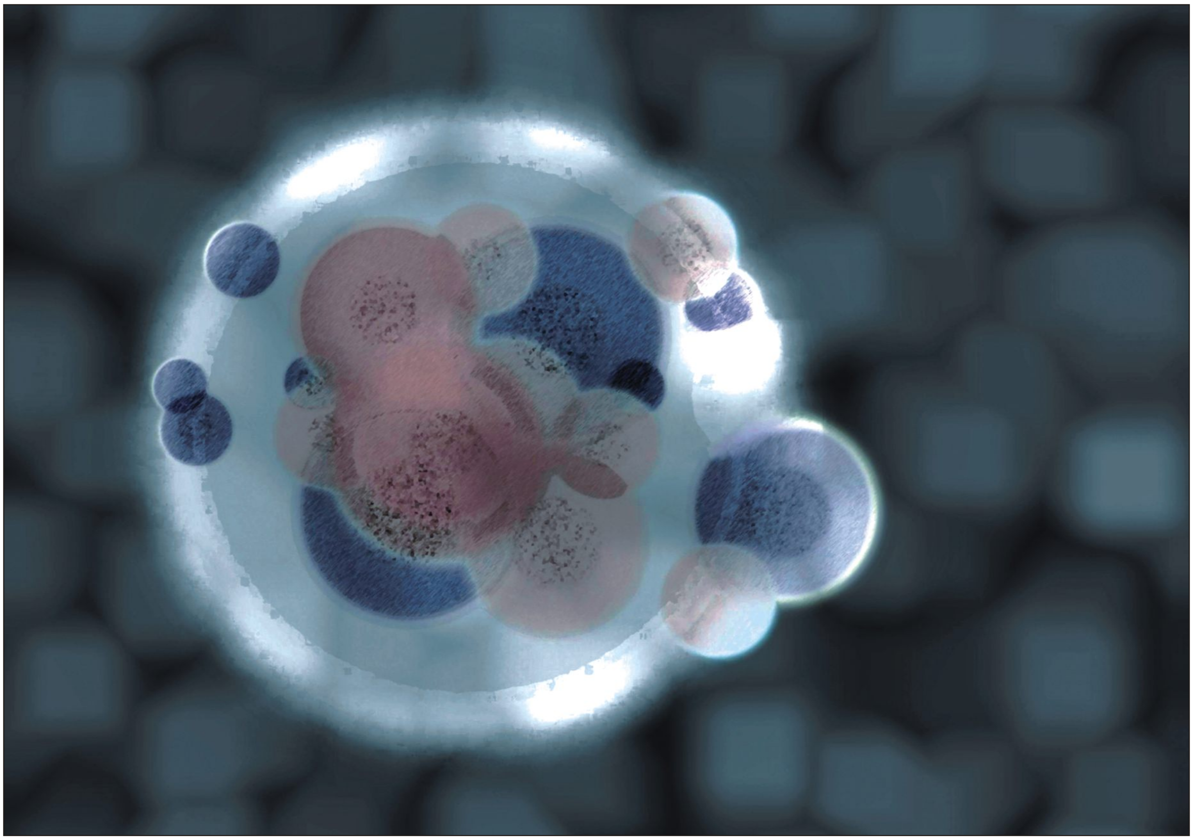

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Research Article
In vitro Rumen-fermentation and Gas Production of Pomegranate Peel with or Without Polyethylene Glycol

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Abstract
Background and Objective: Rising production of pomegranate juice causes to the gathering a novel by-product, i.e., pomegranate peel, which have attracted attention for their obvious wound-recovering characteristics. This paper aimed to evaluate the best percentage (%) addition of pomegranate peel (PP), detanninated pomegranate peel (DPP) and pomegranate peel with polyethylene glycol) (PP+PEG) on *in vitro* characteristics. **Materials and Methods:** The treatments were control has a total mixed ration consisted of concentrates feed mixture, Egyptian clover and wheat straw (2:1:1), from Ration 1 (R1) to R5 have control+PP, R6-R10 have control+DPP 1, 2, 3, 4 and 5% DPP, respectively, finally R11-R15 have control+PP with 20 g PEG. The experimental rations were incubated *in vitro* with rumen liquor for 24 h and the amount of the production of gas has been measured. **Results:** The findings demonstrated that the dry matter digestibility in R11, R12, R13 and R6 were the highest insignificant. The gas production volume values were highly significant of R11, R12, R13, R14 and R15, the polyethylene glycol (PEG) supplementation demonstrated the highest significant impact on organic matter digestibility (OMD), short-chain fatty acids (SCFA), metabolisable energy (ME) and net energy for lactation (NEL) between the experimental rations. Addition the PEG for both 1, 2 and 3% pomegranate peel and utilized 1% detanninated pomegranate peel raised dry matter digestibility (DMD), OMD and could utilize them as ruminants feed supplement full of a valuable nutritional element at an awfully low expense. **Conclusion:** Pomegranate peel+polyethylene glycol (PEG) and detanninated pomegranate peel have a possibility relative nutritive value in farm animals under the condition of *in vitro* studies.

Key words: Pomegranate peel, detanninated pomegranate peel, polyethylene glycol, *in vitro* gas production, digestibility, short-chain fatty acids

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pomegranate (*Punica granatum* L.) has been cultivated all over in tropical and subtropical areas including Iraq, Iran, California, Turkey, Egypt, Italy, India, Chile and Spain[1]. Pomegranate fruit has consisted of only three components: the seeds, which represents 3% of weight of the fruit; the fruit drink, which represents 30% of the fruit weight and peels, that involves husk and internal membranes. Pomegranate peels are vanished after producing juice and becoming equipped to eat[2]. Rising production of pomegranate juice causes to the gathering a novel by-product, i.e., pomegranate peel.

Pomegranate peels consist of an enormous number of polyphenols[1]. Pomegranate peel parts have attracted attention for their obvious wound-recovering characteristics[3], immunomodulatory activity[4], antibacterial activity[5] and antiatherosclerotic and antioxidative capacities[6].

Tannins are high molecular weight phenolic component, that exists in numerous plants, including pomegranate fruit and by-product. Shabtay *et al.*[7] indicated that utilizing pomegranate peel up to 20% in calves diet has negative effect on fattening performance and consequently average daily gain were increased.

Low and moderate of condensed tannins in the diet (2-4.5%) have improved production effectiveness in ruminants, via raising the flow of non-ammonia nitrogen and essential amino acids from the rumen.

While polyphenolic compounds may improve animal health, they can reduce proteolytic activity, so in the current research, we utilized detanninated pomegranate peel and Polyethylene glycol (PEG), as a tannin-binding agent to reduce the impact of polyphenolic compounds.

Polyethylene glycol (PEG) that binds tannins irreversibly, decreasing the adverse impacts of tannins on food intake[8], digestibility[9] and preferences[10]. Polyethylene glycol (PEG) has been successfully utilized for nutritional evaluation of condensed tannins (CT)-rich foods[11]. It has a high affinity for binding condensed tannins and preventing the formation of tannin-protein complexes. So, PEG raises the intake of high tannin forages by ruminants vi mitigating adverse impacts of tannins[6,10,12]. Salem *et al.*[13] pointed that polyethylene glycol (PEG, mol. wt. 4000) inactivated acacia condensed tannins, thus improved microbial protein synthesis, acacia intake and digestion and growth of sheep. According to Decandia *et al.*[14], the inactivation of tannins via PEG raised the availability of nutrients and reduced microbial inhibition, leading to raised degradability of nutrients and best animal performance.

The purpose of this research was to estimate the addition of pomegranate peel (PP), detanninated pomegranate peel (DPP) and pomegranate peel with polyethylene glycol (PEG) on *in vitro* gas production characteristics, dry matter digestibility (DMD), fiber fractions, pH, ammonia concentration (NH_3N), organic matter digestibility (OMD), metabolisable energy (ME), net energy for lactation (NEL) and short-chain fatty acids (SCFA) utilizing *in vitro* method of gas production.

MATERIALS AND METHODS

Study area: This study was conducted at Dairy Science Department, National Research Centre, Dokki, Giza, Egypt during March to April, 2019.

Preparation of pomegranate peel: Dried pomegranate peels were acquired from the by-product unites in Fayoum, Egypt. Peels were crushed in a grinder to reduce it to coarse size peel. Detanninated pomegranate peel prepared according to Kushwaha *et al.*[15], the prepared peel was percolated in distilled water (1:1 w/v) and kept at ambient condition for 12 h then whole material squeezed and filtered through muslin cloth and collecting solid residue leftover muslin cloth and were placed in a hot air oven at 60°C for 18 h to obtain dried material. The dried materials were crushed by food grinder into powder form up to completely pass through 0.5 mm size sieve. Both detanninated and dried pomegranate peel powder transferred in polyethylene bags for chemical analysis and *in vitro* gas production.

Polyethylene glycol (PEG): Polyethylene glycol 4000p produced by Chem-Lab NV, Industriezone "De Arend" 2, Belgium.

Chemical analysis: Dried and detanninated pomegranate peel powder were dried at 105°C and assayed for DM, crude protein (CP), ether extract (EE), crude fiber (CF) and Ash content according AOAC[16]. Nitrogen free extract (NFE) was calculated by subtracting the summation percentages of CP, EE and CF from organic matter (OM), while OM was calculated by subtracting the percentage of ash from one hundred. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin[17]. The tannin contents were determined by Folin denis reagent as described by Makkar *et al.*[18].

***In vitro* gas production:** Batch fermentation culture experiment was conducted according to Ismail *et al.*[19] to evaluate the effect of pomegranate peel, detanninated pomegranate peel and pomegranate peel+polyethylene glycolon rumen fermentation characteristics. A total mixed ration consisted of 50% concentrates feed mixture, 25% Egyptian clover and 25% wheat straw were used as a substrate. For obtaining of the rumen microorganisms (inoculum), rumen fluid was collected from the rumen of slaughtered rams fed berseem hay ration. The tested rations are in Table 1. Each tested rations were tested in 3 replicates accompanied by 3 blank vessels (no substrate). The tested rations (400 mg) were added separately to the 125 mL incubation vessels. Each vessel was filled with 40 mL of a mixture of 1:3 (v/v) rumen fluids: buffer solution (292 mg of $K_2HPO_4{\cdot}3H_2O$, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4{\cdot}7H_2O$, 64 mg of $CaCl_2{\cdot}2H_2O$, 4 mg of Na_2CO_3 and 600 mg of cysteine hydrochloride per liter). All vessels were sealed and incubated at 39°C for 24 h. After 24 h of incubation, all vessels were filtered in fiber filter bags 25-micron porosity (ANKOM- USA). The residues in the bags were dried at 70°C in the oven for 48 h to analyze dry matter (DM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility. Rumen fluid pH was measured using (pH-meter). The overall volume of the produced gases was determined using Hohenheim Syringes (100 mL) as described by Navarro-Villa *et al.*[20]. Quantitative analysis of ammonia concentration was carried out by a modified Nessler's method[21]. The short chain fatty acids (SCFA) concentration was calculated according to the equation of Makkar[22]. The ME, NEL and OMD of pomegranate peels were calculated using equations of Menke and Steingass[23] as:

$$\text{Metabolisable energy (ME) (MJ kg}^{-1}\text{ DM)} = 2.20+0.136\times GP+0.0057\times CP$$

$$\text{Net energy for lactation (NEL) (MJ kg}^{-1}\text{ DM)} = 0.115\times GP+0.0054\times CP+0.014\times EE-0.0054\times CA-0.36$$

$$\text{OMD (g kg}^{-1}\text{ DM)} = 14.88+0.889\times GP+0.45\times CP+0.0651\times CA$$

where, GP is 24 h net gas production volume (mL/200 mg DM) and CP, EE, CA are crude protein, ether extract and crude ash (g kg^{-1} DM), respectively.

Statistical analysis: Statistical analyses were made via the general linear model procedure through SPSS[24]. Duncan test[25] was carried out to separate among means.

RESULTS

Chemical composition: Data in Table 2 revealed that chemical composition of pomegranate peel and ingredients. The detanninated pomegranate peel (DPP) contained lower OM, EE, NFE and tannins compared with dried pomegranate peel (PP), while DPP recorded higher content of DM, CP, CF, NDF, ADF, ADL, hemicelluloses, cellulose and lignin than the dried Pomegranate peel's values.

Table 1: Formulation of the tested rations (on DM basis)

Tested ration	Ingredients (%) CFM*	EC	WS	PP	DPP	PEG (g)
Control	50	25	25	-	-	-
R1	50	25	25	1	-	-
R2	50	25	25	2	-	-
R3	50	25	25	3	-	-
R4	50	25	25	4	-	-
R5	50	25	25	5	-	-
R6	50	25	25	-	1	-
R7	50	25	25	-	2	-
R8	50	25	25	-	3	-
R9	50	25	25	-	4	-
R10	50	25	25	-	5	-
R11	50	25	25	1	-	20
R12	50	25	25	2	-	20
R13	50	25	25	3	-	20
R14	50	25	25	4	-	20
R15	50	25	25	5	-	20

*Concentrates feed mixture, composed of 60% yellow corn, 20% soybean meal, 17.5% wheat bran, 1.5% limestone, 0.2% dicalcium phosphate, 0.3% premix and 0.5% NaCl, EC: Egyptian clover, WS: Wheat straw, PP: Pomegranate peel, DPP: Detanninated pomegranate peel, PEG: Polyethylene glycol

Table 2: Chemical composition of ingredients that used in the tested rations

Items	Ingredients CFM	EC	WS	PP	DPP
DM	87.56	93.54	92.36	90.85	96.75
OM	83.46	78.27	73.04	87.21	84.12
CP	16.45	18.39	4.11	4.84	5.89
EE	2.08	4.50	0.91	4.69	1.62
CF	4.18	16.14	32.62	15.53	18.87
NFE	60.76	39.24	35.39	62.15	57.72
Ash	4.10	15.27	19.32	3.64	12.64
NDF	11.50	49.45	57.53	20.58	30.10
ADF	5.16	40.99	49.76	19.46	29.17
ADL	1.20	12.40	12.18	7.68	12.09
Hemicelluloses	6.33	8.47	7.75	1.11	1.82
Cellulose	3.96	28.58	37.57	11.78	17.07
Lignin	0.53	10.32	5.6628	4.15	6.89
Tannins	-	-	-	15.28	14.65

CFM: Concentrates feed mixture, EC: Egyptian clover, WS: Wheat straw, PP: Pomegranate peel, DPP: Detanninated pomegranate peel, DM: Dry matter, OM: Organic matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, NFE: Nitrogen free extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin

Table 3: Gas production characteristics of the tested rations

Tested ration	DMD (%)	NDFD (%)	ADFD (%)	GP (mL/200 mg)	pH	NH_3N (µmol mL^{-1})
C	49.81^{abc}	39.99^{c}	33.83^{ab}	56.95^{b}	6.87^{a}	3.40^{abc}
R1	48.66^{bcd}	38.32^{d}	30.91^{cd}	54.87^{de}	6.68^{b}	3.25^{abcd}
R2	47.80^{d}	37.31^{de}	28.65^{de}	57.31^{b}	6.66^{b}	3.67^{a}
R3	47.33^{d}	41.65^{bc}	28.35^{def}	56.50^{bc}	6.72^{b}	3.51^{ab}
R4	44.46^{e}	32.55^{f}	25.96^{fg}	56.29^{bcd}	6.38^{cd}	3.40^{abc}
R5	41.96^{f}	30.53^{g}	26.10^{efg}	53.39^{f}	6.43^{c}	3.32^{abcd}
R6	50.04^{ab}	36.22^{e}	30.58^{cd}	55.20^{cde}	6.35^{d}	3.39^{abc}
R7	48.36^{bcd}	36.46^{e}	28.83^{d}	56.70^{bc}	6.37^{cd}	3.65^{a}
R8	44.75^{e}	32.15^{f}	28.40^{def}	56.85^{b}	6.35^{d}	3.43^{abc}
R9	41.82^{f}	29.44^{g}	25.68^{g}	54.39^{ef}	6.24^{e}	3.44^{abc}
R10	38.77^{g}	25.74^{h}	21.75^{h}	53.26^{f}	6.19^{e}	2.97^{defg}
R11	51.54^{a}	44.14^{a}	35.92^{a}	62.74^{a}	5.61^{i}	3.08^{cdef}
R12	51.09^{a}	43.69^{a}	35.22^{a}	62.38^{a}	5.71^{h}	3.12^{bcde}
R13	50.79^{a}	41.77^{b}	33.88^{ab}	62.34^{a}	5.84^{g}	2.74^{efg}
R14	47.90^{cd}	40.15^{c}	31.95^{bc}	62.05^{a}	5.88^{g}	2.69^{fg}
R15	47.54^{d}	37.02^{de}	28.33^{def}	61.57^{a}	5.96^{f}	2.65^{g}
±SE	0.54	0.75	0.57	0.49	0.05	0.05

Average in the same column having different superscripts are differ significantly ($p<0.05$), DMD: Dry matter digestibility, NDFD: Neutral detergent fiber digestibility, ADFD: Acid detergent fiber digestibility, GP: Gas production volume, NH_3N: Ammonia concentration, SE: Standard error

Table 4: Parameters estimated of the tested rations

Tested ration	OMD (%)	SCFA (mmol)	ME (g kg^{-1} DM)	NEL (g kg^{-1} DM)
C	72.44^{b}	1.26^{b}	10.02^{b}	6.24^{b}
R1	70.59^{de}	1.24^{de}	9.74^{de}	6.00^{de}
R2	72.76^{b}	1.27^{b}	10.07^{b}	6.28^{b}
R3	72.03^{bc}	1.25^{bc}	9.96^{bc}	6.18^{bc}
R4	71.85^{bcd}	1.25^{bcd}	9.93^{bcd}	6.16^{bcd}
R5	69.27^{f}	1.18^{f}	9.54^{f}	5.83^{f}
R6	70.88^{cde}	1.22^{def}	9.79^{cde}	6.04^{cde}
R7	72.21^{bc}	1.25^{b}	9.99^{bc}	6.21^{bc}
R8	72.35^{b}	1.26^{b}	10.01^{b}	6.23^{b}
R9	70.16^{ef}	1.20^{ef}	9.68^{ef}	5.95^{ef}
R10	69.16^{f}	1.18^{f}	9.52^{f}	5.82^{f}
R11	77.58^{a}	1.39^{a}	10.81^{a}	6.91^{a}
R12	77.27^{a}	1.38^{a}	10.76^{a}	6.87^{a}
R13	77.22^{a}	1.38^{a}	10.75^{a}	6.86^{a}
R14	76.97^{a}	1.37^{a}	10.72^{a}	6.83^{a}
R15	76.54^{a}	1.36^{a}	10.65^{a}	6.77^{a}
±SE	0.43	0.01	0.07	0.06

Average in the same column having different superscripts are differ significantly ($p<0.05$), OMD: Organic matter digestibility, SCFA: Short chain fatty acids, ME: Metabolisable energy, NEL: Net energy for lactation, SE: Standard error

***In vitro* gas production of experimental rations:** Table 3 showed DMD, NDFD, ADFD, gas production (GP), pH, NH_3N and calculated amounts of organic matter digestibility (OMD), GP volume, SCFA, ME and NEL of the tested rations. Dry matter digestibility in R11, R12, R13 and R6 was insignificantly higher than that of control. Also, R11 and R12 were the highest significant NDFD and ADFD. However, pH was significant highest of control and lowest for R11, R12, R13, R14 and R15. The PEG supplementation has the highest significant effect on GP, OMD, SCFA, ME and NEL between the tested rations.

Also, OMD, SCFA, ME and NEL showed in Table 4 organic matter digestibility recorded high significant values in R11, R12, R13, R14 and R15, respectively compared with the control ration and the same trend of SCFA, ME and NEL.

DISCUSSION

Chemical constituents of dried peels are nearly similar to the outcomes of Kushwaha *et al.*[15] and Taher-Maddah *et al.*[26] founded that CP, EE, ash, CF, NDF and ADF of PP were 3.95, 4.9, 5.49,12.61, 17.83 and 14.55%, respectively, Also Sadq *et al.*[27] indicated that CP, EE, ash and CF of PP were 5.1, 4.9, 3.7 and 11.22% correspondingly. Some variations are founded between chemical compositions of PP in the present research in comparison to those shown by Mirzaei-Aghsaghali *et al.*[28], Ebrahimi[29] and Delavar *et al.*[30]. These variations in the chemical constitution of PP is possibly caused by dissimilar unique materials, growing conditions (geographic, seasonal differences, climatic changes and land properties) and the degree of unfamiliar materials, contaminations, differences, dissimilar processing and measuring systems.

Chemical compositions of DPP were in agreement with Kushwaha *et al.*[15], which they reported that DM (17.63%), ash (3.29%), EE (1.43%), CP (6.43%), CF (24.36%), NDF (28.54%), ADF (26.11%) and lignin (7.59%). Higher chemical compositions in DPP than PP indicate retention and accumulation of the above compositions during the detannination process but in case of lower test values in DPP than PP indicated that losses of the above components during the detannination process.

Gas volume is a good scale from which to predict digestibility and microbial protein synthesis of the materials by rumen microbes in *in vitro* test[31]. It also have shown a close correlation with feed intake[32] and the growth rate in cattle[33]. Kinetics of gas production is depended on at relative proportions of soluble, insoluble but degraded and undegradable materials of the feed[34]. Kamalak *et al.*[35] found that total and soluble condensed tannins were negatively correlated with the volume of gas production. Gas production in the current research are consistent with those of Feizi *et al.*[36] who reported that pomegranate peel tannins had a negative effect on *in vitro* fermentation. Besharati and Ghezeljeh[37] reported that increased gas production with addition of PEG to pomegranate seed[34,38-40]. Pomegranate peel is rich in tannins[41] and tannins bind to the protein and reduce the accessibility of proteins to rumen microorganisms, so in our study, we used DPP and added polyethylene glycol to decreased effect of tannins on intake, digestion and animal performance. Tannins have both detrimental and beneficial effects in ruminant animals, which high concentrations of tannins may decrease intake, digestibility of protein and carbohydrates and then animal performance through their negative effect on digestion[42]. By preventing bloat and increasing the flow of non-ammonia nitrogen and essential amino acids from the rumen[43], while low and moderate (20-45 mg g^{-1} DM) concentrations of condensed tannins in the diet improved production efficiency in ruminants, without increasing feed intake[7]. The increase in gas production in the presence of PEG is possibly due to an increase in the available nutrients to rumen micro-organisms, especially the available nitrogen.

The DMD, OMD, GP, NH_3N, SCFA, ME and NEL values of PP was higher than reported by Shabtay *et al.*[7], Taher-Maddah *et al.*[26], Feizi *et al.*[36] and Besharati and Ghezeljeh[37], the different result may be due to preparation method of PP, differences in variety, environment conditions, estimation methods .

In vitro dry matter and OMD were shown to have high correlation with gas volume[31] and Menke and Steingass[23] suggested that gas volume at 24 h after incubation has been relationship with metabolisable energy in feedstuffs, also, Maheri-Sis *et al.*[44] reported that amount of gas production at 24 h incubation is important because of its high positive correlation by energetic value of feedstuffs.

In farm animals, SCFAs including C_2, C_3, C_4, iso-C_4, C_5 and iso-C_5, that has been in the rumen by fermentation of diets by microbes (e.g. fiber), supply up to 80% of their maintenance requirements from energy. The principal SCFAs; C_2, C_3 and C_4; have been cagerly absorbed as a nutrient cause by the ruminant. The SCFAs are about 50-70% of catable energy intake. Accordingly, it is obvious that higher SCFAs in the gas production system is the consistent index of gas production and energy content of tested diets[28].

The values of pH of PP in our study were similar to Delavar *et al.*[30], who indicated that the pH of PP was 6.48. The values of pH decrease in ration which have PEG may be due to raised passing of obtainable carbohydrate and a raise in the total bacterial population, fermentation and VFA production-regrettably, this outcomes in a lowering in pH value[45]. It can be said that pomegranate peel+polyethylene glycol (PEG) and detanninated pomegranate peel have a possibility relative nutritive value in farm animals under the condition of *in vitro* studies.

CONCLUSION

The outcomes of present research depend on chemical composition, DMD, OMD, GP, ME, NEL and SCFA indicated that addition 20 g Polyethylene glycol (PEG) for both 1, 2 and 3% pomegranate peel and used 1% detanninated pomegranate peel increased DMD and can use them as ruminants feed supplement full of valuable nutritional constituent at very low charge. Pomegranate peel+ and Polyethylene glycol (PEG) and detanninated pomegranate peel have a possibility relative nutritive value in farm animals under the condition of *in vitro* studies. On the other hand,, it is necessary to *in vivo* researches to affirming these outcomes.

SIGNIFICANCE STATEMENT

This study discovers that pomegranate peel can be improves by using polyethylene glycol or water to be detanninated and then it have a possibility relative nutritive value in farm animals under the condition of *in vitro* studies. This study will help the farmers to use it in ruminants feeding and decreasing the pollution in our country.

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