Biochemistry Applications (2023) Volume 1 Issue 1
doi: 10.54517/ba.v1i1.2177

ORIGINAL RESEARCH ARTICLE

Enhancement in secondary metabolites and arbutin content via gamma irradiation elicitation in *Bergenia ciliata* (Haw.) Sternb. callus

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ABSTRACT

In recent times, plants are known to be the novel sources for obtaining pharmacologically active drugs which are produced by them in response to the stress which in turn facilitate mutualistic and antagonistic interactions of the plant with its changing environment. Keeping in view the medicinal importance and vulnerability status of *Bergenia ciliata* in vitro cultures combined with mutagenesis prove to be better option for improvement of this plant. The reddish green callus obtained on Gamborg's half strength medium supplemented with TDZ (2 mg/L) and NAA (2 mg/L) were irradiated with different doses of radiations and evaluated for various parameters. The maximum carotenoid content was found in 0.5 Gy treated callus while as maximum carbohydrates were present in 1.0 Gy treated callus (7.36 mg/g) as compared to non-irradiated callus (6.30 mg/g). The higher doses of gamma radiations (4.0 to 5.0 Gy) showed enhanced carbohydrate levels as compared to non-irradiated callus. The highest protein content was found in 3.0 Gy treated callus (41.1 mg/g) followed by 2.5 Gy treated callus (39.9 mg/g). The methanolic extracts of non-irradiated and irradiated callus were subjected to DPPH radical scavenging activity with maximum activity in 1.0 Gy treated sample (29.44%) followed by 1.5 Gy (23.86%). In the present study, the antioxidant enzyme activities were also evaluated (glutathione reductase, superoxide dismutase and ascorbate peroxidase) with the highest activity in 2.0 Gy treated callus as compared to non-irradiated callus. The dried callus was examined for quantification of alkaloids and tannins. Both alkaloids and tannins were significantly higher in 1.5 Gy treated callus. Gamma irradiations showed overall increase in the phenol content with highest concentration in 0.5 Gy treated callus (0.35 mg/mL). With regard to flavonoids, lower doses of gamma irradiation showed inhibitory effect on the flavonoid synthesis in the present study. The highest flavonoid content was found in 4.5 Gy (0.26 mg/mL) as compared to control (0.08 mg/mL). The maximum arbutin was found in 3.0 Gy treated sample (366.27 µg/g) while as maximum bergenin was found in non-irradiated samples (2.47 µg/g) which was analysed via HPLC.

Keywords: gamma elicitation; DPPH radical scavenging; polyphenols; alkaloids; tannins; flavonoids; arbutin

1. Introduction

In recent times, plants are known to be the novel sources for obtaining pharmacologically active drugs which are produced by them in response to the stress which in turn facilitate mutualistic and antagonistic

ARTICLE INFO
Received: 19 June 2023 | Accepted: 17 August 2023 | Available online: 12 October 2023

CITATION
Rafi S, Kamili AN, Ganai BA, et al. Enhancement in secondary metabolites and arbutin content via gamma irradiation elicitation in *Bergenia ciliata* (Haw.) Sternb. callus. *Biochemistry Applications* 2023; 1(1): 2177. doi: 10.54517/ba.v1i1.2177

interactions of the plant with its changing environment. Plant cell cultures with the help of optimum mutagenic treatments assimilate higher amounts of secondary metabolites via mass growth under controlled conditions bypassing factors like seasonal harvesting and cross contamination in field conditions[1–3]. Keeping in view the vulnerability of *Bergenia ciliata* in its natural habitat[4], other alternative ways are to be utilised to get the benefit from this medicinal plant so that market demand for the drug is fulfilled vis-a-vis reducing the load on natural habitat also. In this regard, in vitro cultures combined with mutagenesis prove to be better option for improvement of vegetatively propagated plants and continues to be a forefront subject for scientific enquiry having implications on health care and also for areas such as agricultural biotechnology[5]. It is considered feasible to introduce phenotypic variations via mutagens in controlled growth systems (e.g., callus culture) for enhancing the secondary metabolites, increasing potency, reducing toxin levels, increasing uniformity and predictability of extracts[1]. The genetic variations obtained by tissue culture techniques are currently becoming a valuable outcome for crop improvement and are considered as valuable resource for understanding the processes governing plant development[4,6]. Ionizing radiations generate reactive oxygen species (ROS) which on reacting with nucleic acids and their precursors cause detectable deletions, major rearrangements and point mutations[7–10] which in turn affect the morphology, anatomy, biochemistry and physiology of plants differentially depending on the irradiation level. Changes like dilation of thylakoid membranes, alteration in photosynthesis, modulation of the anti-oxidative system and accumulation of phenolic compounds has been reported because of interaction of ionising radiations with plant cell systems[11–14]. Numerous reports are available which show genetic variability for several desired characters due to interaction of irradiations with plant cells[15–17]. Gamma rays have gained popularity in recent decades as a novel elicitation technique for the enhanced production of secondary metabolites in plant organ and cell cultures because of convenient operation, short cycle, and high mutation frequency[18,19]. Therefore, the present study was undertaken to evaluate the efficiency of gamma irradiations on the callus of *Bergenia ciliata* in terms of biochemical and phytochemical aspects.

2. Radiation dose and explants selected

The current study was undertaken on the reddish green leaf callus of *Bergenia ciliata* grown on B_5 ½ medium supplemented with TDZ (2 mg/L) and NAA (2 mg/L)[4] after 3rd sub-culturing. The callus was irradiated after two weeks of 3rd sub-culturing just to overcome stress during sub-culturing and was kept under dark conditions post treatment for two weeks to avoid photo-inducible DNA repair so that maximum mutations are being inherited[20]. The series of doses from 0.0 Gy (control), 0.5 Gy, 1.0 Gy, 1.5 Gy, 2.0 Gy, 2.5 Gy, 3.0 Gy, 3.5 Gy, 4.0 Gy, 4.5 Gy and 5.0 Gy were given from Bhaba Atomic Research Centre, Zakura, Srinagar from cobalt-60 source. The irradiated callus was further sub-cultured every 4–6 weeks to get enough material for analysis.

3. Methodology

The biochemical parameters including chlorophyll and carotenoid content, total carbohydrate content, soluble protein content were evaluated using the standard methodology: acetone method[21], phenol-sulphuric method[22] and Bradford's method[23] respectively against the respective standard solutions viz-a-viz glucose solution for carbohydrate estimation and bovine serum albumin for protein estimation. The L-proline content was determined by the method of Bates et al.[24] using standard solution of L-proline (Sigma). DPPH radical scavenging property of extracts was carried out according to the method of Parray et al.[25]. The anti-oxidative enzyme analysis of GR, SOD and APX was done using the standard methodology of Foyer and Halliwell[26], Dhindsa et al.[27] and Nakano and Asada[28] respectively. The standard methodology was also followed for the quantitative analysis of various phytocompounds: Alkloids[29], phenolics[30], tannins[31] and flavanoids[32] using

the standard solutions of rutin for flavonoids and gallic acid for phenols and tannins. The values are taken as mean + S.D., and calculated in triplicate samples.

4. HPLC analysis

Arbutin and bergenin was purchased from Sigma (Germany). All chemicals were of analytical reagent grade. Solvent (methanol) was of HPLC grade and purchased from Ranbaxy Fine Chemicals Limited (Okhla, New Delhi). The instrument used was Thermo finnigan HPLC system consisting of HPLC pump (P 4000), an auto sampler (AS 3000), a column oven, a Diode array detector (UV 6000 LP), Vacuum membrane degasser (SCM 1000) and System Integrator (SN 4000). Chrom-Quest 4.0 software was used for data analysis and processing. The measurements were carried out on C18 column (250 mm × 4.6 mm; particle size 5 µm; Merck, Germany) at 30 ℃.

For sample preparation, callus was shade dried and powdered. 1–2 g of each sample was extracted in a soxhlet with 200 mL methanol. The solvent was removed under reduced pressure to furnish a semi-solid gum. 1 mg from the extract was dissolved in HPLC grade methanol and the resulting solution was filtered through 0.22 µm filter membrane for HPLC analysis. The analysis was carried out using an isocratic solvent system consisting of a mixture of acetonitrile and water (HPLC Grade) (70:30 v/v) and the flow rate was 1.0 mL/min and the pressure was 122 kgf/cm^2. The HPLC method used by Boros et al.[33] has been followed with some modification. The mobile phase composition has been changed to meet the requirements for the development of completely resolved chromatogram. 20 µL of the sample for bergenin and 0.5 µL for arbutin were injected to the system. The detection was made at 280 nm. Using peak height and area of the standards, the amount of arbutin and bergenin was calculated in each sample.

5. Statistical analysis

The experiments were performed in completely randomized block design (CRD). Results were evaluated as mean ± SD. Significant differences ($P < 0.05$) for multiple comparisons were determined by one way ANOVA using SPSS 17.0.

6. Results and discussion

Gamma irradiations have been used by many researchers for crop improvement via somaclonal variants and are reported to improve economically important traits through cytological, biochemical, physiological and morphogenetic changes in cells. The lower exposures are reported to be more stimulating which improved the economically important traits. Moreover, gamma radiations were reported to raise the flavinoids, alkaloids, phenolics and antioxidant activities also[34].

Carotenoids, being photosynthetic pigments as well as endogenous antioxidants, absorb surplus energy and quench active oxygen in addition to protection of chlorophyll by absorption of photon energy. The lower doses of gamma radiations have shown stimulatory effect on the carotenoid content in the present study. The maximum carotenoid content (0.192 mg/g) was found in 0.5 Gy treated callus. With the increase in doses beyond 2.5 Gy, the concentration of carotenoids showed decreased trend (**Figure 1**). Gamma radiations have previously resulted in increase in carotenoid content in *Cajanus cajan* and *Onobrychis viciifolia*[34,35]. Soluble sugars accumulate in plants growing under stress and reduce the internal osmotic potential thus prevent denaturation of macromolecules by helping retain their natural configuration. In the present study, gamma irradiations showed enhanced effect on carbohydrate content of irradiated callus. The maximum carbohydrates were present in 1.0 Gy treated callus (7.36 mg/g) as compared to non-irradiated callus (6.30 mg/g). The higher doses of gamma radiations (4.0 to 5.0 Gy) also showed enhanced carbohydrate levels as compared to non-

irradiated callus (**Figure 1**). This behaviour confirms that increase in osmolyte/osmoprotectant contents being another defensive mechanism via the activation of genes leading to protection for up-regulating enzymes involved in the anabolism of these contents[36]. The proline content was also evaluated in non-irradiated and irradiated samples. Gamma radiations were unable to increase the proline levels in irradiated callus. Few doses (1.0, 1.5, 2.0 Gy) showed proline levels similar to that present in non-irradiated samples (0.45 mg/g) while as rest of doses did not show any proline levels. The results are in agreement with the results obtained in rice calli by Falahati et al.[37] and reason being ascertained that radiations may have increased the level of antioxidants and consequently there would be no need for extra proline to cope with the same problem of oxidative reagents.

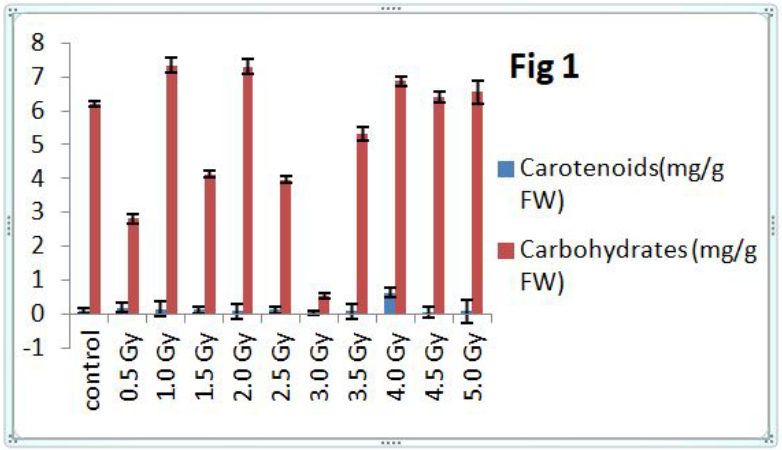

Figure 1. Carotenoids and carbohydrate content of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

Gamma radiations showed overall stimulatory effect on the soluble protein levels of *Bergenia* callus. The highest protein content was found in 3.0 Gy treated callus (41.1 mg/g) followed by 2.5 Gy (39.9 mg/g) treated callus (**Figure 2**). Gamma radiations have shown a linear increase in soluble protein content in *Cajanus cajan* under in vitro conditions with increasing doses of gamma radiation[35]. Gamma radiations have proven successful in increasing protein content in *Eurycoma longifolia* callus also[38]. Gamma irradiation did not induce significant loss in water soluble components such as total soluble proteins[39]. Gamma irradiation has shown to prompt oxidative stress with over production of reactive oxygen species that may damage to macromolecules such as DNA, proteins and lipids. The methanolic extracts of non-irradiated and irradiated callus were subjected to DPPH radical scavenging activity. There was slight increase in the % inhibition in almost all treated samples as compared to non-irradiated samples except 0.5 Gy treated sample which showed low activity as compared to control. The maximum activity was found in 1.0 Gy treated sample (29.44 %) followed by 1.5 Gy (23.86 %) (**Figure 3**). Generation of ROS, particularly H_2O_2 had been suggested to be part of the signalling cascades that conduct to protection from stresses[40]. The general strategy assumed by plants against oxidative stresses is to induce antioxidant enzyme system and over expression of antioxidant production under irradiation has shown enhancement of the anti-oxidative defence[40]. In the present study, the antioxidant enzymes, i.e., GR, SOD, and APX showed overall enhanced activities in irradiated samples, the highest activity of all the above mentioned enzymes shown by 2.0 Gy treated callus as compared to non-irradiated callus (**Figure 2**). The results are in line with the findings of Esfandiari et al.[41] who reported that certain doses of gamma irradiation promotes the level of antioxidants. Gamma radiation at higher doses induce oxidative stress with overproduction of ROS such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, which react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids causing disturbance of cellular metabolism[35].

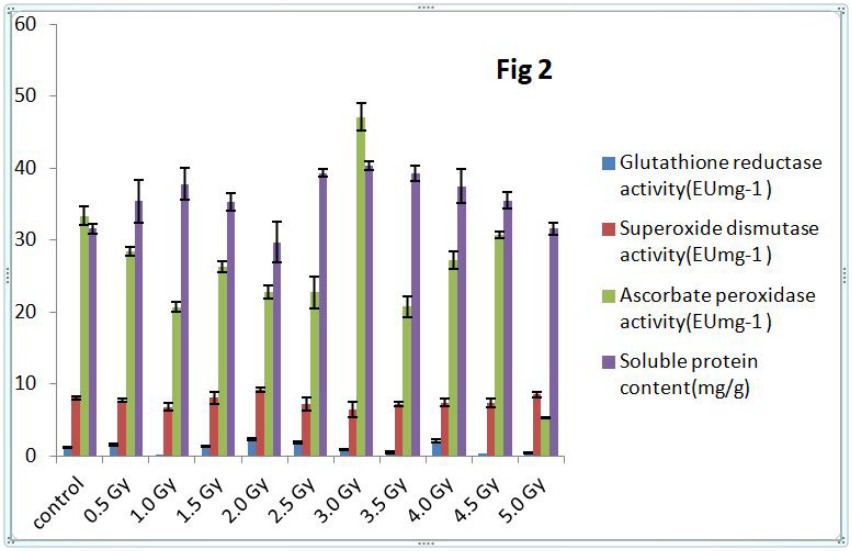

Figure 2. Soluble protein and anti-oxidant enzyme activities of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

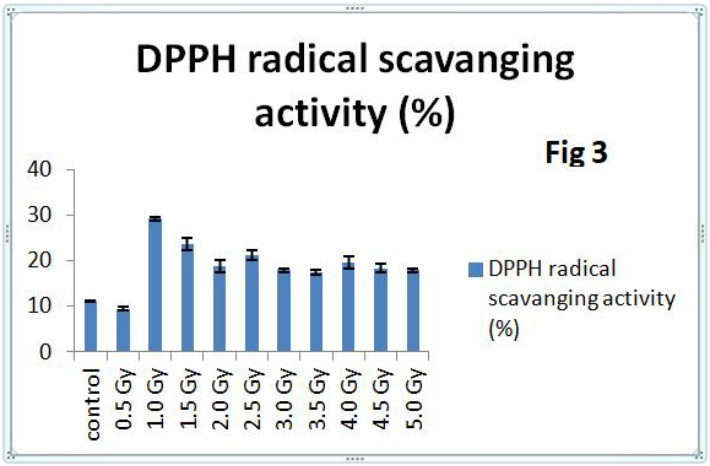

Figure 3. DPPH radical savanging activity of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

Flavonoids are the widespread group of natural phenolics which possess a wide range of biological activities and have high economical and pharmaceutical values. These compounds often accumulate in small quantities and sometimes in specific cells[40]. For quantification of phenolics and flavanoids, the methanol extracts of dried callus were used. Gamma radiations showed overall increase in the phenol content with highest concentration in 0.5 Gy treated callus (0.35 mg/mL DW). With regard to flavinoids, lower doses of gamma radiation showed inhibitory effect on the flavinoid synthesis in the present study. The highest flavinoid content was found in 4.5 Gy which showed highest flavinoid content (0.26 mg/mL DW) as compared to control (0.08 mg/mL) (**Figure 4**). Gamma irradiation is reported to enhance positively total phenolic and flavonoid accumulation in rosemary callus cultures and *Culantro* plants respectively[42,43]. Enhancement of polyphenols and flavinoids via gamma radiations has been reported by many other authors also[3,43–45]. Gamma radiations are reported to enhance the polyphenol and flavinoid content in tissues either by degradation of larger phenolic compounds into smaller ones[43] or due to higher extractability by depolymerization and dissolution of cell wall polysaccharides[46] or stimulate the activity of phenylalanine ammonia lyase, which is responsible for the synthesis of polyphenolic acids[44]. The dried callus was examined for quantification of alkaloids and tannins. Both alkaloids and tannins were significantly higher in 1.5 Gy treated callus. The alkaloids were significantly low in all other treatments as well as in non-irradiated callus (**Figure 5**). Patil et al.[47] studied elicitation via gamma irradiations in *Artemisia annua* callus cultures and the study showed enhanced antioxidant activity,

phenol and flavanoid content at low dose of gamma irradiation from 5 to 25 Gy whereas high dose such as 30 and 35 Gy shown inhibitory effect on biological activity. Total phenolic content and total flavonoid content were also enhanced in quiona flour irradiated with gamma irradiations[48]. A higher level of different types of metabolites, including phenolics, flavonoids, terpenoids, and alkaloids, has been reported in many medicinal plants by γ-irradiation[3].

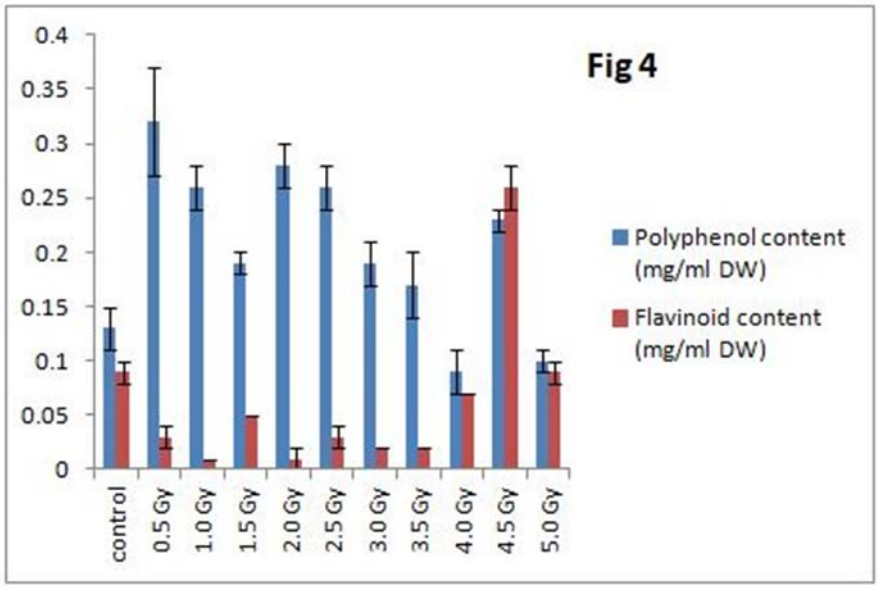

Figure 4. Polyphenol and flavinoid content of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

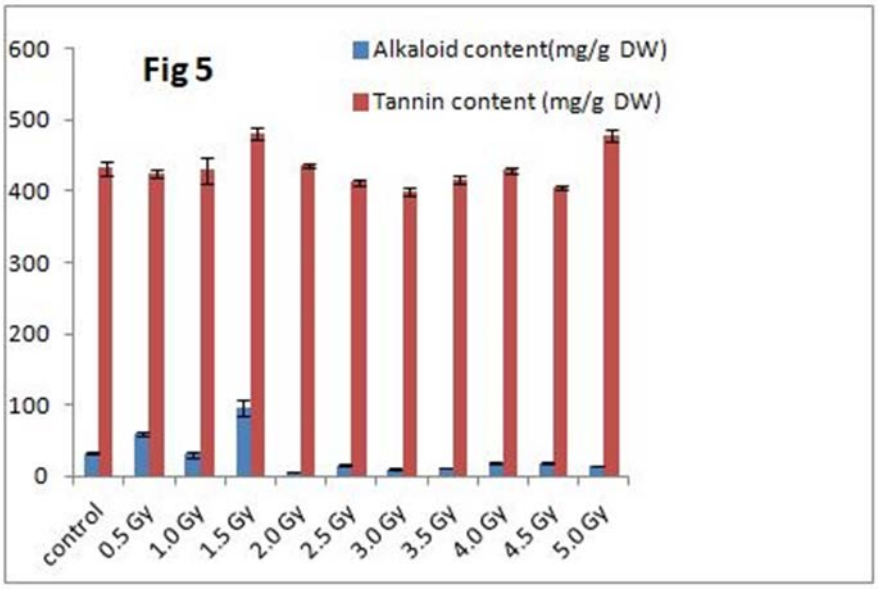

Figure 5. Alkaloid and tannin content of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

The methanol extracts of both non-irradiated as well as irradiated callus were subjected to HPLC analysis for arbutin and bergenin content. Both these compounds are very important medicinal compounds present in *Bergenia ciliata*. The present doses of irradiation showed enhancement in the concentration of arbutin in samples with maximum concentration found in 3.0 Gy treated samples (366.27 µg/g) as compared to control (228.52 µg/g) (**Figure 6**). Regarding the bergenin, the maximum concentration was found in non-irradiated/ control samples (2.47 µg/g) while as irradiated samples showed lesser concentrations than irradiated ones. Enhancement in medicinally important secondary metabolites via gamma irradiation elicitation have been

studied by various authors. Patil et al.[47] reported ten-fold increase in artemesinin content at 15 Gy dose (7.04 µg/gm dry callus weight) compared to non-treated sample (0.700 µg/gm) in *Artemisia annua*. While studying effect of gamma radiations on callus and shoots of *Gentiana kurroo* Royle, Alphonse et al.[19] revealed that low doses favoured growth and showed increased gentiopicroside content in the callus cultures during M_1V_1 generation, but both parameters were reduced in the M_1V_2, M_1V_3 and M_1V_4 generations. However, shoots irradiated at low doses boosted the growth and GPD level during M_1V_2, M_1V_3 and M_1V_4 generations. *Panax ginseng* mutant lines were developed with high biomass and ginsenoside content using gamma irradiation of adventitious root cultures by Le et al.[18]. Also, it was revealed that long-term callus and adventitious root cultures of *P. ginseng* were more sensitive to gamma rays than short-term cultures.

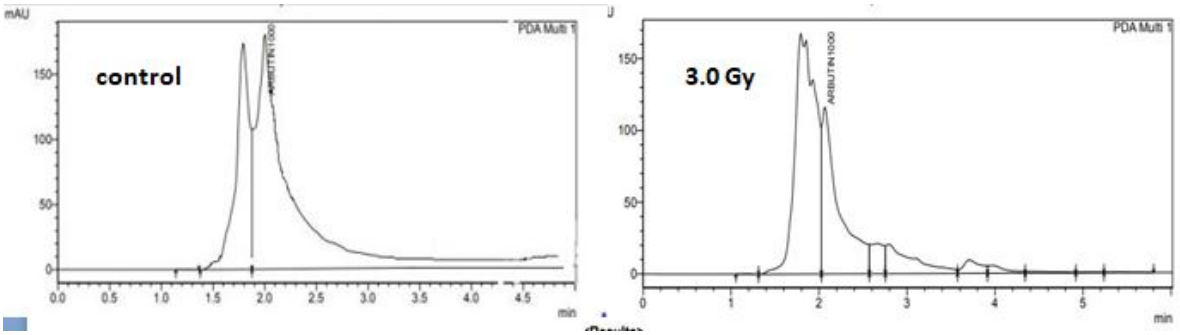

Figure 6. HPLC analysis of arbutin of gamma irradiation treated callus of *B. ciliata* (Haw.) Sternb.

7. Conclusion

In conclusion, it can be said that gamma irradiation doses employed in the current study succeeded in inducing superior cell types with significant alterations in metabolism of the cells regarding the enhancement of arbutin. The work requires further investigation in terms of using slightly higher doses of γ-irradiation for enhancing the bergenin concentration from this medicinal plant so that commercial requirement of drugs can be fulfilled via this technique.

Author contributions

Formal analysis, SR and SJ; writing—original draft preparation, SR; writing—review and editing, NAW; supervision, ANK and BAG; project administration, ANK and BAG. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The study was supported by DST, GOI, New Delhi, INSPIRE fellowship vide letter no. DST/INSPIRE-Fellowship/2014/21 Dtd. 15/09/2014, the assistance of which is highly acknowledged. The author wishes to thank Director, CORD, University of Kashmir for providing laboratory facilities and the necessary support. The author is thankful to Peerzada Raashid Hussain, Nuclear Research Laboratory, Bhaba Atomic Research Centre, Zakura, Srinagar for providing gamma irradiation facility.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

TDZ	Thidiazuron

NAA	Napthalene acetic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
GR	Glutathione reductase
HPLC	High performance liquid chromatography

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