The Effect of Gamma Irradiation on *In vitro* Total Phenolic Content and Antioxidant Activity of *Ferula gummosa* Bioss.

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Received: 20 Jan. 2016 **Accepted:** 2 May 2016

Abstract

Background: ***Ferula gummosa*** **Boiss. is a medicinal and economical plant belonging to Apiaceae family. It is a native herb of Iran and is in serious danger of extinction.**

Objective: **Gamma irradiation is an environmental stress and can consider as a new tool to change the content of secondary metabolites in medicinal plants. The effect of gamma irradiation on phenolic content and radical scavenging activity of *F. gummosa* callus was investigated in this research.**

Methods: **Embryogenic axes were removed from seed coats and were cultivated on ½ Murashige and Skoog medium. After 2 weeks, roots were used as source of plant material for callus induction. Root explants were transferred into MS medium containing NAA (1.5, 3 and 4.5 mg/L) and BA (0, 0.5, 1 and 2 mg/L), and the best medium of callus establishment was selected for gamma irradiation. Sub-cultured calli were irradiated at different doses of gamma irradiation from 0 to 25 Gray (Gy).**

Results: **The obtained data showed that MS medium supplemented with 1.5 mg/L NAA and 2 mg/L BA was the best medium for callus induction. Under gamma irradiation, a significant decline was observed in the fresh and dry weight of irradiated-calli as compared to control one. Phenolic content increased under gamma irradiation especially at 20 and 25 Gy, and the best dose was 20 Gy according to growth response. Aqueous extract showed higher phenolic content than methanol extract. The 50% inhibitory concentration (IC_{50}) obtained in the DPPH radical test decreased significantly under gamma irradiation espacially at 20 and 25 Gy.**

Conclusion: **According to the obtained results, gamma radiation could increase radical scavenging activity by induction of phenolic compounds, and the aqueous extract identified as a proper extraction solvent for free radical scavenging activity in *F. gummosa*.**

Keywords: ***Ferula gummosa*****, Callus tissue, Gamma irradiation, Plant growth regulators, Total phenolic content**

Introduction

Gamma irradiation is one of the environmental stresses that has considered as a rapid and new method to change the qualitative and quantitative characteristics of plants for several years, and has widely used in biology and medicine in terms of biological effects from low dose stimulation to high-dose inhibition [1]. Low-dose ionizing irradiation can affect on cell growth, proliferation, germination rate, enzyme activity as well as stress resistance [2, 3]. Gamma radiation interacts with atoms and molecules to create oxidative stress with overproduction of reactive oxygen species [4], that are able to modify important components of medicinal plant cells [5].

Bioactive compounds also can promote 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 [6, 7] and have high economical and pharmaceutical values. These compounds often accumulate in small quantities and sometimes in specific cells [8]. Gamma irradiation at 20 Gy enhanced positively total phenolic and flavonoid accumulation in rosemary callus culture [9]. Doses of 10 Gy gamma irradiation increased phenolic acid content in cinnamon [10].

Ferula gummosa Boiss. (Apiaceae), known as Barijeh is a perennial plant native to central Asia, and grows in the northern and western parts of Iran [11, 12]. In Iranian traditional medicine, the gum obtained from the aerial parts of Galbanum has been used for stomach pain, chorea, epilepsy and as a wound ointment [11, 12]. Several reports have been previously showed that the extract and essential oil of *F. gummosa* have an anti-nociceptive, antibacterial [15] and antioxidant activities [16]. According to our knowledge there is no information about effect of gamma irradiation on antioxidant activity of *Ferula gummosa* callus. Hence, the present study was designed to investigate the effect of different doses of gamma radiation on growth, phenolic content and antioxidant activity of *F. gummosa* callus.

Materials and Methods

Plant materials

The mature seeds of *F. gummosa* were prepared by Isfahan Pakan Bazr Company and were washed thoroughly under running tap water. The soaked seeds were treated with 70% ethyl alcohol for 2 minutes and rinsed with sterile water three times. The seeds were then surface sterilized in 2.5% sodium hypochlorite solution for 20 minutes and were washed three times in succession with sterile water.

Embryo culture

Embryogenic axis was separated from seed coats and were cultivated on ½ MS [17] medium supplemented with 30 $g1^{-1}$ sucrose and 7 gl^{-1} agar. The cultures were incubated at 23 ± 2 °C under a 16-hour lighting and 8-hour darkness photoperiod with 80 µ Mol m^{-2} s^{-1} irradiance provided by cool fluorescent lamps. The cultures were used as a source of plant material for the establishment of explants before initiating the experiments.

Callus induction

Root explants from two weeks old seedlings were cultured on MS medium, treated with 0, 1.5, 3, 4.5 mg/L α-naphthalene acetic acid (NAA) and 0, 0.5, 1, 2 mg/L benzyl adenine (BA) for callus induction. After 6 weeks of cultivation, callus formation was evaluated. All the calli obtained were subcultured into medium containing the same concentrations of regulators for callus proliferation.

Irradiation treatment

Irradiation of the calli was conducted in the Dosimetry Laboratory, Atomic Energy Organization of Tehran, using a Gamma cell 220 Excel Irradiator. The source of gamma rays was Cobalt 60 (60 Co). Prepared calli were acutely irradiated at dose levels of 10, 15, 20, 25 Gy at room temperature (25 ± 1°C). After 3 weeks, callus fresh weight were measured, calluses were placed in oven with 34 °C and after 3 days callus dry weight were measured.

Extract Preparation

The irradiated calluses were placed in oven at 30° C and dried for chemical analysis. 10 mg dried calluses were milled and eroded with 10ml of desired solvent (methanol and 0.2 M acetate buffer, 5/5 = pH, and water) after that, calli were pulverized in a mortar. The mixture was centrifuged (1 minute and G1500) and supernatant was filtrated subsequently by No. 1 filter paper, wrapped in foil and were placed in the dark at 4°C.

Total phenol content measurement

Total phenolic content was measured using a spectrophotometer (Spectronic Genesys-5, Thermo Electron, USA) by Folin-Ciocalteu colorimetric method [18]. Samples (100 µl of each methanolic or aqueous extract) were mixed with 1480µl deionized water and 40µl Folin-Ciocalteu reagent. After 8 minutes, 300µl of 20% sodium carbonate were added and mixed together. After the mixture has been allowed to stand for 60 minutes at room temperature, the absorbance was measured at 740 nm. All the measurements were taken in triplicates and mean values were calculated. Concentration of total phenolics in both extracts (control and irradiated) was determined and results were expressed as miligram of gallic acid equivalents per 1 gram of dry weight (mg GAE /g DW).

DPPH radical scavenging activity

Radical scavenging effects of the extracts were determined based on reducing the 2, 2-diphenyl-2-picryl hydrazyl (DPPH) solution. Dried extracts of each sample were used for this test. Different concentrations of the callus extract (8.3, 16.6, 33.2, 66.4 and 99.6 µg/ml of extract equivalent to 50, 100, 200, 400 and 600 µl of extract solution, respectively) were added to 2 ml of a methanol solution of DPPH. 300 µl of a 0.2 mM DPPH methanol solution was added to 2.7 ml of different concentrations sample solution. A solution containing methanol and sample solutions was used as blank and 300µl DPPH solution plus 2.7 ml of methanol was used as negative control. Because DPPH is sensitive to light, it was exposed to the minimum possible light and allowed to react for 30 minute. The absorbance values were measured at 517 nm. Absorbance of each sample was converted into the radical scavenging activity (RSA) percent using the following equation:

$$\text{RSA\%} = \left(\frac{1-(Sample\ absorbance-Blank\ absorbance)}{Control\ absorbance}\right) \times 100$$

The IC_{50} was calculated by linear regression of plots. The required concentration of samples to scavenge 50% of DPPH (IC50) were reported [17].

Statistical analysis

The experiments were carried out by one-way analysis of variance (ANOVA) using SPSS, version 18 (Chicago, IL, USA). The data were subjected to analysis of variance as a factorial experiment laid out in a completely randomized design with three replications with four NAA and four BA as the two factors. The means comparisons were separated by Fisher's least significant difference (LSD) at 0.05 levels of probability.

Results

Callus induction and growth measurement

The embryos cultured on ½ MS medium devoid of any plant growth regulator were germinated normally (Fig. 1A), and developed into seedlings with roots and shoots without any callus formation. Roots derived from 14-days-old seedlings cultured on MS medium with different levels of NAA and BA for callus induction. The optimum callus induction (about 94.5%) was obtained at 1.5 mg/L NAA and 2 mg/L BA (Table 1), and the first sign of callus growth from root explants was noticeable between 7-10 days after the sub-culturing in the fresh medium (Fig. 1B). Callus induction was initiated above the surface of the root explant (Fig. 1 B, C). The calli were light and watery with soft surface (Fig. 1C).

The callus from root explants became brownish after 6 weeks in medium with more than 3 mg/L NAA or BA alone. Also calli induced in MS basal medium looked dark brown after one week of culture. Callus formation was significantly influenced by the concentration of growth regulators (Table 1). The medium supplemented with 1.5 mg/L NAA and 2 mg/L BA was the best medium for callus formation of root explants (Table 1). There was a significant interaction between BA and NAA for callus induction in *F. gummosa* (Table 2).

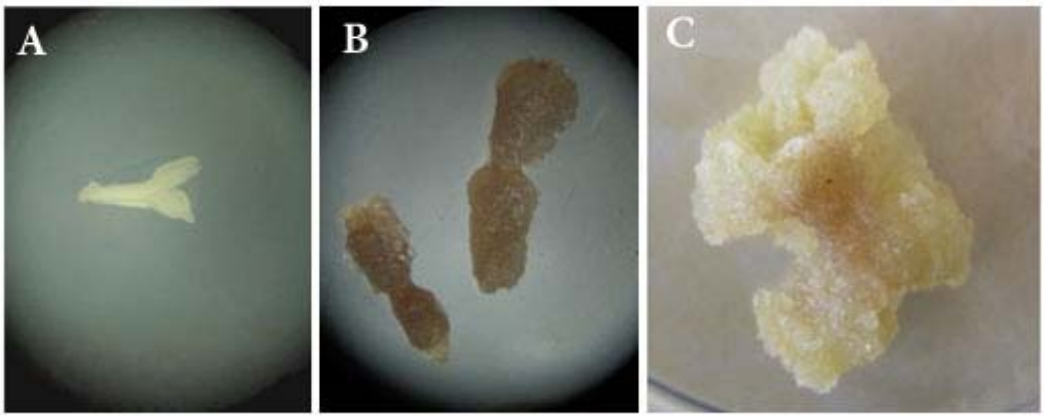

Fig. 1- Embryogenic axis of *F. gummosa* seed (A), and callus induction of root explants in 0.5 mg/L BA after 7 days (B), and 1.5 mg/LNAA and 2 mg/L BA after 6 weeks (C)

Growth response of callus was significantly influenced by irradiation dose. A significant decline was observed in fresh and dry weight of irradiated calluses compared to control. The lowest fresh and dry weights were obtained at 25 Gy, and a 15.6 and 48.7% decrease were observed for fresh and dry weight as compared to control, respectively (Table 3).

Table 1- Effects of different concentrations of NAA and BA on callus induction of root explants in *F. gummosa*

Growth regulators (mg/L)		Callus induction (%)
NAA	BA	
0	0	6.4 ± 1.12 ^{g}
0	0.5	25.3 ± 3.82 ^{ef}
0	1	48.7 ± 4.25 ^{cd}
0	2	45.9 ± 3.92 ^{d}
1.5	0	31.8 ± 2.12 ^{e}
1.5	0.5	41.2 ± 4.25 ^{d}
1.5	1	66.6 ± 3.57 ^{b}
1.5	2	94.5 ± 5.50 ^{a}
3	0	25.9 ± 4.76 ^{e}
3	0.5	38.2 ± 3.44 ^{d}
3	1	51.3 ± 2.93 ^{c}
3	2	29.4 ± 4.45 ^{e}
4.5	0	21.7 ± 5.36 ^{ef}
4.5	0.5	34.7 ± 6.63 ^{de}
4.5	1	27.3 ± 4.37 ^{e}
4.5	2	12.6 ± 2.74 ^{f}

Mean ± SE based on three replicates with 5 callus pieces in each are presented. Different letters indicate significant differences at P ≤0.05 (15 replications for each treatment).

Table 2- Analysis of variance for callus induction in *F. gummosa*

S.O.V.	df	Mean Square
BA	3	2636.16***
NAA	3	1802.60**
BA × NAA	9	795.92***
Error	32	1

: significant at P ≤ 0.01, *: significant at P ≤ 0.001

Table 3- Effect of gamma irradiation on fresh and dry weight of *F. gummosa*

Gamma irradiation (Gy)	Fresh weight (g)	Dry weight (g)
0	5.1±0.35^{a}	0.43±0.12 ^{a}
10	4.9±0.25 ^{a}	0.34±0.15 ^{bc}
15	4.2±0.22 ^{b}	0.35±0.13 ^{b}
20	4.3±0.11 ^{b}	0.36±0.12 ^{b}
25	4.04±0.11 ^{b}	0.22±0.11 ^{c}

Mean ± SE based on three replicates are presented. Different letters indicate significant differences at P ≤ 0.05

With increasing of gamma radiation dose, the color of callus turned darker and became brown and the structure of the callus tissue was relatively poor compared to control (Fig. 2 B-D).

Total phenolic compounds content

Total phenolic content of *F. gummosa* calli were affected by different doses of gamma irradiation is illustrated in Table 4. Gamma irradiation increased phenolic significantly content in *F. gummosa* calli, and the highest content was observed in aqueous extract as compared to methanolic extract. With the increase of gamma doses, total phenolic content increased, and 20 and 25 Gy showed a 36.5 and 38.9% increase of total phenolic content as compared to control, respectively (Table 4).

Radical scavenging activity

The DPPH and IC50 value of the gamma irrigated and control callus extracts are presented in Fig. 3. Gamma irradiation increased DPPH content significantly and the highest DPPH was found at 20 Gy (Fig. 3 A). In contrast to DPPH, IC50 content decreased significantly under gamma irradiation. The lowest IC50 was obtained at 20 and 25 Gy, and a 54.4 and 51.2% decrease of IC50 were observed as compared to control, respectively (Fig 3. B).

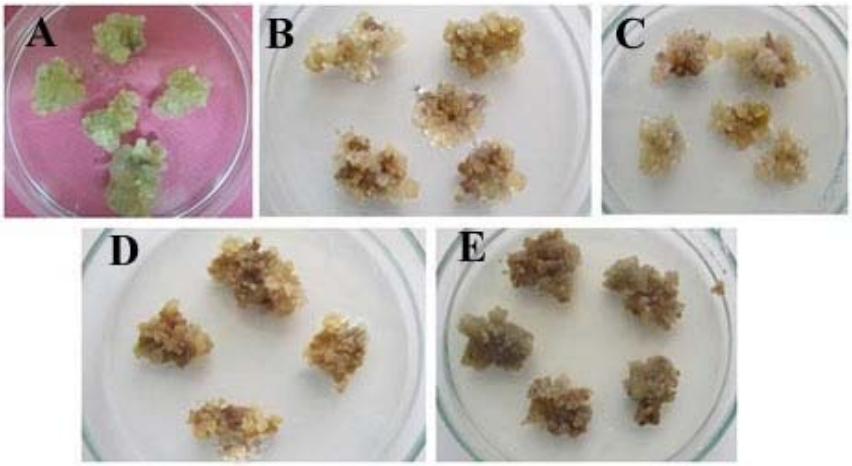

Fig. 2- Effect of gamma radiation on callus morphology on MS medium containing 1.5 mg /L NAA and 2 mg/L BA in control (A), 10 (B), 15 (C), 20 (D) and 25 (E) Gy

Table 4- Effect of gamma irradiation on total phenolic content of *F. gummosa*

Gamma irridation (Gy)	Phenolic content (mg GAE/g DW)	
	Methanolic extract	Aqueous extract
0	5.12±0.35 ^{b}	6.14±0.12 ^{c}
10	5.27±0.25 ^{b}	6.27±0.15 ^{c}
15	5.62±0.22 ^{b}	6.78±0.13 ^{bc}
20	6.41±0.11 ^{a}	9.68±0.12 ^{a}
25	6.93±0.11 ^{a}	10.06±0.11 ^{a}

Mean ± SE based on three replicates are presented. Different letters indicate significant differences at $P \leq 0.05$

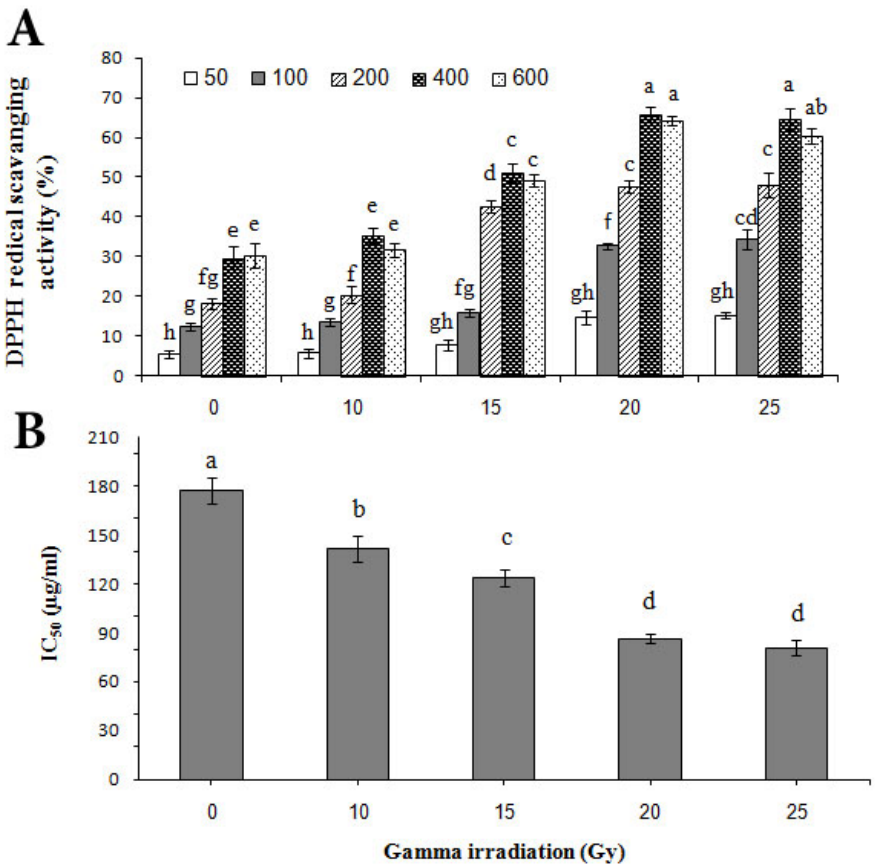

Fig. 3- Effect of different gamma irradiation doses (0, 10, 15, 20 and 25 Gy) on DPPH (A) and IC50 values (B) of *F. gummosa* calluses

Discussion

Embryo culture requires separating and growing an immature or mature zygotic embryo under sterile conditions on a medium to obtain a seedling and overcome the seed dormancy. In the present study, maximum callus induction was observed in 1.5 mg/L NAA and 2 mg/L BA (Table 1). Presence of NAA and BA in the medium for callus formation of *F. gummosa* was previously reported [20]. The calluses became brownish in medium with high concentration of NAA, that may be due to existence of the secondary metabolites and effective compounds in callus tissues [21].

Gamma irradiation significantly decreased callus fresh and dry weights with the increase of gamma doses (Table 3). Similar results have been previously observed in *Gerbera jamesonii* [3], *Zea mays* [22], *Nicotiana tabacum*, *Antirrinum majus* [23] and *Helianthus annus* [24]. Gamma irradiation can affect cell membrane and endogenous auxin in plants [20]. Kiong *et* al (2008) showed that gamma radiation caused breakdown or lack of growth regulator synthesis, especially the cytokinin and auxin, and finally reduction of growth parameters [25].

Solvent extraction significantly affected the concentration of total phenols in control and

irradiated *F. gummosa*. Aqueous extract showed higher phenolic content as compared to metanolic extract. It can be inferred from these results that methanol is the most efficient solvent for extracting phenolic compounds from *F. gummosa* calli. Phenolic content increased with increase of gamma radiation dose. Therefore, the 20 and 25 Gy showed the higher phenolic content in constant to control (Table 4). Increased total phenols and flavonoids in irradiated plants was also reported by El-Beltagi at al. (2011) in rosemary [9]. Gamma irradiation can induce the release of phenolic compounds from glycosidic components and degradate the larger phenolic compounds into smaller ones, and finally can increase total phenolic content [26]. Irradiation causes radiolysis of water and help to the production of free radicals such as hydroxyl radicals, hydroperoxide radicals and hydrated electrons. These radicals may break the glycosidic bonds of procyanidin trimer, tetramer and hexamer that are present in plants, which increase the total phenolic and total flavonoids content in irradiated plants [27]. In the other hand, Abou-Zeid *et al*. (2014) found that the increase in phenylalanine ammonia lyase (PAL) concentration and activity was happened in *Triticum aestivum* L. seedlings with increasing the doses of γ-irradiation [28]. PAL is a key enzyme in the first step of the phenylpropanoid pathway responsible for the synthesis of plant phenylpropanoids or phenolics [29]. Increased phenolic content under gamma radiation could relate to change in enzyme activity of phenolic biosynthesis pathway [30].

Gamma irradiation increased DPPH content and decreased IC50 significantly in *F. gummosa* calli (Fig. 3). The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant [31]. Lower IC50 indicates to higher DPPH free radical scavenging activity. It seems that the decrease of IC50 by gamma irradiation especially at 20 and 25 Gy could be due to induction of polyphenolic compounds under gamma irradiation. Gamma irradiation was shown to prompt oxidative stress with over production of reactive oxygen species that may damage to macromolecules such as DNA, proteins and lipides [32]. Generation of ROS, particularly H_2O_2 had been suggested to be part of the signaling cascades that conduct to protection from stresses. Induction of antioxidant enzyme activities was reported to be a general strategy assumed by plants to overcome oxidative stresses [33]. In plant tissues, many phenolic compounds are potential antioxidants: flavonoids, tannins and lignin precursors may work as ROS-scavenging compounds [35]. Over expression of antioxidant production under irradiation can show the enhancement of the antioxidative defence.

Conclusion

Results showed that aqueous extract of *F. gummosa* calli possesses significant antioxidant activity as compared to its methanolic extract. Gamma irradiation decreased growth parameters significantly, and increased phenolic content. Treatment of 20 and 25 Gy showed the highest phenolic

content, while growth at 20 Gy was higher than 25 Gy. Then, 20 Gy can be used as tools for phenolic induction in *F. gummosa* calli. Gamma radiation decreased IC50 content and increased the free radical scavenging activity with decrease of IC50. Thus gamma radiation could be useful to improve secondary metabolite induction in *F. gummosa*. However, the elaborated mechanism in which secondary metaboilte induced under gamma radiation remains unclear and needs to be investigated further.

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