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Evaluation of Effect of Gamma Radiation on Total Phenolic Content, Flavonoid and Antioxidant Activity of *In Vitro* Callus Culture of *Artemisia annua*

Anita Surendra Patil[1*], **Pooja Suryavanshi**[1] **and Devanand Fulzele**[2]

[1]*Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India*

[2]*Department of Plant Biotechnology and Secondary Metabolites Section, Bhabha Atomic Research Centre, Mumbai, India*

[*]**Corresponding author:** Anita Surendra Patil, Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India, Tel: 91- 9881735354; E-mail: anitapatil@sgbau.ac.in

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Abstract

Gamma irradiations are physically induced stresses that has been considered as the rapid and fast method to enhance the quality and quantity of plant characteristics. Phenolic compounds and flavonoids possess a wide range of biological activities such as antioxidant, anti-inflammatory, anti-aging, anti-bacterial and anti-tumor functions and have high economical and pharmaceutical values. In current study *In vitro* callus cultures were exposed to gamma irradiation at different doses 5, 10, 15, 20, 25, 30 and 35 Gy. After 25 days of gamma irradiation the gamma irradiated callus (M0T0) culture were further subcultured on 0.5 mg/L napthalein acetic acid (NAA) and benzyl amino purine (BAP) till M5T5 generation. The free radical scavenging activity of gamma irradiated methanolic callus extract was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method. The highest free radical scavenging activity was found in 15 Gy dose of M2T2 generation which is 7.1 ± 0.29 mg mL^{-1} as compared to control which is 6.71 ± 1.15 mg ml^{-1}. Irradiation dose of 15 Gy showed highest amounts of flavonoid content 103.78 mg ml^{-1} in M2T2 generation. Gamma irradiation dose of 15 Gy showed high amounts of phenols that is 10.64 ± 1.08 mg/gm as compared to control which is 1.87 mg/g of dry callus.

Keywords: *Artemisia annua;* Callus culture; Gamma irradiation; Antioxidant activity; Total phenol; Total flavonoid

Introduction

Gamma irradiations are the physically induced stress that has been considered as the rapid and fast method to enhance the quality and quantity of plant characteristics and it has been widely used in plant biology as an elicitor as effects from low dose stimulation to high-dose inhibition [1]. Bioactive compounds such as phenolics and flavonoids can be induced under proper dose of radiation. Phenolic compounds and flavonoids possess a wide range of biological activities such as antioxidant, anti-inflammatory, anti-aging, anti-bacterial, and anti-tumor functions [2,3] and have high economical and pharmaceutical values. g-irradiation enhances the production of ROS in a variety of cells resulting in oxidative stress. ROS are the by-products of many degenerative reactions, which will affect the regular metabolism by damaging the cellular components. oxidative stress demonstrated that exposure of plants or cell cultures to adverse environmental conditions induces the production of ROS, such as superoxide radical (O^{-2}), H_2O_2 and hydroxyl radical in plant cells. The plant evolved cellular adaptive responses like up-regulation of oxidative stress protectors and accumulation of protective solutes. Plants exposed to g-radiation produces various defense and anti-oxidant enzymes many of which produce secondary metabolites which in turn activates the induction of oxidative stress conditions. Gamma irradiation exerts its effects by inducing oxidative stress in the cells which increases the phenolics due to the release of phenolic compounds from glycosidic components and the degradation of complex phenolics into simple ones. These free radicals may break the glycosidic bonds of procyanidin trimers, tetramers and hexamers that leading to the formation of procyanidin monomers, which increase the total phenolic content. However, the key enzyme in the flavonoid biosynthesis is the chalcone synthase (CHS) which catalyzes the formation of chalcones from malonyl-coA and coumaroyl-coA. Chalcones are the important intermediate in the flavonoid pathway which gives rise to various classes of flavonoids. Phenolic compounds are the metabolites that can be derived from pentose phosphate, shikimate and phenylpropanoid pathways [4,5] most importantly they work as an antioxidant compounds having the ability to scavenge free radicals and inhibits the oxidative stress in plant. They play an important role by interacting with signal transduction pathways and cell receptors [6,7]. Free radicals, reactive nitrogen species (RNS), and other reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide are an entire class of highly reactive species derived which can be generated from normal oxygen metabolism. According to oxidative damage to most cellular molecules induced by ROS is responsible for several human diseases such as cancer, cardiovascular diseases, atherosclerosis, Alzheimer diseases and ageing [8,9]. The current research shows the effect of g-irradiation on *in vitro* callus cultures of *A. annua* and to investigate the effect of gamma radiation on total phenolic, flavonoids content and antioxidant activity of the metanolic extract of gamma irradiated callus culture.

Materials and Methods

Collection of plant materials

Artemisia annua variety CIM-Arogya. Received from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, by Material transfer Agreement (MTA) between SGBAU and CIMAP.

***In vitro* seed germination:** The seeds of *A. annua* was washed thoroughly with sterile distilled water. It was rinsed in 2% aqueous $HgCl_2$ solution for 5 min, again it was washed with sterile distilled water three times. It was inoculated on Murashig and Skoog medium containing 2% sucrose and kept in plant growth chamber at 24°C.

Establishment of callus cultures and media condition: Leaves of size (0.5-1.0 cm long) were excised from 4-week old *in vitro* germinated seedling and cultured on M.S. medium supplemented with 0.5 mg/l of NAA (Napthalin acetic) and BAP (Benzyl amino purine) and 3% w/v sucrose. The pH of the medium was adjusted to 6.8 ± 0.2. Cultures were incubated at 25 ± 1°C under 16/8 h light and dark photoperiod conditions. The cultures were maintained on same media compositions and sub-cultured after every 4 weeks.

Gamma irradiation to callus cultures: Callus induced from *A. annua* leaf was exposed to gamma irradiation for elicitation purpose at BARC, Mumbai. The 4 week old callus were placed in irradiation chamber of the gamma cell and it was irradiated at different dose level of g-irradiation (5, 10, 15, 20, 25, 30 and 35 Gy. at room temperature (25 ± 1°C). g-Irradiation was performed using a gamma cell 4000 apparatus equipped with a 60Co g-source (dose rate, 0.23 Gy/s) at Bhabha atomic research centre Trombay, Mumbai. The gamma-irradiated callus cultures were labeled as G0M0 generation. After 25 days of gamma irradiation the g-irradiated callus (M0T0) culture were subcultured on freshly prepared M.S. medium containing 2% sucrose and 0.5 mg/L napthalein acetic acid (NAA) and benzyl amino purine (BAP). It was labeled as M1T1 generation of callus. Non treated (non g-irradiated) callus were also kept and labeled as control. After M1T1 generation the same protocol was followed after 30 days for subculture of M2T2, M3T3, M4T4 and M5T5 generation [10].

Methanolic extraction of gamma irradiated callus culture: *In vitro* callus of M1T1, M2T2, M3T3, M4T4 and M5T5 generations were harvested and analyzed for the determination of antioxidant activity, total phenol and flavonoid contents. Dried callus (1 gm) were powdered in mortor and pistel and mixed with 10 ml of methanol and centrifuged at 10,000 rpm. Supernatant was removed and again diluted to 1 mg/ml with methanol and used for further study.

Determination of antioxidant activity of gamma irradiated callus: DPPH Assay and evaluation of IC90: The free radical scavenging activity of gamma irradiated methanolic callus extract was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method. Different concentration of callus extract was prepared such as 10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100 µg respectively in 96 well plate and volume was make upto 100 µl by methanol. 150 µl 10 mM DPPH was added in each reaction and incubated for one hour and absorbance was measured at 517 nm. Ascorbic acid was used as standard [11].

% Inhibition was calculated using formula

$$\% \text{ Inhibition} = \frac{(\text{Ab of Control} - \text{Ab of sample})}{\text{Absorbance of control}} \times 100$$

Quantitative estimation of total flavonoid content in gamma irradiated callus: Quantitative estimation of total Flavonoid content from gamma irradiated callus extract was determined by using (Biotek Synergy H1 Microplate reader) aluminium chloride method in 96 well plate. 100 µl of callus extract was taken in 96 well plate and 10 l of aluminium chloride was mixed with it followed by 10 µl of potassium acetate was added and volume was make up with 100 µl of distilled water and absorbance was measured at 435 m. Quercetin was used as standard [12].

Quantitative estimation of total phenol content in gamma irradiated callus: Quantitative evaluation of total phenol content of gamma irradiated callus extract was determined by using (Biotek Synergy H1 Microplate reader) Folin-Ciocalteu method in 96 well plate. 100 µl of each extract was added in well followed by 100 µl of Folin-Ciocalteu reagent was added, then 50 µl (2.5%) aqueous Na_2CO_3 was added an absorbance was measured at 765 nm. Gallic acid was used as standard [13].

Results

Antioxidant activity of gamma irradiated callus by DPPH assay

Antioxidant activity of methanolic extract of gamma irradiated callus was determined by DPPH Assay. Free radical scavenging is one of the mechanisms of inhibiting free radicals formation in plants. The DPPH free radical inhibiting activity was represented in terms of ascorbic acid equivalent to mg/g of dry callus. The Y equation obtained from the calibration curve is y=0.409x+55.74 R^2=0.872 as shown in Figure 1.

(%) Inhibition and IC90

The 90% inhibitory concentration (IC90) was used to measure the percent Free Radical Inhibitory Activity of methanolic extract of gamma irradiated callus. IC90 of the standard ascorbic acid was found 83.76 mg/mL^{-1}. The highest free radical scavenging activity was found in 15Gy gose of M2T2 generation which is 7.01 ± 0.29 mg/mL^{-1} as compared to control which is 6.17 ± 1.15 (Figure 2 and Table 1).

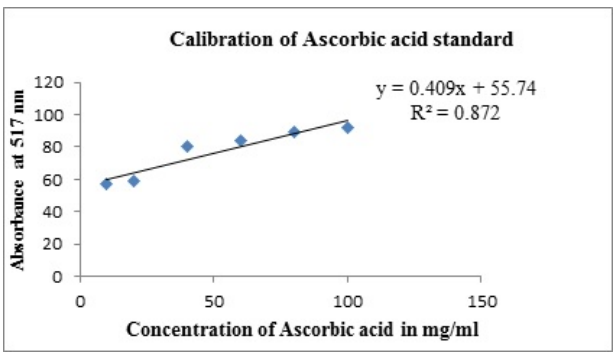

Figure 1: Calibration graph of Ascorbic acid (1 mg/ml).

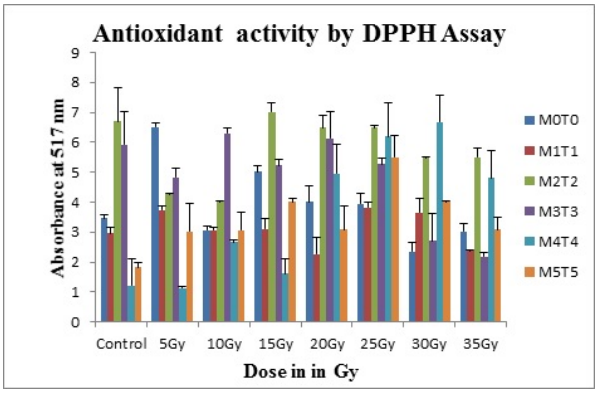

Figure 2: Antioxidant activity of callus culture by DPPH assay in different doses of gamma irradiation from M1T1 to M5T5 generation in mg/dry weight of callus. The results were obtained from triplicate determinations Mean (± SE).

Estimation of total flavonoid content (µg/ml) in gamma irradiated callus

Flavonoids are secondary metabolite having antioxidant properties [14]. The total flavonoid content was expressed in terms of quercetin equivalent in mg/ gm of dry callus. The calibration curve obtained from y equation y=0.023x+0.010 R^2=0.999 was used to determine Flavonoid content as shown in Figure 3. The total flavonoid content of the various extract of gamma irradiated callus are represented in Figure 4 and Table 2. Irradiation dose of 15 Gy showed highest amounts of flavonoids content 103.78 ± 0.8 in M2T2 generation.

	M0T0	M1T1	M2T2	M3T3	M4T4	M5T5
Control	3.47 ± 0.12	2.97 ± 0.19	6.71 ± 1.13	5.92 ± 1.11	1.22 ± 0.91	1.82 ± 0.16
5 Gy	6.51 ± 0.12	3.74 ± 0.15	4.27 ± 0.01	4.84 ± 0.31	1.14 ± 0.03	3.02 ± 0.92
10 Gy	3.6 ± 0.16	3.06 ± 0.09	4.04 ± 0.01	6.31 ± 0.19	2.67 ± 0.08	3.06 ± 0.62
15 Gy	5.01 ± 0.19	3.11 ± 0.36	7.01 ± 0.29	5.23 ± 0.19	1.61 ± 0.49	4.02 ± 0.11
20 Gy	4.02 ± 0.54	2.26 ± 0.58	6.5 ± 0.39	6.13 ± 0.91	4.95 ± 0.97	3.08 ± 0.81
25 Gy	3.95 ± 0.34	3.83 ± 0.19	6.5 ± 0.08	5.27 ± 0.21	6.19 ± 1.12	5.51 ± 0.71
30 Gy	2.36 ± 0.28	3.65 ± 0.49	5.5 ± 0.01	2.71 ± 0.91	6.68 ± 0.91	4.02 ± 0.04
35 Gy	3.02 ± 0.28	2.37 ± 0.03	5.5 ± 0.29	2.8 ± 0.12	4.8 ± 0.91	3.09 ± 0.41

Table 1: Antioxidant activity (mg Ascorbic acid/g) of dried gamma irradiated callus culture.

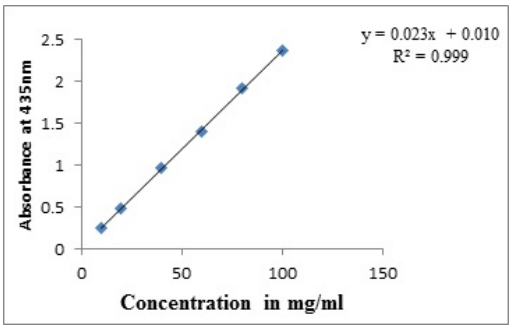

Figure 3: Calibration graph of Quercetin (1 mg/ml).

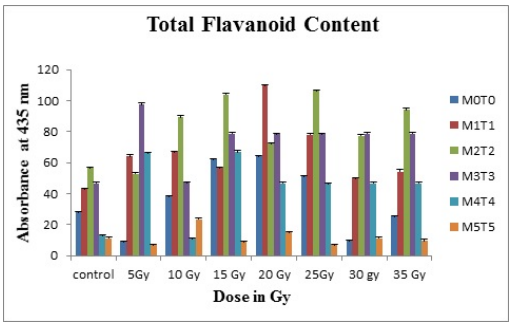

Figure 4: Total Flavonoid content of callus culture in different doses of gamma irradiation from M1T1 to M5T5 generation in mg/g of dry weight of callus. The results were obtained from triplicate determinations Mean (± SE).

	M0T0	M1T1	M2T2	M3T3	M4T4	M5T5
control	28.39 ± 0.08	43.18 ± 0.4	56.62 ± 0.5	46.48 ± 0.97	12.82 ± 0.8	10.92 ± 0.9
5 Gy	8.87 ± 0.50	64.11 ± 0.8	52.85 ± 0.6	97.47 ± 0.9	65.95 ± 0.39	6.89 ± 0.3
10 Gy	38.27 ± 0.17	67.13 ± 0.2	89.25 ± 0.9	46.86 ± 0.4	11.09 ± 0.24	23.44 ± 0.7
15 Gy	61.88 ± 0.27	56.64 ± 0.4	103.78 ± 0.8	78.61 ± 0.82	66.72 ± 0.94	8.87 ± 0.10
20 Gy	64.39 ± 0.16	109.80 ± 0.5	72.31 ± 0.2	50.41 ± 0.3	46.66 ± 0.9	15.27 ± 0.1
25Gy	51.11 ± 0.39	78.03 ± 0.59	106.11 ± 0.6	47.36 ± 0.4	40.56 ± 0.4	6.68 ± 0.20
30 gy	9.73 ± 0.01	50.0 ± 0.39	77.37 ± 0.83	43.91 ± 0.7	59.12 ± 0.5	11.09 ± 0.8
35 Gy	25.44 ± 0.11	54.19 ± 1.4	93.91 ± 0.92	78.13 ± 0.9	30.21 ± 0.7	9.48 ± 0.90

Table 2: Total Flavonoid content (mg Qurcetin/g) of dried gamma irradiated callus culture.

Quantitative estimation of total phenol content in gamma irradiated callus

Phenolics are important constituents of plants which are responsible for scavenging the free radical due to the presence of hydroxyl groups and it directly responsible for the antioxidative potential [15]. In current study total phenol content is represented in terms of gallic acid equivalent in milligram (mg) per gram of dry callus. The calibration curve (the standard curve: y=0.015x+0.359 R^2=0.821) was used to determine total phenolic content as shown in Figure 5. High values of phenol content were found in 15 Gy of M2T2 generation mg GAE/g. Gamma irradiation dose of 15 Gy showed high amounts of phenols that is 10.64 as compared to control which is 5.96 ± 1.49 mg/g of dry callus (Figure 6 and Table 3).

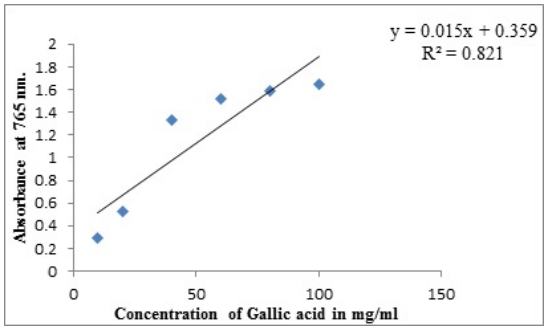

Figure 5: Calibration graph of Gallic acid standard (1 mg/ml).

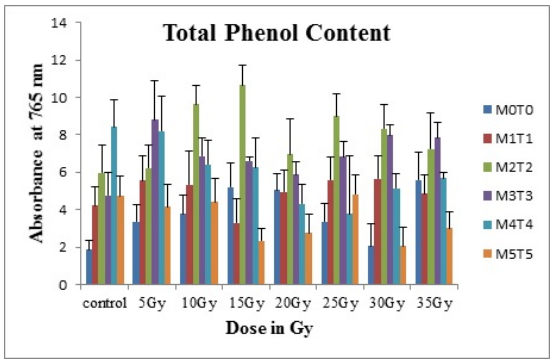

Figure 6: Total phenol content of callus culture in different doses of gamma irradiation from M1T1 to M5T5 generation in mg/dry weight of callus. The results were obtained from triplicate determinations Mean (± SE).

	M0T0	M1T1	M2T2	M3T3	M4T4	M5T5
control	1.87 ± 0.5	4.22 ± 1.02	5.96 ± 1.49	4.76 ± 1.2	8.41 ± 1.45	4.71 ± 1.10
5 Gy	3.36 ± 0.9	5.55 ± 1.30	6.22 ± 1.2	8.79 ± 2.1	8.19 ± 1.86	4.14 ± 1.20
10 Gy	3.76 ± 1	5.32 ± 1.8	9.64 ± 1.02	6.84 ± 0.98	6.40 ± 1.29	4.40 ± 1.29
15 Gy	5.19 ± 1.3	3.30 ± 1.30	10.64 ± 1.08	6.60 ± 0.23	6.25 ± 1.59	2.32 ± 0.69
20 Gy	5.03 ± 0.88	4.93 ± 1.20	6.97 ± 1.9	5.86 ± 0.67	4.32 ± 1.02	2.75 ± 0.99

25 Gy	3.36 ± 0.99	5.58 ± 1.20	8.99 ± 1.2	6.84 ± 0.78	3.7 ± 3.10	4.83 ± 0.99
30 Gy	2.05 ± 1.2	5.64 ±1.2	8.32 ± 1.3	7.97 ± 0.56	5.15 ± 0.80	2.04 ± 0.98
35 Gy	5.57 ± 1.5	4.85 ± 0.98	7.24 ±1.9	7.85 ± 0.83	5.68 ± 0.30	2.98 ± 0.89

Table 3: Total phenol content (mg GAE/g) of dried gamma irradiated callus culture.

Discussion

The increased antioxidant activity, total phenol and flavonoid content of *A. annua* callus culture at a low dose of gamma irradiation might be due to the formation of free radicals, which shown to have greater antioxidant effect. In current study low dose of gamma irradiation from 5 to 25 Gy have shown the positive effect whereas high dose such as 30 and 35 Gy shown inhibitory effect on Biological activity. In M2T2 generation 15 Gy dose shown highest biological activites such as antioxidant, total phenol and flavonoid when compared with control. Low dose of gamma irradiation induces the breakdown of glycosides and releases phenolic compound which increases the antioxidant activity [16,17]. Whereas higher dose of gamma irradiation generates a flux of free radicals which causes more deleterious effects and decreases antioxidant activity [18]. Improvement in the genetic trait of plant by using gamma irradiation needs an essential study to distinguish how it can affect plant cell [19]. It is reported that high gamma doses had a negative influence on the morphology of tomato and okra seedlings [20]. Some researchers reported that high dose of gamma irradiation is inhibitory, where as low doses of gamma irradiation could be used as safer and ecofriendly tools to improve quality of plants [21,22]. Some scientists stated that low doses of gamma irradiation stimulates the growth by changing the hormonal influence in the plant cells. In addition, low dose improved the antioxidative capability of plant cell [23].

Conclusion

The current study conclude that low doses of gamma irradiations are ecofriendly and a low- cost method that can be used for the elicitation of total phenol, flavonoid and antioxidant activity. In the present study, it was found that low dose of gamma irradiation are effective strategy to enhance the yield of phytochemical content of *A. annua*. The highest free radical scavenging activity by DPPH method was found at 15 Gy dose of M2T2 generation which is 7.01 ± 0.29 mg/mL^{-1} as compared to control which is 6.71 ± 1.15 mg/mL^{-1}. The highest amounts of flavonoids contents were found at 15 Gy dose which is 103.78 ± 08 mg/mL^{-1} in M2T2 generation as compared with control which is 56.62 ± 0.5. Gamma irradiation dose of 15 Gy showed high amounts of phenols that is 10.64 ± 1.08 mg/g as compared to control which is 5.96 ± 1.49 mg/g of dry callus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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