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Supplementation of grass (*Eragrostis spp.*) hay with *Vachellia karroo* leaves: effect on chemical composition and *in vitro* ruminal fermentation

Steve Kgotlelelo Mahlake[1] and Caven Mguvane Mnisi[1,2,*]
[1]School of Agricultural Science, North-West University, Mafikeng-2745, South Africa
[2]Faculty of Natural and Agricultural Science, North-West University, Mafikeng-2745, South Africa
*Corresponding author e-mail: mnisiecm@gmail.com
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Abstract
This study was designed to investigate the effect of supplementing a grass (*Eragrostis spp*.) hay basal diet with increasing levels of *Vachellia karroo* leaves (VKL) on chemical composition and *in vitro* ruminal fermentation. Fresh VKL were harvested, dried (60ºC), milled and used to supplement grass hay at a rate of 0 (VKL0), 5 (VKL5), 10 (VKL10), 15 (VKL15), 20 (VKL20) and 25% (VKL25). The effect of VKL condensed tannins on *in vitro* ruminal fermentation was assessed with the aid of a tannin-binding compound, polyethylene glycol (PEG Mr 4400), using the Reading Pressure Technique. VKL25 had the highest CP, soluble condensed tannin (SCT) and total soluble phenolics (TSPh) contents ($P<0.05$) than VKL0. Supplementing grass hay with VKL reduced ($P<0.05$) NDF and ADF contents but had no influence on ADL ($P>0.05$). With or without PEG inoculation, VKL0 had the highest ($P<0.05$) rate of gas production. Cumulative gas production was higher ($P<0.05$) for the inoculation with PEG, but no differences ($P>0.05$) were observed among the treatments. PEG inoculation had ($P<0.05$) a shorter lag phase and lower Pgas but had higher *in vitro* ruminal organic matter degradability (ivOMD), effective gas production (Egas) and partitioning factors (PF). VKL0 had the highest gas production rate constant for the slowly fermentable fraction *c* (0.007 %/h) compared to the other treatments, which did not differ ($P>0.05$). VKL25 had the lowest Egas (43.7 ml/g OM) than VKL0, VKL5 and VKL10. It was concluded that *Vachellia karroo* leaves could be used as a potential protein source and, with the aid of PEG, the leaves could improve ruminal fermentation efficiency.

Keywords: Browse leaves, Chemical composition, Grasses, *In vitro* fermentation, Polyethylene glycol

Introduction

Ruminant production plays important nutritional and socio-economic roles for people residing in semi - arid and arid regions of the world (Ben Salem and Smith, 2008). However, during the dry seasons, feed supply in these localities fluctuates both in quality and quantity resulting in reduced animal performance (Shinde and Mahanta, 2020). The use of commercial supplements is prohibitive to most communal farmers due to a lack of capital, and as a consequence, poor quality forages are fed to ruminants (Ramantsi *et al*., 2019). Malik *et al*. (2016) reported that feeding of highly fibrous forages prolongs ruminal microbial fermentation, which causes an increase in greenhouse gas emissions. The enteric gaseous emissions represents a major energy loss from the basal diet (Opio *et al*., 2013), indicating a reduction in efficiency of energy and nitrogen utilization (Bhatta *et al*., 2015). During the dry periods, the crude protein content of many grass species is very low to meet the animal's nutritional requirements for optimum performance (Ismail *et al*., 2014; Ravhuhali *et al*., 2018). Alternatively, browse trees can be used as potential foliage sources (Mnisi and Mlambo, 2017; Nag *et al*., 2017). *Vachellia karroo* browse trees are abundantly distributed in tropical rangelands of Africa (Abdulrazak *et al*., 2001) and can withstand harsh environmental conditions (extreme temperatures and low rainfall). Browse leaves have high crude protein compared to grasses (Goodchild and McMeniman, 1994; Singh *et al*., 2015), but their utilisation may be limited by the presence of condensed tannins (CT). Overconsumption of CT can cause gastrointestinal haemorrhages, toxicities and eventually result in death (Carulla *et al*., 2005; Mnisi and Mlambo, 2017). Nonetheless, tannin-rich feeds have the potential to reduce rumen fermentation gases (Carulla *et al*., 2005; Patra and Saxena, 2010) and rumen degradable protein as they bind to proteins and other nutrients (Makkar *et al*., 1999).

Thus, there is a need to explore the use of a tannin-binding compound to ameliorate the negative effects of CT. Polyethylene glycol (PEG) is one such compound

that has high affinity to CT (Frutos *et al*., 2004) and can be used to inactivate CT by forming PEG-CT complexes (Kumanda *et al*., 2019). The use of PEG can be an effective strategy to improve the utilization of *Vachellia karroo* leaves (VKL) as alternative and least-cost protein supplements in pasture-based feeding systems. Indeed, there is scanty information on the use of VKL as potential protein sources in a grass hay basal diet and its effect on ruminal fermentation when treated with PEG. This study was, therefore, designed to determine the proximate constituents and *in vitro* ruminal fermentation (with or without PEG) of VKL as supplements in a grass hay based diet. The hypothesis tested that supplementing a grass hay basal diet with increasing levels of VKL improves chemical composition and *in vitro* ruminal fermentation efficiency.

Materials and Methods

Description of study site: The study was conducted at the North-West University Research Farm (Molelwane), a private farm with a very low number of ruminants that do not rely entirely on the rangeland. This site is situated in the semi-arid region of the north west province of South Africa, at an altitude of 1226 m above sea level and geographical coordinates of 25°86' 00" S latitude and 25°64' 32" E longitude. The annual rainfall ranges from 300 to 600 mm, and annual temperatures range between 3°C and 39°C.

Harvesting, processing and formulation: Bulk leaf samples from *V. karroo* were harvested by hand within a marked area (100 × 100 m) in Molelwane farm. A grass (*Eragrostis spp*.) hay offered to the animals was sampled from the storage. Both the leaf and hay samples were simultaneously oven-dried at 60°C until constant weight and, thereafter, ground (Polymix PX MFC 90 D) to pass through a 1-mm sieve before treatment formulations. The treatment samples were formulated by supplementing the grass hay with VKL at a rate of 0 (VKL0), 5 (VKL5), 10 (VKL10), 15 (VKL15), 20 (VKL20) and 25% (VKL25). Each of the six treatments was replicated six times, with each replicate sample being treated independently of each other.

Chemical analysis: The treatment samples (VKL0, VKL5, VKL10, VKL15, VKL20 and VKL25) were analysed for dry matter (DM) by oven-drying at 105ºC for 12 h. Organic matter (OM) content was determined after ashing samples in a muffle furnace set at 550°C overnight. Crude protein (CP) was determined through the Kjeldahl method (AOAC, 2005, method no. 984.13). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to the method of Van Soest *et al*. (1991) in which samples were refluxed with neutral detergent and acid detergent solutions for 60 min and 75 min, respectively, using the ANKOM^{2000} Fibre Analyzer (ANKOM Technology, Fairport, NY, USA) and expressed inclusive of residual ash. A heat stable *α*-amylase was used for NDF analysis. Acid detergent lignin (ADL) was determined by treating ADF residue in ANKOM F57 bags with 72% sulphuric acid and estimated after drying (105°C) for 12 h. Total phenolics were determined using Fautholin–Ciocalteau methods and expressed as tannic acid equivalent (g TAE /kg DM) (Makkar, 1995). Condensed tannin (CT) was determined using the butanol–HCl method (Porter *et al*., 1986) and expressed as absorbance unit at 550 nm wavelength ($AU_{500\ nm/\ 200\ mg}$).

In vitro ruminal fermentation: The *in vitro* ruminal microbial fermentation was evaluated with the aid of PEG (Mr 4400, Associated Chemical Enterprises (LTD), South Africa), using the Reading Pressure Technique (RPT) (Trantech, TT26D, ZA) developed by Mauricio *et al*. (1999). A total of 72 samples (1 g each) were weighed into individual RPT bottles (125 ml capacity). An ANKOM buffer solution (90 ml per bottle) without PEG was added to 36 RPT bottles (including two blanks for each treatment). To the remaining 36 RPT bottles, PEG-dissolved (400 mg PEG/90 ml buffer) buffer solution was added. The bottles were then sealed with rubber stoppers and thereafter transferred into an incubator set at 39°C for 12 h prior to inoculation with rumen fluid. Rumen fluid was collected from the cannulated Bonsmara cow (~600 kg), which had unrestricted access to the grass hay and clean water. The procedures for use and care of the donor cow (approval number NWU-00126-13-A9) were as described by Mnisi and Mlambo (2017). The rumen fluid was collected into two pre-warmed thermos flasks and quickly taken to the laboratory, where it was blended and strained through two layers of warm muslin cloths. Strained rumen fluid was held at 39°C under a stream of carbon dioxide gas. The processed rumen fluid (25 ml) was then inoculated into each bottle and incubated at 39°C. Gas pressure was measured using a pressure transducer at 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after inoculation. The gas pressure measured was converted into millilitres (ml) using the following equation: $y = 0.034x^2 + 6.2325x + 1.8143$, set for the RPT and used to calculate cumulative gas production (ml/g OM) until 96 h. To determine *in vitro* fermentation kinetics, cumulative gas data were fitted to the non-linear model (Ørskov and McDonald, 1979):

$Y = a + b\ (1 - e^{-c\ (t - tl)})$

Where, Y = gas produced at time t; a = gas production from the rapidly fermentable fraction; b = gas production from the slowly fermentable fraction; c = gas production rate constant for fraction b; t = incubation time (h); lt = lag time.

Potential gas production (Pgas) was calculated as a summation of a and b, whereas effective gas production (Egas) determined using the equation: $Egas = a + \frac{bc}{K + c}$, where K is the rumen outflow rate (assumed to be 2 %/ h). Rate of gas produced was calculated by dividing the volume of gas produced for each period by the amount of hours within that period. *In vitro* ruminal organic matter degradability (ivOMD) was determined at 96 h post-inoculation through the recovery of fermentation residue by filtration through glass-sintered crucibles (100-160 µm porosity, Pyrex, Stone, UK). Partitioning factors (PF) were calculated as a ratio of cumulative gas production at 96 h post-incubation over ivOMD.

Statistical analysis: Chemical composition data were analysed using the general linear model (GLM) procedure of SAS (2010), within a completely randomised design (CRD), using the following linear statistical model:

$Y_{ij} = \mu + VKL_i + E_{ij}$

Where, Y_{ij} = dependant variable, μ = population mean, VKL_i = effect of *V. karroo* levels, and E_{ij} = random error associated with observation *ij*, assumed to be normally and independently distributed.

The *in vitro* ruminal fermentation data were analysed using the GLM procedure of SAS (2010), based on a two-way factorial treatment design within a CRD. Main effects were *V. karroo* levels (0, 5, 10, 15, 20 and 25%) and PEG (0 and 400 mg/sample). The following linear model was used:

$Y_{ijk} = \mu + VKL_i + PEG_j + (VKL \times \text{PEG})_{ij} + E_{ijk}$

Where, Y_{ijk} = dependant variable, μ = population mean, VKL_i = effect of VKL, PEG_j = effect of PEG, $(VKL \times PEG)_{ij}$ = effect of interaction between VKL and PEG, and E_{ijk} = random error associated with observation *ijk*, assumed to be normally and independently distributed. For all statistical tests, significance was declared at P<0.05. Least squares means (LSMEANS) were compared using the probability of difference option in the LSMEANS statement of SAS.

Results and Discussion

Chemical composition: One sustainable way of improving the utilization of poor quality pastures is though supplementation with leguminous forages or conventional sources of protein (Babiker and Abdulla, 2015). Supplementing grass hay with VKL had significant effects on all chemical components, except for ADL (P>0.05) (Table 1). VKL20 had the lowest DM (952.0 g/ kg) and the highest was from VKL5 and VKL10, which did not differ (P>0.05). VKL5 had the highest OM (918.5 g/kg DM) and the lowest was from VKL25 (907.2 g/kg DM). According to Mnisi and Mlambo (2017), one of the major drawbacks of browse trees as a nutritious source of feed is the presence of antinutritional factors such as polyphenolic compounds. The CP content of the treatments increased with increasing levels of VKL but so was the total soluble phenolics (TSPh) and condensed tannins (CT) concentrations. VKL25 had the highest CP and TSPh contents (P<0.05) and the lowest was from the VKL0. VKL0 had the lowest SCT (0.44 $AU_{550\ nm/200mg}$) and the highest was from VKL20 and VKL25, which did not differ (P>0.05). This indicated that VKL could be a potential source of proteins but the presence of antinutritional factors may restrict its utilisation in ruminant diets.

According to Robinson *et al*. (1998), the NDF content of a feed can be used to predict feed intake, whereas the ADF gives an expected digestibility and energy intake (Wright and Lackey, 2008). In this study, the NDF content declined with VKL inclusion, where VKL0 and VKL5 had the highest NDF (P<0.05) and the lowest was from VKL20 and VKL25. A similar trend was observed for ADF content, where VKL20 and VKL25 had the lowest ADF values and the highest values were from VKL0, VKL5 and VKL10. This showed that the use of these browse leaves as supplements in a grass hay basal diet might increase voluntary feed intake and ruminal digestibility. ADL content did not differ (P>0.05) across all treatment levels. On a DM basis, grasses and leguminous plants (i.e. browse leaves) were reported to have the same lignin concentration, therefore it was not surprising that supplementing grass hay with VKL had no significant influence on ADL content. However, even though the lignin content was similar, a larger negative effect would be on the digestibility of grasses.

Table 1. Chemical composition (g/kg DM, unless stated otherwise) of grass hay supplemented with incremental levels of *Vachellia karroo* leaves

[2]Components	[1]*Vachellia karroo* levels						
	VKL0	VKL5	VKL10	VKL15	VKL20	VKL25	[3]SEM
Dry matter (g/kg)	956.2^{bc}	961.2^{d}	960.0^{d}	958.6^{cd}	952.0^{a}	953.2^{ab}	0.920
Organic matter	913.1^{bc}	918.5^{d}	916.8^{cd}	915.7^{cd}	909.8^{ab}	907.2^{a}	0.935
NDF	814.7^{c}	801.3^{c}	761.3^{bc}	729.6^{b}	661.8^{a}	630.3^{a}	16.46
ADF	430.3^{b}	420.9^{b}	421.5^{b}	388.3^{ab}	365.3^{a}	362.4^{a}	12.39
ADL	93.7	102.2	121.2	89.1	71.7	87.4	14.71
Crude protein	60.6^{a}	68.6^{b}	72.9^{c}	74.9^{d}	76.9^{e}	79.5^{f}	0.498
SCT ($AU_{550/200mg}$)	0.44^{a}	1.31^{b}	2.27^{c}	2.51^{c}	2.88^{d}	2.92^{d}	0.062
TSPh (g TAE/kg DM)	51.3^{a}	75.0^{b}	100.7^{c}	131.4^{d}	166.1^{e}	210.4^{f}	2.901

[a,b,c,d,e,f] In a row, means with common superscripts did not differ (P>0.05)

[1]Vachellia karroo levels: Grass hay supplemented with Vachellia karroo leaves at a rate of 0, 5, 10, 15, 20 and 25% (VKL0, VKL5, VKL10, VKL15, VKL20 and VKL25)

[2]Components: NDF = Neutral detergent fibre; ADF = Acid detergent fibre; ADL = Acid detergent lignin; SCT = Soluble condensed tannins; TSPh = Total soluble phenolics

[3]SEM = Standard error of mean

Table 2. Significance levels (*P*-value) of the main factors on the rate of gas produced per period (ml/h OM) and cumulative gas production (ml/g OM) of grass hay supplemented with incremental levels of *Vachellia karroo* leaves

[2]Source	[1]*In vitro* ruminal gas production parameters							
	Rtgas12	Rtgas24	Rtgas36	Rtgas48	Cumgas12	Cumgas24	Cumgas36	Cumgas48
VKL	NS	***	***	***	NS	NS	*	**
PEG	***	***	***	***	**	***	***	***
VKL × PEG	**	***	***	***	NS	NS	**	***

[1]Parameters: Rtgas12 = Rate of gas produced at 12 h post-inoculation; Rtgas24 = Rate of gas produced at 24 h post-inoculation; Rtgas36 = Rate of gas produced at 36 h post-inoculation; Rtgas48 = Rate of gas produced at 48 h post-inoculation; Cumgas12 = Cumulative gas production at 12 h post-inoculation; Cumgas24 = Cumulative gas production at 24 h post-inoculation; Cumgas36 = Cumulative gas production at 36 h post-inoculation; Cumgas48 = Cumulative gas production at 48 h post-inoculation

[2]Source: VKL = Levels of Vachellia karroo leaves; PEG = Polyethylene glycol; VKL × PEG = Interaction effect of Vachellia karroo levels and polyethylene glycol

*NS = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001*

Table 3. Effect of polyethylene glycol and *Vachellia karroo* levels on rate of gas produced per period (ml/h OM)

[2]Parameters		[1]*Vachellia karroo* levels						
	[3]PEG	VKL0	VKL5	VKL10	VKL15	VKL20	VKL25	[4]SEM
Rtgas12	-PEG	1.39	1.44	1.44	1.13	1.21	1.10	0.124
	+PEG	1.52	1.57	1.47	1.73	1.86	1.84	
Rtgas24	-PEG	1.25^{c}	0.97^{bc}	1.00^{bc}	0.60^{a}	0.67^{ab}	0.49^{a}	0.089
	+PEG	1.77^{b}	1.46^{ab}	1.33^{a}	1.58^{ab}	1.68^{ab}	1.44^{ab}	
Rtgas36	-PEG	1.60^{c}	1.51^{c}	1.31^{bc}	0.87^{ab}	0.94^{ab}	0.61^{a}	0.121
	+PEG	1.81	1.82	1.88	1.80	1.85	1.75	
Rtgas48	-PEG	1.46^{bc}	1.65^{c}	1.43^{bc}	1.20^{b}	1.14^{ab}	0.73^{a}	0.107
	+PEG	1.76	1.78	1.91	1.88	1.87	1.79	

[a,b,c] In a row, means with common superscripts did not differ (P>0.05)

[1]Vachellia karroo levels: Grass hay supplemented with Vachellia karroo leaves at a rate of 0, 5, 10, 15, 20 and 25% (VKL0, VKL5, VKL10, VKL15, VKL20 and VKL25)

[2]Parameters: Rtgas12 = Rate of gas produced at 12 h post-inoculation; Rtgas24 = Rate of gas produced at 24 h post-inoculation; Rtgas36 = Rate of gas produced at 36 h post-inoculation; Rtgas48 = Rate of gas produced at 48 h post-inoculation

[3]PEG (Polyethylene glycol): -PEG = Inoculation without polyethylene glycol; +PEG = Inoculation with polyethylene glycol

[4]SEM = Standard error of mean

Rate of gas produced per period: Martin *et al*. (2010) stated that most browse species contain plant secondary metabolites such as CT, which have both beneficial and detrimental effects on the animals. Thus to ameliorate the negative effects of CT, the use of PEG was proposed (Nsalhai *et al*., 2011), and this phenomenon was also employed in VKL. The increasing VKL levels had an effect (P<0.05) on the rate of gas produced at 24, 36 and 48 h of incubation as well as on cumulative gas production at 36 and 48 h post-inoculation (Table 2). PEG had an effect (P<0.05) on the rate of gas produced and cumulative gas production at 12, 24, 36 and 48 h post-inoculation. With PEG, VKL0 had the highest Rtgas24 (1.77 ml/h OM) whereas VKL10 had the lowest (1.33 ml/h OM) (Table 3). For the inoculation without PEG, VKL0 had the highest (P<0.05) Rtgas24, Rtgas36 and Rtgas48 and the lowest was from VKL25.

Cumulative gas production and fermentation kinetics: The inoculation with PEG had higher (P<0.05) cumulative gas production at 12, 24, 36 and 48 h post-incubation than the one without PEG (Fig 1). Significant interaction effect were observed between VKL and PEG treatment on cumulative gas production at 36 and 48 h post-inoculation. For the inoculation without PEG, VKL0 had the highest (P<0.05) Cumgas36 and Cumga48 and the lowest was from VKL25. However, no differences (P>0.05) were observed among the treatments in terms of Cumgas36 and Cumgas48 for the inoculation with PEG (Fig 2). These results indicated that PEG inoculation shortens the period of gas production, which could be the reason the lag phase was shorter for the PEG inoculation. Moreover, VKL0 had the highest cumulative gas production for the inoculation without PEG, which could be due the higher fibre and low CT content in VKL0. This could be explained from the findings of Nsalhai *et al*. (2011) that the negative effects of CT on digestion also interferes with the activity of fibrolytic enzymes, which subsequently reduce ruminal gas production in tannin-rich plants. For the inoculation with PEG, no differences were observed between VKL0 and the highest supplementation rate in terms of rate of gas production and cumulative gas production. This showed that, indeed, PEG inactivated CT, which was supposed to manipulate ruminal microbial environment and reduced gas production (Abdulrazak *et al*., 2000).

The increasing VKL levels had an effect (P<0.05) only on effective gas production (Egas; (Table 4). Treatment with PEG had a significant effect on all *in vitro* fermentation kinetics, except for the immediately fermentable fraction '*a*'. Significant interaction effects were observed on fraction *c* and Egas. PEG treatment increased the gas production rate constant for the slowly fermentable fraction *b* (*c*), Egas and partitioning factors (PF). but reduced (P<0.05) potential gas production (Pgas) and *in vitro* ruminal organic matter degradability (ivOMD) (Table 5). The PEG inoculation also promoted a shorter lag than the one without PEG. Furthermore, there were significant differences among treatments on fraction *c* and Egas for the inoculation without PEG. VKL0 had the highest fraction *c* (0.007 %/h) compared to the other treatments, which did not differ (P>0.05). VKL0, VKL5 and VKL10 had a higher (P<0.05) Egas value than VKL25. According to Reis *et al*. (2016), a higher Egas value means that there would be higher availability of nutrients for rumen synthesis of short chain fatty acids as well as microbial protein, hence PEG improved PF, a measure of fermentation efficiency. However, it must be noted that PEG does not bind non-tannin phenolics meaning that these compounds might still interfere with ruminal fermentation.

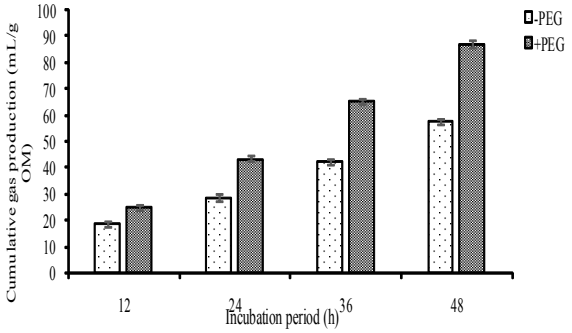

Fig 1. Effect of polyethylene glycol on cumulative gas production (ml/g OM) of grass hay supplemented with *Vachellia karroo* leaves

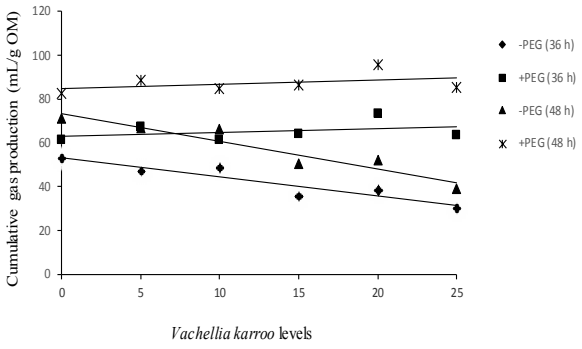

Fig 2. Interaction effect of polyethylene glycol and *Vachellia karroo* levels on cumulative gas production (ml/h OM) at 36 and 48 h of incubation

Table 4. Significance levels (*P*-value) of the main factors on *in vitro* ruminal gas production kinetics of grass hay supplemented with incremental levels of *Vachellia karroo* leaves

[2]Source	*In vitro* ruminal gas production kinetics[1]							
	a	***b***	***c***	**Lag phase**	**Pgas**	**Egas**	**ivOMD**	**PF**
VKL	NS	NS	NS	NS	NS	***	NS	NS
PEG	NS	*	***	***	**	***	***	***
VKL × PEG	NS	NS	*	NS	NS	***	NS	NS

[1]Kinetics: a = The immediate fermentable fraction; b = The slowly fermentable fraction; c = Fermentation rate of fraction b; Pgas = Potential gas production; Egas = Effective gas production; ivOMD = In vitro ruminal organic matter degradability; PF = Partitioning factors

*[2]Source: VKL = Levels of Vachellia karroo leaves; PEG = Polyethylene glycol; VKL * PEG = Interaction effect of Vachellia karroo levels and polyethylene glycol*

*NS = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001*

Table 5. The effect of polyethylene glycol on in vitro ruminal gas production kinetics of grass hay supplemented with *Vachellia karroo* leaves

[2]Kinetics	[1]Polyethylene glycol	
	-PEG	**+PEG**
b (ml/g OM)	301.1	220.0
c (%/h)	0.004[a]	0.010[b]
Lag phase (h)	7.29[b]	5.27[a]
Pgas (ml/g OM)	339.3[b]	231.2[a]
Egas (ml/g OM)	60.6[a]	80.6[b]
ivOMD (g/kg OM)	845.8[b]	633.5[a]
Partitioning factor (ml/g OM)	119.8[a]	229.5[b]

[a,b]In a row, means with common superscripts did not differ (P>0.05)

[1]Polyethylene glycol: -PEG = Inoculation without polyethylene glycol; +PEG = Inoculation with polyethylene glycol

[2]Kinetics: b = The slowly fermentable fraction; c = Fermentation rate of fraction b; Pgas = Potential gas production; Egas = Effective gas production; ivOMD = In vitro organic matter degradability

Conclusion

It was concluded that *Vachellia karroo* leaves could be utilised as a potential protein source for ruminants fed a grass hay basal diets, but the presence of antinutrional factors (condensed tannin) might be detrimental to the animals. Therefore, polyethylene glycol treatment of *Vachellia karroo* leaves was recommend to negate the effects of tannins and improve rumen fermentation efficiency.

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