influenced by factors such as radiation dose, plant genotype, and tissue sensitivity (Saha *et al*., 2019). Previous research on medicinal plants suggests that controlled doses of gamma radiation can lead to increased biomass, improved secondary metabolite production, and enhanced environmental resilience (Kumari *et al*., 2021). However, excessive radiation can have adverse effects such as reduced germination, poor growth, and increased mortality (Jain *et al*., 2018).

EMS is a chemical mutagen, functions as an alkylating agent that induces point mutations by modifying nucleotide sequences in the DNA (Patil and Gaikwad, 2022). These mutations can result in diverse phenotypic variations, including improved phytochemical accumulation, enhanced resistance to diseases and modified morphological traits (Choudhary *et al*., 2021). The impact of EMS depends on factors such as concentration, duration of exposure, and the genetic makeup of the plant (Sharma *et al*., 2017). In Senna, EMS treatment could create genetic variations leading to increased production of beneficial bioactive compounds and improved medicinal attributes (Kumar *et al*., 2020). The effects of gamma radiation and

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EMS on plant growth are largely dose-dependent. Low to moderate doses tend to stimulate germination, seedling vigour, and overall metabolic functions, whereas high doses may induce oxidative stress, reducing seed viability and stunting growth (Goyal and Sharma, 2019). Various studies on medicinal plants have demonstrated that carefully optimized mutagen treatments can enhance essential oil content, alkaloid levels, and phenolic compounds (Verma *et al*., 2021). Similarly, in Senna, mutation breeding can help optimize sennoside production, thereby increasing its pharmacological efficacy (Bhatt *et al*., 2022).

Figure 1: Senna plant with flowers and pods.

Beyond yield improvements, induced mutagenesis can enhance the medicinal potential of plants by modifying their secondary metabolic pathways (Rana *et al*., 2021). These biochemical changes can lead to increased antioxidant, antimicrobial, and anti-inflammatory properties (Meena *et al*., 2020). Research in medicinal plants has revealed that mutations induced through radiation and chemical treatments can alter phytochemical profiles, leading to novel and enhanced therapeutic properties (Das *et al*., 2018). In Senna, mutation induced genetic diversity could result in superior genotypes with better pharmacological value (Pandey *et al*., 2019). A key aspect of mutation breeding is the identification and selection of desirable mutant lines. This involves extensive screening using morphological evaluation, biochemical analysis, and molecular markers (Saxena *et al*., 2020). The M_1 generation, being the initial population of mutants, plays a vital role in assessing the effectiveness of mutagenic treatments (Kundu *et al*., 2019). Observing growth patterns, yield parameters and phytochemical compositions in this generation helps in identifying promising variants for further development (Prasad *et al*., 2018). The demand for high-quality medicinal plants is increasing globally due to the growing interest in natural health solutions (Raj *et al*., 2021). To meet this demand, there is a need to develop improved plant varieties with enhanced therapeutic benefits. Mutation breeding provides an efficient and sustainable approach to enhance genetic diversity, optimize phytochemical production and improve overall plant resilience. By leveraging gamma radiation and EMS-induced mutagenesis, researchers can develop superior Senna variants that cater to the evolving needs of the pharmaceutical industry (Yadav and Sharma, 2022).

This study aims to explore the impact of gamma radiation and EMS on the phytochemical and pharmacological characteristics of Senna in the M_1 generation. By evaluating factors such as chlorophyll mutationsand phytochemical profiles, this research seeks to identify mutant lines with enhanced medicinal value. The findings will contribute to a deeper understanding of mutation breeding in medicinal plants and open new avenues for the development of high-quality herbal products.

2. Materials and Methods

2.1 Authentication of plant material

Dr. R. Ramasubbu, Associate Professor, Department of Biology, Gandhigram Rural Institute, Gandhigram, Dindigul, conducted the entire botanical authentication and identification of the plant specimen. The Voucher Specimen is Catalogued under Collection 395 and stored at the GUD Herbarium.

2.2 Sample material and experimental site

The present studywas conducted at the Botanical Garden, Department of Medicinal and Aromatic Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Coimbatore, India. The field trials and laboratory analyses were carried out under controlled conditions to ensure accuracy and reproducibility. The plant material used in this study comprised seeds of the Tinnevelly Senna (KKM-1) variety, collected from Tuticorin district, Tamil Nadu, India. The seeds were selected based on uniformity, maturity, and viability before undergoing mutation treatments. Standard agronomic practices and intercultural operations were followed to provide optimal growth conditions. KKM-1 is a widely cultivated variety known for its bushy and spreading growth habit, high rejuvenation capacity and crop duration of 110-130 days. The plants begin flowering within 60-70 days and pod formation occurs around 90 days after sowing. The variety is commercially significant due to its high export potential, with a sennoside content ranging from 1.5% to 3%. The experimental field was composed of clay loam soil and composite soil samples were collected from various locations before sowing to analyse their physical and chemical properties. The soil was properly prepared through ploughing and levelling to ensure a uniform seedbed. The treated seeds were sown at an optimal depth with proper spacing between plants and rows. Standard irrigation, weed management, and plant protection measures were consistently followed for all treatments.

2.3 Mutagenic treatment

The study utilized two types of mutagens. Gamma radiation as a physical mutagen and ethyl methane sulfonate (EMS) as a chemical mutagen. The gamma radiation treatments were administered using a 1000 Curie Co-60 Gamma Cell at the Centre for Plant Breeding and Genetics (CBPG), TNAU, Coimbatore. The EMS, obtained from Sigma-Aldrich Corporation, had a molecular weight of 124.6 g/mol and was used for seed treatment. The seeds were subjected to different mutagenic doses to induce genetic variation, with EMS treatments applied at concentrations ranging from 0.5% to 5.0%, while gamma radiation doses varied between 1 kR and 10 kR.

2.4 Imposing treatment

The seeds were exposed to gamma irradiation at varying doses based on different time durations. Three sets, each containing 100 seeds, were subjected to gamma rays at doses of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 Gy to determine the LD_{50} (lethal dose for 50% germination) value. For EMS treatment, three sets of 100 seeds were initially soaked in water overnight to enhance uniform absorption. After pre-soaking, the seeds were shade-dried before exposure to the chemical mutagen. Ethyl methane sulfonate (EMS) was applied at varying concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0%. The mutagenic solutions were prepared using the required quantity of EMS in a phosphate buffer solution. To ensure even absorption, the volume of the mutagenic solution was maintained at approximately five times the volume of the seeds.The pre-soaked seeds were then immersed in the EMS solution for one hour under controlled conditions at 25 ± 2°C, with intermittent shaking to ensure uniform exposure. Following the treatment, the seeds were thoroughly washed under running water for 10 min to remove residual chemicals. An equal quantity of untreated seeds, soaked in water alone, was used as a control. Both the treated and control seeds were sown in sterilized media under controlled conditions. The LD_{50} values for gamma rays and EMS treatments were determined based on the number of seeds that germinated by the 15th day after sowing.

2.5 Biochemical parameters

The biochemical and phytochemical parameters of Senna were analysed using standardized laboratory procedures to assess its medicinal and nutritional value. The estimation of chlorophyll content was carried out following Arnon's method (1949). Fresh leaf samples were ground in 80% acetone, and the extract was filtered. The absorbance was measured at 645 nm and 663 nm using a UV-Vis spectrophotometer. The total chlorophyll content was calculated using the standard formula and expressed in milligrams per gram of fresh weight. For protein estimation, Lowry's method (1951) was employed. Fresh leaf samples were homogenized in phosphate buffer and centrifuged. The supernatant was mixed with alkaline copper sulfate reagent, followed by the addition of Folin-Ciocalteu reagent. The reaction mixture was incubated for 30 min, and the absorbance was measured at 660 nm. The protein content was quantified using a standard curve of bovine serum albumin (BSA) and expressed in milligrams per gram of fresh weight. Carbohydrate content was determined using Anthrone's method. The dried leaf powder was hydrolyzed with hydrochloric acid and subjected to a reaction with Anthrone reagent in sulfuric acid. The mixture was incubated in a boiling water bath for 10 min, cooled, and absorbance was recorded at 620 nm. The carbohydrate content was determined using a standard curve prepared with glucose and expressed in milligrams per gram of dry weight. The proline content, an important indicator of stress tolerance, was estimated using Bates *et al.* (1973) method. Fresh leaves were homogenized in 3% sulfosalicylic acid and filtered. The extract was treated with acid ninhydrin reagent and incubated in a boiling water bath for one hour. After cooling, the mixture was extracted with toluene, and absorbance was measured at 520 nm. The proline content was calculated using a standard curve and expressed in micrograms per gram of fresh weight.

2.6 Antioxidant enzyme activity

The antioxidant enzyme activity of Superoxide dismutase (SOD), Catalase (CAT) and Peroxidase (POD) was estimated using spectrophotometric methods. SOD activity was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), and absorbance was recorded at 560 nm. Catalase activity was assessed based on its ability to decompose hydrogen peroxide, with absorbance measured at 240 nm. Peroxidase activity was determined using guaiacol as a substrate, and the reaction product was measured at 470 nm (Awang Daud *et al*., 2022).

2.7 Secondary metabolites

For total phenolic content (TPC) estimation, the Folin-Ciocalteu method was employed. The methanolic extract of Senna leaves was mixed with Folin-Ciocalteu reagent, followed by the addition of sodium carbonate solution. The reaction mixture was incubated in the dark for 30 mins, and absorbance was recorded at 760 nm. The phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight. The total flavonoid content (TFC) was estimated using the aluminium chloride colorimetric assay. The methanol extract was mixed with aluminium chloride reagent and incubated for 10 mins. The absorbance was measured at 415 nm, and the flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of dry weight. The tannin content was estimated using the Folin-Denis method. The methanol extract was mixed with Folin-Denis reagent and sodium carbonate solution. The reaction mixture was incubated for 30 mins, and absorbance was recorded at 725 nm. The tannin content was calculated using a standard curve of tannic acid and expressed in milligrams per gram of dry weight (Twaij *et al*., 2022).

2.8 Sennosideestimation

2.8.1 Preparation of reagents and solutions

To prepare stock solutions for sennoside estimation, a precise quantity of 2.50 mg was dissolved in acetonitrile and diluted to 5 ml. Intermediate stock solutions were then prepared using a solvent mixture of acetonitrile and water (40:60 v/v). The mobile phase for chromatographic analysis was formulated by combining acetonitrile (ACN) with 0.05% v/v acetic acid in water (60:40 v/v) to facilitate the separation of compounds.

2.8.2 Sample preparation

Plant extracts were prepared using 70% methanol, with continuous stirring at room temperature for 30 min to ensure maximum solubility of alkaloids. The extract was then filtered using Whatman No. 1 filter paper, and the filtrate was stored at 4°C. Before subjecting the sample to LC-MS analysis, it was passed through a 0.45 µm microporous filter to remove particulate matter.

2.8.3 Standard preparation

Sennoside A and Sennoside B reference standards were procured from Sigma Aldrich India Pvt. Ltd. for comparative analysis. The mobile phase used for HPLC analysis consisted of acetonitrile (ACN) and 0.05% acetic acid in water (60:40 v/v) to optimize compound separation and detection.

2.8.4 HPLC analysis

Chromatographic analysis was conducted using a Thermo Finnigan HPLC system equipped with a Genesis C18 100 RP column (100 mm × 4.6 mm i.d., 4 µ). The mobile phase composition remained acetonitrile and 0.05% v/v acetic acid in water (60:40 v/v), delivered at a flow rate of 0.500 ml/min. Each sample run lasted 5.50 min, with the column oven temperature maintained at 40°C. An injection volume of 20 µl was used for sample introduction. The presence of sennosides and hydrochlorothiazide was confirmed using LC-MS/MS detection. The LC-MS/MS analysis was performed using a Thermo Finnigan LC module equipped with a TSQ Quantum Discovery Max Triple Quadrupole mass spectrometer operating in negative ionization mode. Nitrogen and argon were used as sheath gas (40 psi) and auxiliary gas (20 psi), respectively. The electrospray ionization (ESI) source was set at 4500 V, with the capillary temperature at 350°C. The mass analyser was optimized for detecting negative ions, with sennoside and hydrochlorothiazide monitored at m/z 386.50 and m/z 223.00, respectively, using collision energies of 10 V and 11 V (Shreedhara*et al.*, 2013).

Calculation of sennoside content

The percentage of Sennoside A in the sample was calculated using the formula:

$$\text{Sennoside A} = \frac{m_2 \times A_1 \times 5 \times p}{A_2 \times m_1}$$

where:

A_1 = Area of the peak for Sennoside A in the test solution

A_2 = Area of the peak for Sennoside A in the reference solution

m_1 = Mass of the test sample in grams

m_1 = Mass of the Sennoside A reference standard in grams

p = Percentage purity of Sennoside A

Similarly, the percentage of Sennoside B was determined using the formula:

$$\text{Sennoside B} = \frac{m_2 \times A_1 \times 5 \times p}{A_2 \times m_1}$$

A_1 = area of the peak due to sennoside B obtained with the test solution

A_2 = area of the peak due to sennoside B obtained with there ference solution

m_1 = mass of the drug to be examined in the test solution in grams

m_2 = mass of sennoside B in there ference solution in grams

p = percentage content of sennoside B.

2.9 Statistical analysis

The experiment was laid out in a randomized block design (RBD) with three replications. Each treatment, including a control group (untreated seeds), was allocated to specific plots. All data collected were statistically analysed using Analysis of Variance (ANOVA) to determine the significance of treatment effects. Duncan's multiple range test (DMRT) at a 5% probability level was employed for mean separation to identify the most promising mutant lines with enhanced biomass and phytochemical traits (Mishra and Slater, 2012).

3. Results

3.1 Biochemical parameters

Chlorophyll plays a crucial role in photosynthesis and is an indicator of plant health. The chlorophyll content in control plants was 1.87 mg/g fresh weight (FW). It gradually increased with gamma irradiation up to 40 Gy (2.30 mg/g FW), suggesting an initial stimulatory effect on chlorophyll synthesis. However, at higher doses (above 50 Gy), chlorophyll levels declined significantly, reaching 0.65 mg/g FW at 100 Gy. This reduction can be attributed to radiation-induced oxidative stress, which damages chlorophyll pigments and affects chloroplast function. Similar to gamma irradiation, EMS treatment led to an increase in chlorophyll levels at low concentrations, with a peak at 2.0% EMS (2.25 mg/g FW). However, at higher concentrations (above 2.5%), chlorophyll content declined, reaching 1.14 mg/g FW at 4.0% EMS. This suggests that excessive EMS exposure disrupts chlorophyll biosynthesis and photosynthetic efficiency.

Protein is an essential macromolecule that determines plant growth, metabolism, and enzymatic activity. In untreated plants, the protein content was 12.4 mg/g FW. Moderate doses of gamma irradiation (10-40 Gy) enhanced protein synthesis, with the highest recorded value at 40 Gy (17.3 mg/g FW). However, protein levels declined sharply at doses beyond 50 Gy, indicating damage to cellular proteins and impaired biosynthetic pathways. At 100 Gy, the protein content dropped to 6.4 mg/g FW, confirming the inhibitory effects of excessive radiation exposure. The protein content in untreated plants was 12.4 mg/g FW, and it increased at lower EMS concentrations, reaching 16.9 mg/g FW at 2.0% EMS. However, at concentrations above 2.5%, protein synthesis was negatively affected, with levels dropping to 9.0 mg/g FW at 4.0% EMS. This reduction may be due to mutations affecting protein-coding genes or inhibition of enzyme activity.

Carbohydrates serve as an energy reserve and structural component in plants. In control plants, the carbohydrate content was 45.2 mg/g dry weight (DW). Low to moderate doses of gamma irradiation stimulated carbohydrate accumulation, peaking at 40 Gy (54.7 mg/g DW). This increase may be due to enhanced photosynthetic efficiency and improved enzymatic activity at optimal radiation exposure. However, excessive radiation (above 50 Gy) resulted in a decline in carbohydrate levels, with 27.5 mg/g DW recorded at 100 Gy, likely due to damage to photosynthetic machinery. Carbohydrate levels followed a similar trend, increasing from 45.2 mg/g DW in control plants to 54.2 mg/g DW at 2.0% EMS. However, excessive EMS exposure resulted in a decline, with 32.7 mg/g DW at 4.0% EMS, indicating metabolic disruptions caused by mutagenic stress. Proline is an important osmolyte that accumulates under stress conditions to mitigate oxidative damage. In untreated plants, proline content was 3.87 µg/g FW. As gamma irradiation increased, proline levels also increased, with the highest accumulation at 40 Gy (5.35 µg/g FW). Beyond 50 Gy, proline levels declined, indicating that excessive radiation disrupted normal cellular function, reducing the plant's ability to counteract stress. Proline levels increased with EMS treatment, reaching a peak at 2.0% EMS (5.05 µg/g FW). Higher EMS concentrations caused a reduction in proline accumulation, suggesting that excessive mutations hinder stress tolerance mechanisms (Table 1 and Table 2).

Table 1: Effects of gamma irradiation (Gy) on biochemical parameters

Gamma dose (Gy)	Chlorophyll (mg/g FW)	Protein(mg/g FW)	Carbohydrates(mg/g DW)	Proline (µg/g FW)
Control (0 Gy)	1.87 ± 0.12	12.4 ± 0.45	45.2 ± 1.21	3.87 ± 0.18
10 Gy	1.92 ± 0.13	13.1 ± 0.48	47.5 ± 1.25	4.15 ± 0.20
20 Gy	2.05 ± 0.14	14.5 ± 0.50	49.3 ± 1.30	4.68 ± 0.22
30 Gy	2.22 ± 0.15	16.0 ± 0.52	52.8 ± 1.35	5.10 ± 0.24
40 Gy	2.30 ± 0.16	17.3 ± 0.55	54.7 ± 1.40	5.35 ± 0.25
LD_{50} (~50 Gy)	1.78 ± 0.12	13.8 ± 0.47	43.5 ± 1.18	4.92 ± 0.23
60 Gy	1.56 ± 0.11	11.7 ± 0.44	39.6 ± 1.12	4.45 ± 0.21
70 Gy	1.32 ± 0.10	10.5 ± 0.40	37.1 ± 1.10	4.02 ± 0.19
80 Gy	1.10 ± 0.09	9.3 ± 0.38	34.2 ± 1.05	3.65 ± 0.18
90 Gy	0.89 ± 0.08	7.8 ± 0.35	30.9 ± 1.00	3.22 ± 0.17
100 Gy	0.65 ± 0.07	6.4 ± 0.32	27.5 ± 0.95	2.85 ± 0.16
SE.d	0.08	0.34	1.10	0.13
SE.m	0.05	0.21	0.69	0.08
CD (*p*=0.05)	0.18	0.72	2.15	0.27

Table 2: Effects of ethyl methane sulfonate (EMS) on biochemical parameters

EMS concentration (%)	Chlorophyll (mg/g FW)	Protein (mg/g FW)	Carbohydrates (mg/g DW)	Proline (µg/g FW)
Control (0%)	1.87 ± 0.12	12.4 ± 0.45	45.2 ± 1.21	3.87 ± 0.18
0.5%	1.95 ± 0.13	13.0 ± 0.48	47.1 ± 1.25	4.10 ± 0.20
1.0%	2.08 ± 0.14	14.4 ± 0.50	49.5 ± 1.30	4.52 ± 0.22
1.5%	2.19 ± 0.15	15.7 ± 0.52	52.1 ± 1.35	4.85 ± 0.23
2.0%	2.25 ± 0.16	16.9 ± 0.55	54.2 ± 1.38	5.05 ± 0.24
LD_{50} (~2.5%)	1.80 ± 0.12	13.5 ± 0.46	43.8 ± 1.20	4.75 ± 0.22
3.0%	1.60 ± 0.11	11.8 ± 0.44	39.8 ± 1.12	4.40 ± 0.21
3.5%	1.38 ± 0.10	10.4 ± 0.40	36.2 ± 1.08	4.00 ± 0.19
4.0%	1.14 ± 0.09	9.0 ± 0.37	32.7 ± 1.05	3.55 ± 0.18
4.5%	0.92 ± 0.08	7.6 ± 0.34	29.4 ± 1.00	3.20 ± 0.17
5.0%	0.72 ± 0.07	6.2 ± 0.31	26.1 ± 0.95	2.85 ± 0.16
SE.d	0.08	0.35	1.05	0.12
SE.m	0.05	0.22	0.68	0.08
CD (*p*=0.05)	0.17	0.75	2.05	0.25

3.2 Antioxidant enzyme activity

The antioxidant enzyme activities, including SOD, CAT and POD, were significantly influenced by gamma irradiation. SOD activity in control plants was recorded at 3.85 U/mg protein, which increased to a maximum of 5.10 U/mg protein at 40 Gy. However, beyond 50 Gy, SOD activity gradually declined, reaching 2.05 U/mg protein at 100 Gy. A similar pattern was observed in CAT activity, which increased from 6.12 U/mg protein in control plants to a peak of 7.85 U/mg protein at 40 Gy but decreased to 4.00 U/mg protein at 100 Gy. Peroxidase (POD) activity also followed a comparable trend, with an increase from 4.35 U/mg protein in the control to a peak of 5.80 U/mg protein at 40 Gy, but declined to 2.50 U/mg protein at 100 Gy. The antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), were also significantly affected by EMS treatments. SOD activity in untreated plants was 3.85 U/mg protein, which increased to a maximum of 4.85 U/mg protein at 2.0% EMS. This suggests an upregulation of antioxidant defence mechanisms in response to moderate oxidative stress induced by EMS. However, beyond 2.5% EMS, SOD activity progressively declined, reaching 2.10 U/mg protein at 5.0%, indicating a breakdown of enzymatic defence mechanisms under high mutagenic stress. A similar trend was observed for CAT activity, which increased from 6.12 U/mg protein in the control to 7.35 U/mg protein at 2.0% EMS, but dropped to 4.10 U/mg protein at 5.0%. Catalase plays a crucial role in detoxifying hydrogen peroxide, and its enhanced activity at lower EMS concentrations suggests a protective response to oxidative damage. Peroxidase (POD) activity also followed a comparable trend, with an increase from 4.35 U/mg protein in control plants to a peak of 5.55 U/mg protein at 2.0% EMS. This increase in POD activity at moderate EMS concentrations suggests an enhanced lignification process, which strengthens plant cell walls against oxidative stress. However, at higher EMS concentrations, POD activity decreased, reaching 2.55 U/mg protein at 5.0%. This decline may be due to enzyme inhibition or degradation under excessive oxidative stress. The overall trend in antioxidant enzyme activities suggests that moderate EMS concentrations induce a beneficial stress response, enhancing antioxidant defence mechanisms, but excessive doses disrupt enzymatic activity, leading to cellular and metabolic impairment (Table 3 and Table 4).

Table 3: Effects of gamma irradiation (Gy) on antioxidant enzyme activity

Gamma dose (Gy)	SOD (U/mg protein)	CAT (U/mg protein)	POD (U/mg protein)
Control (0 Gy)	3.85 ± 0.18	6.12 ± 0.22	4.35 ± 0.19
10 Gy	4.10 ± 0.20	6.45 ± 0.25	4.60 ± 0.20
20 Gy	4.38 ± 0.22	6.78 ± 0.28	4.95 ± 0.22
30 Gy	4.65 ± 0.24	7.10 ± 0.30	5.30 ± 0.24
40 Gy	4.92 ± 0.25	7.42 ± 0.32	5.65 ± 0.25
LD_{50} (~50 Gy)	3.62 ± 0.18	5.95 ± 0.20	4.10 ± 0.18
60 Gy	3.28 ± 0.16	5.62 ± 0.18	3.85 ± 0.17
70 Gy	3.05 ± 0.15	5.30 ± 0.16	3.50 ± 0.16
80 Gy	2.80 ± 0.14	4.92 ± 0.15	3.15 ± 0.15
90 Gy	2.50 ± 0.13	4.60 ± 0.12	2.90 ± 0.14
100 Gy	2.28 ± 0.12	4.20 ± 0.10	2.60 ± 0.13
SE.d	0.16	0.24	0.21
SE.m	0.10	0.17	0.12
CD (*p*=0.05)	0.31	0.42	0.36

Table 4: Effects of ethyl methane sulfonate (EMS)on antioxidant enzyme activity

EMS concentration (%)	SOD (U/mg protein)	CAT (U/mg protein)	POD (U/mg protein)
Control (0%)	3.85 ± 0.18	6.12 ± 0.22	4.35 ± 0.19
0.5%	4.05 ± 0.19	6.38 ± 0.24	4.55 ± 0.20
1.0%	4.35 ± 0.21	6.72 ± 0.27	4.88 ± 0.22
1.5%	4.60 ± 0.23	7.05 ± 0.29	5.22 ± 0.23
2.0%	4.85 ± 0.24	7.35 ± 0.31	5.55 ± 0.25
LD_{50}(~2.5%)	3.58 ± 0.17	5.80 ± 0.19	4.05 ± 0.18
3.0%	3.20 ± 0.16	5.45 ± 0.17	3.75 ± 0.17
3.5%	2.92 ± 0.15	5.12 ± 0.15	3.40 ± 0.16
4.0%	2.60 ± 0.14	4.75 ± 0.13	3.10 ± 0.15
4.5%	2.38 ± 0.13	4.42 ± 0.12	2.85 ± 0.14
5.0%	2.10 ± 0.12	4.10 ± 0.10	2.55 ± 0.13
SE.d	0.18	0.22	0.19
SE.m	0.12	0.15	0.13
CD (*p*=0.05)	0.30	0.40	0.35

3.3 Secondary metabolites

Phenolics and flavonoids are secondary metabolites that contribute to plant defence mechanisms, antioxidant activity, and stress tolerance. The total phenolic content in control plants was 28.9 mg gallic acid equivalent (GAE)/g DW, while the total flavonoid content was 19.7 mg quercetin equivalent (QE)/g DW. These values increased significantly at 40 Gy (35.8 mg GAE/g DW and 24.1 mg QE/g DW), reflecting an enhancement in secondary metabolite production as a stress response. However, at higher doses, phenolic and flavonoid levels declined, suggesting mutagenic damage to biosynthetic pathways. Phenolic and flavonoid content also showed an initial increase with EMS exposure, peaking at 2.0% EMS (34.7 mg GAE/g DW and 24.0 mg QE/g DW, respectively). However, higher concentrations led to a decrease, indicating impaired secondary metabolism.

Tannin content showed a similar trend, with a steady increase up to 40 Gy, where it reached a maximum of 16.2 mg/g DW compared to 12.5 mg/g DW in the control. Tannins play a crucial role in plant defence and exhibit strong antioxidant properties, suggesting that moderate gamma irradiation enhances their biosynthesis as a protective response. However, at higher doses, tannin content declined significantly, with the lowest recorded value at 100 Gy (6.0 mg/g DW). Tannin content followed a similar pattern, with a steady increase up to 2.0% EMS treatment, reaching a maximum of 15.6 mg/g dry weight (DW) compared to 12.5 mg/g DW in the control. Tannins are known for their antioxidant and antimicrobial properties, and their increased accumulation at moderate EMS concentrations might indicate an adaptive response to stress. However, as EMS concentration exceeded 2.5%, tannin content began to decline, with the lowest value recorded at 5.0% (6.2 mg/g DW). This reduction could be attributed to oxidative damage or impairment of secondary metabolite biosynthetic pathways at higher EMS levels. The decline in sennosides and tannins at higher mutagenic doses suggests that excessive EMS exposure disrupts normal physiological and biochemical processes in Senna.

The exposure of Senna seeds to gamma rays at doses ranging from 10 to 100 Gy led to differential effects on Sennoside A and Sennoside B content. In the control plants, Sennoside A content was recorded at 2.85%, which increased to a peak of 3.40% at 40 Gy, indicating an enhancement in biosynthesis due to low-dose radiation-induced stress. Similarly, Sennoside B content was 1.76% in untreated plants but increased to a maximum of 2.10% at 40 Gy. However, beyond this radiation dose, both sennoside levels showed a declining trend, with the lowest values observed at 100 Gy (1.50% for Sennoside A and 0.85% for Sennoside B). This suggests that while low to moderate gamma irradiation doses stimulate secondary metabolite production, higher doses have a suppressive effect, likely due to DNA damage and metabolic disruptions. Sennoside A and Sennoside B, the primary bioactive compounds responsible for Senna's medicinal properties, displayed a notable increase up to 2.0% EMS concentration, after which their content gradually declined. The control plants exhibited a Sennoside A content of 2.85%, which increased to a peak value of 3.30% at 2.0% EMS. Similarly, Sennoside B content was 1.76% in untreated plants but increased to 2.05% at 2.0% EMS, indicating an enhancement in biosynthesis due to mild mutagenic stress. However, beyond this concentration, a decline in both Sennoside A and Sennoside B was observed, with the lowest levels recorded at 5.0% EMS (1.55% and 0.92%, respectively). This suggests that higher EMS concentrations may have induced metabolic disruptions or inhibited the enzymatic pathways responsible for sennoside biosynthesis (Table 5 and Table 6).

Table 5: Effects of gamma irradiation (Gy) on secondary metabolites

Gamma dose (Gy)	Total phenolics (mg GAE/g DW)	Total flavonoids (mg QE/g DW)	Tannin (mg/g DW)	Sennoside A (%)	Sennoside B (%)
Control (0 Gy)	28.9 ± 1.05	19.7 ± 0.87	12.5 ± 0.45	2.85 ± 0.12	1.76 ± 0.08
10 Gy	30.2 ± 1.10	20.5 ± 0.90	13.0 ± 0.48	2.98 ± 0.13	1.84 ± 0.09
20 Gy	31.8 ± 1.12	21.2 ± 0.95	13.8 ± 0.50	3.12 ± 0.14	1.92 ± 0.10
30 Gy	34.6 ± 1.18	22.8 ± 1.00	14.5 ± 0.52	3.27 ± 0.15	2.01 ± 0.11
40 Gy	35.8 ± 1.20	24.1 ± 1.05	15.3 ± 0.55	3.45 ± 0.16	2.12 ± 0.12
LD_{50} (~50 Gy)	29.5 ± 1.08	20.1 ± 0.88	11.8 ± 0.44	2.75 ± 0.12	1.70 ± 0.08
60 Gy	25.4 ± 1.00	18.6 ± 0.85	10.9 ± 0.42	2.55 ± 0.11	1.58 ± 0.07
70 Gy	23.7 ± 0.98	16.8 ± 0.80	9.5 ± 0.40	2.32 ± 0.10	1.42 ± 0.06
80 Gy	21.5 ± 0.95	15.4 ± 0.75	8.7 ± 0.38	2.10 ± 0.09	1.28 ± 0.05
90 Gy	19.2 ± 0.92	14.1 ± 0.70	7.9 ± 0.35	1.88 ± 0.08	1.12 ± 0.04
100 Gy	17.6 ± 0.90	12.9 ± 0.68	7.2 ± 0.32	1.65 ± 0.07	0.98 ± 0.03
SE.d	1.02	0.85	0.42	0.11	0.08
SE.m	0.64	0.53	0.26	0.07	0.05
CD (*p*=0.05)	2.05	1.72	0.85	0.21	0.16

Table 6: Effects of ethyl methane sulfonate (EMS) on secondary metabolites

EMS concentration (%)	Total phenolics (mg GAE/g DW)	Total flavonoids (mg QE/g DW)	Tannin (mg/g DW)	Sennoside A (%)	Sennoside B (%)
Control (0%)	28.9 ± 1.05	19.7 ± 0.87	12.5 ± 0.45	2.85 ± 0.12	1.76 ± 0.08
0.5%	29.8 ± 1.10	20.4 ± 0.90	13.1 ± 0.48	2.92 ± 0.13	1.82 ± 0.09
1.0%	31.2 ± 1.12	21.3 ± 0.92	13.9 ± 0.50	3.05 ± 0.14	1.90 ± 0.10
1.5%	33.5 ± 1.15	22.6 ± 0.98	14.8 ± 0.53	3.18 ± 0.15	1.98 ± 0.11
2.0%	34.7 ± 1.18	24.0 ± 1.00	15.6 ± 0.55	3.30 ± 0.16	2.05 ± 0.12
LD_{50} (~2.5%)	28.4 ± 1.06	19.9 ± 0.88	11.5 ± 0.43	2.68 ± 0.12	1.66 ± 0.08
3.0%	26.0 ± 1.02	18.2 ± 0.84	10.2 ± 0.41	2.42 ± 0.11	1.48 ± 0.07
3.5%	23.8 ± 0.99	16.7 ± 0.80	8.9 ± 0.38	2.15 ± 0.10	1.32 ± 0.06
4.0%	21.3 ± 0.95	15.1 ± 0.75	7.8 ± 0.35	1.90 ± 0.09	1.18 ± 0.05
4.5%	18.9 ± 0.92	13.6 ± 0.72	7.0 ± 0.33	1.72 ± 0.08	1.05 ± 0.04
5.0%	17.0 ± 0.90	12.2 ± 0.68	6.2 ± 0.30	1.55 ± 0.07	0.92 ± 0.03
SE.d	0.95	0.80	0.45	0.12	0.08
SE.m	0.62	0.50	0.28	0.08	0.05
CD (*p*=0.05)	1.85	1.60	0.92	0.25	0.18

4. Discussion

The study on the biochemical and phytochemical responses of Senna (*C. angustifolia*) to mutagenic treatments using ethyl methane sulfonate (EMS)and gamma irradiation revealed significant variations in secondary metabolites and antioxidant enzyme activities. The differential effects of these treatments suggest that both chemical and physical mutagenesis can influence the biosynthetic pathways of key bioactive compounds, but the extent of these changes depends on the dosage applied (Kumar *et al*., 2023; Sharma *et al*., 2022). Chlorophyll content, a critical indicator of photosynthetic efficiency, showed a slight increase at lower doses of gamma radiation and EMS but declined at higher concentrations. The highest chlorophyll content was recorded at 40 Gy for gamma irradiation and 2.0% for EMS treatment, suggesting that mild stress can stimulate chlorophyll biosynthesis, possibly due to an upregulation of pigment-related genes or enhanced enzymatic activities (Rahman *et al*., 2021; Zhang *et al*., 2020). However, at higher doses, the reduction in chlorophyll levels indicates that excessive mutagenic stress leads to chlorophyll degradation or inhibition of biosynthetic pathways (Ghosh *et al*., 2022). This could be attributed to oxidative damage, structural alterations in chloroplasts, or disruption of pigment-associated enzymes (Ali *et al*., 2023).

Protein content exhibited a similar trend, increasing at moderate doses and decreasing beyond the LD_{50} threshold (~50 Gy for gamma irradiation and 2.5% for EMS). The initial increase in protein levels suggests enhanced metabolic activity, potentially due to an upregulation of stress-responsive proteins and enzymes involved in adaptive mechanisms (Nadeem *et al*., 2021). However, at higher doses, the decline in protein content could be a result of protein degradation, enzyme inhibition, or reduced biosynthesis due to DNA and cellular damage (Das *et al*., 2020; Hasan *et al*., 2023). Carbohydrate content followed a dose-dependent pattern, with an initial increase and subsequent decline at higher mutagenic stress levels. The maximum carbohydrate accumulation at 40 Gy and 2.0% EMS suggests an enhanced photosynthetic activity and carbon metabolism under moderate stress conditions (Sharma *et al*., 2022). However, beyond these levels, the decline in carbohydrate content indicates that higher doses interfere with energy metabolism, possibly by impairing enzymatic activities related to carbohydrate synthesis and storage (Patel and Meena, 2023; Iqbal *et al*., 2021). The reduction at extreme doses could also be due to increased utilization of carbohydrates for stress mitigation processes such as osmo protection and cellular repair (Singh *et al*., 2023). Proline, a well-known osmo protectant and stress marker, exhibited a consistent increase with rising doses of gamma radiation and EMS. The highest proline accumulation at 40 Gy and 2.0% EMS highlights its role in mitigating oxidative and mutagenic stress by stabilizing proteins, membranes, and scavenging reactive oxygen species (ROS) (Mishra *et al*., 2021; Verma *et al*., 2023). The continuous increase in proline levels with higher doses suggests that Senna plants employ osmotic adjustment as a primary defence mechanism against mutagen-induced stress (Gupta and Reddy, 2022).Total phenolic and flavonoid contents showed a similar pattern, increasing at lower doses and declining beyond the optimum threshold. The enhanced production at moderate stress levels suggests that secondary metabolite biosynthesis is activated as a protective mechanism against oxidative stress (Choudhary *et al*., 2020; Wang *et al*., 2023). Phenolics and flavonoids are known to possess strong antioxidant properties, which help neutralize ROS and mitigate cellular damage (Fernandez *et al*., 2022; Kumar *et al*., 2024). However, at excessive mutagenic doses, the decline in these compounds could be due to metabolic disruption, enzymatic inhibition, or diversion of metabolic resources towards survival rather than secondary metabolite synthesis (Yadav *et al*., 2023; Roy *et al*., 2021).

The increase in Sennoside A and Sennoside B content at moderate EMS (2.0%) and gamma irradiation (40 Gy) treatments indicates a stimulatory effect on the metabolic pathways responsible for their biosynthesis (Ali *et al*., 2023). This suggests that controlled stress conditions can enhance the synthesis of these medicinally valuable compounds, likely due to an adaptive response by the plant (Das *et al*., 2021). Previous studies have also indicated that mild stress conditions can upregulate secondary metabolite production as a defence mechanism (Gupta and Singh, 2022). However, at higher doses (above 2.5% EMS and 50 Gy gamma irradiation), the decline in sennoside content suggests that excessive mutagenic stress disrupts enzymatic activities involved in their biosynthesis, possibly through DNA damage or metabolic imbalances (Iqbal *et al*., 2021; Verma *et al*., 2023). Tannins, known for their antioxidant and antimicrobial properties, exhibited a similar trend, with increased accumulation at moderate mutagenic doses followed by a decline at higher concentrations (Mishra *et al*., 2021). The enhanced production of tannins at 2.0% EMS and 40 Gy gamma irradiation could be linked to stress-induced activation of phenylpropanoid pathways, which are responsible for polyphenol biosynthesis (Raza *et al*., 2022). However, at higher doses, oxidative stress may impair enzymatic activities or divert metabolic resources towards stress mitigation rather than secondary metabolite synthesis, leading to reduced tannin levels (Fernandez *et al*., 2022).

The significant increase in antioxidant enzyme activities (SOD, CAT and POD) at moderate doses of EMS and gamma irradiation suggests that these treatments induce oxidative stress, prompting an upregulation of enzymatic defence mechanisms (Patel *et al*., 2023; Wang *et al*., 2023). The peak levels of these enzymes at 2.0% EMS and 40 Gy gamma irradiation indicate that plants can efficiently manage oxidative stress up to a certain threshold. SOD plays a crucial role in neutralizing superoxide radicals, while CAT and POD help in scavenging hydrogen peroxide, preventing cellular damage (Rahman *et al*., 2021). The decline in enzyme activities at higher mutagenic doses suggests that excessive oxidative stress overwhelms the plant's defence mechanisms, leading to enzymatic inhibition or degradation (Singh *et al*., 2023).Statistical analysis further confirms that the observed variations in sennosides, tannins, and antioxidant enzymes were significant, validating the effects of EMS and gamma irradiation on Senna's biochemical profile (Kumar and Roy, 2024). The LD_{50} values for EMS and gamma irradiation (2.5% and 50 Gy, respectively) indicate that these doses represent the threshold beyond which mutagenic stress becomes detrimental to biochemical processes (Sharma *et al*., 2023). Overall, the findings suggest that controlled exposure to gamma irradiation and EMS can enhance the biochemical composition of *Senna* seeds by stimulating secondary metabolite production and stress-adaptive mechanisms (Raza *et al*., 2021; Sharma *et al*., 2023). However, higher doses lead to metabolic suppression, highlighting the importance of optimizing mutagenic treatments for maximum benefits in medicinal plant improvement (Das *et al.*, 2023).

5. Conclusion and future prospects

The present study demonstrates that controlled exposure to EMS and gamma irradiation can significantly influence the biochemical and phytochemical profile of Senna (*C. angustifolia*). Moderate doses (2.0% EMS and 40 Gy gamma irradiation) enhanced chlorophyll, protein, carbohydrate, and secondary metabolite content, indicating a stimulatory effect on biosynthetic pathways. The increased accumulation of sennosides, tannins, flavonoids, and phenolics at these levels suggests an adaptive response that boosts the plant's medicinal properties. However, excessive mutagenic stress (beyond 2.5% EMS and 50 Gy gamma irradiation) led to a decline in these biochemical parameters, likely due to oxidative damage, enzymatic inhibition, and metabolic disruptions. The study also highlights the role of antioxidant enzymes in mitigating oxidative stress, with SOD, CAT and POD showing peak activity at optimal doses before declining at higher concentrations.Future research should focus on refining mutagenic treatment strategies to maximize beneficial phytochemical responses while minimizing adverse effects. Molecular studies exploring gene expression changes in response to EMS and gamma irradiation would provide deeper insights into the regulatory mechanisms of secondary metabolite biosynthesis. Additionally, long-term field trials are necessary to assess the stability and agronomic performance of treated Senna plants under natural conditions. Further investigation into the combined effects of mutagenic treatments with bio stimulants or elicitors may offer new approaches for enhancing medicinal plant productivity. By optimizing these strategies, mutagenesis could be a valuable tool for improving the pharmacological potential of Senna and other medicinal crops.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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