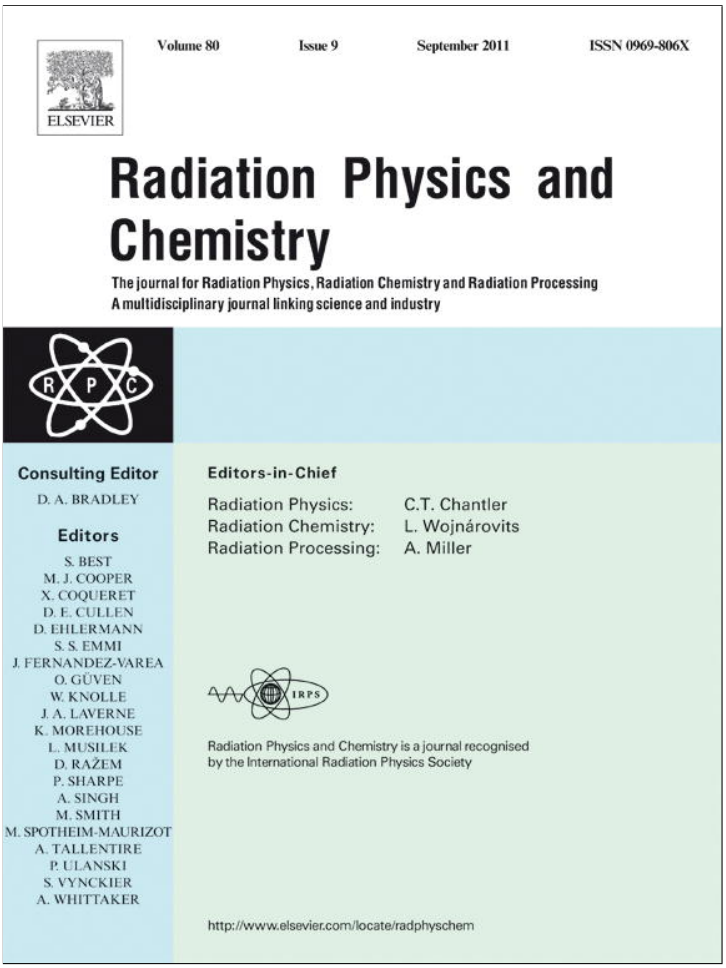

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Gamma radiation effects on phenolics, antioxidants activity and *in vitro* digestion of pistachio (*Pistachia vera*) hull

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ABSTRACT

The effect of gamma radiation (10, 20, 30, 40, 50 and 60 kGy) on tannin, total phenolics, antioxidants activity and in vitro digestion of pistachio hulls has been investigated in this study. The possibility of using the radial diffusion method based on software measurement of the rings area has also been investigated in this study. The software based method in radial diffusion method showed a higher r^2 (0.995) value when compared to the traditional method. Irradiation reduced the tannin content ($P<0.01$) and activity of antioxidants ($P<0.05$) of pistachio hull extracts but increased the total phenolic content ($P<0.05$). There was no effect of gamma irradiation on the *in vitro* digestion of the pistachio hull. Irradiation decreased the digestion rate of the pistachio hull at the dose of 40 kGy when compared to the control. This study showed that gamma irradiation decreased tannin and antioxidants activity of pistachio hull.

1. Introduction

In the recent years high amounts of pistachio by-products have been produced (at an average rate of about 310,000 metric tons annually) in Iran and cause an environmental problem. Pistachio by-products are produced during de-hulling of pistachio nuts after harvesting and contain a high level of pistachio epicarp (53.5% of dry matter) and to a lesser extent peduncles, leaves, mesocarp and kernel (27.7%, 9.5%, 5.3% and 4.0% of dry matter, respectively) of the pistachio plant (Bohluli et al., 2008a). Most of the pistachio by-products are considered as agricultural waste and is often mixed with soil and to a lesser extent are used as feedstuff by local livestock farmer and a small portion is used as herbal medicine and in human foods (mainly as pistachio hull jam). The chemical composition, phenolics and digestion of pistachio hull is varied and is largely dependent upon variations in pistachio cultivars (Bohluli et al., 2008b), harvesting time (Hashami et al., 2008), drying and de-hulling processes.

Like pistachio nut and pistachio skin (Tomaino et al., 2010), pistachio hull (Goli et al., 2005) is a good source of natural phenolics and antioxidants. Phenolics content and antioxidant activity of pistachio hull are more than those of the skin and nuts. Bohluli et al. (2008a) found a higher tannin content (one of the natural antioxidant molecule) in pistachio hull compared to pistachio skin (4.5% and 0.5% of dry matter, respectively).

Due to physiological importance of phenolic compounds such as their cardioprotective (Tosca and Fernandez, 2005) and vasodilation capacity (Padilla et al., 2005), pistachio hull has been considered as a source of natural antioxidants in herbal medicine. High natural antioxidants of pistachio hull can make it a good substitute for synthetic antioxidants (Goli et al., 2005).

It has been reported that gamma irradiation and electron beam could increase phenolics and antioxidant levels in almond skin (Harrison and Were, 2007) and citrus pomaces extracts (Kim et al., 2008). Although many researchers have observed that gamma irradiation decreased phenolics (De Toledo et al., 2007), an increase in phenolics may be partly due to the higher extractability of these materials after irradiation.

Pistachio hulls contain a high level of phenolic compounds and tannins, which can affect their nutrient utilization by animals (Reed, 1995). Gamma irradiation and electron-beam have been proven to be successful in detannification and improvement of overall qualities of food and agricultural commodities (El-Niely, 2007; Shawrang et al., 2011).

The radial diffusion method (Hagerman, 1987) is a simple protein precipitation method for quantification of tannin. In this method the amount of tannins is proportional to the square diameter of the formed ring on the gels containing bovine serum albumin. The limitation of this method is using a ruler or caliper to measure ring areas and sometimes non-uniformity of wells on the gels that causes the rings not to advance in the uniform circle

Abbreviations: PH, pistachio hull; E, tannin contained extract; TFE, tannin-free extract

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shape. In such situations, determining the areas of the rings is not possible.

The main objectives of this study were to evaluate the effects of gamma irradiation on tannin, total phenolics, antioxidants activity and *in vitro* digestion of PH. The present study also intended to measure the ring areas in the method of radial diffusion assay using a software based method.

2. Material and methods

2.1. Pistachio hull (PH)

Pistachio hull (Ohadi variety) was obtained from the pistachio processing factory, near Bardaskan city, in Iran, during the summer of 2009 and was air dried before it was used in this study.

2.2. Gamma irradiation

PH was irradiated by ^{60}Co radiation in a gamma cell (Issledo Vatel Gamma Cell Facility, Model PX-30) at a dose rate of 0.8 kGy h^{-1} at a absorbed dose of 10, 20, 30, 40, 50 and 60 kGy in polyethylene bags (10×10 cm^2) under the same conditions of temperature and humidity. Samples were treated uniformly by packing them in small sizes.

2.3. Extraction of tannins and other phenolics compound

Aqueous acetone (70%) was used for the extraction process. Briefly, 200 mg of finely ground PH was measured in a glass beaker of 25 ml capacity, 10 ml of aqueous acetone (70%) was added to the beaker, it was suspended in an ultrasonic water bath (JENCONS, 2800) and subjected to ultrasonic treatment for 20 min at room temperature. The resulting mixture was then transferred to centrifuge tubes and centrifuged for 10 min at approximately 3000*g* at 4 °C. The Supernatant was collected and stored at 4 °C until further analysis (extract named as E).

2.4. Removal of tannin from the extracts

100 mg of polyvinyl polypyrrolidone (PVPP) was transferred into 100×12 mm^2 test tubes; 1.0 ml distilled water and 1.0 ml of the pistachio extract were added to them. The tubes were vortexed and kept at 4 °C for 15 min, vortexed again and centrifuged (3000*g* for 10 min) for supernatant collection. The supernatant contained phenolics except tannins (named as tannin-free extract, TFE).

2.5. Radial diffusion assay

Protein precipitation by tannins in the PH extract (E) was assayed by the radial diffusion method (Hagerman, 1987) with a little modification in the calculation of ring areas based on computer software. Wells were punched in the gel plates (internal diameter 8.5 cm), spaced 2 cm apart. Using a micropipette, prepared solutions (standards and E extract) were added to the wells (in duplicate) as 20 µl aliquots. As the solution was absorbed by the gel, other successive 20 µl aliquot was added (totally 40 µl of extracts). Petri dishes were covered and sealed with Parafilm, and incubated at 30 °C for 96 h. For ring area measurement at the end of incubation, gel plates were subjected to digital imaging at actual size and images imported in an image processing software (ImagJ 1.42q, National Institute of Mental Health, Bethesda, Maryland, USA). The ring areas (mm^2) of standards (10–40 ppm) and irradiated PH extracts in the plates were determined automatically by the software and the area of standard rings were regressed against known quantities of tannic acid (mg) as standards.

2.6. Determination of total phenolic content

Total phenolic content was determined based on a modified method by Slinkard and Singleton (1997). 200 µl of the samples was mixed with 1.4 mL distilled water and 100 µL of the Folin–Ciocalteu reagent. After at least 30 s (but not exceeding 8 min), 300 µL of 20% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h. The absorbance was measured at 765 nm with a spectrophotometer (Cecil, CE 1021). Standard solutions of tannic acid (6–16 ppm) were treated similarly to prepare the calibration curve. Results were expressed as mg tannic acid equivalent per 100 g dry sample.

2.7. DPPH radical scavenging activity

The scavenging activity of PH extracts (E and TFE) on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined using the method described by Huang et al. (2006). 1 mL of PH extracts was mixed with 1 mL of freshly prepared 80 ppm DPPH in methanol. The mixture was kept in dark for 30 min. The absorbance was then measured at 517 nm using the spectrophotometer (Cecil, CE 1021). Percent activity was calculated using the following equation:

$$\text{percent activity} = (1-(A_{\text{sample}}/A_{\text{blank}})) \times 100\%$$

2.8. Reducing power

The reducing power of the PH extracts (E and TFE) was determined by the method of Singh and Rajini (2004). 1 mL aliquot of the extracts was added to 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% (w/v) $K_3Fe(CN)_6$. The mixture was incubated at 50 °C for 20 min. 10 percent (10% w/v) trichloroacetic acid (1 mL) was added to the resulting mixture, and then centrifuged at 1750*g* for 10 min. 1 mL of the supernatant was taken and 1 mL of distilled water and 0.2 mL of 0.1% (w/v) $FeCl_3$ solution were added to it. The absorbance was measured at 700 nm using the spectrophotometer (Cecil, CE 1021).

2.9. In vitro digestion

In vitro digestion was determined using the gas production (GP) assay according to Menke et al. (1979). Approximately 200 mg of dry matter (DM) of each treatment was placed in triplicate 100 ml syringes and the incubation medium was then added to the syringes. The incubation medium contained 477 ml of buffer (7.9 g NH_4HCO_3 and 70.6 g $NaHCO_3$ in 2 l of distilled water), 237 ml of macromineral solution (11.4 g Na_2HPO_4, 12.4 g KH_2PO_4 and 1.2 g $MgSO_4 \cdot 6H_2O$ in 2 l of distilled water), 0.237 ml of a micromineral solution (13.2 g $CaCl_2 \cdot 2H_2O$, 10 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$, 8 g $FeCl_3 \cdot 6H_2O$ in 100 ml of distilled water), 0.297 ml of resazurine, 0.297 g Na_2S, 0.318 ml of 6 M NaOH, 529 ml distilled water and 501 ml rumen fluid collected from the cannulated sheep. Rumen digesta was squeezed through four layers of cheese cloth. Gas production was measured at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h and a set of appropriate blanks (i.e., without PH) was included. Gas produced from each treatment was calculated by subtracting the gas produced in a control blank.

2.10. Statistical analysis

Gas production results (ml per 0.2 g dry matter) were fitted using the NLIN option of SAS (Statistical Analysis System) (2001) to the model $G=b(1-e^{-k(t)})$, where G is the volume of gas production at

time *t*, *b* the asymptotic gas production (ml per 0.2 g DM) and *k* the rate of gas production (per hour) from the slowly fermentable feed fraction *b*. The data were analyzed by analysis of variance (ANOVA) through the *F* test. Duncan's multiple range tests were used to determine the differences amongst samples. Significant levels were defined as those with probabilities of 0.05 or less.

3. Results and discussion

3.1. Radial diffusion assay and total phenolics

Fig. 1 shows a good relationship ($r^2=0.995$) of ring area (mm^2) with tannic acid concentration. In the present study the use of software based method resulted in higher r^2 value compared to the studies of Hagerman (1987) and Gedir et al. (2005) in which area calculation was based on using calipers (0.94 and 0.97). Using the software based method we can easily calculate ring areas that may not advance in the uniform circle shape. According to our data tannin content of PH extract was 5.77 mg/ml, which is close to the reported value of tannin content in PH (5.17 mg/ml) in the study of Ghasemi et al. (2011, unpublished data).

The effects of gamma irradiation on the ring areas (mm^2), tannin concentration and total phenolics are shown in Table 1. Gamma irradiation decreased ($P<0.01$) the ring area (mm^2) and tannin equivalent of the PH extracts. Tannin equivalent values show a decrease with increasing absorbed dose, but the decrease was much higher for the doses 10–40 kGy compared to the doses 50 and 60 kGy. Same results were noted for the area of formed ring (mm^2). At the absorbed dose of 10 kGy approximately 33% reduction in tannin was observed (Table 1).

Although the effects of electron beam and gamma irradiation on tannin contents of some materials have been reported, there is no information available in literature on the effect of ionizing irradiation on tannin contents of PH. Reduction of tannin by gamma irradiation in the present study is consistent with some previous studies using gamma irradiation (El-Niely, 2007; De Toledo et al., 2007) and electron-beam (Bhat and Sridhar, 2008; Shawrang et al., 2011), but Stajner et al. (2007) observed tannin content of soybean seeds to increase(21.6%) by gamma irradiation at the dose of 1 kGy.

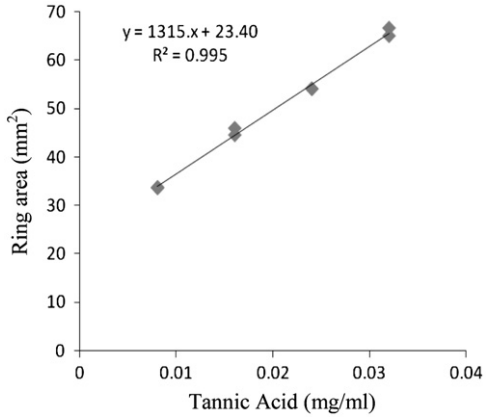

Fig. 1. Calibration curve for tannic acid. Ring areas (mm^2) are regressed against known tannic acid concentrations (mg/ml).

In the present study, absorbed dose of 10 kGy decreased total phenolics but at the dose greater than 10 kGy (20–60 kGy) the level of total phenolics numerically increased compared to the control. As mentioned above generally irradiation resulted in the degradation of tannin (Variyar et al., 1998) and a change in its molecular conformation (Topuz and Ozdemir, 2004). In most of the studies using ionizing irradiation a decrease in the level of tannin was accompanied with an increase in total phenolic compounds (Stajner et al., 2007; Harrison and Were, 2007; Kim et al., 2008) or an increase in gallic acid content (Variyar et al., 1998). In contrast, some studies reported an increase in the amount of tannin (Stajner et al., 2007; Harrison and Were, 2007) or decrease in the amount of total phenolic content (Bhat and Sridhar, 2008) by irradiation. Some studies have found that the effects of ionizing irradiation on tannin and phenolic compounds are dose dependent. De Toledo et al. (2007) observed that gamma radiation (up to 4 kGy) significantly decreased phenolic compounds; however in this study the irradiation at the dose of 8 kGy significantly increased phenolics in soybean grains, as compared to the control samples. The differences in the effect of ionizing radiation on phenolic compounds may be partly due to the higher extractability of these compounds in irradiated samples as a result of alternations in cellular compounds and release of bound or insoluble phenolics especially at high doses of irradiation.

3.2. Antioxidant assay

The antioxidant content of PH extracts (E and TFE) is affected by absorbed dose and is shown in Table 2. The scavenging activity of antioxidants in PH was determined by the DPPH assay. In this method, the antioxidants were able to reduce the stable radical DPPH and decrease its absorbance at 517 nm, i.e. higher absorbance shows lower activity. We could not find any data regarding the scavenging activity in PH extracts especially treated by gamma irradiation. Data of scavenging activity shows higher activity in control PH (53.96%) than those of irradiated ones (10–60 kGy). The same trend was observed when TFE was used as extract for determination of scavenging activity. As shown in Table 2 tannins to some extent contributed to the scavenging activity of E (approximately 10%).

The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. Activity is measured as the change in the absorbance at 700 nm relative to the blank solution. In the present study, reducing power (as absorbance at 700 nm) of E decreased by irradiation of PH at doses of 30 and 60 kGy. For the TFE, irradiation decreased reducing

Table 1
Effect of gamma irradiation on ring area, tannin equivalent and total phenolic content of pistachio hulls.

	Control	Absorbed dose (kGy)					
		10	20	30	40	50	60
Ring area (mm^2)	84.15 ± 0.02^{a}	64.33 ± 4.20^{cd}	65.95 ± 2.47^{d}	61.33 ± 1.57^{d}	64.35 ± 1.82^{d}	72.12 ± 2.45^{cb}	77.14 ± 3.52^{b}
Tannin equivalent (mg/ml)	5.77 ± 0.02^{A}	3.89 ± 0.39^{C}	4.05 ± 0.23^{C}	3.61 ± 0.15^{C}	3.89 ± 0.17^{C}	4.64 ± 0.23^{B}	5.11 ± 0.33^{B}
Total phenolics (mg/ml)	7.86 ± 0.38^{a}	6.49 ± 0.77^{b}	8.10 ± 0.58^{a}	8.13 ± 1.03^{a}	8.39 ± 0.89^{a}	8.90 ± 1.07^{a}	8.45 ± 0.59^{a}

[abcd] In the same row, means with different superscript are statistically different ($P<0.05$).
[ABC] In the same row, means with different superscript are statistically different ($P<0.01$).

Table 2
Effect of gamma irradiation on antioxidants contents of PH extracts.

	Control	Absorbed dose (kGy)					
		10	20	30	40	50	60
Scavenging activity (%)							
E	53.96 ± 0.64^{a}	49.40 ± 0.64^{b}	48.94 ± 1.30^{b}	49.85 ± 2.15^{b}	47.42 ± 1.30^{b}	47.11 ± 0.86^{b}	47.88 ± 0.64^{b}
TFE	48.48 ± 0.64^{a}	41.49 ± 0.64^{bc}	41.49 ± 1.07^{bc}	43.77 ± 2.15^{b}	40.73 ± 1.30^{dc}	38.6 ± 0.64^{dc}	38.45 ± 0.64^{d}
Reducing power (absorbance)							
E	0.550 ± 0.07^{a}	0.539 ± 0.02^{ba}	0.489 ± 0.01^{bac}	0.467 ± 0.01^{bc}	0.500 ± 0.01^{bac}	0.513 ± 0.02^{ba}	0.426 ± 0.02^{c}
TFE	0.156 ± 0.01^{a}	0.106 ± 0.01^{cd}	0.130 ± 0.01^{bc}	0.104 ± 0.01^{d}	0.123 ± 0.01^{bcd}	0.121 ± 0.01^{bcd}	0.139 ± 0.01^{ba}

[abcd] In the same row, means with different superscript are statistically different ($P < 0.05$).

Table 3
GV (ml/200 mg DM of pistachio hulls) and GP parameters (%) at 96 h of incubation of treatments.

	Control	Absorbed dose (kGy)					
		10	20	30	40	50	60
Gas volume	27.72 ± 2.12	25.26 ± 2.31	27.42 ± 0.66	24.34 ± 0.46	24.45 ± 1.32	27.86 ± 1.71	25.35 ± 2.63
GP parameters							
b	27.63 ± 1.56^{a}	25.58 ± 1.81^{bac}	26.94 ± 0.32^{bac}	24.58 ± 0.46^{bc}	24.42 ± 1.15^{c}	27.20 ± 1.15^{ba}	24.97 ± 2.09^{bc}
k	5.91 ± 0.41^{bac}	4.65 ± 0.51^{c}	6.69 ± 0.63^{a}	5.15 ± 0.29^{bc}	6.08 ± 0.31^{bac}	7.26 ± 0.48^{a}	6.40 ± 1.62^{ba}

[abc] In the same row, means with different superscript are statistically different ($P < 0.05$).

power of extract up to 50 kGy. As shown in Table 2 most of the absorbance in E is attributed to tannins (0.550 in E compared to 0.156 in TFE of control group). Absorbed dose of 60 kGy induced more reduction in reducing power (0.426); when the tannin was removed from the extract the same reduction was noted at absorbed dose of 40–60 kGy. The decrease of reducing power and radical-scavenging activity with gamma irradiation in the present study is consistent with the reduction in tannin content. Gamma irradiation (5–30 kGy) significantly decreased the DPPH radical-scavenging activity and reducing power of ground black pepper extracts (Suhaj et al., 2006).

In contrast to the present study, increase in phenolic contents and antioxidant activity was observed in almond skin extract (ethanol extract) irradiated at doses greater than 4 or 12.7 kGy (Harrison and Were, 2007). Similarly Kim et al. (2008) showed that electron-beam irradiation of citrus pomaces could increase polyphenolic compounds and scavenging activity of citrus pomaces extracts in methanol, ethanol and water at the absorbed doses of 3.6–37.9 kGy. In this study on increasing the dose of irradiation, scavenging activity in 70% acetone extract of citrus pomaces was decreased.

An explanation of decrease in antioxidants level in irradiated PH in the present study might be due to the presence of lipids (approximately 7% of DM) in PH (Behgar et al., 2009). If oxygen is present (as the condition of the present study) autooxidation induced by high-energy radiation (direct or indirect) of lipid molecules takes place (Nawar, 1986). However, decrease in lipid oxidation was observed in the study of Harrison and Were (2007) when soybean oil was treated by almond skins extract. These authors attributed the effect of almond skins extract to the presence of phenolic compounds in the almond skins.

The difference in the effect of ionizing radiation on antioxidant contents in studies may be due to variation in the extraction solvent, existence of fat or other chemicals and different doses of irradiation.

3.3. *In vitro* digestion and gas production parameters

Cumulative gas volume (GV) and gas production (GP) parameters (*b* and *k*) are shown in Table 3. GV parameters were not affected by absorbed dose and averaged 26.06 mm among treatments. Irradiation at the doses of 30, 40 and 60 kGy caused a decrease ($P < 0.05$) in potential GP (i.e., fraction *b*) compared to the control. But there were no differences in potential GP of irradiated PH at the doses of 10, 20 and 50 kGy compared to the control. Although low (i.e., *k*) and high rates of GP were observed, respectively, in PH at absorbed doses of 10 and 50 kGy ($P < 0.05$), the differences were not significant when compared to the control. No data were found by authors regarding the effect of irradiation on detannification and *in vitro* GP of PH. Generally it is accepted that tannins negatively affect digestion of feedstuff (Mohmmadabadi et al., 2010) or growth and morphology of rumen microorganisms (O'Donovan and Brooker, 2001). Kamalak et al. (2004) studied the correlation of GP parameters with chemical composition of tree leaves and condensed tannins and found a negative correlation of fraction *b* with NDF, ADF, total condensed tannin and soluble condensed tannin. In the present study, irradiation significantly decreased the tannin level in PH; however, this result is not consistent with the effects of irradiation on *b* fraction of GP assay. Irradiation resulted in the degradation of tannin and an increase of free phenolics that are toxic and suppressed the growth of the cellulolytic microorganism in the rumen (Chesson et al., 1982). The decrease in fraction *b* in the present study might be related to an increase of total phenolic content as shown in Table 1. The intake of feed is mostly explained by the rate of GP (*k*), which affects the passage rate of feed through the rumen. The rate of GP in the study of Kamalak et al. (2004) was related neither to chemical composition nor condensed tannins. Reductions in gas volume, fraction *b* and rate of GP were observed when tannic acid was added to the sunflower meal (Mohmmadabadi et al., 2010). There is no obvious explanation for the effects of irradiation on the rate of GP in the present study.

4. Conclusion

The use of software based method in radial diffusion assay provided more precise results when compared to the traditional

technique. Gamma irradiation decreased tannin equivalent (approximately 33% reduction) and antioxidants level of PH extracts. Some part of antioxidant activity in extracts was due to the tannin molecule. Further studies are needed to evaluate the definite effect of gamma radiation on tannic acid and phenolics using pure molecules.

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