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The Effect of Tannins in Grape Pomace and Oak Leaf on the *in vitro* Organic Matter Digestibility and *in situ* Disappearance of Sheep

Research Article

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ABSTRACT

The effect of grape pomace (*Vitis vinifera*) and in oak leaf (*Quercus libani*) tannins by three runs of *in vitro* gas production on the organic matter digestibility (OMD) and *in situ* dry matter (DM) and crude protein (CP) disappearance were studied in four sheeps. Polyethylene glycol (PEG) was used to deactivate the tannins. *In vitro* gas production was recorded at 2, 4, 6, 8, 12, 48, 72, 96 and 120 h of incubation. The chemical composition (g/kg DM) of grape pomace and oak leaf were 940, 940 organic matter (OM); 94, 116 crude protein (CP); 568, 515 neutral detergent fiber (NDFom); 467, 316 acid detergent fiber (ADFom); 242, 93; (lignin (sa)); 70.5, 82 total phenols (TP); 49.7, 73 total tannins (TT); 79, 5.4 condensed tannin (CT) and 40, 70 hydrolysable tannin (HT). Using grape pomace and oak leaf decreased OMD, short chain fatty acids (SCFA), insoluble but fermentable fraction (b) and fermentation rate (c) comparing to control ($P<0.05$). The addition of PEG increased *in vitro* gas production (IVGP) at all times of incubation. Kinetics of gas production, OMD and SCFA were also increased by PEG incorporation ($P<0.05$). The increase in gas production (%) (IGP) in grape pomace (GP) diet was higher than those in oak leaf (OL) diet. The amounts of total protozoa, *Isotricha*, *Dasytricha*, subfamily of *Entodiniinae*, *Diplodiniinae* and *Ophrioscolecinae* were decreased by addition of grape pomace and oak leaf. The addition of PEG increased total protozoa, subfamily of *Entodiniinae*, *Diplodiniinae* and *Ophrioscolecinae* populations in grape pomace diet ($P<0.05$), but increased *Isotricha*, *Dasytricha* and subfamily of *Diplodiniinae* in oak leaf diet ($P<0.05$). The effective degradability (ED) (g/kg DM) of DM and CP for alfalfa, grape pomace and oak leaf were (646.6, 357.7 and 362.3) and (821, 227.3 and 202), respectively based on *in situ* fermentation. In conclusion, using grape pomace and oak leaf have positively modified NH_3-N concentration and protozoa population. Diet containing grape pomace and oak leaf had lower fermentability than the diet containing alfalfa. Supplementation of PEG in GP and OL diets improved the fermentability of these diets.

KEY WORDS grape pomace, oak leaf, polyethylene glycol, rumen, sheep, tannin.

INTRODUCTION

Grape pomace (*Vitis vinifera*) is produced in vast amounts in many parts of the world (Spanghero *et al.* 2009) and in Iran; production of this by-product exceeds 50000 ton / year (Abarghuei *et al.* 2010). Also, approximately 3 million ha of forest are covered by various oak species, mainly dominated by *Quercus persica*, *Quercus infectoria* and *Quercus libani*, in the west of Iran (Fatahi, 1995). In this region, oak leaf is the main source of forage for goats and sheep, since scarcity of animal feed is the major constraint to animal production in this area. In addition, dry climatic conditions and shortage of water resources in many countries, has led to a scarcity in the quantity and quality of consistent year-round supplies of conventional ruminant feeds. Therefore, the potential use of grape by-products and oak leaf can

overcome not only environmental issues, but balancing food shortage and malnutrition of ruminants in the Middle East and in other vineyard regions across the globe. However, a major limitation of using this feeds as a ruminant feed is the presence of high condensed and hydrolysable tannins content (Abarghuei *et al.* 2010; Abarghuei *et al.* 2011; Yousef Elahi and Rouzbehan, 2008).

Tannins are naturally occurring plant secondary compounds that are present in many species commonly consumed by ruminants. Tannins are generally defined as water soluble polymeric phenolics that precipitate proteins (McSweeney *et al.* 2001b).

They are broadly classified into hydrolysable and condensed tannins. Hydrolysable tannins are gallic acid and ellagic acid esters of a core molecule that consists of polyols including sugars and phenolics (e.g. catechin), whereas condensed tannins consist of oligomers of flavan-3-ols and related flavanol residues, which produce an thocyanidins on acid degradation (McSweeney *et al.* 2001a).

A unique chemical property of tannins is their affinity to bind to feed proteins and thereby reduce excessive breakdown of protein in rumen (Getachew *et al.* 2000) and increase availability of high quality protein for absorption in the lower gut of ruminants (Waghorn *et al.* 1987). In addition to protecting feed proteins from rumen degradation, tannins also play significant roles in the prevention of bloat in ruminants, suppressing intestinal parasites (Min *et al.* 2002) and increasing amino acid absorption (Waghorn *et al.* 1987). In contrast, similar levels of these tannins had a negative effect on rumen fermentation (McSweeney *et al.* 2001a; Min *et al.* 2002).

Information reported by these authors suggested that the effect of tannins on ruminal parameters depended on their level, type (condensed or hydrolysable tannin) and nature of plant (Abarghuei *et al.* 2010).

Polyethylene glycol (PEG, MW 6000) which possesses a very high affinity for tannins, has been used to deactivate them (Makkar, 2003). Although the technique of PEG inclusion is quite useful, success of its adoption depends on the cost:benefit ratio (Makkar, 2003). PEG is produced from oil, and in Iran, one of the largest oil producer in the world, the production capacity of PEG exceeds 7000 MT per year (Abarghuei *et al.* 2010).

Inclusion of PEG increased *in vitro* organic matter digestibility, metabolizable energy, microbial protein biomass and ammonia in grape pomace (Alipour and Rouzbehan, 2007) and in oak leaf (Yousef Elahi and Rouzbehan, 2008). The *in vitro* gas production is a suitable technique for rapid evaluation of additive-feed interactions and fermentation kinetics (Makkar, 2010).

Therefore, the purpose of this study was to assess the effect of replacing alfalfa forage with grape pomace and oak leaf on IVOMD using *in vitro* and *in situ* DM and CP disappearance of this feed.

MATERIALS AND METHODS

Grape pomace and oak leaf

Grape pomace (*Vitis vinifera*) was obtained from two main factories in Urmia city, which were using similar grape varieties and processing methods. Grape pomace (contains skins, pulp, seeds) was obtained after taking juice. The collected grape pomade was mixed and used for sun-drying. Oak leaf (*Quercus libani*) was obtained from Kurdistan province, in Baneh city of Iran. Baneh is located at 35.9975 (latitude in decimal degrees), 45.8853 (longitude in decimal degrees) at an altitude of meters. The average altitude of Baneh is 1503 meters. Leaf was harvested by hand branches were randomly sampled from at least 10 plants per species.

Leaves were removed from branches, pooled to five samples per species and air dried in the shade to minimize changes in tannin content and activity (Makkar and Singh, 1991).

In vitro fermentation

For the diet samples, gas production kinetics and OM digestibility (IVOMD) were determined as described by Menke and Steingass (1988) and Makkar (2004) in three runs of *in vitro* gas production.

Rumen fluid was obtained from four healthy mature rumen-cannulated sheep (Ghazel breed, twelve months of age with live body weight of 61.8±2.9 kg) fitted with permanent 70 mm rumen cannula that were fed a daily ration of a mixture of 390 g/kg DM alfalfa hay, 250 g/kg DM barely grain, 83 g/kg DM wheat bran, 277 g/kg DM Wheat straw divided into equal meals at 8:00 and 16:00 h daily.

Sheep had free access to water throughout the experiment. Samples of rumen fluid were collected prior to their morning feeding, strained through two layers of cheesecloth, transferred into prewarmed CO_2 filled thermos bottles and the fluid samples were combined prior to *in vitro* fermentation.

The temperature of the rumen fluid was maintained at 39 ˚C throughout the preparation of the incubation medium. Syringes were pre-warmed (39 ˚C) for 1 h before addition of 30 mL of rumen buffer mixture (ratio of reduced buffer medium:rumen fluid, 2:1) into each syringe, and incubated in a water bath maintained at 39 ± 0.1 ˚C as described by Menke and Steingass (1988). Reduced buffer medium composition, per liter, was $NaHCO_3$, 35.00 g; NH_4HCO_3, 4.00 g; Na_2HPO_4, 5.7 g; KH_2PO_4, 6.2 g; $MgSO_4$·7H2O, 0.6 g; $FeCl_2.6H_2O$, 0.80 g; $CaCl_2·H_2O$, 13.2 g; $MnCl_2·4H_2O$, 10 g; $CoCl_2·6H_2O$, 1 g and sodium resazu-

rin, 0.01 g and, 60 mL freshly prepared reduction solution containing 580 mg $Na_2S·9H_2O$ and 3.7 mL 1 *M* NaOH. Diet samples (375±0.20 mg) were incubated in 30 mL of incubation medium with or without polyethylene glycol (PEG), molecular weight (MW, 6000, Merck Schuchardt, Hohenbrunn, Germany) (Makkar, 2004). Five diet samples were used that including (Table 1): control (alfalfa hay, barley grain, wheat chaff, wheat straw), GP diet (grape pomace, barley grain, wheat chaff and urea) and GP diet + PEG, OL diet (oak leaf, barley grain, wheat chaff and urea) and OL diet + PEG.

Analyses were completed in triplicate with readings of gas production recorded after incubation. Differences in the composition and activity of rumen fluid inoculum without substrate was controlled by parallel measurements within incubation of buffered ruminal fluid (Blank test Gb0) and incubation of a standard hay meal (Hohenheim hay standard), which should give a mean gas production of 44.16 mL at 24 hours (GbH). From these measurements, each series of determinations was corrected using 44.16/(GbH-Gb0).

Volume of gas produced was recorded at incubation times of 3, 6, 8, 12, 16, 24, 48, 72, 96 and 120 h. Cumulative gas production data were fitted to the exponential equation as follow:

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

Where:
Y: gas produced at t time.
b: gas production after 120 h from the insoluble but fermentable fraction (mL/g OM).
c: gas production rate constant for b and t the incubation time.

The organic matter digestibility (OMD) (g/kg DM) and metabolisable energy (ME) (MJ/kg DM) in oak leaf grape pomace and alfalfa were estimated by equations of Menke and Steingass (1988), based on 24 h gas production (Gas, mL) and CP content (g/kg DM) as:

$$OMD\ (g/kg\ OM)= 148.8 + 8.89\ GAS + 4.5\ CP + 0.\ 651\ XA$$

$$ME\ (MJ/kg\ DM)= 2.20 + 0.136\ GAS + 0.057\ CP + 0.0029\ CP^2$$

Where:
OMD: OM digestibility.
ME: metabolisable energy.
CP: crude protein in g/100 g DM.
XA: ash in g/100 g DM.
GAS: net gas production (mL) for 200 mg of sample.

After 24 h of incubation, for the second set of syringes the volume of gas production was recorded and a subsample for protozoal counts was taken with 2 mL of syringe content pipetted into a screw-capped test tube containing 10 mL of formalinized physiological saline (containing 20 mL formaldehyde in 100 mL distilled water) and were stored at 4 ˚C prior to measuring of protozoa (Dehority, 2003). Then, the content of syringes were transferred to tubes centrifuge and centrifuged at 20000 × g for 20 min at 4 ˚C. Supernatants were stored at -20 ˚C prior to analysis of ammonia.

In situ DM disappearance and estimated parameters

Approximately 5 g of sample (alfalfa or grape pomace or oak leaf) was weighed into 10 cm × 20 cm polyester bags (53±10 µm pore size; Bar Diamond, Inc., Parma, ID) in triplicate. Bags were attached on semi-rigid stalks to ensure immediate insertion within the liquid of the rumen contents while allowing free movement. After withdrawing the bags from the rumen, they were washed in a washing machine for 1 h using cold water and dried for 48 h at 50 ˚C. The degradability value at t= 0 was obtained by washing two bags in a washing machine for 1 h using cold water. For each bag, the residue was analyzed for DM. Degradability at each incubation time was calculated by taking the values obtained from the three bags (i.e., n=3). The ruminal degradability (Y) of DM at time (t) was obtained from an exponential curve of the type:

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

This was fitted to the experimental data by iterative regression analysis (Ørskov and McDonald, 1979). In this equation, the constant a represents the soluble and very rapidly degradable component and b represents the insoluble but potentially degradable component which degrades at a constant fractional rate (c) per unit time. The effective degradability of DM in each species was then estimated (Ørskov and McDonald, 1979) by the equation:

$$Effective\ degradability\ (g/kg\ DM)= a + bc / c + k.$$

Where:
k: fractional outflow rate of small particles from the rumen. A value of 0.05 fraction/h was used for k.

Analytical methods

The fresh grape pomace and oak leaf were analyzed according to AOAC (1990) for dry matter (DM, method 930.15), ash (method 924.05) and N (method 984.13). Ash-free neutral detergent fiber (NDFom) was determined according to Van Soest *et al.* (1991). ADFom was determined and expressed exclusive of residual ash (AOAC, 1990).

Lignin (sa) was determined by solubilisation of cellulose with sulphuric acid as described by Robertson and Van Soest (1981).

Nitrogen in feed was determined by the Kjeldahl (AOAC, 1990; method 954.01). Short chain fatty acids (SCFA) were calculated (Getachew *et al.* 2001) as:

SCFA (mmol/200 mg DM)= 0.0222 GP - 0.00425

Total phenolics (TP) were measured using the Folin-Ciocalteau method (Makkar, 2000). Dried plant material (200 mg) was extracted with acetone:water (10 mL; 70:30, v/v) in ultrasonic bath for 20 min. Contents were centrifuged (4 ˚C, 10 min, 3000×g) and the supernatant was kept on ice until analysis. Non-tannin phenols (NTP) were determined using absorption to insoluble polyvinylpyrrolidone.

The insoluble polyvinylpyrrolidone (PVPP; 100 mg) was weighted into 100 mm × 12 mm test tubes. Distilled water, 1 mL, and then 1 mL tannin containing extract were added and vortexed. The tube was kept at 4 ˚C for 15 min, vortexed again, then centrifuged (3000×g) for 10 min and supernatant collected. The phenolic content of the supernatant was measured by the folin-ciocalteau reaction and this was accepted as the NTP. Total tannins were calculated as the difference between TP and NTP (Makkar, 2000). Tannic acid (Merck GmbH, Darmstadt, Germany) was used as the standard to express the amount of TP and TT. Condensed tannins were measured by the HCL-butanol method (Makkar, 2000).

An aliquot from the above acetone: water extract (0.5 mL; although this extract occasionally needed diluting with the extractant, acetone: water, if final absorbance at 550 nm exceeded 0.6 absorbance units) plus HCL-butanol (3 mL) and ferric ammonium sulfate (0.1 mL) reagents were heated in a boiling water bath for 60 min. Absorbance was read at 550 nm.

Hydrolysable tannins were analyzed using Rhodanine assay according to Makkar (2000). The results were expressed as gallotannin. The concentration of NH_3-N in supernatants was determined using the phenol-hypochlorite method.

For counting protozoa, two drops of brilliant green dye (2 g brilliant green and 2 mL glacial acetic acid diluted to 100 mL with distilled water) was added to the test tube containing 1 mL sampled syringe fluid, mixed thoroughly and allowed to stand overnight at room temperature. Total and differential counts of protozoa were made in 30 microscopic fields at a magnification of 20 × in a Haemocytometer (Neubauer improved, Marienfeld, Germany).

Statistical analysis

Incubation was done in three separates *in vitro* run with three replicates test feed samples. Data of each of the three runs within sample were averaged. All data was analyzed using the SAS (2001) with treatment (with or without PEG). Duncan's multiple-range test (Duncan, 1955) was used to separate means within species of plant extracts. For *in situ* and *in vitro* gas production estimates data, the following statistical model was fitted:

Table 1 Ingredients and nutrient composition (g/kg DM) as stated for the experimental diets (g/kg DM)

Ingredients	Diets				
	Control	GP	GP + PEG	OL	OL + PEG
Alfalfa hay	390	-	-	-	-
Grape pomace	-	762	676	-	-
Oak leaf	-	-	-	709	629.2
Wheat bran	83	114	110	206.8	189.3
Barley	250	120	120	70.9	65.3
Wheat straw	277	-	-	-	-
Urea	-	4	4	13.3	11.75
PEG	-	-	100	-	100
Nutrient composition					
DM (g/kg fresh weight)	941.6	965.4	932.9	934	933
OM	965.5	965.5	968.5	947	965
Ash	34.5	34.5	31.5	53	35
ME (MJ/d)	5.5	5.5	5.5	5.5	5.5
MP (g/d)	44	43	43	48	49
ERDP:FME ratio	11.1	11.5	11.5	11.1	11.1
NDFom	442.9	502.4	437.5	472	372
TP	-	40.7	40.7	57.6	57.6
TT	-	28.7	28.7	50.9	50.9
CT	-	24.3	24.3	3.8	3.8
HT	-	15.1	15.1	69.7	69.7

DM: dry matter; OM: organic matter; ME: matabolizable energy; MP: metabolizable protein; ERDP: effective rumen degradable protein; FME: fermentable matabolizable energy; NDFom: neutral detergent fiber; TP: total phenolic compounds; PEG: polyethylene glycol; TT: total tannin; CT: condensed tannin; HT: hydrolysable tannin; GP grape pomace and OL: oak leaf.

$Y_{ij} = \mu + T_i + e_{ij}$

Where:
Y_{ij}: general observation.
μ_{ij}: general mean.
T_i: treatment.
e_{ij}: standard error term.

RESULTS AND DISCUSSION

Ingredients and nutrient composition and phenolics

Ingredients and nutrient composition (g/kg DM) as stated for the experimental diets (g/kg DM) had shown at Table 1. Grape pomace contains high level of condensed tannin but oak leaf had the high level of HT (Table 2). The chemical composition (g/kg DM basis) of grape pomace and oak leaf were 940, 940 OM; 94, 116 CP; 568, 515 NDFom; 467, 316 ADFom; 242, 93; lignin (sa); 70.5, 82 TP; 49.7, 73 TT; 79, 5.4 CT; and 40, 70 HT.

Table 2 Phenolic compounds (g/kg DM) of grape pomace and oak leaf

Feed	Phenolics compounds			
	TP	TT	CT	HT
Grape pomace	70.5	49.7	79	40
Oak leaf	82	73	5.4	70

TP: total phenolic compounds; TT: total tannin; CT: condensed tannin and HT: hydrolysable tannin.

IVGP and estimated parameters and protozoa population

Gas production characteristics (i.e., b, c and OMD) during the fermentation period are in Table 3. Using grape pomace and oak leaf decreased OMD, SCFA, b and c comparing to control ($P<0.05$). The addition of PEG increased IVGP at all times of incubation. Values of b and c, OMD and SCFA were also increased by PEG incorporation ($P<0.05$). The IGP in GP diet was higher than those in OL diet. NH_3-N concentrations decreased with using grape pomace and oak leaf and addition of PEG increased NH_3-N concentration ($P<0.05$) (Table 3).

The amounts of total protozoa, *Isotricha*, *Dasytricha*, subfamily of *Entodiniinae*, *Diplodiniinae* and *Ophrioscolecinae* were decreased by addition of grape pomace and oak leaf (Table 3). The addition of PEG increased total protozoa, subfamily of *Entodiniinae*, *Diplodiniinae* and *Ophrioscolecinae* populations in grape pomace diet ($P<0.05$), but PEG increased *Isotricha*, *Dasytricha* and subfamily of *Diplodiniinae* in oak leaf diet ($P<0.05$).

***In situ* DM disappearance and estimated parameters**

Characteristics of the DM and CP disappearance of the alfalfa, grape pomace and oak leaf are in Table 4. The soluble component (a), the degradation rate of b (c), the potential degradability (a+b) and the effective degradability (ED) of the grape pomace and oak leaf were decreased comparing to alfalfa.

Ingredients and nutrient composition and phenolics

The amount of TP, TT and CT in grape pomace was similar with study by Alipour and Rouzbehan (2007) and Abarghuei *et al.* (2010), but higher than other studies (Baumgärtel *et al.* 2007). Levels of TP and TT in the oak leaf were higher than in *Quercus hartwissiana* (Yildiz *et al.* 2005), *Quercus coccifera* (Ben Salem *et al.* 2003; Ben Salem *et al.* 2000), alike to *Quercus rotundifolia* (Khazaal *et al.* 1994), but lower than *Quercus coccifera* (Khazaal *et al.* 1993). The level of HT is high in oak leaf. Similarly, some researchers have reported that oak leaf is rich in HT (Abarghuei *et al.* 2011; Yousef Elahi and Rouzbehan, 2008; Makkar, 2003).

However; others noted that levels of HT in oak leaf are low (Yildiz *et al.* 2005; Singh *et al.* 2005). The variations between our grape pomace and oak leaf and other species in the phenolics contents is probably due to any or all of the vegetative stage (Makkar and Singh, 1993), method of storage (Makkar and Singh, 1993), drying conditions (Makkar and Singh, 1991), species (Makkar and Singh, 1991; Makkar *et al.* 1991) and habitat (Goncalves-Alvim *et al.* 2004).

IVGP and estimated parameters and protozoa population

The *in vitro* gas production and OMD was significantly lower in GP and OL diets than control diet. This reduction could be due the direct inhibition of the micro-organisms through tannin interactions with the cell wall and secreted catabolic enzymes or reduced substrate availability due to complexing of tannin with carbohydrate, protein and minerals (McSweeney *et al.* 2001b; Goel *et al.* 2005). Similar results were obtained by Alipour and Rouzbehan (2007) and Yousef Elahi and Rouzbehan (2008) when using grape pomace and oak leaf as an *in vitro* gas technique. The decrease in SCFA, with the GP and OL diets comparing to control could be due to level of fiber in diet (Anele *et al.* 2009; Van Soest, 1994). This finding is in agreement with earlier observations (Getachew *et al.* 2001; Anele *et al.* 2009). *In vitro* gas production, OMD and SCFA were increased due to adding PEG. PEG has a very high affinity for binding to tannins (Makkar, 2003). This increment suggests a negative influence of tannins on digestibility (Makkar, 2003). Inactivation of tannins through PEG binding raises availability of nutrients resulting in increased microbial activity and gas production (Makkar, 2003). Increases in gas production due to inclusion of PEG to tanniniferous feeds have been reported by several authors (e.g., Getachew *et al.* 2001; Vitti *et al.* 2005). Inclusion of PEG increased IGP, but this increase was higher in GP diet comparing to OL diet. This deference may be due to level

and type of tannin. The higher tannin content would result in the higher PEG neutralization on effect of PEG (Moujahed *et al.* 2000). However Vitti *et al.* (2005) showed that using effect of PEG for deactivating of tannin in two different plant with similar level of tannin was dissimilar, that either tannins bind with varying degrees to PEG or that fermentation processes and gas production are affected in a complex fashion by tannins (Vitti *et al.* 2005). Indeed, improved fermentation by PEG depends on the amount and form of secondary compounds, especially tannins (Ben Salem *et al.* 2003).

Secondary compounds in different plants, even with an equal amount, have different effects on the rate of gas production and digestibility, that it confirms the relationship between structure and activity of the tannins (Makkar, 2003). In addition, influence of PEG depended on plant species (Yildiz *et al.* 2005), synchronization of the energy and nitrogen (Frutos *et al.* 2004; Getachew *et al.* 2001), PEG molecular weight, application method of PEG and amount of tannin and animal species (Frutos *et al.* 2004).

NH_3-N concentration in the experimental diets was within the optimum range (Table 3). Reduced NH_3-N may be due to tannins ability to bind to plant protein, to diminish the activity of microbial enzymes, and to reduce the growth rate of proteolytic bacteria (Molan *et al.* 2001), and finally to decline of NH_3-N (Min *et al.* 2005). Additionally, decreased NH_3-N is usual when protozoa are inhibited (Williams and Coleman, 1991), presumably as a consequence of depressed bacterial lysis (Hristov *et al.* 1999). Belanche *et al.* (2012) proved that the protozoa of *Entodinium* were responsible for most ruminal bacterial breakdown.

In the present study, deceasing *Entodinium* by grape pomace and oak leaf could have led to the decrease NH_3-N concentration. Similarly, McSweeney *et al.* (2001b) has been shown that tanniniferous feeds containing 2.5% condensed tannin decreased NH_3-N. However, in present work, addition of PEG increased NH_3-N concentration which may show more fermentation of the dietary protein by inactivation of tannin (Makkar, 2003).

Table 3 *In vitro* gas production parameters and protozoa population (log_{10}/g digesta) of experimental diets

Parameters	Diets					SEM	Significant
	Control	GP	GP+PEG	OL	OL+PEG		
$IVGP_{24}$	44.4^{a}	26.1^{c}	32.6^{b}	27.5^{c}	32.5^{b}	0.871	*
IGP	-	-	25	-	18		-
b	52.2^{a}	32.4^{b}	35.1^{b}	32.9^{b}	31.1^{b}	1.3	*
c	0.093^{a}	0.07^{b}	0.088^{ab}	0.063^{b}	0.06^{b}	0.006	*
OMD	610^{a}	442^{d}	499^{bc}	490^{c}	512^{b}	6.25	*
SCFA	1.00^{a}	0.56^{c}	0.73^{b}	0.60^{c}	0.72^{b}	0.001	*
NH_3-N (mg/dL)	34.03^{a}	22.43^{d}	29.67^{b}	24.65^{c}	30.50^{b}	0.213	*
Protozoa population							
Total	6.25^{a}	5.75^{d}	5.86^{c}	5.95^{bc}	6.06^{b}	0.033	*
Isotricha	5.05^{a}	2.29^{c}	1.25^{e}	1.68^{d}	3.64^{b}	0.041	*
Dasytricha	5.30^{a}	5.23^{a}	2.86^{d}	3.96^{c}	4.09^{b}	0.033	*
Entodiniinae	5.95^{a}	5.44^{c}	5.76^{b}	5.87^{ab}	5.9^{ab}	0.052	*
Diplodiniinae	5.00^{a}	1.78^{b}	3.35^{b}	0.00^{e}	1.61^{d}	0.048	*
Ophrioscolecinae	1.84^{a}	0.34^{c}	1.79^{a}	0.73^{b}	0.83^{b}	0.038	*

$IVGP_{24}$: *in vitro* gas production at 24 h; IGP: increase in gas production (%); b: insoluble but fermentable fraction (mL); c: rate constant of gas production during incubation (mL/h); OMD: organic matter digestibility (g/kg DM); SCFA: shortchain fatty acid (mmol/200 mg DM); GP grape pomace; OL: oak leaf and POG: polyethylene glycol. SEM: standard error of the means.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 4 *In situ* disappearance parameters of alfalfa hay, grape pomace and oak leaf

Dry matter	Alfalfa hay	Grape pomace	Oak leaf	SEM	Significant
a	289.23^{a}	101.03^{b}	25.73^{c}	16.58	*
b	421.3	382.77	418.07	18.07	NS
a + b	710.53^{a}	483.80^{b}	443.50^{c}	8.33	*
c	0. 551^{a}	0.042^{b}	0.153^{b}	0.077	*
ED	646.6^{a}	357.7^{b}	362.3^{b}	4.69	*
Crude protein					
a	540.33^{a}	43.70^{c}	89.10^{b}	11.30	*
b	353.83^{a}	371.93^{a}	168.63^{b}	25.26	*
a + b	894.2^{a}	575.0^{b}	228.0^{c}	91.01	*
c	0.119^{a}	0.020^{b}	0.109^{a}	0.025	*
ED	821^{a}	227.3^{b}	202.0^{b}	8.12	*

a: water-soluble fraction (g/kg DM); b: insoluble but fermentable fraction (g/kg DM); c: the degradation rate of b (/h); a + b: the potential degradability (g/kg DM) and ED: the effective degradability of dry matter calculated for an outflow rate of 0.05/h (g/kg DM).
SEM: standard error of the means and NS: non significant.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

In consistent with this research Alipour and rouzbehan, (2007) found that addition of PEG to grape pomace led to an increase in the rumen ammonia level. The decrease in the protozoa populations is probably due to the presence of tannins (Abarghuei *et al.* 2010). The aniprotozoal influence was most likely due to the phenolic structure of active compounds (i.e., tannin and saponins). These compounds may interrupt the protozoal membrane, inactivation of protozoal enzymes and remove of substrates and metal ions which are vital for cell metabolism (Calsamiglia *et al.* 2007; Goel *et al.* 2005). In contrast, some studies indicated that total protozoa number was unaffected in the rumen of sheep fed diet containing tanniniferous feed contain low level of CT (i.e. 15 g condensed tannin/kg DM). Also, Benchaar *et al.* (2008) illustrated that addition of quebracho condensed tannin (150 g condensed tannin/d/cow) and *Yucca schidigera* saponin extracts (60 g/d/cow) to dairy cow diet had no effect on the populations of total, *Isotricha* spp., *Dasytricha*, *Entodinium* and *Diplodinium*. Such discrepancies may be due to the diet type, animal variability, sampling methods some studies, level and type of plant metabolites (Patra and Sexena, 2011) and the variability in the adaptation of the protozoa to active compounds, the previous experience of the animal to this compounds, or both (Wallace *et al.* 2002; Abreu *et al.* 2004).

***In situ* DM disappearance and estimated parameters**

The ED of the DM and CP of grape pomace and oak leaf in this experiment (357.7 g/kg and 362.3 g/kg; 227.3 g/kg and 202.0 g/kg) was different compared to other studies (Yousef Elahi and Rouzbehan, 2008; Besharati *et al.* 2009). Discrepancy in degradability may be dependent on the stage of growth (Kaitho *et al.* 1993) and also on their content of tannins (Salawu *et al.* 1997). The values of a, a + b and ED of the grape pomace and oak leaf were decreased comparing to alfalfa. Since reviewed by McSweeney *et al.* (2001a), tannins are polyphenolic compounds of plant origin, with two different types, condensed tannin and hydrolysable tannins. The presence of tannins in nutritionally important forage trees, shrubs, legumes, cereals and grain often limits the feed application. The tannin compounds can have toxic or anti-nutritional properties on animals due to the complexing ability with dietary protein, which then inhibits the growth of microorganisms (McSweeney *et al.* 2001a). Similar results were obtained by Salawu *et al.* (1997) and Yousef Elahi and Rouzbehan (2008) when using tanniniferous plant and oak leaf as an *in situ* disappearance. However Khazaal *et al.* (1993) did not get a relationship between the phenolic content of plants and their dry matter loss upon incubation *in situ*. Most probably their chemical analysis involved compounds that washed out of the bags upon incubation in the rumen, so that effects such as toxicity to microbes or binding of the tannins to the microbial enzymes would be very small due to the dilution in the large rumen volume. However, the capability of tannins to reduce the activities of rumen microbes, and to interact with proteins and carbohydrates, depends on the types of tannin, the molecular mass, degree of polymerization, pH and protein: tannin ratios (McSweeney *et al.* 2001a; Reed, 1995).

CONCLUSION

Inclusion of grape pomace and oak leaf has shown to positively manipulate rumen fermentation parameters mainly decreased NH_3-N concentration and protozoa population. Diet containing grape pomace and oak leaf had lower IVOMD than the diet containing alfalfa. Grape pomace diet had lower IVOMD comparing to oak leaf diets. Supplementation of PEG in GP and OL diets improved the fermentability. Although addition of PEG illustrates a positive influence on most of *in vitro* parameters, the addition of 100 g of this supplement per day is not a suitable amount in terms of cost-benefit response under the Iranian condition. Oak leaf is recommended as medium-quality food for ruminants. Further research is needed to assess alternative ways to overcome negative effects of tannins in grape pomace and oak leaf, as well as to assess their impacts on the animal performance.

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