U. of K. J. Agric. Sci. 20(1), 98-112, 2012

Effect of Gamma Irradiation on Some Biochemical Components of Seed Extracts and Microbial Load of Fenugreek (*Trigonella foenum-graecum* L.) Seeds*

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Abstract: The work was carried out to study the effects of gamma irradiation on microbial load and the content of some biochemical constituents of fenugreek (*Trigonella foenum-graecum*) seeds. It was conducted at the Department of Botany and Agricultural Biotechnology, Faculty of Agriculture, University of Khartoum and Institute of Botany, Technische Universität Dresden. Seeds were treated with gamma irradiation at the doses of 5, 10 and 15 kGy. Bacterial load was reduced from 1.44 x 10^6, in untreated seeds, to 1.5 x 10^4, 9.0 x 10^3 and 5.0 x 10^2 cfu/g, in seeds treated with5, 10 and 15 kGy, respectively. The stability of glycosides, phenols and tannins constituents of treated seeds was evaluated using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The results of TLC analysis revealed that application of 15 kGy doses of gamma radiation to fenugreek seeds methanol-extract had no effect on glycosides patterns, and slight reduction in total phenol and increase in tannins content were observed. Analysis of methanol extractable compounds by HPLC revealed variations in the metabolite profile at different wavelengths after irradiation. While the content of several compounds increased slightly after gamma irradiation that of some decreased and some constituents showed no change. Accordingly, there were no major differences in chemical constituents between the irradiated and un-irradiated samples.

Key words: Gamma irradiation; fenugreek; glycosides; phenols; tannins; microbial load

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*Part of a Ph.D. thesis by the first author, the University of Khartoum, Sudan

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INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is an annual plant belonging to the family Leguminosae. It is cultivated predominantly in Asia, the Mediterranean, and North Africa. The seeds are commonly used in oriental countries as condiment and seasoning in food preparations due to their strong flavour and aroma (Eidi *et al.* 2007). It is also used in some countries as a traditional medicine for treatment of many diseases such as abdominal disturbance and diabetes. Fenugreek seed contains 5% to 6% saponins and 2% to 3% alkaloid, and potential biologically-active components, including galactomannan, steroidal saponins, and 4-hydroxyisoleucine (Rao *et al*. 1996).

Fenugreek seeds, like other plant materials, could carry various microbial contaminants due to the environmental factors (soil, air and water), during pre-harvesting, post-harvesting and handling or transport and may, thus, pose different hazards to the consumer (Soriani *et al*. 2005). Seeds can have microbial populations as great as millions per gramme. The most common bacteria are *Bacillus* spp., *Clostridium* spp. and *Salmonella* spp. (McKee 1995). The major method of decontamination of plant materials is fumigation with gaseous ethylene oxide, whose use is now prohibited or being increasingly restricted in most advanced countries for health, environmental or occupational safety reasons (Uijl 1992). Currently, the use of gamma irradiation is one of the most effective techniques of microbial decontamination of dried herbs and plant materials (Migdal *et al*. 1998). However, the use of this technique may involve changes in the chemical constituents of seeds.

Fenugreek seeds contain a number of compounds, such as flavonoids and glycosides, which have received increasing attention for their potential role in the prevention of human diseases as well as in food quality improvement (Chattak and Ihsanullah 2009). The plant phenolics are of considerable interest in the field of food chemistry, pharmacy and medicine due to a wide range of favourable biological effects including antioxidant properties (Kaviarasan *et al*. 2007). Fenugreek seed extract is reported to have appreciable anti-cancer and anti-diabetic activities (Amin *et al.* 2005).

For the emerging pharmaceutical industry in Sudan and, also, for export purposes, decontamination of plant material using gamma irradiation may enhance their value. There are few reports on microbial loads and the characteristics of irradiated fenugreek seeds commonly used for medicinal purposes in Sudan, especially when high irradiation doses (up to 15 kGy) are employed for decontamination. The objective of this study was, therefore, to investigate the effect of different doses of gamma radiation on the microbial load and some biochemical constituents of these seeds mainly flavonoids and glycosides.

MATERIALS AND METHODS

Seeds of fenugreek (*Trigonella foenum-graecum* L**.)** were purchased from the local market in Khartoum, Sudan. The seeds were cleaned, and freed from any extraneous matters, and kept in polyethylene bags. All the solvents and other chemicals used were of analytical grade, and the solutions were prepared with bi-distilled water.

Irradiation treatment

Samples (50 g each) packed in biaxially oriented polypropylene bags were irradiated at different doses of gamma irradiation (0, 5, 10, and 15 kGy), at room temperature and atmospheric pressure, using a cobalt-60 source (Nordion gamma cell 220-Excell) at the Sudan Atomic Energy Commission, Khartoum. The activity of the source was 6.345 Kci and the energy 1.25 MeV. At a dose rate of 5 kGy h^{-1}, the irradiation time was 1, 2 and 3 hours for the 5, 10 and 15 kGy, respectively. Irradiated and non-irradiated samples of fenugreek seeds were ground to pass through a 0.4 mm screen and kept in glass bottles at room temperature for analysis.

Determination of microbiological load

For microbiological analysis, estimation of the total viable count of bacteria on all irradiated fenugreek seeds in comparison with the control was carried out using the pour plate count method (Harrigan 1998). Ten grammes of the powdered sample were weighed, under aseptical conditions, and homogenized in 90 ml of sterile diluents (0.1% peptone solution) to give a 1:10 dilution (10^{-1}). Five test tubes each containing exactly 9 ml of sterile diluents were prepared. One ml of the dilution 10^{-1} was aseptically removed and transferred to a tube containing 9 ml of the

diluents yielding the dilution 10^{-2}. The procedure was repeated to prepare serial dilutions up to 10^{-6}. From each of the six dilution tubes, 1 ml was transferred aseptically to each of six sterile empty Petri dishes beginning with the dilution 10^{-6}. To each plate 15 ml of sterile plate count agar were added. The plates were then incubated at 37° for 48 hours. The plates containing between 30 and 300 colonies were counted using a colony counter (Scientific and Cook Electronics L.T.D, United Kingdom, London)**,** then the viable count of bacteria in 1 ml of the original sample was calculated from the colony count and the respective dilution. The results were presented as colony forming units per g dry weight (cfu/g).

Preparation of seed extract

Fifty grammes of each plant sample were macerated, using 500 ml of 80% methanol. The mixture was allowed to shake for 4 hours, and then further incubated without shaking for 72 hours. The extracts were filtered, using Whatman No. 1 filter paper. The solvent was evaporated under reduced pressure, using a rotary evaporator (Büchi Labortechnik GmbH, Switzerland). The extracts were placed in Petri dishes and then air-dried until the solvent was completely evaporated. The different dried extracts were stored at 4°C. The dried extracts were further processed by addition of 1 ml of 80% methanol to 1 g of the crude extract, and then 4 ml of methanol were added to obtain a suitable dilution (1:5) for the samples. From these samples, a 1:10 dilution was prepared using 80% methanol which was stored at -20°C.

Glycosides and flavonoids analysis

Thin layer chromatography was used to analyze glycosides, according to Slimestad *et al.* (1994) and flavonoids according to Nitzsche *et al.* (2004) using silica gel plates 20×20 cm (Silica gel F254, Merck, Darmstadt, Germany). From the extract of fenugreek seeds, 10 µl were spotted on the plate. The thin layer chromatography (TLC) solvent system for glycosides was butanol/acetic acid/H_2O (4:1:1; v/v/v) and for flavonoids was formic acid/H_2O/ethyl acetate (6:9:90; v/v/v). For both methods, the plate was dried for 10 minutes at 100^0C-105^0C and then sprayed with diphenylboryloxy-ethylamine (0.25 g DPBA in 25 ml methanol). After 30 minutes yellow-brown spots were visualized under UV light at 366 nm.

Total phenol analysis

The total phenol content of the material sample was determined according to the method described by Makkar *et al*. (2007). For preparing the phenol standard curve, 0, 1, 2, 3, 5 and 10 ml of the gallic acid stock solution (500 mg/100 ml) were added into 100 ml volumetric flasks, and then diluted to the final volume with water followed by detection with Folin-Ciocalteau micro method. These solutions had final concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid. From each calibration solution, 20 µL were pipetted into separate cuvettes, and to each 1.58 ml water and 100 µL of Folin-Ciocalteau reagent were added, and then 300 µL of sodium carbonate solution after 8 minutes. The cuvettes were shaken until mixing, and then left at 2 ºC for 2 hours. The absorbance for each solution was determined at 765 nm against the blank (the "0 ml" solution) using a spectrophotometer (UNICOM UV/Vis spectrometer), then plotted against concentration. A calibration curve was created with the standards and used to determine the levels in the samples. Twenty µL from each plant sample were pipetted into separate cuvettes and, thereafter, treated in the same manner as for the calibration solutions. The results are reported as gallic acid equivalents. The observed concentrations were multiplied by the dilution factor (1:30).

Analysis of tannins

The concentration of tannins in the sample was estimated according to the protein precipitation method described by Hagerman and Butler (1978). For the preparation of standard curve, 50 to 300 µl of catechin solutions were taken and the volume adjusted to 875 µl with buffer C (Five ml of 5% triethanolamine (v/v) and 5g sodium dodecyl sulphate were dissolved in 100 ml of H_2O, and the pH adjusted to 9.4 with HCl). One hundred and twenty-five microlitres of the ferric chloride reagent (0.2703 g $FeCl_3$ was dissolved in 1 ml HCl (0.01 N), and 99 ml H_2O) was added and mixed. A zero tannin sample was made with 875 µl buffer C and 125 µl of ferric chloride reagent. The absorbance value was recorded at 510 nm using a spectrophotometer (UNICOM UV/Vis spectrometer).

The standard samples and the zero tannin were incubated at room temperature for 10 minutes, then the absorbance was determined. The samples were diluted with buffer B in the ratio of 1:1 (e.g. 750 µl sample: 750 µl buffer B) (Buffer B was 5g/L potassium sodium-tartrate tetrahydrate $C_4H_4KNao_6.H_2o$, 12% EtOH, pH adjusted to 3.3 with HCl). For each sample, 1 ml of the protein solution was pipetted into a microfuge tube with 500 µl of the diluted sample and incubated for 15 minutes with slow agitation (300 rpm) in a thermo-mixer (Eppendorf, Hamburg, Germany). The samples were centrifuged for 5 minutes in micro-centrifuge at 14 000 rpm (Eppendorf, Hamburg, Germany). The supernatants were carefully poured off, retaining the pellet in the microfuge tube. Two hundred and fifty micro litres (250 µl) of buffer A (washing buffer, 200 mM acetic acid, 170 mM NaCl, pH adjusted to 4.9 with NaOH) were slowly added to the pelleted samples, and then centrifuged for 1 minute (14 000 rpm). The last three steps were repeated to wash the pellet a second time. The supernatants were poured off and 875 µl of buffer C added, and the tubes were incubated for 10 min in the thermo-mixer (950-1000 rpm) for complete mixing. Ten minutes after dissolving the pellet, the absorbance was read at 510 nm and the value was recorded. Then, 125 µl of ferric chloride reagent were added, the sample mixed, incubated for 10 minutes and the absorbance was re-read at 510 nm. Values are given as mg/L Catechin equivalents.

HPLC separation of metabolites

The high performance liquid chromatography (HPLC) profiles, representing the chemical constituents of fenugreek seeds as influenced by gamma irradiation, were determined from the seeds extract (Diluted 1:10 in methanol). The profiles were determined using an HPLC system [Jasco PU 2080 plus pumps and Jasco DE 2080-53 degasser; coupled to a Jasco AS-1550 auto sampler, with a 4 × 250 mm reverse phase column Hyper clone ODS C_{18} 5µ (Phenomenex, Aschaffenburg, Germany), and a Multi-wavelength Diode Array Detector (Jasco MC-919)]. The HPLC equipment was linked to a computer (IBM ThinkPad) using the Jasco LC-Net II/ADC. For software, the Jasco ChromPass Chromatography Data System software was used (Jasco, Gross-Umstadt, Germany). A linear gradient of aqueous 1% acetic acid and 100% methanol was used. The gradient was started with 20% methanol/80% aqueous acetic acid and run

within 70 minutes to 100% methanol. Then, the initial conditions were re-established over a period of 10 minutes, and samples were analyzed at different wavelengths. The wavelength of the highest absorption was used for the chromatograms.

Statistical analysis

Each sample was analyzed in triplicate following a factorial design. Data were assessed by the analysis of variance as described by Sndecor and Cochran (1987).

RESULTS AND DISCUSSION

Effect of irradiation on the microbial load of fenugreek seeds

Gamma irradiation at 5, 10 and 15 kGy caused significant reduction in microbial load of fenugreek seeds proportionate to the delivered dose (Fig.1). The reduction was 1.5 x 10^4, 9.0 x 10^3 and 5.0 x 10^2 cfu/g, for 5, 10 and 15 kGy, respectively. Little information in the literature is available on the effect of gamma irradiation on the microbial load of fenugreek seeds. Reduction in microbial load of other plant materials following gamma irradiation was reported by some workers. Al-Bachir (2007) reported that a dose of 10 kGy is sufficient for microbial sterilization of aniseeds. Kim *et al.* (2000) found that gamma irradiation at 5–10 kGy inactivated contaminating microorganisms in twenty-one kinds of Korean medicinal herbs.

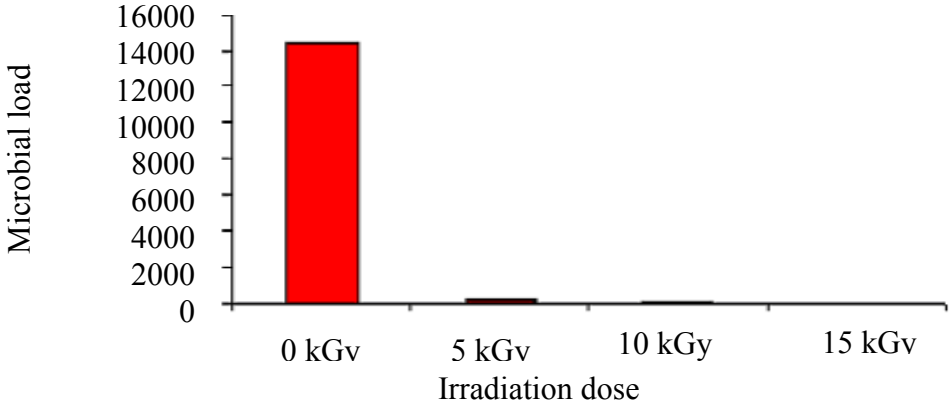


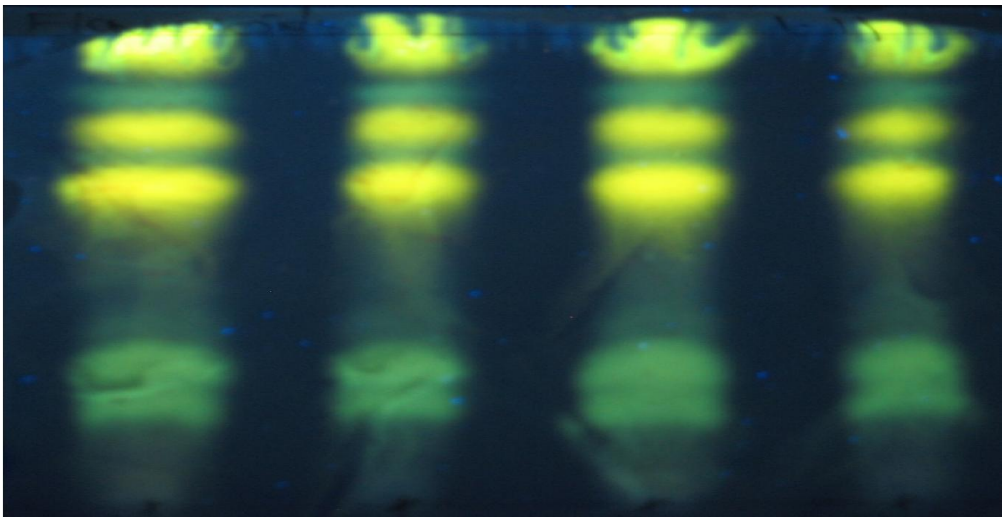
Fig. 1. Microbial load (CFU×$10^2 g^{-1}$) of fenugreek seeds irradiated with different doses of gamma radiation

Effect of irradiation on glycosides and flavonoids of fenugreek seeds

Selected groups of compounds were investigated for possible qualitative and quantitative changes after irradiation in seeds. Total glycosides and flavonoids were chosen because of their known beneficial properties, i.e. antioxidants (Kaviarasan *et al*. 2007). Fig. 2 shows the effect of gamma irradiation on the glycosides profile of fenugreek seeds. The results indicated no differences in the Rf (Rate of flow) values among the irradiated and un-irradiated samples of seeds in the qualitative analysis of glycosides. Similarly, the pattern of flavonoid aglycones was not altered (data not shown). These results support previous findings by some other workers. Oliveira *et al.* (2009) reported that the total isoflavone content in plant materials remained almost unchanged with the increase of radiation dose up to 10 kGy. On the other hand, Chatterjee *et al*. (2009) reported that gamma-radiation processing resulted in dose dependent breakdown of phenyl glucopyranoside, which accounted for 90% of the total glycosides in fenugreek seeds, with a reduction of almost 30% at a dose of 10 kGy.

Effect of irradiation on total phenol and tannins of fenugreek seeds

Fig. 3 illustrates the effect of the application of gamma radiation dose of 15 kGy on the concentration of total phenol and tannins in fenugreek seeds. The results revealed no significant change in total phenol and a slight increase in tannins content. The result for total phenol support the finding of Koseki et *al*. (2002) who reported that total phenol content showed a small change in rosemary after gamma irradiation of 10 kGy. However, Variyar *et al*. (1998) reported that a 10 kGy dose of radiation induced significant increase in some phenolic acids and significant decrease in others, while some others remained unchanged. The contradictory reports may have either resulted from differences in concentrations of the metabolites or, possibly, differences in the irradiation protocols. Harrison and Were (2007) found an increase in tannin content of irradiated clove and nutmeg that have appreciable amounts of hydrolysable tannins compared with the compressed tannins present in cinnamon and other spices. The increased tannin content in the present study may have, thus, been due to an increase in hydrolysable tannins.



P0 P5 P10 P1

P0: Control sample
P5, P10, P15: irradiated samples at 5, 10, 15 kGy

Fig. 2. Glycosides profile of *Trigonella foenum-graecum* L. by TLC method

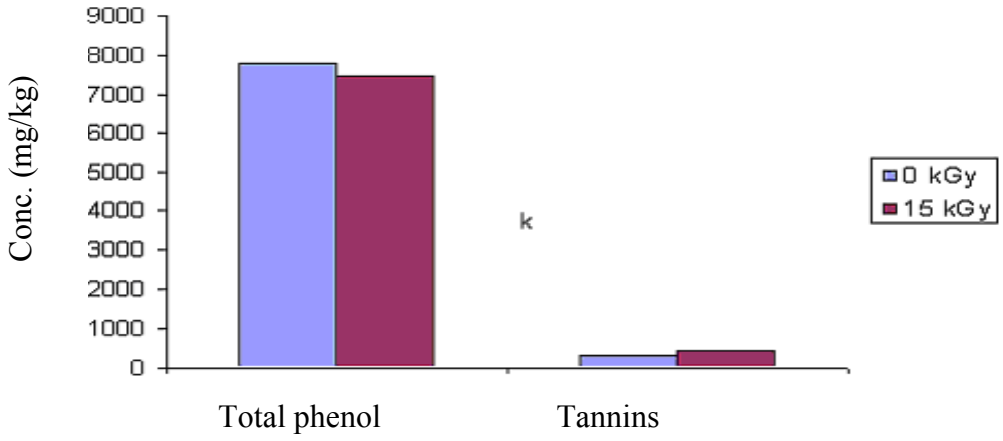


Fig. 3. Concentration of total phenol and tannins in fenugreek seeds irradiated with 15 kGy of gamma radiation

Effect of irradiation on methanol extractable fenugreek seeds constituents

The influence of gamma radiation dose of 15 kGY on the stability of the major constituents of fenugreek seeds is presented as HPLC retention times (min) at different wave lengths (Table 1). The results indicated that the compounds of retention times 2.67, 12.17 (280 nm), 13.36 (320 nm), 17.44, 20.81 and 16.81 min (370 nm), remained unchanged, while the compound of retention time 16.27 min (320 nm) was not detected. Slight changes could be observed in the other chemical constituents. The response of compounds to irradiation was variable. While the content of several compounds increased after gamma irradiation, the content of some major compounds decreased. Despite the minor changes observed, there were no significant differences in the chemical constituents between the irradiated and un-irradiated samples. The observed changes in the relative proportions of some of the constituents and the complete elimination of others could presumably be due to gamma irradiation sensitivity of these compounds (Gyawali *et al.* 2008). The increase in the quantity of some constituents, as affected by gamma irradiation, may be attributed to the release of some natural chemical constituents from their precursors due to the degradation resulting from irradiation (Adamo *et al*. 2004). The decrease may have resulted from the conversion from one form into another. Oliveira *et al.* (2009) reported that data from some plant cultivars suggest that increasing radiation doses induced a decrease in the phytochemical contents. They found that decrease in glycosides content was followed by an increase in their aglycone forms and suggested that these variations could be ascribed to a conversion from the glycoside forms into aglycones

Table1. Retention times and quantities (%) of some compounds recorded at different wave lengths (nm) as percentage areas of treated and un-treated methanolic extracts of *Trigonella foenum- graceum* L. seeds

Wave length (mm)	Major compounds (Unknown)	Retention time	Quantities (%) radiation dose(kGy)	
			0.0	15
17.86	16.76	17.45	1	280
18.89	15.61	15.13	2	
12.10	12.38	2.67	3	
9.73	12.27	20.81	4	
8.04	7.29	12.17	5	
8.27	6.75	16.78	6	
7.33	6.31	13.34	7	
3.98	4.67	27.30	8	
2.42	3.53	16.27	9	
3.19	2.60	29.30	10	
19.46	17.77	17.45	1	320
19.55	16.93	15.12	2	
13.52	14.37	20.81	3	
8.78	8.86	12.17	4	
6.67	6.90	13.36	5	
8.19	6.81	16.78	6	
4.92	6.32	27.23	7	
4.62	5.66	29.30	8	
-	4.42	16.27	9	
3.01	3.66	28.15	10	
25.46	25.98	17.44	1	370
16.37	16.59	15.13	2	
13.87	13.44	20.81	3	
8.92	8.50	12.17	4	
7.50	7.72	16.81	5	
-	7.17	16.29	6	
6.01	7.07	13.34	7	
3.95	4.09	27.23	8	
2.34	2.63	28.15	9	
1.58	2.19	2.24	10	

- : Not detected

CONCLUSION

Gamma irradiation dose of up to 15 kGy is effective in ensuring microbiological safety of the fenugreek seeds without affecting their glycosides concentrations.

ACKNOWLEGEMENT

The authors wish to acknowledge the financial support of the DAAD and the technical and scientific assistance of the members of the Laboratory of Plant Physiology, Institute of Botany, Technische Universität Dresden, Germany.

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تأثير أشعة جاما على بعض المكونات البيوكيميائية والحمل الميكروبي لبذور الحلبة (*Trigonella foenum-graecum* L.)

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المستخلص: أجرى هذا البحث بهدف دراسة تأثير أشعة جاما على الحمل الميكروبي وبعض المكونات البيوكيميائية لبذور الحلبة (*Trigonella foenum-graecum* L.) في قسم النبات والتقانة الحيوية الزراعية، كلية الزراعة، جامعة الخرطوم، ومعهد علم النبات، جامعة درسدن التقنية، المانيا. عوملت البذور بالجرعات التالية من أشعة جاما: 5 و 10 و15 kGy. وجد أن الحمل الميكروبي للبذور قد إنخفض من 1.44×10^6 فى البذور غير المعاملة إلى 1.5×10^4 و 9.0×10^3 و5.0×10^2 cfu/g فى البذور المعاملة بالأشعة 5 و 10 و15 kGy على التوالي. تم تقييم ثبات مكونات البذرة من الجليكوسيدات والفينولات والتانينات باستخدام كروماتوجرافيا الطبقة الرقيقة (TLC) وكروماتوجرافيا السائل عالية الأداء (HPLC). أوضحت نتائج التحليل بواسطة TLC أن معاملة مستخلص بذور الحلبة بجرعة 15 kGy من أشعة جاما لم يكن له تأثير على الجليكوسيدات وأدى الى انخفاض طفيف فى الفينولات الكلية وزيادة طفيفة في محتوى التانينات. وقد أظهرت نتائج HPLC لتحليل المركبات المستخلصة بواسطة الكحول الميثيلي تبايناً في الأيضيات عند موجات الضوء المختلفة بعد المعاملة بأشعة جاما، فبينما زاد محتوى عدد من المركبات، انخفض محتوى بعضها ولم يتغير محتوى البعض الآخر. وبناءً على ذلك فلم تكن هنالك اختلافات كبيرة فى المكونات الكيميائية بين العينات المعاملة و غير المعاملة بالأشعة.

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