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Crude Protein Fractions and *in vitro* Gas Production of Alfalfa Silages Treated with Pistachio by-Products Extract

Research Article

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ABSTRACT

Two experiments were conducted to evaluate the crude protein fractionation, *in vitro* gas production and the biological effects of tannins in alfalfa silage treated with different levels of pistachio by-products (PB) extract. PB was soaked in water with a ratio of 1:5 (w/v) for 12 h. After filtering and concentrating, the crude extract was added to fresh alfalfa to a final concentration of 1%, 2% and 3% tannin dry matter (DM) and then ensiled for 60 days. Four treatments were as follows: alfalfa silage without addition of PB extract as a control (T0), PB tannin added at 1% (T1), 2% (T2) and 3% DM (T3). Treating alfalfa silage decreased the formation of non-protein nitrogen (NPN) compounds and increased crude protein fraction B2 during ensilage. Cumulative *in vitro* gas production after 96 h incubation, organic matter digestibility (OMD) and metabolizable energy (ME) decreased in T2 and T3 compared to the control. The gas production and short chain fatty acids (SCFA) of treated silages increased in the presence of polyethylene glycol (PEG) which indicates that the presence of phenolic compounds and tannins depressed the gas production. It can be concluded that treating alfalfa silage with PB extract can reduce the nitrogen losses during ensilage and degradability of organic matter (OM) by microbial inhibition.

KEY WORDS crude protein fractionation, *in vitro* gas production, pistachio by-products, polyethylene glycol, tannin bioassay.

INTRODUCTION

It is well documented that the nutritive value of ensiled leguminous forage such as alfalfa is limited by rapid and excessive degradation of protein during ensiling (Albrecht and Muck, 1991) and rumen fermentation (Broderick, 1995). Improving forage quality by reducing proteolysis during ensiling will improve N utilization by ruminants (Givens and Rulquin, 2004). There has been increasing interest in reducing the rate and extent of protein degradation during ensiling and rumen fermentation by using natural products such as tannins. Tannins are secondary plant metabolites that react with proteins, carbohydrates and minerals thus retard their digestion (McSweeny *et al.* 2001). Pistachio (*Pistacia vera*) by-products (PB) contain phenolic compounds and tannins ranging from 9.06-14.57% and 4.97-8.67% DM, respectively, depending on the method of extraction including solvent, particle size and time of extraction (Mokhtarpour *et al.* 2014). The *in vitro* gas production method is widely used to evaluate the nutritive value of foodstuffs (Getachew *et al.* 2002) and is closely related to digestibility and energetic value (Menke and Steingass, 1988). Many papers have been published on the use of the gas production technique as a method to study the anti-nutritive factors (Makkar *et al.* 1995; Getachew *et al.* 2008; Kumara Mahipala *et al.* 2009) and is seemed to be more

efficient than other *in vitro* techniques in determining the nutritive value of feeds containing tannins (Getachew *et al.* 2002).

The potential of PB extract addition to alfalfa during silage making and its effect on gas production has not yet been exploited. Therefore, the aims of this study were to evaluate the effect of treating alfalfa silage with PB extract on *in vitro* gas production and assaying the biological effect of tannin by using PEG.

MATERIALS AND METHODS

Pistachio by-products extract preparation

Sun-dried pistachio by-products (PB) were obtained from a pistachio de-hulling factory in Feizabad (Khorasan Razavi Province, Iran) which is located on the north east part of Iran at 35 ˚01′ N latitude and 58 ˚78′ E longitude. Pistachio by-products were soaked in water by a ratio of 1:5 (w/v) at room temperature for 12 h. The contents were filtered through 4 layers of cheesecloth and then were concentrated by a rotary evaporator at 40 ˚C.

Preparation of alfalfa silages

A second regrowth of alfalfa was harvested at early bloom. The alfalfa was chopped to a length of 3 cm with a cutter. Alfalfa herbage was ensiled in 12 plastic buckets with the capacity of 500 g. PB extract was added to fresh alfalfa at 0, 40, 80 and 120 mL/kg of fresh weight to achieve final concentrations of 0%, 1%, 2% and 3% tannin as tannic acid equivalent (24% DM), respectively. Three replicate treatments were set up and all were ensiled for 60 days at room temperature (25 ˚C±1) in a dark place.

***In vitro* gas production**

Approximately 200 mg dry weight of the samples were weighed in triplicate and placed in 120 mL gas-tight culture bottles following the gas pressure transducer technique of Theodorou *et al.* (1994). The culture bottles were filled with 30 mL of buffered rumen fluid. Rumen fluid was obtained from three fistulated bulls before the morning feeding. The donor animals were fed with corn silage and concentrate with a ratio of 70:30 twice daily. The rumen fluid collection and composition of buffer were performed according to the Menke and Steingass (1988) procedure. The culture bottles containing samples and mixture of rumen fluid and buffer were incubated in a water bath at 39 ˚C. Gas pressure in the head-space of each bottle was read from the display unit after 2, 4, 8, 12, 24, 48, 72 and 96 hours' incubation and the corresponding gas volume was determined by recording the volume of gas displaced into the syringe barrel. Bottles were shaken gently after each gas production reading without removing them from the water bath. This procedure was performed twice. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to the model of Ørskov and Mcdonald (1979):

$$Y= b(1-e-ct)$$

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

The OM digestibility, ME and NE_L were calculated to the following equations according to Menke and Steingass (1988):

$$OMD\ (\%)= 14.88 + 0.8893\ GP + 0.448\ CP + 0.651\ Ash$$

$$ME\ (MJ/kg\ DM)= 2.20 + 0.1357\ GP + 0.057\ CP$$

$$NEl\ (MJ/kg\ DM)= 0.54 + 0.0959\ GP + 0.038\ CP + 0.001733\ EE2$$

Where:
GP: net production in 24 h in mL/200 mg of DM.
CP: % of DM, EE is % DM and ash is % of DM.

***In vitro* tannin bioassay**

In vitro gas production was carried out using PEG in order to identify the biological effects of tannins as described by Makkar *et al.* (1995). Briefly, 500 mg dry weight of silage samples were incubated without and with 1 g PEG (MW=6000) and filled with 40 mL rumen liquor and buffer mixture in triplicate. Culture bottles were placed in a water bath at 39 ˚C and gas production was recorded after 2, 4, 6, 8, 10, 12 and 24 hours' incubation. Short chain fatty acids were calculated by the equation of Getachew *et al.* (2002):

$$SCFA\ (mmol/40\ mL)= -0.00425 + 0.0222 \times gas\ (mL)$$

Chemical composition

Dry matter content of fresh alfalfa was determined by drying in an oven at 100 ˚C to a constant weight (AOAC 2005). Ash (method 942.05) ether extract (method 920.39) and CP (Kjeldahl N×6.25) content of silages was determined by the block digestion method using copper catalyst and steam distillation into boric acid (method 2001.11) on behr S 5 steam distillation unit (behr Labor Tecknik GmbH, Germany) as described in AOAC (2005). Crude protein fractions of alfalfa silages were determined by the method

of Licitra *et al.* (1996). NPN was determined by precipitation of true protein in tungstic acid solution. Soluble protein was determined using borate phosphate buffer. The amount of insoluble protein in acid detergent and neutral detergent were determined after measuring ADF and NDF (Van Soest *et al.* 1991). For total phenolics (TP) quantification, approximately 200 mg (±0.001) silage samples was extracted in 10 mL 70% aqueous acetone (v/v) in quadruplicate by using an ultrasonic bath for 20 min. After centrifugation (3000×g, 4 ˚C, 10 min) the supernatant was collected and kept in refrigerator (4 ˚C). Non-tannin phenolics (NTP) were determined following absorption of tannins in total phenolic extract to insoluble polyvinylpyrrolidone (PVPP). Total phenolics and non-tannin phenolics were determined by folin-ciocalteu reagent using tannic acid (Merck GmbH, Darmstadt, Germany) as a standard, expressing results as tannic acid equivalent. Total tannins (TT) were calculated as the difference between TP and NTP (Makkar, 2000). Condensed tannins (CT) concentration was measured on phenolics supernatant using the butanol-HCl reagent (Makkar, 2000). The values of CT were expressed as leucocyanidin equivalent.

Statistical analysis

Data from chemical composition and gas production were analyzed using the GLM procedure in SAS (2001) as a completely randomized design. Means were separated by the least squares means procedure (LSMEANS) when a significant (P<0.05) treatment effect was observed. For tannin bioassay, data were analyzed as a completely randomized design with a 4 × 2 factorial arrangement treatment.

The model included the effects of PB tannin level, presence or absence of PEG and the interaction between tannin level and PEG.

RESULTS AND DISCUSSION

The amount of total phenolics, tannins and CP fractions of silages are presented in Table 1. Total phenolics and tannins increased by increasing PB extract. Treating alfalfa silage with PB extract decreased fraction A and increased the concentrations of fractions B1 and B2. The reduction in fraction A (NPN compounds) of treated silages can be attributed to tannin binding to forage proteins and preventing already formed tannin-protein complexes from enzyme hydrolysis or may be due to the action of tannins to bind to plant proteolytic enzymes (Guo *et al.* 2007). Although changes in activity of proteolytic enzymes were not determined in our study, decreased in NPN concentration may not be associated with inhibition of proteolytic enzymes activities as the pH values for all treatments were above 4.5 (data not shown).

Table 1 Phenolic compounds (% DM) and crude protein fractions (% CP) of alfalfa silages

Item	Treatments				
	T0	T1	T2	T3	SEM
Phenolic compounds (% DM)					
Total phenolics	1.42^{d}	2.78^{c}	4.56^{b}	7.01^{a}	0.791
Total tannins	0.61^{d}	1.11^{c}	2.21^{b}	3.17^{a}	0.375
Condensed tannins	ND	0.12^{c}	0.25^{b}	0.35^{a}	0.043
CP fractions (% CP)					
A	62.76^{a}	57.02^{b}	58.01^{b}	58.26^{b}	0.883
B1	0.45^{b}	0.97^{b}	1.96^{a}	2.52^{a}	0.310
B2	28.80^{b}	33.25^{a}	32.40^{a}	32.36^{a}	0.686
B3	5.08	5.07	4.66	3.72	0.248
C	2.93^{b}	3.69^{a}	2.99^{b}	3.15^{ab}	0.151

T0: control; T1: 1% of DM PB tannin; T2: 2% of DM PB tannin; T3: 3% of DM PB tannin.
SEM: standard error of the means; ND: not detected.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Henderson (1993) stated that the optimal pH for plant peptidase activity is between 5-7 and it will be reduced at pH below 4. Our results are consistent with Tabacco *et al.* (2006) and Guo *et al.* (2007) who reported that addition of tannic acid to alfalfa decreased the NPN concentration of silages. An increase in fraction B1 and B2 was associated with a reduction in fraction A of silages treated at 2% and 3% tannin.

However, a trend (P=0.06) for more concentrated fraction B1 was observed in T1 than T0. The concentration of fraction B3 was not affected by treatments. Treating alfalfa silage at 1% tannin (T1) increased fraction C compared with T0 and T2. Fraction C are heat-damaged proteins, proteins associated with lignins and tannin-protein complexes which are not degradable in the rumen and are indigestible in the intestine (Krishnamoorthy *et al.* 1982; Licitra *et al.* 1996). *In vitro* gas production and fermentation characteristics of alfalfa silages are shown in Table 2.

Table 2 cumulative *in vitro* gas productions (mL/200 mg DM) after 24 and 96 h of incubation and estimated parameters of alfalfa silages

Item	Treatments				
	T0	T1	T2	T3	SEM
Cumulative gas production					
24 h	48.7^{a}	44.8^{ab}	41.2^{bc}	35.2^{c}	1.75
96 h	78.3^{a}	76.4^{a}	67.6^{b}	54.5^{c}	2.93
Estimated parameters					
b	76.8^{a}	75.3^{a}	65.7^{b}	52.6^{c}	2.98
c	0.044^{b}	0.038^{c}	0.042^{b}	0.049^{a}	0.001
OMD (%)	73.3^{a}	70.8^{ab}	65.3^{bc}	60.8^{c}	1.73
ME (MJ/kg DM)	9.94^{a}	9.31^{ab}	8.44^{bc}	7.55^{c}	0.306
NEl (MJ/kg DM)	6.04^{a}	5.60^{ab}	4.98b^{c}	4.36^{c}	0.215

T0: control; T1: 1% of DM pistachio by-products tannin; T2: 2% of DM pistachio by-products tannin; T3: 3% of DM pistachio by-products tannin.
b: gas production from the insoluble but fermentable fraction (mL/200 mg DM); c: gas production rate constant (mL/h); OMD: organic matter digestibility; ME: metabolizable energy; NEl: net energy for lactation; 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).

There were significant differences among the treatments. Addition of PB extract (tannin) lowered gas productionwith a decrease in gas production from the insoluble but fermentable fraction along with both OMD and nutritive value of silages. However, PB tannin at 1% DM had no effect on these parameters. These results are in accordance with those obtained by Tabacco *et al.* (2006) who reported that treating alfalfa silage with hydrolysable tannin (chestnut) at the rate of 2%, 4% and 6% DM decreased gas production and estimated parameters including OMD, ME and NE_L. The rate constant of gas production (c) decreased in T1, but increased in T3. The lower gas production and OMD in treated silages could be attributed to the inhibitory effects of phenolic compounds, especially tannins to the rumen microorganisms' activity. It is suggested that high concentration of condensed tannin (more than 5.5% DM) has negative effects on digestibility *in vivo* (Min *et al.* 2003). In the present study, using culture bottles, low levels of tannins (2%, 3% DM) inhibited rumen fermentation and it seems that rumen microbial population could not counteract the effects of tannins.

This is because the *in vitro* gas production technique is a static system compared to a dynamic system (*in vivo*). As 200 mg alfalfa silage was incubated in 30 mL medium, the rumen microorganisms were able to utilize alfalfa silage as a sole energy source. However, in an *in vivo* experiment, a tannin-treated feed is in combination with other feeds and a large population of microorganisms (Tabacco *et al.* 2006). Getachew *et al.* (2002) reported that over 90% of the variation in gas production was explained by SCFA produced by incubation of browsed leaves. Hence, the amount of gas production reflects the extent of feed degradability. The higher gas production in control and silage treated with 1% tannin compared to other treatments indicated the higher extent of fermentation of OM. Tabacco *et al.* (2006) reported that the gas production decreased by 10.2%, 7.9% and 10.2% when chestnut tannin was added to alfalfa silage at the rate of 2%, 4% and 6% DM respectively. In our experiment, the gas production decreased by8.0%, 15.4% and 27.7% in T1, T2 and T3 respectively.

This difference may be due to higher inhibitory effect of PB tannin than chestnut tannin. Moreover, other factors such as concentration of phenolic compounds may affect rumen microorganisms' activity or interfere with their enzyme secretion (Yanez Ruiz *et al.* 2004). Correlation between phenolic compound and gas production and estimated parameters are shown in Table 3. A decreased in gas production, OMD and ME in treated silages suggests a strong negative relationship between gas production, OM and ME and total phenolic and total tannins.

Table 3 Correlation coefficients (r) of phenolic compounds and *in vitro* gas production characteristics of alfalfa silages

Item	Characteristics				
	GV	b	c	OMD	ME
Total phenolics	-0.998**	-0.982*	0.636	-0.994**	-0.998**
Total tannins	-0.989**	-0.981*	0.642	-0.999**	-0.998**

GV: cumulative gas volume at 24 h of incubation; b: gas production from the insoluble but fermentable fraction; c: gas production rate constant; OMD: organic matter digestibility and ME: metabolizable energy.
* (P<0.05) and ** (P<0.01).

Addition of PEG increased the gas production in treated silages, but had no effect on control silage (Table 4). At 24 h of incubation, an increase in gas production as a result of PEG inclusion was 20.9%, 24.4% and 28.7% for T1, T2 and T3 respectively. Increase in gas production by the addition of PEG revealed a negative influence of tannins on digestibility (Makkar, 2005). Polyethylene glycol is a polymer that binds to tannins irreversibly and thereby increase the availability of nutrients (Makkar *et al.* 1995; Provenza *et al.* 2000) resulting in increased in microbial activity and gas production (Makkar, 2005). Our results are consistent with Osuga *et al.* (2006), Kumara Mahipala *et al.* (2009) and Yusef Elahi *et al.* (2012). Therefore, an increase in gas production by adding PEG is considered a biological effect of tannins (Makkar *et al.* 1995). Short-chain fatty acids were also increased by PEG inclusion. Getachew *et al.* (2002) found a significant correlation between percent changes in gas production and SCFA by PEG addition and phenolic compounds.

Table 4 *In vitro* gas production at 24 h of incubation (mL/0.5g DM) and estimated SCFA (mmol/40mL) without and with polyethylene glycol of alfalfa silages

Item	Tannin level								SEM
	T0		T1		T2		T3		
	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	
GV	84.4	96.0	82.0	99.1	78.2	97.3	71.7	99.2	2.18
SCFA	1.88	2.14	1.82	2.20	1.84	2.17	1.60	2.21	0.049

Item	Significance of effects		
	TL	PEG	TL × PEG
GV	0.09	< 0.001	0.01
SCFA	0.09	< 0.001	0.01

T0: control; T1: 1% of DM pistachio by-products tannin; T2: 2% of DM pistachio by-products tannin and T3: 3% of DM pistachio by-products tannin.
TL: tannin level; GV: cumulative gas volume at 24 h of incubation (mL/0.5g DM); SCFA: short-chain fatty acids (mmol/40mL) and PEG: polyethylene glycol.
SEM: standard error of the means.

CONCLUSION

The results of our study showed that even lower level of PB tannin (1% DM) inhibited proteolysis during ensiling resulting in lower concentration of NPN compared to untreated silage. At moderate levels of tannins (2-3% DM) the gas production at 24 h and 96 h of incubation, ME and OMD decreased. PEG inclusion increased cumulative gas production at 24 h of incubation in PB extract treated silage. Therefore, it can be concluded that phenolic compounds especially tannins in PB can interfere with rumen microorganisms' activity leading to decreased proteolysis and OM fermentation.

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بخش بندی پروتئین خام و تولید گاز در سیلاژهای یونجه عمل‌آوری شده با عصاره محصولات فرعی پسته در شرایط آزمایشگاه

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چکیده

به منظور بررسی بخش‌های مختلف پروتئین خام و تولید گاز در شرایط آزمایشگاه در سیلاژهای یونجه عمل‌آوری شده با سطوح مختلف عصاره محصولات فرعی پسته و ارزیابی ارزش زیستی تانن‌های آن، دو آزمایش انجام شد. محصولات فرعی پسته با نسبت ۱:۵ (وزن به حجم) به مدت ۱۲ ساعت در آب خیسانده شدند. پس از صاف کردن و تغلیظ، عصاره خام به علوفه تازه یونجه اضافه شد تا به ترتیب سطوح ۱، ۲ و ۳ درصد ماده خشک یونجه، تانن به دست آید و سپس به مدت ۶۰ روز سیلو شدند. چهار تیمار به این ترتیب بود؛ سیلاژ یونجه بدون عمل‌آوری با عصاره محصولات فرعی پسته، به عنوان شاهد (T0)، اضافه کردن تانن محصولات فرعی پسته در سطح ۱ درصد (T1)، ۲ درصد (T2) و ۳ درصد ماد خشک (T3). عمل‌آوری سیلاژ یونجه، میزان ترکیبات نیتروژنه غیر پروتئینی را کاهش و بخش B2 را در طی سیلو شدن، افزایش داد. تولید گاز تجمعی پس از ۹۶ ساعت انکوباسیون، قابلیت هضم ماده آلی (OMD) و انرژی قابل متابولیسم (ME) در تیمار T2 و T3 در مقایسه با تیمار شاهد کاهش پیدا کرد. میزان تولید گاز و اسیدهای چرب کوتاه زنجیر (SCFA) در سیلاژهای عمل‌آوری شده در حضور پلی اتیلن گلیکول (PEG) افزایش یافت که نشان می‌دهد وجود ترکیبات فنولی و تانن‌ها باعث کاهش تولید گاز شد. می‌توان نتیجه گرفت که عمل‌آوری سیلاژ یونجه با عصاره محصولات فرعی پسته از طریق ممانعت میکروبی، می‌تواند منجر به کاهش هدرروی نیتروژن در طی سیلو شدن و کاهش تجزیه پذیری ماده آلی شود.

کلمات کلیدی پروتئین خام، تولید گاز، محصولات فرعی پسته، پلی اتیلن گلیکول، ارزش زیستی تانن.