The aim of this experiment was to determine the effect of polyethylene glycol (PEG) on in vitro gas production (GP) kinetics of Prosopis cineraria leaves at different growth stages. The samples of total phenol (TPH), total tannin (TT) and condensed tannin (CT) were determined. Effects on in vitro organic matter digestibility (OMD), metabolisable energy (ME) and effective dry matter digestibility were assessed by PEG tannin bioassay. No significant differences (P>0.05) were observed for TPH content, however, the stage of flowering had the highest (P<0.05) content of both TT and CT. No interaction effects (P>0.05) were observed between the growth stage and PEG addition for in vitro GP and its parameters. Addition of PEG increased (P<0.05) GP, OMD and ME in all stages. In conclusion, adding PEG to P. cineraria leaves can improve their nutritive value and could be considered as a potential feed for ruminants.

Introduction

Tree legume forages play an important role in livestock nutrition in many parts of the tropics. One of the commonly used tree species in south of Iran is Prosopis spp. (mesquite). The genus Prosopis belongs to the family Leguminosae (subfamily Mimosaceae). About 43 species of this genus are known; three of them are commonly found in Iran. These species are P. cineraria (Iranian Kahor), P. koelsziana (Valley Kahor) and P. juliflora (Pakestanian Kahor). Mesquite trees and shrubs are vigorous, drought- and heat-tolerant plants that are able to survive in many arid parts of the world. In some of these regions mesquite leaves and pods are principal sources of forage during dry seasons (Folkler, 1979).

Prosopis cineraria is found in the arid areas of Arabia, Indian sub-continent, Iran and Afghanistan where it provides fodder, fuel, shade and improvement of soil and stabilises sand dunes. Kahor leaves and pods are consumed by many animal species. However, Prosopis sp. has been reported to contain levels of anti-nutrients such as tannins. Therefore, the nutritive value of these leaves as feed for ruminants is offset by tannin potential negative effect on protein utilisation. Tannins are known to affect the availability of nutrients by formation of soluble and insoluble complexes. Their effects on the digestibility of nutrients will vary depending on tannin content and astringency (McNeill et al., 1998). As it would take a considerable effort to screen for all possible anti-nutritive factors by conventional chemical methods, there is interest in the possible use of simple bio-assays as potential screening methods. The use of polyethylene glycol (PEG) to neutralise condensed tannins (CTs) has proved useful in further elucidating the specific nutritional consequences of dietary CT as it displaces protein-tannin complexes. As a consequence, CTs interact more strongly with PEG than they do with protein (Mangan, 1988). Thus, supplementation with PEG has been used to alleviate the negative effects of tannins on livestock (Landau et al., 2000). Palmer and Jones (2000) have shown that PEG improved the in vitro digestibility of nitrogen in Calliandra and most other legumes containing tannins. The treatment with tannin binding agents can be highly effective in overcoming the anti-nutritive effects of tannins leading to improved animal performance. However, their effects can be variable which may relate to the nature of the tannins and the nature of the tannin-feedstuff complexes which can form.
Chemical analysis

Standard methods as described in AOAC (1990) were used for determination of dry matter (DM) (method #930.15), ash (method #924.05) and N (method #984.13). Ash-free neutral detergent fibre (NDF) was determined using sodium sulphite according to the method of Van Soest et al. (1991), while ash-free acid detergent fibre (ADF) (method #973.18) was determined based on AOAC (1990).

Total phenols determination

Samples were analysed for CT using the method of Makkar (2000). Briefly, 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), then the supernatant was kept on ice until analysis. Total phenols were determined with the Folin-Ciocalteau reagent, and detected at 725 nm. A calibration curve was prepared using tannic acid (Merck GmbH, Darmstadt, Germany). Total phenols were calculated as tannic acid equivalents and expressed as equivalents g/kg DM.

Tannins determination

Samples were analysed for total extractable tannin (TT) using HCL-butanol as described in Makkar (2000). Briefly, an aliquot from the above acetone:water extract (0.5 mL; although this extract occasionally needed diluting with the extract of acetone:water, if final absorbance at 550 nm exceeded 0.6 absorbance units) plus HCL-butanol (3 mL) and ferric ammonium sulphate (0.1 mL) reagents were heated in a boiling water bath for 60 min. Absorbance was read at 550 nm. The colorimetric data (in absorbance units) were converted to leucocyanidin equivalents for 60 min. Absorbance was read at 39°C, using a magnetic stirrer fitted on a hotplate.

Effects of tannins on in vitro organic matter digestibility (OMD) were assessed by incubating approximately 375 mg (DM bases) of triplicate test feed samples with or without 750 mg PEG bioassay was performed according to Makkar et al. (1995) and revised by Makkar (2000). Preparation of an in vitro mineral buffer media for the gas test was completed as described by Menke and Steinengaas (1988). Reduced buffer medium composition, per liter, was 70.0 g of NaHCO3, 4.00 g of NH4HCO3, 5.7 g of Na2HPO4, 6.2 g of KH2PO4, 0.6 g of MgSO4·7H2O, 0.52 g of Na2S, 13.2 g of CaCl2·2H2O, 10.00 g of MnCl2·4H2O, 1.00 g of CoCl2·6H2O, 0.01 g of sodium resazurin, 60 mL of freshly prepared reduction solution containing 580 mg of Na2S·9H2O, and 3.7 mL of 1 M NaOH. The mixture was kept stirred, under CO2 flushing at 39°C, using a magnetic stirrer fitted on a hotplate.

Gas production

Rumen fluid for the in vitro digestibility tannin bioassay was obtained from two mature male native cattle with live weight of 346±11.5 kg fitted with permanent 70 mm rumen cannulae. Cattle were fed a ration divided into equal meals at 08:00 a.m. and 04:00 p.m. daily. Cattle had free access to water throughout the experiment. Rumen fluid was obtained from the two cattle in the morning before feeding (07:00 a.m.), flushed with CO2 filtered through three layers of cheesecloth and mixed (1:2, v/v) with an anaerobic mineral buffer solution as described by Makkar et al. (1995) and revised by Makkar (2000). Preparation of an in vitro mineral buffer media for the gas test was completed as described by Menke and Steinengaas (1988). Reduced buffer medium composition, per liter, was 70.0 g of NaHCO3, 4.00 g of NH4HCO3, 5.7 g of Na2HPO4, 6.2 g of KH2PO4, 0.6 g of MgSO4·7H2O, 0.52 g of Na2S, 13.2 g of CaCl2·2H2O, 10.00 g of MnCl2·4H2O, 1.00 g of CoCl2·6H2O, 0.01 g of sodium resazurin, 60 mL of freshly prepared reduction solution containing 580 mg of Na2S·9H2O, and 3.7 mL of 1 M NaOH. The mixture was kept stirred, under CO2 flushing at 39°C, using a magnetic stirrer fitted on a hotplate.

Statistical analysis

Data of chemical composition and tannin content were subjected to analysis using the General Linear Model procedure of SAS (2002), based on the statistical model: Yijk=μ+Si+Pj+Tij+Eijk, where Yijk is the general observation on chemical composition and tannin content, μ is the general mean, Si is the ith effect of growth stage on the observed parameters, and Eijk is the random standard error. Means were tested using Duncan test.

For in vitro GP and estimated parameters of digestibility and ME, the following statistical model was fitted: Yijk=μ+Sij+Pj+Pij+Eijk, where Yijk is the observation on GP and digestibility estimates, Sij is the jth effect of growth stage and Pij is the jth effect of PEG on the observed parameters. The (SP)ij term represents pth and jth interaction effects of growth stage and PEG on in vitro GP, and Eijk is the random standard error.

Results

Primary and secondary compounds

The chemical composition of P. cineraria at different growth stages is shown in Table 1. There were variations in chemical compositions between phenological stages of P. cineraria. The CP content of the different stages ranged (P<0.05) from 9.26 to 11.79% for seed ripening and vegetative stages, respectively. The NDF and ADF content ranged (P<0.05) from 9.26 to 11.79% for seed ripening and vegetative stages. The content of TPH was almost the same at 45.12 to 46.51 and 28.14 to 30.48%, respectively for seed ripening and vegetative stages. The content of TPH was almost the same (P>0.05) between different stages. However, the stage of flowering had the highest (P<0.05) content of both TT and CT compared to seed ripening and vegetative stages, respectively (Table 1).

Gas production

No interaction effects (P>0.05) were observed between the growth stage and PEG addition for in vitro GP and GP parameters.
Discussion

In our study, leaves of Prosopis cineraria had CP content more than 80 g/kg DM (range: 92.6 to 117.9 g/kg DM), which according to Norton (2003) should provide ruminal ammonia levels above the minimum required by rumen microorganism to support optimum growth for maintenance, production and optimum activity. However, the CP content of leaves of seed ripping stage was lower than required by microorganisms in the rumen to support optimum activity (Norton, 2003).

In tree leaves, tannins are tightly bound to the cell wall (NDF and ADF) and cell protein (Reed et al., 1990). Tannins may form a less digestible complex with dietary proteins and may bind and inhibit the endogenous protein, such as digestive enzymes (Kumar and Sing, 1984). Tannin can also adversely affect the microbial and enzyme activities (Silanikove et al., 1996a; Makkar et al., 1995).

There was a relationship between TPH and TT which is similar to findings of Makkar et al. (1993) who noted a high positive correlation between content of TT and TPH compounds. Levels of TPH and TT in the growth stages were higher for flowering than vegetative stage compared to seed ripping stage. The variation in the phenolic compound contents was probably due to growth stage (Makkar and Singh, 1993).

Table 1. Chemical composition and phenolic compounds of Prosopis cineraria at different growth stages.

| Growth stage | SEM |
|--------------|-----|
| Vegetative   |     |
| Flowering    |     |
| Seed ripping |     |
| DM, %        |     |
| DM           | 93.5^a | 93.5^a | 93.2^a | 0.61 |
| OM, %        | 85.5^a | 86.2^a | 85.2^a | 0.80 |
| Ash, %       | 14.5^a | 13.8^a | 14.8^a | 0.78 |
| CP, %        | 11.8^a | 10.7^a | 9.3^a  | 0.47 |
| Ether extract| 3.6^a  | 4.7^a  | 4.8^a  | 0.35 |
| ADF, %       | 30.5^a | 29.0^a | 28.1^a | 0.51 |
| NDF, %       | 46.5^a | 45.8^a | 45.1^a | 0.39 |
| TPH, %       | 8.8^a  | 8.8^a  | 8.5^a  | 0.23 |
| TT, %        | 7.67^ab | 7.76^a | 7.15^b | 0.26 |
| CT, %        | 3.01^b | 4.23^a | 3.60^a | 0.32 |

DM, dry matter; OM, organic matter; CP, crude protein; ADF, acid detergent fibre; NDF, neutral detergent fibre; TPH, total phenolic; TT, total tannin; CT, condensed tannin. *Means in the same row with different superscript letters differ (P<0.05).

Table 2. Gas production characteristics of Prosopis cineraria leaves without or with polyethylene glycol at different growth stages.

| Growth stage | Significance |
|--------------|--------------|
| Without PEG | With PEG |
| Without PEG | With PEG |
| Without PEG | With PEG |
| Without PEG | With PEG |

| Incubation time, h | Vegetative | Flowering | Seed ripping | SEM | Stage | PEG | Stage*PEG |
|--------------------|------------|-----------|--------------|-----|-------|-----|----------|
| 4                  | 11.9       | 15.6      | 10.9         | 14.4 | 11.9  | 16.2 | 0.52     |
| 6                  | 16.1       | 21.6      | 15.2         | 20.4 | 16.0  | 22.6 | 0.65     |
| 8                  | 17.9       | 20.1      | 17.2         | 21.4 | 18.0  | 26.2 | 0.57     |
| 12                 | 21.8       | 31.3      | 21.5         | 30.8 | 22.3  | 33.1 | 1.11     |
| 24                 | 26.3       | 37.5      | 26.4         | 38.3 | 26.6  | 39.6 | 1.74     |
| 48                 | 29.8       | 41.2      | 30.3         | 43.0 | 29.0  | 42.9 | 2.13     |
| 72                 | 32.7       | 44.0      | 33.1         | 46.2 | 31.6  | 44.5 | 2.17     |
| 96                 | 34.5       | 45.5      | 35.7         | 47.8 | 33.6  | 45.6 | 2.22     |

GP parameters

| b, ml/200 mg dry matter | 30.9 | 42.8 |
|-------------------------|------|------|
| c, ml/h                 | 0.096 | 0.106 |
| OMD, %                  | 52.9 | 62.9 |
| DOMD, %                 | 44.7 | 53.8 |
| ME, MJ/kg DM            | 6.8 | 8.4 |

PEG, polyethylene glycol; GP, gas production; b, insoluble but fermentable fraction; DM, dry matter; c, rate constant of gas production during incubation; OMD, organic matter digestibility; DOMD, digestible organic matter in dry matter; ME, metabolisable energy. *Means with different letters within stages differ (P<0.05); ns, not significant.
nutrient digestibility and N retention (Kumar and Vaithiyananthan, 1990; Silanikove et al., 1996b). Total tannin content of forages in the range of 60 to 100 g/kg DM depresses intake and growth of animals (Barry et al., 1984). The tannin content of leaves obtained in this experiment fell into this range. Therefore, supplementation of PEG can be recommended to reduce the detrimental effect of tannin in leaves. Pritchard et al. (1998) showed that giving PEG to sheep fed with mulga markedly increased feed intake, weight gain and wool growth. Gilboa et al. (2000) also showed that a single daily oral dose of PEG substantially improve feed intake and efficiency of utilisation by sheep and goats consuming tannin rich forages (Gurbuz, 2007). The effects of tannins seem to be dependent on several factors including forage species, chemical nature and structure of tannins, and biochemical interaction among tannins and proteins, than the tannins level itself. On the other hand, in palm leaves, addition of PEG had small effect despite their relatively high tannin content (Arhab et al., 2009).

The OMD was higher when PEG was added to the different growth stages. Increased in vitro GP and OMD due to addition of PEG suggest a negative influence of tannins on digestibility (Makkar, 2003). Inactivation of tannins through PEG binding increases availability of nutrients resulting in increased microbial activity and GP (Makkar, 2003).

Addition of PEG caused different increments in GP between different growth stages. These variable responses of GP could be due to variations in tannin content between different stages. The increase in the GP in the presence of PEG can be due to an increase in the availability of nutrients to rumen microorganisms, especially N (Bakhshizadeh and Taghizadeh, 2013). Increased degradability of samples at different growth stages treated with PEG was reflected in greater GP at different time intervals.

The leaves of P. cineraria at the stage of flowering provide more soluble fractions, which is a fermentable energy source within time. Gas production is associated with volatile fatty acids production following fermentation of substrate; therefore, more fermentation of a substrate will result in a greater GP (Blümmel and Ørskov, 1993). Differences between GP could be explained by the differences in total VFA production and molar proportion of VFA as a result of fermentation (Beuvink and Spoelstra, 1992). Doane et al. (1997) found a significant correlation between GP and VFA production. Quickly soluble carbohydrates produce relatively higher propionate as compared to acetate and vice versa when slowly fermentable carbohydrates are fermented (Getachew et al., 1998).

The increased GP when samples were incubated with PEG were also reported for different forages by other authors. Basha et al. (2013) noted that the addition of PEG overcomes the inhibitory effect of tannins on rumen microbes. Arhab et al. (2009) evaluated the influence of tannins present in arid zone forages, like limited available N for ruminal microbiota, the higher NDF, ADF and lignin contents, and the saponins may limit fermentation (Ndlovu and Nherera, 1997). Blümmel et al. (2003) suggested selection of forages for high degradability but proportionally low GP. The theoretical range for PF values for tannins free plants was suggested by Blümmel et al. (1997) to be between 2.75 and 4.41. According to Blümmel and Becker (1997), plants with high PF are in general highly digestible and the values correlate well with DM intake in ruminants. Thus, these results could suggest that these forages had a potential nutritive value which tends to enhance microbial synthesis rather than GP.

The effect of PEG addition is more pronounced on potential GP, measured at 96 h of incubation. The effects of tannins on nutrient degradability depends essentially on the formation of complexes between tannins and the components of diets, primarily proteins and to a lesser extent with amino acids, polysaccharides and minerals, as well as on their effects on the microbial population and on its enzy-

Figure 1. In vitro gas production of Prosopis cineraria leaves with or without polyethylene glycol at different growth stages (P<0.05): a) vegetative, b) flowering, and c) seed ripping stage.
matic activity (McSweeney et al., 2001). Guimarães-Beelen et al. (2006) noted that if the rate of GP is reduced, the bacteria colonization is restricted. This could suggest that complexes forming between tannins and PEG generate steric obstruction which does not permit and/or limit the fixation of adherent bacteria to the feeds.

**Conclusions**

The addition of PEG, which has a relatively low cost in Iran, improves nutritional value of *Prosopis cineraria* testified by increased GP and OMD. It appears that a PEG/GP assay is a useful first screen for evaluating nutritional value of tannin-rich feeds. However, further research is needed to assess their impact on animal performance.

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