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Effects of Graded Levels of Tannin-Containing Pomegranate Peel (*Punica granatum*) by Irradiation on In Vitro Methane Production and Population of Protozoa

Mohsen Zarei[1*], Jamal Seifdavati[1], Parvin Shawrang[2] and Farokh Kafilzadeh[3]

[1]*Department of Animal Science, Mohaghegh Ardabili University, Ardabil, Iran*
[2]*Agricultural Research School, Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran, Karaj, Iran*
[3]*Department of Animal Sciences, Razi University, Kermanshah, Iran*
[*] *Corresponding Email: m.zarei@uma.ac.ir*

ABSTRACT

This study was conducted to determine the effect of pomegranate peel tannin on in vitro CH_4 production and protozoa population. Electron beam and gamma ray irradiation exposed to pomegranate peel at doses of 5, 10, 15 and 20 kGy to evaluate condensed tannin (CT). Three ruminally fistulated rams used to obtain ruminal fluid for in vitro CH_4 production and protozoa population. Data were analyzed by using the GLM procedure. The results showed that both gamma and electron beam irradiation significantly decreased condensed tannin than control. There was no difference between gamma and electron beam irradiation concerning condensed tannin reduction. Irradiation did not change methane production. Irradiation had no significant effect on total population and five genuses of protozoa (Diplodinium, Entodinium, Dasytricha, Isotricha and Ophryoscolex). Although, there was no correlation between condensed tannin and CH_4 production, a significant negative correlation coefficients between methane production and total protozoa population and between gas volume at 24 h and total protozoa population of gamma irradiated pomegranate peel were observed ($P<0.05$). In conclusion, the results showed that degrading and reducing of condensed tannin polymer of pomegranate peel by irradiation was not essentially related to the methanogenesis and protozoa population.

Keywords: Condensed tannin, Irradiation, Methane, Protozoa.

INTRODUCTION

The emission of greenhouse gases such as carbon dioxide (CO_2) and methane (CH_4) is considered to be one of the most important global environmental issues [1]. Animals, particularly ruminants, produce CH_4 from anaerobic fermentation in their gastro-intestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. Ruminant animals are responsible for about 15-20% of the total anthropogenic emission of CH_4 [2] and the CH_4 produced from their enteric fermentation is not only related to environmental problems, but also associated with energy losses and, hence reductions of energy usage. Typically 6–8%, but up to 12%, of the gross energy (GE) in feed is converted to CH_4 during microbial digestion in the rumen [3]. Therefore, decreasing CH_4 production from ruminants is desirable for reducing greenhouse gas emissions and increasing utilization of the digested energy. Plant secondary metabolites (PSM) have been suggested as effective alternatives to antibiotics to suppress rumen methanogenesis through their antimicrobial activity [4-5]. There has been increased interest in use of plants and plant extracts to mitigate enteric ruminal CH_4 emissions [6-7].

As such, very extensive screening of a large range of plants and their secondary compounds, such as saponins and tannins, is now underway in several laboratories [8-9]. Early indications of the ability of CT to suppress CH_4 were

given by Hayler et al. (1998) [10], who tested *in vitro* the rumen fluid from sheep fed different CT plants. Several studies indicate that tannin have anti-methanogenic activity, either by direct inhibition on methanogenesis or indirectly though inhibition on protozoa [11-12,5]. An even larger pool of plant sources of tannins exists, and these are often tropical shrub legumes such as pomegranate peel. Based on their structure and chemical properties, tannins are divided into hydrolysable tannins and condensed tannins (CT) or proanthocyanidines, which have no carbohydrate core and are derived by condensation of flavonoid precursors or polymers of flavonoids [13]. Due to the lower risk of toxicity, research has focused on CT rather than on hydrolysable tannins. The huge diversity in tannin structures may explain their variable effects on methanogenesis and rumen function with observed responses depending on source, type and level of tannin [14,9].

Tannins also reduce ruminal CH_4 production when included either as temperate legumes [7] or as purified tannin extracts [15]. The higher extractability of these compounds in irradiated samples was observed [16]. Gamma ray and electron-beam irradiation have been proven to be successful in detannification and improvement of overall qualities of food and agricultural commodities [17-18]. Irradiation processing has been used as a method to inactivate these antinutritional factors, alternations in cellular compounds and release of bound or insoluble phenolic compounds especially at high doses of irradiation [17-16]. Generally irradiation resulted in the degradation of tannins [19] and a change in its molecular conformation [20]. However, there are no reports on potential differences in the activities of electron and gamma on condensed tannin of pomegranate peel and subsequent effects on CH_4 production and ciliated protozoal populations. The correlation between methane production and protozoa population and the antimethanogenic potential of pomegranate peel condensed tannin have not yet been explored. This study was carried out to determine the effect of graded levels of tannin-containing pomegranate peel, on the *in vitro* methane suppression and protozoa population in order to determine their correlation.

MATERIALS AND METHODS

Samples preparation and irradiation treatments

Pomegranate peel was obtained from the Neyriz Green Farm pomegranate juice factory, in Fars province, Iran, during the pomegranate harvest season and dried before it used in this study. Irradiations of samples were done in radiation applications research school, nuclear science and technology research institute, atomic energy organization of Iran. Gamma-ray (GR) irradiation was completed by using a cobalt-60 irradiator at 20°C. The dose rate determined by Fricke dosimetry was 0.36 Gy/s. Three-paper packages of samples were irradiated to total doses of 5, 10, 15 and 20 kGy in the presence of air. After irradiation and prior to sealing the plastic bags, samples were allowed to air equilibrate for 2 h.

Three poly-ethylene packages of samples were exposed to 10 MeV electron beam (EB) of a Rhodotron accelerator model TT-200 (IBA Co., Belgium), Radiation Applications Research School (of Atomic Energy Organization of Iran) to various doses (5, 10, 15 and 20 kGy). All irradiations were performed at room temperature in air, with 4 mA beam of 10 MeV electrons and single sided irradiation has been used because the samples packages had low thickness. The required doses were delivered to the samples by adjusting the conveyer speed when each of sample batches passed under the beam. Condensed tannins (CT) were determined according to Galyean (1997) [21] procedure and results are expressed as catechin equivalents (mg of CE/g of dry sample).

Measurement of methane production

For measuring methane production, after 24 hours of incubated samples in glass syringe of *in vitro* gas production techniques, 2 mL of NaOH (10 M) were introduced to estimate methane production following the method by Fievez et al. (2005) [22]. NaOH (10 M), which was then introduced into the incubated contents, thereby avoiding gas escape. Mixing of the contents with NaOH allowed absorption of CO_2, with the gas volume remaining in the syringe considered to be CH_4 [23]. Data were obtained on volume of gas and methane (CH_4) produced. Net methane and gas productions were calculated by the differences of the methane and gas in the test syringe and the corresponding blank; the methane concentration was determined as [5]:

$$\text{Methane concentration} = \frac{\text{Net Methane Production}}{\text{Net Gas Production}} \times 100$$

Protozoa population

Ruminal fluid from three Sanjabi fistulated rams which were fed at maintenance level was diluted anaerobically in the anaerobic dilution solution. According to Dehority [24] the media were placed in a culture tube containing 10 mL of medium and substrate added. The tube was closed anaerobically and incubated in a 39°C incubator. Whole serum bottles contents for protozoal counts were preserved by diluting with an equal volume formalin solution (185 ml formaldehyde/l distilled water). Total numbers and generic composition of ciliate protozoa were determined according to the procedures described by Dehority [25]. This procedure was as follows: Using a 1.0-ml-wide orifice (3 -mm) pipette, a 1.0-ml aliquot of the fixed rumen contents was pipetted into a 16 ✕ 150 mm culture tube. Three drops of brilliant-green dye were added and the tube was allowed to stand overnight. Nine milliters of 30% glycerol were added and the sample was pipetted into a Sedgewick-Rafter chamber with a wide-orifice pipette (the chamber is calibrated to hold exactly one ml). Protozoa were counted at a magnification of 100X with a 0.5-mm-square counting grid mounted into the eyepiece. A total of 50 grids evenly spaced over the entire chamber were counted. The chamber was then turned 180", another 50 grids were counted, and the two counts were averaged. Dilutions giving counts between 100 and 150cells per 50 grids are the most satisfactory for counting. Where required, further dilutions were made with 30% glycerol. The protozoa numbers were calculated according to Kamra et al. (1991) [26].

Statistical analyses

Data were analyzed by analysis of variance using the general linear model (GLM) procedure. All statistical analysis was carried out using SAS software (SAS v. 9.1; Statistical Analysis System). Comparison of irradiation groups and control and between ionizing radiation (gamma and electron) was conducted by orthogonal comparison. The least significant difference (LSD) was used to compare and estimate the differences between irradiation treatments dose and un-irradiated pomegranate peel (control).

RESULTS

Orthogonal contrast of methane production and condensed tannin of pomegranate peel before and after irradiation are shown in Table 1. Orthogonal comparisons indicated that both GR and EB irradiation significantly decreased CT content of PP than control ($P<0.01$). There was no difference between GR and EB irradiation. Irradiation did not change methane production, but the effects of irradiation on gas production volume at 24 hour incubation (GV_{24}) were significantly different between low and upper doses of gamma radiation and between gamma and electron beam irradiation in low doses ($P<0.05$).

Table 1. Orthogonal contrast (mean square) of gas production, condensed tannin and methane concentration of pomegranate peel in different dose of irradiation

Treatments	CH_4	CT	GV_{24}
Irradiation vs. control	21.46	19.14**	1.79
GR vs. control	69.31	18.17**	113.87
5 and 10 GR vs. control	110.11	2.83**	622.69
15 and 20 GR vs. control	22.15	37.2**	29.92
5 and 10 vs. 15 and 20 GR	50.22	29.26**	1388.47*
EB vs. control	0.21	16.30**	66.08
5 and 10 EB vs. control	6.30	3.04**	77.21
15 and 20 EB vs. control	2.76	31.68**	36.66
5 and 10 vs. 15 and 20 EB	26.10	22.64**	11.19
GR vs. EB	154.48	0.12	883.67
5 and 10 GR vs. 5 and 10 EB	95.59	0.005	1707.66*
15 and 20 GR vs. 15 and 20 EB	60.84	0.33*	0.51

GR: Gamma Ray, EB: Electron Beam, GV_{24}: Gas Volume at 24 hour,
$^{*}P<0.05$. $^{**}P<0.01$.

Table 2 showed that methane production of electron irradiated pomegranate peel at a dose of 15 kGy was significantly lower than gamma irradiated of pomegranate peel at a dose of 10 kGy. Decrease in CT was dose dependent. Gamma ray and electron beam irradiation at the doses of 5, 10, 15 and 20 kGy significantly decreased condensed tannin compared to control by 11%, 38%, 81% and 98% for GR and by 4%, 46%, 76% and 89% for EB respectively. Maximum and minimum level of condensed tannin content of pomegranate peel observed at a doses of 5 kGy and 20 kGy electron beam (5.58 and 0.07 g/100 g dry matter) respectively.

Table 2. In vitro gas production volume, condensed tannin and methane concentration of pomegranate peel

Treatments	CH_4 (%)	CT	GV_{24}
Control	17.72^{ab}	5.87^{a}	174.13^{ab}
Gamma			
5 kGy	22.96^{ab}	5.20^{b}	149.66^{b}
10 kGy	27.32^{a}	3.63^{c}	163.32 ^{ab}
15 kGy	21.29^{ab}	1.11^{e}	166.36 ^{ab}
20 kGy	20.80^{ab}	0.07^{g}	189.64^{a}
Electron			
5 kGy	19.11^{ab}	5.58^{ab}	179.05 ^{ab}
10 kGy	19.88^{ab}	3.15^{d}	181.65 ^{ab}
15 kGy	14.91^{b}	1.37^{e}	179.34 ^{ab}
20 kGy	18.18^{ab}	0.63^{f}	177.49 ^{ab}
LSD*	11.99	0.42	35.04
P-value	0.6397	0.0001	0.1289
SEM	1.29	0.51	3.26

Means in the same column without a common superscript are different (P<0.05), LSD: Least Significant Difference, GV_{24}: Gas Volume at 24 hour (mL/g), CT (mg of CE/g of dry sample), SEM, Standard Error of Mean.

Total population and five genuses (*Diplodinium*, *Entodinium*, *Dasytricha*, *Isotricha* and *Ophryoscolex*) of protozoa observed (Table 3 and 4). The results showed that irradiation had no significantly effect on total population and five genus of protozoa.

Table 3. Effects of irradiated pomegranate peel on ruminal protozoa concentration

Treatments	df	Protozoa					
		Total	Diplo	Ento	Dasy	Iso	Ophryo
Irradiation vs. control	1	8.05×10^{10}	2.3×10^{8}	8.1×10^{10}	4.7×10^{6}	2.5×10^{7}	1.8×10^{7}
GR vs. control	1	5.7×10^{10}	9.2×10^{7}	5.7×10^{10}	1.7×10^{7}	1.7×10^{7}	7.5×10^{6}
5 and 10 GR vs. control	1	9.5×10^{10}	6.3×10^{6}	9.8×10^{10}	5.6×10^{7}	6.3×10^{6}	6.3×10^{6}
15 and 20 GR vs. control	1	1.6×10^{10}	4.04×10^{8}	1.5×10^{10}	0.00	2.5×10^{7}	6.3×10^{6}
5 and 10 vs. 15 and 20 GR	1	4.9×10^{10}	7.6×10^{8}	5.4×10^{10}	8.5×10^{7}	9.4×10^{6}	0.00
EB vs. control	1	8.9×10^{10}	3.7×10^{8}	9.09×10^{10}	0.00	3.03×10^{7}	3.03×10^{7}
5 and 10 EB vs. control	1	1.5×10^{11}	7.6×10^{8}	1.6×10^{11}	2.5×10^{7}	2.5×10^{7}	6.3×10^{6}
15 and 20 EB vs. control	1	2.2×10^{10}	5.6×10^{7}	1.9×10^{10}	2.5×10^{7}	2.5×10^{7}	5.6×10^{7}
5 and 10 vs. 15 and 20 EB	1	9.2×10^{10}	6.06×10^{7}	1.08×10^{11}	1.5×10^{8}	0.00	3.7×10^{7}
GR vs. EB	1	8.7×10^{9}	2.3×10^{8}	9.6×10^{9}	4.2×10^{7}	4.7×10^{6}	1.8×10^{7}
5 and 10 GR vs. 5 and 10 EB	1	1.1×10^{10}	$1.3\times10^{9*}$	1.3×10^{10}	9.4×10^{6}	9.4×10^{7}	0.00
15 and 20 GR vs. 15 and 20 EB	1	6.06×10^{8}	2.3×10^{8}	$4.\times10^{8}$	3.7×10^{7}	0.00	3.7×10^{7}

GR: Gamma Ray, EB: Electron Beam, Diplo= Diplodinium, Ento= Entodinium, Dasy= Dasytricha, Iso= Isotricha, Ophry= Ophryoscolex, $^{*}P<0.05.$, $^{**}P<0.01.$

Table 4. Effects of irradiated pomegranate Peel on ruminal protozoa concentration

Treatments	Protozoa					
	Total	Diplodinium	Entodinium	Dasytricha	Isotricha	Ophryoscolex
Control	7.8×10^{5}	7.1×10^{3}	7.3×10^{5}	2.1×10^{4}	3.5×10^{3}	0
Gamma						
5 kGy	5.1×10^{5}	7.1×10^{3}	4.6×10^{5}	1.4×10^{4}	0	0
10 kGy	6.08×10^{5}	3.5×10^{3}	5.5×10^{5}	1.7×10^{4}	$3.\times10^{3}$	3.5×10^{3}
15 kGy	6.2×10^{5}	1.06×10^{4}	5.9×10^{5}	1.7×10^{4}	0	3.5×10^{3}
20 kGy	7.6×10^{5}	3.2×10^{4}	7.0×10^{5}	2.4×10^{4}	0	0
Electron						
5 kGy	5.1×10^{5}	3.2×10^{4}	4.5×10^{5}	1.4×10^{4}	0	0
10 kGy	4.8×10^{5}	2.1×10^{4}	4.3×10^{5}	2.1×10^{4}	0	3.5×10^{3}
15 kGy	7.0×10^{5}	7.1×10^{3}	6.5×10^{5}	2.8×10^{4}	0	7.1×10^{3}
20 kGy	6.5×10^{5}	1.7×10^{4}	6.08×10^{5}	2.1×10^{4}	0	3.5×10^{3}
*LSD	3.6×10^{5}	2.4×10^{4}	3.5×10^{5}	2.5×10^{4}	4.9×10^{3}	7.8×10^{3}
P-value	0.64	0.16	0.57	0.95	0.55	0.1050
SEM	3.9×10^{4}	3.08×10^{3}	3.8×10^{4}	2567.33	547.85	869.69

Means in the same column without a common superscript are different (P<0.05), LSD: Least Significant Difference, SEM, Standard Error of Mean.

The Correlation coefficient between experimental parameters of gamma and electron irradiated pomegranate peel are presented in table 5 and 6.

Table 5. Correlation coefficient (*r*) of the relationship between experimental parameters of gamma irradiated pomegranate Peel

	Total	Diplo	Ento	Dasy	Iso	Ophryo	CH_4	CT	GV_{24}
CH_4	-0.56*	-0.09	-0.57*	-0.38	-0.13	-0.31	1	-0.07	-0.10
CT	-0.30	-0.46	-0.30	-0.10	-0.06	-0.06	-0.07	1	-0.44
GV_{24}	-0.52*	-0.47	-0.53*	-0.10	-0.12	-0.32	-0.10	-0.44	1

** Significance levels: 0.05.*

Table 6. Correlation coefficient (r) of the relationship between experimental parameters of electron beam irradiated pomegranate Peel

	Total	Diplo	Ento	Dasy	Iso	Ophryo	CH_4	CT	GV_{24}
CH_4	-0.15	0.46	-0.18	-0.05	-0.04	-0.49	1	0.16	-0.12
CT	-0.29	0.04	-0.28	-0.25	.	-0.59	0.16	1	-0.03
GV_{24}	-0.04	-0.01	0.00	-0.34	-0.60*	0.36	-0.12	-0.03	1

** Significance levels: 0.05.*

DISCUSSION

Methane production (CH_4)

Condensed tannin content of irradiated pomegranate peel was significantly decreased, but irradiation did not change methane production and gas volume at 24 significantly. Decrease in CT was dose dependent and methane production (CH_4%; Table 2) ranged from 14% and 27%, which almost increased with irradiation. This is in agreement with the result reported by several authors [27-28,9] that measured gas and CH_4 production. Generally it is accepted that tannins are a secondary compound with a high capacity to reduce CH_4 production in the rumen [29]. Evidently some sources of CT are not effective in reducing CH_4 production, as shown for Schinopis quebracho CT in cattle by Beauchemin [30]. Pellikaan et al. (2011) [31] reported that addition of polyethylene glycol (PEG) to chestnut, tara and myrabolan tannins only caused a modest increase in gas and CH_4 production, whereas PEG inclusion with tea tannins decreased CH_4 and gas production. Some authors have suggested that the molecular weight of CT has a direct effect on CH_4 production with the impact more pronounced at higher molecular weight [32]. Thus, similar to Pellikaan et al. (2011) [31] it seems that responses in gas and CH_4 production to irradiation processing differed among tannin sources.

Population of protozoa

The number of *Diplodinium* was significantly different between low dose of gamma and electron irradiated pomegranate peel. Generally, the results showed that total population and five genera of protozoa did not changed by irradiation treatment (P>0.05). similarly Abarghuei et al. (2013) [33] reported that pomegranate peel extract (PPE) had no effect on populations of *Dasytricha*, *Diplodinium*, *Eudiplodinium*, *Stracodinium*, *Polyplastron* and *Ophryoscolex*, Total number of protozoa, genus *Isotricha* and *Entodinium* in cows offered PPE diet was lower than in those fed the control diet without PPE. Researches on the effect of plant secondary metabolites (PSM) on ruminal protozoa population were not consistent, i.e. either no effect [34], decreases [35] or increases [36]. Such discrepancies may be due to the diet type, animal variability, sampling methods [37], level and type of plant metabolites [38], variability in the adaptation of the protozoa to plant secondary metabolites (PSM), and previous experience of animal to PSM [39-40].

Correlation between CH_4 production, tannin content and population of protozoa

Although a significant correlation between condensed tannin and CH_4 production did not observe, the correlation was negative in gamma ray irradiated pomegranate peel. This could be due to lower decreasing condensed tannin in electron radiation compared to gamma radiation and resulted in higher methane production. The lowest methane production (14.91%) in 15 kGy of electron radiation treatment supports this. Therefore electron irradiation treatments could display suppression potential of methane production of condensed tannin in pomegranate peel. Tannin could induce methane reduction because of inhibition of fibre degradation, reduction in protozoa and/or methanogenic archaea population. Many studies have shown that feeds containing tannins reduce CH_4 emissions from ruminants [41-42,11]. However, Behatta et al. [43] found that T. Chebula containing tannin showed lower methane suppression property in relation to other samples investigated. The reason for this discrepancy may be due

to the different CT, and/or that the level of tannin to cause a reduction in CH_4 production [44]. This result showed that suppression effects of electron irradiated of condensed tannin pomegranate peel was better than gamma ray and the control group on methane production.

In the present study, a significant negative correlation coefficients were observed between methane production and total protozoa population and also between gas volume at 24 h and total protozoa population of gamma irradiated pomegranate peel ($P<0.05$). Bhatta et al. [12] reported that graded levels of the tannin source were incubated with the basal diet there was no increase in archaea bacterial counts despite the suppression of protozoa. Tannins suppressed methanogenesis directly through their antimethanogenic and indirectly through their antiprotozoal property [12]. Methanogenic archaea are associated symbiotically with the ciliate protozoa on the surface (ectosymbionts) and inside the protozoa (endosymbionts [45]); hence, a reduction in protozoal counts may decrease archaeal counts as well. In the presence of tannin, both protozoal and archaeal numbers were reduced, which likely led to a compensatory increase in the population of other ruminal bacteria [46]. But, Soliva et al. [47] did not find an increase in the bacterial population after suppression of protozoa, and this was attributed to an adverse effect of tannins on some bacterial species as well. Furthermore, Dohme et al. [48] showed that medium chain fatty acids (MCFA) supplementation via coconut oil suppressed methanogenesis in both faunated and defaunated ruminal fluid. Bhatta et al. [43] failed to record this association between methane production and protozoa population. Probable reasons could be that effects of tannin on protozoal numbers were variable and some of the tannin might have direct effect on methanogenic archaea, which are not associated with the protozoa. Our results are in agreement with recent meta-analysis report of Jayanegara et al. [49] that there was no direct relationship between condensed tannin and protozoa counts.

CONCLUSION

The results showed that both gamma and electron beam irradiation significantly decreased condensed tannin than control. Irradiation did not change methane production. The results showed that irradiation had no significantly effect on total population and five genuses of protozoa (*Diplodinium*, *Entodinium*, *Dasytricha*, *Isotricha* and *Ophryoscolex*). Although a significant correlation between condensed tannin and CH_4 production did not observe, the correlation was negative in gamma ray irradiated pomegranate peel. Result showed that suppression effects of electron irradiated pomegranate peel condensed tannin at a dose of 15 kGy was better than gamma ray and the control group on methane production. While the variability in response tannin sources may be viewed as a constraint, it also provides an opportunity to select highly efficient sources. Research is needed to find the balance between reducing CH_4 production and the potentially anti-nutritional side-effects associated with condensed tannin.

A significant negative correlation coefficients between methane production and total protozoa population and between gas valume at 24 h and total protozoa population of gamma irradiated pomegranate peel were observed ($P<0.05$). Our results are in agreement with recent meta-analysis report of Jayanegara et al. [49] that there is no direct relationship between condensed tannin and protozoa counts. Therefore, further investigation of this plant is warranted. A systematic evaluation is needed to determine suitable levels of supplementation in order to attain a maximal depressing effect on enteric CH_4 production [50]. Further, in this study it was also established that methanogenesis was not essentially related to the protozoa population.

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