IJAS Iranian Journal of Applied Animal Science

In vitro and *in situ* Ruminal Degradability of Oak Leaves (*Quercus persica*) as Affected by Growth Stage during Spring Season and Polyethylene Glycol Application

Research Article

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Received on: 23 Dec 2021
Revised on: 7 Mar 2022
Accepted on: 31 Mar 2022
Online Published on: Sep 2022

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Online version is available on: www.ijas.ir

ABSTRACT

This study was conducted to identify the nutritive and anti-nutritive composition, *in situ* rumen degradability, and the kinetics of *in vitro* gas production of Persian oak (*Quercus persica*) leaves harvested at three growth stages during the spring season. A tannin bioassay was also performed using polyethylene glycol (PEG 6000) as a tannin-complexing agent in a gas production test. Leaves were harvested in monthly intervals in spring, starting on April when leaves were at the early vegetative stage, and then May and June. As the leaf maturity progressed, crude protein decreased but total phenols, total tannins, and hydrolysable tannins increased. Condensed tannin concentration was not affected by maturity stage (Average=13.0 mg leucocyanidin equivalent/g dry matter (DM)). As leaf maturity increased, the rapidly degradable *A* fraction of DM increased. *In vitro* gas production, metabolizable energy, *in vitro* DM degradability, ruminal NH_3-N, and short-chain fatty acid concentrations were greatest in leaves harvested at the early vegetative stage (April) compared with other months. Application of PEG increased *in vitro* gas production, metabolizable energy, *in vitro* DM degradability, and NH_3-N and short-chain fatty acid production in the rumen fluid compared with no addition of PEG. Overall, oak leaves harvested at the early vegetative stage appeared to be a good source of forage for ruminants. However, as leaf maturity increased, ruminal fermentability decreased, which was improved with PEG addition.

KEY WORDS growth stage, *in situ* degradability, *in vitro* gas production, oak leaf, tannin.

INTRODUCTION

With growth of human population there is a growing demand for animal products, necessitating extensive research into expanding feed resources from unconventional low-quality biomass (Ben Salem and Znaidi, 2008; Makkar, 2018). Leaves of trees and shrubs could be a source of feed for small ruminants (Meuret *et al*. 1990). The north-west of Iran, a semi-arid region, is densely forested with different oak species (nearly 3 million ha), with *Quercus persica* being one of the dominant species (Fatahi, 1995; Azizi *et al*. 2013). Oak leaves in these regions can potentially provide a good source of forage for small ruminants during the critical period of year when higher quality forages are less

available, thereby contributing to sustainable animal production in these areas (Yousef Elahi and Rouzbehan, 2008; Abarghuei *et al*. 2015). However, high content of tannins/phenols in *Quercus* species may impair nutrient utilization by ruminants (Makkar and Singh, 1991; McSweeney *et al*. 2001; Makkar, 2003). Moreover, their feeding value in terms of anti-nutritional components largely differs by the maturity stage (Al-Masri and Mardini, 2013).

Polyethylene glycol (PEG) is a synthetic polymer produced from oil that is available at a cheap price (Makkar *et al*. 1995; Abarghuei *et al*. 2010). Owing to its high affinity to tannins, PEG forms tannin-PEG complexes, which liberates protein from tannin-protein complexes or inhibits their complex formation (Barry and Manley, 1984; Mangan, 1988; Makkar *et al*. 1995). Earlier studies reported that the detanninification of tannin-rich feeds using PEG treatment increased nutrient availability and microbial activity, which resulted in improvement of ruminal fermentation and animal performance (Silanikove *et al*. 1997; Makkar, 2003). However, the level and structural nature of tannins are important factors that determine the effectiveness of PEG in improving nutrient availability (Vitti *et al*. 2005; Abarghuei *et al*. 2010). Vitti *et al*. (2005) found different PEG/gas responses with Brazilian fodder legumes (*Cajanus cajan*, *Leucaena leucocephala* and *Sesbania sesban*) with similar tannin contents, which is possibly related to the chemical structure of tannins as well as the nature of the tannin-feed complexes (Ammar *et al*. 2005). Although past studies (Yousef Elahi and Rouzbehan, 2008; Yousef Elahi, 2010) investigated the nutritive value of the leaves obtained from the different oak species, few information exists with regard to the rumen fermentability and the effectiveness of PEG on the ruminal fermentation in *Q. persica* leaves harvested at the different growth stages. Therefore, the objectives were to i) evaluate nutritive and anti-nutritive composition as well as *in situ* rumen degradability of the oak leaves harvested at different stages of maturity during the spring season, and ii) assess the efficacy of PEG in an *in vitro* gas production test.

MATERIALS AND METHODS

Sample preparation and chemical characterization

Oak leaves (*Q. persica*) were collected from the Zagros region (33˚ 3´ N latitude and 46˚ 46´ E longitude and an elevation of 1373 meters above sea level) located in the Ilam Province in Iran. Leaves were harvested for three consecutive months in spring, starting on April when leaves were at the early vegetative stage. Thirty trees were chosen at random from various locations, branches were selected, and leaves were randomly divided into five allotments (with in each growth stage). The leaves were dried at 55 ˚C in a forced-air convection oven for 72 h. For phenolic composition analyses, a fraction of the leaves was air-dried at room temperature (27±2 ˚C) in a dark, well-ventilated room until dry matter (DM) content was increased to about 900 g/kg. For compositional analysis, the representative samples (about 500 g) were milled to pass a 1.0-mm sieve (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA). Standard procedures recommended by the Association of Official Analytical Chemists (AOAC, 2000) were adopted for measurement of DM, crude protein (CP), crude ash, and ether extract. Organic matter (OM) was calculated as "100 – ash content". Contents of ash-corrected neutral detergent fiber (NDFom; without α-amylase) and acid detergent fiber (ADFom) were measured using an Ankom^{220} Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA) following the procedure of Van Soest *et al*. (1991). Lignin was measured after solubilization with sulphuric acid (Robertson and Van Soest, 1981).

For phenolic composition determination, a 0.2-g sample of dried, finely ground leaf was added to 10 mL of the acetone: water solution (700:300, v/v) and placed in an ultrasonic bath (105 W, 20 min). The mixture was subjected to centrifugation (10 min, 3000×*g*) and the supernatant was collected in subsequent analyses (Yousef Elahi *et al*. 2014). Total phenols were quantified using Folin-Ciocalteu reagent at 765 nm and expressed as mg tannic acid equivalent/g DM (Makkar, 2000). Tannic acid was purchased from Merck Chemical Co. (Merck GmbH, Darmstadt, Germany). Non-tannin phenols were quantified using the modified Folin-Ciocalteu method with polyvinyl polypyrrolidone to separate tannin phenols from non-tannin phenol (Makkar, 2000). Total tannin content was calculated as difference between total phenols and non-tannin phenols and expressed as mg tannic acid equivalent/g DM. Condensed tannins were quantified using the butanol-HCl-iron method at 550 nm (Makkar, 2000). Briefly, 0.5mL of the supernatant was added to HCl-butanol (3 mL) and ferric ammonium sulphate (0.1 mL) reagents. The mixture was placed in a boiling water bath for 1 h. Condensed tannin was expressed as mg leucocyanidin equivalent/g DM. Leucocyanidin was purchased from Merck Chemical Co. (Merck GmbH, Darmstadt, Germany). The rhodanine method was used for quantification of hydrolysable tannins, and expressed as gallotannin equivalent (Makkar, 2000).

In situ experiment

All procedures involving animals were conducted after approval by the Iranian Council on Animal Care (1995). The *in situ* study was performed in 2 separate runs using three cannulated Kurdi rams (body weight=60±3.1 kg) according to the procedures described before (Mjoun *et al*. 2010). Rams were fed at the maintenance level a diet [CP= 125

g/kg DM and 2.5 Mcal gross energy/kg DM] consisting of 400 g/kg alfalfa hay, 100 g/kg wheat straw, 250 g/kg barley grain, 120 g/kg soybean meal, 100 g/kg wheat bran, and 30 g/kg a vitamin-mineral supplement. The leaf samples were ground and passed through a 2-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA). Then, each sample (3.6 g) was put into a Dacron bag (10×20 cm, 50±10-µm pore size; R1020, Ankom Technology, Macedon, NY, USA) and introduced into the rumen. After the designated time of incubations (2, 4, 8, 16, 24, 48, 72, and 96 h), the bags were removed, manually washed, and dried at 55 ˚C for 48 h. The soluble *A* fraction was determined after the 0-h bags were washed in a washing machine (20 min). The *in situ* data were fitted to the following first-order kinetic model (Ørskov *et al.* 1980):

$$P = A + B\,[1 - e^{-c(t-L)}]$$

Where:
P: ruminal disappearance rate at time *t*.
A: immediately soluble fraction.
B: potentially degradable fraction.
t: incubation time.
L: lag time (h).

The effective degradability (ED) of DM and OM was calculated using the following equation (Ørskov and McDonald, 1979):

$$ED = A + B\,[K_d / (K_d + K_p)]$$

Where:
K_p: fractional ruminal passage rate, assuming to be 0.05 h^{-1}.
K_d: degradation rate of the *B* fraction.

***In vitro* ruminal gas production test**

The same cannulated rams in the *in situ* study were used as donor of ruminal fluid. The fluid was collected before feeding in the morning, immediately transferred to the laboratory, squeezed through four layers of gauze, combined among sheep and purged with oxygen-free CO_2 (Rahavi *et al.* 2022). Details for preparation of rumen fluid were as described before (Yousef Elahi *et al.* 2014). The tannin bioassay was preformed following the procedure of Makkar (2000). In brief, each sample (375 mg; DM basis) was incubated without or with PEG (750 mg; Merck Schuchardt, Hohenbrunn, Germany). The head space pressure was measured relative to the atmospheric pressure using a pressure transducer (Testo 512; Testo Inc., Lenzkirch, Germany). The buffered rumen fluid was dispensed into the serum bottles, immediately flushed with CO_2, closed with a 14-mm rubber septum, secured with an aluminum crimp seal, and then placed in a water bath (39±0.1 ˚C). Gas production (GP) was recorded at 2, 4, 8, 12, 24, 48, 72 and 96 h of incubation. All values were blank-corrected. The following equation was used to convert pressure (kPa) to volume unit (mL) (Tagliapietra *et al.* 2011):

$$GP = [(P_1 + P_{atm}) \times V_0] / P_{atm}$$

Where:
P_1: cumulated pressure in headspace (kPa).
P_{atm}: atmospheric pressure.
V_o: head-space volume.

The cumulative GP data were fitted to the exponential model:

$$P = B\,(1 - e^{-ct})$$

Where:
P: GP at time *t*.
B: GP from the insoluble fraction (mL/200 mg DM).
c: GP rate constant for *B* fraction.
t: incubation time (h).

Metabolizable energy (ME; Menke and Steingass, 1988) and short-chain fatty acids (SCFA; Getachew *et al.* 2001) were estimated using the following equations:

$$ME\ (MJ/kg\ DM) = 2.20 + 0.136 \times GP_{24} + 0.0057 \times CP + 0.0029 \times (EE)^2$$
$$SCFAs\ (mmol/200\ mg\ DM) = 0.0222 \times GP_{24} - 0.00425$$

Where:
GP_{24}: 24-h net gas production (mL/200 mg DM).
CP: crude protein (g/100 g DM).
EE: ether extract (g/100 g DM).

In vitro dry matter degradability (IVDMD) was determined using the procedure of Mellenberger *et al.* (1970). In brief, an *in vitro* gas production test was conducted similar to the previous assay. About 500 mg of each sample was placed into each serum bottle and filled with buffered rumen fluid. After 24 h of incubation, the content of each bottle was collected and centrifuged at 3000 × *g* for 20 min. The precipitate was dried at 55 ˚C to a constant weight. The supernatant was analyzed for NH_3-N using the phenol-hypochlorite method (Broderick and Kang, 1980).

Data analysis

Chemical composition data were analyzed using GLM Proc of SAS (2001) as: leaves at 3 growth stages × 5 replicates per each growth stage × 2 analytical replicates (per each

sample), giving a total of 30 observations. The model used for the analysis was:

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

Where:
Y_{ij}: observation.
μ: mean.
G_i: fixed effect of growth stage (i=3).
e_{ij}: error.

The *in situ* data consisted of values from leaves at 3 growth stages × 5 replicates per each growth stage × 2 separate runs, giving a total of 30 observations. The model used for the analysis was:

$Y_{ij}= \mu + G_i + R_j + e_{ij}$

Where:
Y_{ij}: observation.
μ: mean.
G_i: fixed effect of growth stage (i=3).
R_j: effect of incubation run as a random factor (j=2).
e_{ij}: error.

Gas production data were analyzed according to a 2 × 3 factorial arrangement with the following model:

$Y_{ijk}= \mu + R_i + G_j + P_k + (G\times P)_{jk} + e_{ijk}$

Where:
Y_{ijk}: observation.
μ: mean.
R_i: effect of incubation run as a random factor (i=2).
G_j: fixed effect of growth stage (j=3).
P_k: fixed effect of PEG (k=2; with or without PEG).
$(G\times P)_{jk}$= interaction effect.
e_{ijk}: error.

Mean comparisons were performed using Tukey's test, and significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition and phenolic composition of leaves harvested at different growth stages are presented in Table 1. Contents of OM, ether extract, and NDFom were not affected by the growth stage, averaging 943, 26.3, and 621 g/kg DM, respectively. However, as the leaf maturity progressed CP content decreased. The lowest content of ADFom and lignin was found in leaves harvested at the second growth stage. Contents of total tannins, phenols, and hydrolysable tannins increased as the leaf maturity increased. For example, hydrolysable tannins increased from 73.9 at the first growth stage to 189 mg gallotannin equivalent/g DM in leaves at the third growth stage. Condensed tannin concentration was not different among oak leaves at different maturity stages.

The parameters for DM and OM degradation in the leaves collected at different growth stages, as estimated by *situ* rumen incubation are presented in Table 2. The estimate of 53-µm filterable and soluble *A* fraction was least in leaves at the early vegetative stage (304 g/kg DM) compared to those at the second or third stage (average of 331 g/kg DM). However, mean values for the degradable *B* fraction, undegradable *C* fraction, and effective degradability of DM were not affected by the maturity stage. The degradation kinetics of OM followed almost the same trend as that of DM.

The GP kinetics and fermentation metabolites in oak leaves at different growth stages without and with PEG addition, are presented in Table 3. The GP profiles of untreated and PEG-treated leavesduring94-hfermentationare also illustrated in Figure 1. In general, increased leaf maturity resulted in decrease in gas production kinetics, *in vitro* DM degradability, and fermentation metabolites. For example, NH_3-N concentration was lowest in leaves harvested at the third growth stage (6.67 mg/dL), which was approximately 28% lower than that quantified in leaves at the early vegetative stage. The GP rate constant was slowest in the leaves harvested at the second growth stage. Application of PEG was generally resulted in increased gas production, *in vitro* DM degradability, and ruminal NH_3-N and SCFA concentration. However, the GP rate constant remained unaffected by PEG application. No interaction effect was observed between the growth stage and PEG addition for NH_3-N and SCFA concentrations.

Although the phytochemicals in Persian oak leaves have been extensively studied (Yousef Elahi, 2010; Abarghuei *et al*. 2015), we are aware of very little information about the phenolic compounds in oak leaves at various growth stages. Oak leaves in the present experiment had considerable amounts of tannins, particularly hydrolysable tannins that are associated with impairment of productivity and toxicity in ruminants (McSweeney *et al*. 2001; Makkar, 2003). Contrary to our observation that condensed tannin concentration was not affected by the maturity stage, Makkar *et al*. (1991) studied tannin levels in leaves from four oak species at different stages of maturation and identified that condensed tannins increased with maturity advancement in all species studied. A moderate concentration of condensed tannins in ruminant diet is usually associated with improved efficiency of protein digestion and animal health (Min *et al*. 2003).

Table 1 Chemical composition and phenolic compounds of oak leaves at different growth stages

Items	Oak leaf growth stage[1]			Statistics	
	1	2	3	SEM	P-value
Chemical composition, g/kg DM					
Organic matter	941	946	942	2.82	0.50
Ether extract	34.6	23.2	21.0	4.75	0.15
Crude protein	113^{a}	96.7^{b}	96.6^{b}	0.53	< 0.01
Neutral detergent fiber (NDFom)	615	615	632	5.16	0.07
Acid detergent fiber (ADFom)	410^{a}	387^{b}	392^{ab}	3.51	0.01
Lignin	174^{a}	149^{b}	167^{a}	2.42	< 0.01
Secondary compounds					
Total phenols, mg tannic acid equivalents/g DM	151^{c}	244^{b}	267^{a}	3.24	< 0.01
Total tannins, mg tannic acid equivalents/g DM	93.2^{b}	204^{a}	208^{a}	3.11	< 0.01
Condensed tannins, mg leucocyanidin equivalents/g DM	11.2	13.8	14.1	1.46	0.32
Hydrolysable tannins, mg gallotannin equivalents/g DM	73.9^{b}	182^{a}	189^{a}	3.12	< 0.01

1: leaves harvested at early vegetative stage; 2: leaves harvested one month after the vegetative stage and 3: leaves harvested two months after the vegetative stage.
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 *In situ* dry matter and organic matter degradation characteristics of oak leaves at different growth stages

Items	Oak leaf growth stage[1]			Statistics	
	1	2	3	SEM	P-value
Dry matter fractions, g/kg DM					
53-µm filterable and soluble *A* fraction	304^{b}	324^{a}	338^{a}	4.84	< 0.01
Degradable *B* fraction	245	239	233	19.2	0.90
Undegradable *C* fraction	451	437	429	21.7	0.78
A + *B*	549	563	571	21.7	0.78
Effective degradability	401	424	420	6.83	0.07
Organic matter fractions, g/kg DM					
53-µm filterable and soluble *A* fraction	303^{c}	338^{b}	353^{a}	3.12	< 0.01
Degradable *B* fraction	265	266	244	20.2	0.82
Undegradable *C* fraction	432	396	400	25.2	0.62
A + *B*	564	604	599	28.2	0.62
Effective degradability	395^{b}	427^{a}	426^{a}	5.51	< 0.01

1: leaves harvested at early vegetative stage; 2: leaves harvested one month after the vegetative stage and 3: leaves harvested two months after the vegetative stage.
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 Gas production kinetics and fermentation metabolites in oak leaves at different growth stages[1] without (–) or with (+) polyethylene glycol (PEG) application

Items	1		2		3		SEM	P-values		
	–	+	–	+	–	+		Growth stage	PEG	Growth stage × PEG
Gas production kinetics[2]										
b, mL/g DM	216	308	213	227	177	228	12.3	< 0.01	< 0.01	< 0.01
c, h^{-1}	0.053	0.037	0.016	0.036	0.041	0.065	0.009	0.04	0.24	0.10
ME, MJ/kg DM	4.10	4.22	3.48	3.81	3.66	4.05	0.051	< 0.01	< 0.01	0.06
IVDMD, g/kg DM	416	449	305	403	352	456	19.1	< 0.01	< 0.01	0.16
Fermentation metabolites										
NH_3-N, mg/dL	7.82	9.29	7.33	9.40	6.08	6.67	0.32	< 0.01	< 0.01	0.10
SCFA[3], mmol/g DM	2.97	3.41	1.57	2.86	2.21	3.29	0.25	< 0.01	< 0.01	0.16

1: leaves harvested at early vegetative stage; 2: leaves harvested one month after the vegetative stage and 3: leaves harvested two months after the vegetative stage.
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.

Contrary to our findings that the soluble *A* fraction concentration was lower in leaves at the early vegetative stage, previous studies with leguminous shrub plants reported that as the maturity advanced, the rapidly degradable fraction decreased and the slowly degradable fraction increased (Kamalak, 2006).

In the present experiment, total phenols and tannins increased as leaf maturity progressed (Table 1). Tannins are water-soluble compounds (Swain and Bate-Smith, 1962) that may escape the Dacron bag during the washing process, thus causing the immediately soluble *A* fraction concentration to increase.

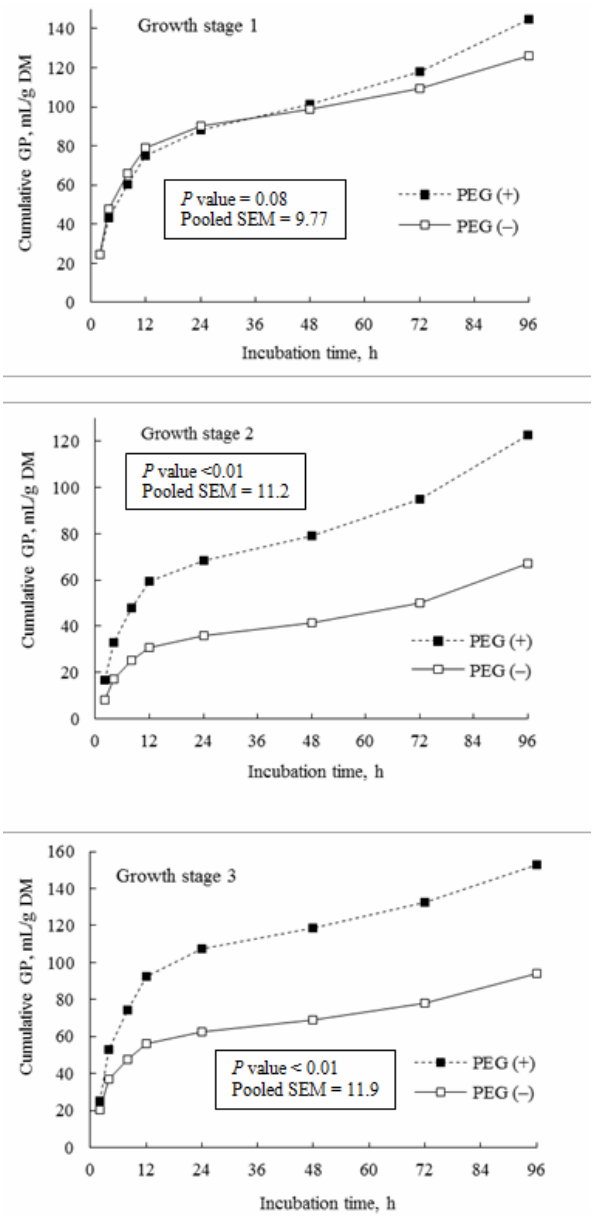

Figure 1 *In vitro* cumulative gas production (GP) kinetics of leaves as affected by growth stage and polyethylene glycol (PEG) application
1 = leaves harvested at early vegetative stage; 2 = leaves harvested one month after the vegetative stage and 3 = leaves harvested two months after the vegetative stage

Effective degradability of Persian oak leaves in the study of Abarghuei *et al.* (2015) was estimated to be 362g/kg DM, while Yousef Elahi (2010) and Yousef Elahi and Rouzbehan (2008) reported that it averaged 300 and 220 g/kg DM, respectively. These differences might be explained by the growth stage of the leaves (Kaitho *et al.* 1993), variety, as well as their phytochemical properties (Salawu *et al.* 1997).

The lower GP potential in mature leaves could be explained by their greater tannin content. Tannic compounds are anti-nutritional factors with known negative effects on *in vitro* ruminal gas production (Ammar *et al.* 2005; Mokoboki *et al.* 2006; Sebata *et al.* 2011). Tannins form complexes with the nutrients and inhibit the activity of ruminal microorganisms as well as enzymes involved in degradation of fibrous materials, thereby declining the efficacy of rumen fermentation (Makkar *et al.* 1995; Silanikove *et al.* 1997; Makkar, 2003). Moreover, tannins can form tight complexes with cell wall fractions (*i.e.*, NDF and ADF), thereby declining degradation fiber fractions in the rumen (Reed *et al.* 1990). The increased GP potential as a result of PEG application is possibly indicative of the negative influence of tannins on ruminal fermentation (Makkar, 2003; Frutos *et al.* 2004; Yousef Elahi *et al.* 2014). The high affinity of PEG with tannins results in formation of the PEG-tannin complex that may improve the accessibility to nutrients, thereby increasing ruminal fermentability (Getachew *et al.* 1998; Makkar, 2003). In support, Getachew *et al.* (2001) also reported that PEG application of tannin-rich browses increased the *in vitro* ruminal fermentability.

The greater CP concentration in leaves harvested at the first growth stage could possibly have resulted in greater NH_3-N concentration in these leaves, as a positive relationship exists between CP concentration and NH_3-N formation in the rumen (Haaland *et al.* 1982; Mohammadi *et al.* 2014). Moreover, tannins, which increased with leaf maturity, hamper the growth rate of proteolytic bacteria, thereby declining the ruminal NH_3-N formation (McSweeney *et al.* 2001; Molan *et al.* 2001; Min *et al.* 2005). Earlier *in vitro* and *in situ* studies demonstrated that the greater tannin concentration was associated with decrease of ruminal NH_3-N concentration, likely because of the negative effect of tannins on protein degradation and bacterial proteolytic activity in the rumen (Min *et al.* 2000; Min *et al.* 2002).

Consistent with our finding that treatment of oak leaves with PEG resulted in the increased NH_3-N production, Getachew *et al.* (2001) also reported that addition of PEG to tannin-rich browses increased ruminal NH_3-N concentration. Salem *et al.* (2007) suggested that the increased NH_3-N production in the rumen with addition of PEG to browse tree leaves was because of greater protein degradability in the rumen that increases availability of large amounts of nitrogen for microbial deamination (Bhatta *et al.* 2002). Poor synchronization between carbohydrate and nitrogen release in the rumen may have occurred in the present experiment, resulting in ammonia accumulation in the rumen fluid with PEG application.

CONCLUSION

The crude protein content of oak leaves decreased as maturity progressed, but concentration of tannins and phenols increased. Oak leaves at the vegetative growth stage could potentially be used as cheap and available source of feed

with high nutritive value in the diet of ruminants. Polyethylene glycol increased the ruminal fermentative kinetics of the leaves at all growth stages, possibly because of its tannin-deactivating properties.

ACKNOWLEDGEMENT

The authors would like to acknowledge Ilam University for the financial support of this study.

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