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Effect of Methanolic Extract of Pomegranate Peel on *in vitro* Rumen Fermentation Kinetics of Oil Seed Meals

Research Article

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ABSTRACT

This study was carried out to determine the effects of supplementing different levels (0, 0.5 and 1% of buffered rumen fluid) of methanolic extract of pomegranate peel, on rumen fermentation kinetics of four oil seed meals (soybean meal, cotton seed meal, rapeseed meal and sunflower seed meal), using *in vitro* gas production technique. The samples were incubated in syringes containing rumen liquor taken from three fistulated Iranian Ghezel rams for 2, 4, 6, 8, 12, 24 and 36 h. Results showed that, addition of methanolic extract of pomegranate peel led to significant increase in gas production volume in all incubation times and all of oil seed meals as well. Amount of gas production, also increased by increasing dose of the extract. Also, amounts of *a* (the gas production from the immediately soluble fraction), *b* (the gas production from the insoluble fraction) and *a* + *b* (the potential gas production) in all of tested oil seed meals increased by increasing pomegranate peel extract doses. Adding pomegranate peel extract resulted in increase volatile fatty acids (VFA) production. Production of VFA increased significantly by the level of the extract supplementation. In conclusion, it is suggested that, adding methanolic extract of pomegranate peel can be lead to higher ruminal fermentation and VFA production in ruminants.

KEY WORDS fermentation, gas production, methanolic extract, oil seed meals, pomegranate peel.

INTRODUCTION

Using cheap, easy available, native and abundant sources of feedstuffs in animal nutrition is one of the most important strategies in developing countries such as Iran. For this purposes, agro-industrial by-products are the first candidate. In addition to nutritional value of these by-products which are usually loosed to the environment (Mirzaei-Aghsaghali and Maheri-Sis, 2008; Delavar *et al.* 2014), some of them are having functional properties owing to possess secondary metabolites. Plant secondary metabolites may be used in order to modulating rumen microbial population and diversity, volatile fatty acids production and proportion and methane production as well as nitrogen metabolism. Applying essential oils can be a useful strategy to improve efficiency of nutrient utilization by ruminants (Benchaar *et al.* 2008; Tajodini *et al.* 2014). Hassanpour *et al.* (2011) also stated that plant secondary metabolites may exert beneficial effects on protein metabolism and decreasing rumen degradation of dietary protein and increasing absorption of amino acids in the small intestine. Although medicinal plants are used due to their health related characteristics such as antimicrobial, antioxidant, anti-inflammatory, antiparasitic and anticancer properties (Sirohi *et al.* 2012a; Sirohi *et al.* 2012b), their production are laborious and expensive. Thus, agro-industrial by-products with same properties are not only being economic but also reducing environmental pollution risk. Pomegran-

ate processing wastes are of the most important by-products with potentially good nutritional value and health benefits in animal production (Ebrahimi *et al.* 2012; Canbolat *et al.* 2014; Delavar *et al.* 2014). Pomegranate (*Punica granatum*) is an important fruit in Iran. Iran is the main origin of this fruit and has the first rank of quality, varieties, cultivation area, production and export in the world (Kohansal and Rahimi, 2013). Worldwide production of pomegranate is approximately 1.5 million tons and Iran produces 47% of world production (FAO/WHO, 2009). Most of pomegranate produced in the country consumed as fresh or processed for producing juice and sauce. Estimated annual production of pomegranate processing by-products exceeds 120000 tons in Iran, where peels constitute approximately 50-60% of fruits weight (Shabtay *et al.* 2008; Mirzaei-Aghsaghali *et al.* 2011).

In recent years, due to production of huge amounts of pomegranate by-products in Iran, many researchers in the country, prompted to investigate feeding value and functional capacity of these by-products for ruminant animals (Feizi *et al.* 2005; Mirzaei-Aghsaghali *et al.* 2011; Modaresi *et al.* 2011; Ebrahimi *et al.* 2012; Taher-Maddah *et al.* 2012a; Taher-Maddah *et al.* 2012b; Delavar *et al.* 2014). Mirzaei-Aghsaghali *et al.* (2011) reported that metabolizable energy, net energy for lactation, organic matter digestibility and short chain fatty acids production of pomegranate peel are higher than that of pomegranate seeds for ruminants. In addition to higher nutritional value, pomegranate peel also contains higher amounts of polyphenolic compounds, stronger biological activities as well as more antioxidant and antimicrobial capacity than the juice and seeds (Negi and Jayaprakasha, 2003; Olaniyi *et al.* 2012; Manuel Viuda-Martos *et al.* 2013). Oliveira *et al.* (2010) reported that pomegranate extracts contain polyphenolic compounds, which have been shown to possess functional properties such as antioxidant and antimicrobial potency. Most recently, Iranian researchers (Abarghuei *et al.* 2013; Abarghuei *et al.* 2014a, Abarghuei *et al.* 2014b) have used pomegranate peel extract in ruminant nutrition due to its rumen modulator properties. Abarghuei *et al.* (2013) found that supplementation of pomegranate peel extract decreased protozoa population, NH_3-N concentration, and increased microbial protein together with milk yield and quality of dairy cows. In another study, Abarghuei *et al.* (2014a) indicated that pomegranate peel extracts successfully manipulate *in vitro* rumen fermentation products, in particular increased propionate concentration and decreased acetate, proportion of acetate to propionate, ammonia nitrogen production and protozoa population in sheep.

Nowadays, oil seed meals are of the main constituents of the dairy and fattening cows' diets. They are supplying major part of energy and protein needs of the ruminants (Nezarati *et al.* 2014). Modulating condition of the rumen may be improves efficiency of protein metabolism and volatile fatty acids production. Because of higher price of oil seed meals as well as their direct involving in supplying energy and protein in the ruminants' diets, we have decided to assess the effect of adding a rumen modulator on their fermentation kinetics. Therefore, the aim of current study was to evaluate the effect of different levels of methanolic extract of pomegranate peel on ruminal gas production parameters and volatile fatty acids production from soybean meal, cotton seed meal, rapeseed meal and sunflower seed meal under *in vitro* condition.

MATERIALS AND METHODS

Samples collection and preparation

Samples of the soybean meal, sunflower meal, rapeseed meal and cotton seed meal were obtained from a commercial unit in Tabriz, Iran. Collected samples were milled through a 1 mm sieve for chemical analysis and gas production procedure. Chemical composition of tested oil seed meals have been reported in in our recently published paper (Nezarati *et al.* 2014).

In vitro gas production

The study was carried out to determine the effects of supplementing different levels (0, 0.5 and 1% of buffered rumen fluid) of methanolic extract of pomegranate peel, on rumen fermentation kinetics of oil seed meals using *in vitro* gas production technique.

Rumen fluid required for *in vitro* incubation obtained from three fistulated Ghezel rams fed twice daily with a diet containing mixture of roughage and concentrate (60:40) before the morning feeding. About 200 mg dry weights of samples (soybean meal, sunflower meal, rapeseed meal and cotton seed meal) were weighed in triplicate into 100 mL calibrated glass syringes following the procedures of Menke and Steingass (1988).

The syringes were pre-warmed at 39 ˚C before the injection of 30 ml rumen fluid-buffer mixture (1:2) into each syringe and incubated in an incubator at 39 ˚C. The samples were incubated in syringes containing rumen liquor taken from three fistulated Iranian Ghezel rams for 2, 4, 6, 8, 12, 24 and 36 h.

All samples were incubated in triplicate. Three syringes containing only rumen fluid-buffer mixture considered as the blank. The net gas productions of samples were determined by correcting gas volumes for blanks. Net gas production data were fitted to the exponential model outlined by Ørskov and McDonald (1979) and model components (*a, b, c*) calculated by FITCURVE software version 6 (Chen, 1995):

$Y = a + b(1-e^{-ct})$

Where:
Y: gas production at time t.
a: gas production from soluble fraction (mL/200 mg DM).
b: gas production from insoluble but fermentable fraction (mL/200 mg DM).
c: gas production rate constant for the insoluble fraction (mL/h).
a + *b*: potential gas production (mL/200 mg DM).
t: incubation time (h).
e: base for natural logarithms (2.718).

Volatile fatty acids (VFA) were calculated by equation of Makkar (2005):

VFA (mmol)= 0.0222 GV – 0.00425

Where:
GV: 24 h net gas production volume (mL/200 mg DM).

Statistical analyses

Data from *in vitro* gas production test were subjected to analysis of variance as a completely randomized design with four treatments including soybean meal, rapeseed meal, cotton seed meal, sunflower seed meal (three replicates for each treatment) using general linear model (GLM) procedure of SAS (2001). Means were compared by Duncan's multiple range tests (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Gas production volume

Effect of supplementing methanolic extract of pomegranate peel on *in vitro* gas production volume of soybean meal, cotton seed meal, rapeseed meal and sunflower seed meal at different incubation times have been illustrated in Tables 1 to 4. Results showed that, addition of methanolic extract of pomegranate peel led to significant increase in gas production in all incubation times and all of oil seed meals as well. In other hand, gas production, also increased by increasing dose of the extract. This is an unexpected result; because we had hypothesized that pomegranate peel extract should decrease gas production due to modifying microbial fermentation. Our results were partly in line with Pashachalandari *et al.* (2014) and Niasati *et al.* (2014). Pashachalandari *et al.* (2014) demonstrated that gas production volume obtained from canola meal initially (till 12 h incubation) decreased and then (24-96 h incubation) increased when supplemented by different levels of methanolic extract of the nettle (*Urtica dioica*). Niasati *et al.* (2014) also obtained same results when added methanolic extract of *Viscum album* on soybean meal.

But they could not interpret gas production enhancement from oil seed meals treated by medicinal plants extracts. Our previous *in vitro* studies by some medicinal plants extracts led to various results (mostly decrease) in gas production from practical diets and oil seed meals (Halimi Shabestari *et al.* 2011; Mirzadeh Ahari *et al.* 2011; Salamat Azar *et al.* 2011; Salamatazar *et al.* 2011; Rezaei *et al.* 2011; Salamat Azar *et al.* 2012; Salamatazar *et al.* 2012).

Salamat Azar *et al.* (2011) showed that *in vitro* addition of *Zataria multiflora* water extract on soybean meal at the level of 0.15 mL/30 mL buffered rumen fluid, could not affect gas production amounts in any incubation times. While in other study, Salamatazar *et al.* (2011), found that supplementation of sunflower meal by the *thyme* methanolic extract, at the same level significantly decreased *in vitro* gas production volume in all incubation times. Kilic *et al.* (2011) indicate that essential oils, doses, and essential oils × dose interactions significantly affected *in vitro* gas production.

They have concluded that gas production kinetics may be affected differently by various essential oils. In their study, *in vitro* gas production was decreased by essential oils of oregano (*Origanum vulgare*), garlic (*Allium sativum*) and anise (*Pimpinella anisum*), unaffected by black seed (*Nigella sativa*), laurel (*Laurus nobilis*) and cinnamon (*Cinnamomum verum*) and increased by cumin (*Cumminum cyminum*).

Maleki Baladi *et al.* (2014) reported that effect of tannin extract obtained from pomegranate pomace on *in vitro* gas production volume of soybean meal at different incubation times is dose dependent. Treating soybean meal by 0, 1.5 and 3% pomegranate pomace extract produced approximately same amount of gas; while addition of 4.5 and 6% extract resulted in significant reduction in gas production volume.

In another study, Abarghuei *et al.* (2014a) have evaluated different levels (0, 15 and 30 mg total phenolic compounds per g dry matter) of pomegranate peel extracted by either water or solvent mixture on *in vitro* gas production and ruminal fermentation patterns and found that gas production at 24 h incubation time, was not significantly affected by the type and levels of pomegranate peel extract.

In spite of the fact that decreased *in vitro* gas production by some essential oils may indicate more efficient utilization of energy due to the controlled loss of energy as methane; it may not easily to interpret gas production enhancement when some medicinal plants derived materials added to the rumen liquor. It is notable that, some other researchers (Kilic *et al.* 2011; Niasati *et al.* 2014; Pashachalandari *et al.* 2014) have also mentioned to the variable effects of medicinal plants extracts on ruminal *in vitro* gas production kinetics.

Table 1 Effect of addition of methanolic extract of pomegranate peel on *in vitro* gas production volume (mL/200 mg DM) of cotton seed meal at different incubation times (h)

Incubation time (h)	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
2	8.49^{c}	20.12^{b}	24.68^{a}	< 0.0001	0.6127
4	13.04^{c}	29.71^{b}	46.55^{a}	< 0.0001	1.1504
6	15.09^{c}	34.91^{b}	59.91^{a}	< 0.0001	1.1664
8	16.98^{c}	39.15^{b}	66.82^{a}	< 0.0001	1.6825
12	21.38^{c}	45.13^{b}	80.50^{a}	< 0.0001	1.5608
24	30.04^{c}	65.26^{b}	104.57^{a}	< 0.0001	2.7302
36	34.91^{c}	84.75^{b}	118.40^{a}	< 0.0001	4.2529
48	37.26^{c}	105.51^{b}	129.56^{a}	< 0.0001	6.2387
72	38.37^{c}	107.71^{b}	152.36^{a}	< 0.0001	6.5513

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Effect of addition of methanolic extract of pomegranate peel on *in vitro* gas production volume (mL/200 mg DM) of soybean meal at different incubation times (h)

Incubation time (h)	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
2	11.79^{c}	23.75^{b}	28.62^{a}	< 0.0001	0.5237
4	18.71^{c}	32.87^{b}	46.07^{a}	< 0.0001	0.6195
6	24.85^{c}	39.32^{b}	60.38^{a}	< 0.0001	0.6343
8	31.13^{c}	44.97^{b}	71.23^{a}	< 0.0001	0.9839
12	39.47^{c}	54.88^{b}	86.01^{a}	< 0.0001	1.2642
24	51.10^{c}	79.72^{b}	115.10^{a}	< 0.0001	1.5540
36	51.42^{c}	102.84^{b}	139.95^{a}	< 0.0001	1.6524
48	52.68^{c}	125.64^{b}	165.26^{a}	< 0.0001	1.7548
72	53.94^{c}	129.73^{b}	214.32^{a}	< 0.0001	2.0978

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Effect of addition of methanolic extract of pomegranate peel on *in vitro* gas production volume (mL200 mg DM) of rapeseed meal at different incubation times (h)

Incubation time (h)	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
2	12.73^{c}	18.55^{b}	20.13^{a}	< 0.0001	0.3479
4	20.59^{c}	35.21^{b}	47.95^{a}	< 0.0001	0.7934
6	26.88^{c}	44.02^{b}	65.40^{a}	< 0.0001	0.7781
8	32.38^{c}	50.31^{b}	71.53^{a}	< 0.0001	1.1097
12	38.83^{c}	58.80^{b}	85.06^{a}	< 0.0001	1.0705
24	47.32^{c}	78.61^{b}	106.75^{a}	< 0.0001	1.5168
36	51.40^{c}	101.25^{b}	127.98^{a}	< 0.0001	2.3369
48	53.45^{c}	127.82^{b}	147.79^{a}	< 0.0001	3.5277
72	54.07^{c}	129.86^{b}	163.51^{b}	< 0.0001	3.8615

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Effect of addition of methanolic extract of pomegranate peel on *in vitro* gas production volume (mL/200 mg DM) of sunflower meal at different incubation times (h)

Incubation time (h)	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
2	4.41^{c}	21.23^{b}	23.59^{a}	< 0.0001	1.0868
4	8.17^{c}	27.51^{b}	45.12^{a}	< 0.0001	0.5545
6	15.25^{c}	30.18^{b}	55.18^{a}	< 0.0001	0.8186
8	17.45^{c}	34.43^{b}	62.57^{a}	< 0.0001	0.8272
12	20.44^{c}	41.19^{b}	70.00^{a}	< 0.0001	0.8916
24	27.36^{c}	60.38^{b}	74.22^{a}	< 0.0001	1.4284
36	28.30^{c}	81.13^{b}	88.99^{a}	< 0.0001	1.8400
48	29.72^{c}	99.21^{b}	105.19^{a}	< 0.0001	1.9990
72	30.66^{c}	99.92^{b}	139.78^{a}	< 0.0001	2.4669

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Most of workers referred this variation to the varied antioxidant, antibacterial, antiprotozoal, antifungal and antiviral potency of medicinal plants (Calsamiglia *et al.* 2007; Benchaar *et al.* 2008; Shabtay *et al.* 2012).

Some others also suggested that the dissimilar results obtained from different functional plant materials on rumen fermentation pattern may be depends on rumen conditions (e.g. pH), chemical and physical nature of the tested feedstuffs, animal species, rumen microbial population and diversity, history of feeds offered to the experimental animals, duration of the experiment period, adaptation time and dose of the utilized medicinal plants as well as preparation methods such as extracting solvent and procedure (Kilic *et al.* 2011; Sirohi *et al.* 2012a; Sirohi *et al.* 2012b; Tajodini *et al.* 2014).

Gas production parameters

Effect of methanolic extract of pomegranate peel on *in vitro* gas production parameters (*a, b, c, a+b*) of soybean meal, cotton seed meal, rapeseed meal and sunflower seed meal have been illustrated in Tables 5 to 8. There are significant differences for *a* (the gas production from the immediately soluble fraction), *b* (the gas production from the insoluble fraction) and *a* + *b* (the potential gas production) between treated and untreated oil seed meals. Amounts of *a, b* and *a* + *b* in all of tested oil seed meals increased by pomegranate peel extract treating; higher dose of extract addition, have led to higher amounts of *a, b* and *a* + *b.* Since, gas production parameters (*a, b* and *a+b*), are subsequent of gas production volume in gas test technique, it was predictable that, increase in gas volume have been conduced to increase in gas production parameters. In spite of harmonic increase in *a, b* and *a* + *b*, the parameter *c* (the gas production rate constant for the insoluble fraction), had different behavior dependent on type of tested oil seed meals. It is unaffected by pomegranate peel extract supplementation, in case of rapeseed meal, and decreased in other oil seed meals. However, it was reduced usually by increasing extract dose. Salamatazar *et al.* (2011) reported that adding thyme methanolic extract at the levels of 0.15 and 0.3 mL/30 mL buffered rumen fluid, decreased all of the *in vitro* gas production parameters (*a, b, c, a+b*) of sunflower meal. While similar to our findings, Niasati *et al.* (2014) and Pashachalandari *et al.* (2014) showed that *in vitro* gas production parameters (*b, c, a+b*) of soybean meal and canola meal increased when rumen liquor supplemented with methanolic extract of *Urtica dioica* and *Viscum album.* However, they have reported that parameter *c* was unaffected or decreased by adding above mentioned extracts, they did not find any interpretation for their results.

Maleki Baladi *et al.* (2014) evaluated the effects of adding 1.5, 3, 4.5 and 6% tannin extracted from pomegranate pomace on *in vitro* gas production parameters of soybean meal. They have reported that *a* + *b* of soybean meal was significantly reduced, when it was treated with 4.5% and 6% of tannin extract; but not by 1.5 and 3%. In their study, amount of parameter *c* remained unaffected by extract of pomegranate pomace. In contrast, Abarghuei *et al.* (2014a) informed that parameter *c* significantly reduced by adding pomegranate peel extract, however they are showed that asymptotic gas production (*a+b*) did not affected by type or level of pomegranate peel extract. Although, various results obtained from supplementing medicinal plants extracts in different *in vitro* studies, but results of current study surprisingly unexpected. In spite of the fact that it can be as a result of gas produced from either alcohol residues or the fermentable main constituents in the methanolic extract of pomegranate peel, we cannot interpret and justify our findings and it is remain unknown. Further knowledge and studies required for recognizing and interpreting the current results.

Volatile fatty acids (VFA) production

Estimated *in vitro* VFA production has been shown in Tables 5 to 8. As it is shown, VFA productions from all of the tested oil seed meals significantly affected by supplementing pomegranate peel extract. Adding pomegranate peel extract has led to increase VFA production. Production of VFA increased significantly by the level of the extract supplementation. It is well known that reduction in gas production associated with increase in VFA production is one of the important desires of ruminant nutritionists. Since VFA contributes to at least 65 to 75% of the total metabolizable energy supply for ruminants; so decreasing gas production (including methane) will be resulted in improved rumen energy efficiency as well as reducing environmental problems. Albeit approaching this goal is not easy, because gas production quantitatively and qualitatively is a result of VFA production. Enhancement of VFA production is a main index of higher digestibility and energetic value (Maheri-Sis *et al.* 2008; Mirzaei-Aghsaghali *et al.* 2011).

Castillejos *et al.* (2006) stated that many of essential oil compounds have important antimicrobial activity and decreased total VFA concentration, although at appropriate doses, these compounds also modified rumen microbial fermentation without decreasing total VFA concentration. Benchaar *et al.* (2008) also stated that effects of secondary metabolites on total VFA concentration and VFA pattern is variable among studies, depending on the dosage and the source of component. Thus exploring and investigating natural compounds and their suitable dose in order to achieving later characteristics may be a useful vector in ruminant nutrition science. Previously, we have found that estimated VFA production of canola meal decreased by *in vitro* ruminal supplementation of *Thymus vulgar* (Salamatazar *et al.* 2012). Kilic *et al.* (2011) deduced that higher dose of thymol and carvacrol decreased and Eugenol increased ruminal VFA production.

Table 5 *In vitro* gas production parameters and estimated VFA production of cotton seed meal affected by methanolic extract of pomegranate peel

Item	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
a (mL)	5.50^{c}	15.99^{b}	23.64^{a}	< 0.0004	1.5219
b (mL)	33.93^{b}	110.21^{a}	127.09^{a}	< 0.0003	7.8479
a + *b* (mL)	39.44^{b}	126.21^{a}	150.74^{a}	< 0.0003	9.3268
c (/h)	0.05^{a}	0.02^{b}	0.04^{a}	< 0.0230	0.005
VFA (mmol)	0.66^{c}	1.44^{b}	2.31^{a}	< 0.0001	0.0605

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
a: the gas production from the immediately soluble fraction; *b*: the gas production from the insoluble fraction; *a* + *b*: the potential gas production and *c*: the gas production rate constant for the insoluble fraction *b* (/h).
VFA: volatile fatty acids.

Table 6 *In vitro* gas production parameters and estimated VFA production of soybean meal affected by methanolic extract of pomegranate peel

Item	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
a (mL)	0.77^{c}	16.65^{b}	32.54^{a}	< 0.0001	0.4321
b (mL)	52.80^{c}	132.95^{b}	263.08^{a}	< 0.0001	1.7890
a + *b* (mL)	53.57^{c}	149.58^{b}	295.63^{a}	< 0.0001	1.9949
c (/h)	0.10^{a}	0.02^{b}	0.01^{c}	< 0.0001	0.0008
VFA (mmol)	1.13^{c}	1.76^{b}	2.55^{a}	< 0.0001	0.0389

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
a: the gas production from the immediately soluble fraction; *b*: the gas production from the insoluble fraction; *a* + *b*: the potential gas production and *c*: the gas production rate constant for the insoluble fraction *b* (/h).
VFA: volatile fatty acids.

Table 7 *In vitro* gas production parameters and estimated VFA production of rapeseed meal affected by methanolic extract of pomegranate peel

Item	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
a (mL)	4.30^{c}	12.62^{b}	23.09^{a}	< 0.0078	2.7531
b (mL)	48.95^{c}	98.73^{b}	142.04^{a}	< 0.0199	16.697
a + *b* (mL)	53.26^{c}	111.35^{b}	165.13^{a}	< 0.0169	19.337
c (/h)	0.10	0.06	0.04	< 0.1227	0.0172
VFA (mmol)	1.04^{c}	1.74^{b}	2.36^{a}	< 0.0001	0.0336

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
a: the gas production from the immediately soluble fraction; *b*: the gas production from the insoluble fraction; *a* + *b*: the potential gas production and *c*: the gas production rate constant for the insoluble fraction *b* (/h).
VFA: volatile fatty acids.

Table 8 *In vitro* gas production parameters and estimated VFA production of sunflower meal affected by methanolic extract of pomegranate peel

Item	Control	Methanolic extract (0.5%)	Methanolic extract (1%)	P-value	SEM
a (mL)	-2.00^{c}	15.04^{b}	36.41^{a}	< 0.0001	0.5397
b (mL)	31.76^{c}	102.22^{b}	135.15^{a}	< 0.0001	7.1147
a + *b* (mL)	33.77^{c}	117.27^{b}	171.57^{a}	< 0.0001	7.2525
c (/h)	0.11^{a}	0.02^{b}	0.004^{c}	< 0.0001	0.0051
VFA (mmol)	0.60^{c}	1.33^{b}	1.64^{a}	< 0.0001	0.0317

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
a: the gas production from the immediately soluble fraction; *b*: the gas production from the insoluble fraction; *a* + *b*: the potential gas production and *c*: the gas production rate constant for the insoluble fraction *b* (/h).
VFA: volatile fatty acids.

Tajodini *et al*. (2014) reviewed that these differing results may be partially explained by the experimental conditions of studies, including type of diets, animal and plant species, type and concentration of active substances and adaptation as well as pH values of rumen fluid.In the study conducted by Maleki Baladi *et al.* (2014), short chain fatty acids and subsequently metabolizable energy and net energy for lactation of soybean meal have significantly reduced when treated by 4.5% and 6%, but not by lower doses of pomegranate pomace extracted tannin. However, Abarghuei *et al.* (2013) declared that concentrations of total VFA and molar proportions of individual VFA were not influenced by supplementation of pomegranate peel extract in the diet, they have suggested that addition of pomegranate peel extract has reduced protozoa population, NH_3-N concentration, and increased microbial protein. In another study, Abarghuei *et al.* (2014a) concluded that inclusion of water or solvent extracts of pomegranate peel kindly manipulate rumen fermentation parameters, particularly increased propionate and decreased acetate and NH_3-N concentration as well as protozoa population. Refat *et al.* (2015) also found that supplementing high grain diets with pomegran-

ate peel extracts in Rusitec system, results in decreasing total and branched-chain VFA and ammonia nitrogen concentration as well.

Effect of natural rumen modifier compounds on VSAs production and their proportions may be varied owing to their antimicrobial and antioxidant capacity (Benchaar *et al.* 2008; Tajodini *et al.* 2014). Pomegranate peel due to higher and diverse phenolic compounds such as tannins is a vigorous antioxidant, antiprotozoal and antibacterial by-product. Olaniyi *et al.* (2012) showed that pomegranate methanolic peel extracts have strong broad-spectrum activity against Gram-positive and Gram-negative bacteria. However, Castillejos *et al.* (2006) cleared that Gram-positive bacteria are generally more sensitive to essential oils than Gram-negative bacteria; so that pomegranate peel extracts may have ionophores like properties on rumen metabolism without their application anxieties. Jami *et al.* (2012) evaluated the effects of pomegranate peel extract addition to the diet of lactating cows at the levels of 1, 2 or 4% on *in vivo* digestibility as well as rumen bacterial population, and stated that when specific bacteria were examined, some of them did not exhibit any significant change (e.g., *Prevotella* spp.), some of species involved in soluble sugar utilization, such as *Succinivibrio dextrinosolvens*, *Eubacterium ruminantium* and *Streptococcus bovis* increased and some others which are mainly known as cellulose degraders (i.e. *Fibrobacter succinogenes* and *Ruminococcus albus*) significantly decreased.

However, cellulose digestibility was not decreased and neutral detergent fibre (NDF) digestibility even increased in the cows fed with highest level (4%) of pomegranate peel extract. In other hand, Abarghuei *et al.* (2013) and Abarghuei *et al.* (2014a) have emphasized on antiprotozoal capacity of pomegranate peel, which in turn it means that, this by-product may exert anti-methanogenic effects in the rumen and subsequently altering energy efficiency and environmental safety. Alternatively, since protozoa populations are bacteria predator, reducing protozoa community may be resulted in higher growth of bacterial cells and causing to higher VFA and microbial protein production. From antioxidant point of view, phenolic compounds of pomegranate peel may acts as free radical scavenger in rumen environment and by this way leads to enhance rumen health and microbial efficiency.

CONCLUSION

In conclusion, it is suggested that, adding methanolic extract of pomegranate peel to the rumen can be lead to higher ruminal fermentation and VFA production from oil seed meals for ruminants. We cannot suggest a clear mechanism for this result. However, in further studies, it should be considered an additional "control" containing rumen fluid-buffer plus extract without tested feedstuffs.

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