The use of certain medicinal plant extracts reduced *in vitro* methane production while improving *in vitro* organic matter digestibility

Abiodun Mayowa AKANMU[1], *Abubeker HASSEN[1]

[1]Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria 0002

Republic of South Africa

Abstract

Some medicinal plants have the tendency to manipulate the rumen microbial ecosystem, which in turn might reduce methane (CH_4) emissions. The anti-methanogenic activities of leaf fraction of P*iper betle, Aloe vera, Carica papaya, Azadirachta indica, Moringa oleifera, Tithonia diversifolia, Jatropha curcas* and *Moringa oleifera* pods were studied at different doses. The plant materials were extracted with pure methanol and subsequently reconstituted at the rate of 25, 50, 75 and 100 mg in 1000 mL distilled water. Four mL of each plant extracts preparation was anaerobically incubated with 400 mg *Eragrostis curvula* hay in four replicates and the experiment was repeated five times. Plant extracts of *P. betle* and *A. vera* significantly increased total gas produced whereas other extracts recorded lesser or similar values to the control group. Leaf extracts of A. *indica, C. papaya, J. curcas, M. oleifera, T. diversifolia* and *M. oleifera* pods all significantly reduced CH_4 volume at dosages of 25 and 50 mg/L due to the activities of their phytochemicals. Total volatile fatty acids and *in vitro* organic matter digestibility values recorded for all extracts were generally superior when compared with the control. Methane yield per unit of total gas were significantly lower in extracts of T. *diversifolia, M. oleifera, A. indica, M. oleifera* pods whereas it is higher in *P. betle* and *A. vera.* It can be concluded from the study that methanolic extracts of A. *indica, C. papaya, J. curcas, M. oleifera, M. oleifera* pods and *T. diversifolia* resulted in reduced CH_4 production, and thus can be used potentially to manipulate rumen condition, improve feed digestibility and reduce enteric CH_4 emission from ruminants. However, the *in vitro* results needs to be verified using in vivo studies by administering concentrated crude extracts at a rate of 25 mg or 50 mg per kg of roughage feed for small ruminants.

Keywords: Methane reduction, Plant extracts, Digestibility, *Eragrostis curvula* hay, volatile fatty acids, Medicinal plants

*Corresponding author email address: Abubeker.hassen@up.ac.za

1. Introduction

The need to produce enough food towards population boom by the year 2050 (Béné *et al.* 2015) and beyond has gathered momentum. A huge world's population do not only require space already evidenced by the current rapid deforestation but needs increased production of meat and milk to meet its bodily needs. Scientist thereby need to find ways of improving animals' feed without having to compete for limited available food for human. Ruminants for many years have been listed as one of the most valuable and renewable resources in terms of utilisation of non-competitive food for meat and milk production. Whereas enteric methane production from ruminants poses a major challenge as it causes energy loss, and a consequent reduction in performance. This emissions from ruminants also contributes towards greenhouse gases which causes global warming.

The use of concentrates was recommended as a strategy for reducing CH_4 production from ruminants (Yan *et al*. 2006; Lovett *et al*. 2005; Holter and Young 1992)**.** The findings of Puchala *et al*. (2005) also suggested feeding animals forages rich in condensed tannins (*Lespedeza cuneata)* to supplement low quality grasses in ruminant diets in order to reduce methane production. However, feeding concentrates are expensive especially in developing countries and diets high in tannins reduces animals' appetites.

The development and implementation of natural feeding and management strategies aimed at reducingto reduce CH_4 emissions and subsequent increase tof dietary energy efficiency use is therefore needed. This feeding strategy should not only reduce greenhouse gas contribution of livestock to the atmospheric budget but enhance production efficiency. This will decrease over-

reliance on antibiotic growth promoters such as monensin that is still in use. The renewed interest in the use of natural feed additives to replace antibiotic ionophores that have been banned in the European Union is as a result of the effect of consuming such meat products on humans.

Several studies (Bodas et al. 2012; Cieslak et al. 2012; Gemeda and Hassen 2015; Pal et al. 2015; Theart et al. 2015; Hassen et al. 2016) have reported the reducing effect of plant secondary compounds on CH4 emission, improvement in performance and reduction in protein degradation in the rumen. P*iper betle, Aloe vera, Carica papaya, Azadirachta indica, Moringa oleifera, Tithonia diversifolia, Jatropha curcas* and *Moringa oleifera* pods were listed in this study for screening based on their traditional uses, presence of phytochemicals, which aids digestion, antimicrobial/ antiprotozoal properties, and laxative effects. These plants were evaluated in order to exploittheir potential as a feed additive with the aim of identifying plant extracts capable of reducing CH4 production without reducing significantly the i*n vitro* gas production and organic matter digestibility of the test substrate. Extracts of all plants used in this study are not toxic to animals.

2. Materials and Methods

2.1 Collection of plant materials

All plants used in this study were harvested fresh from growing and blooming trees. Leaves of *P. betle* were obtained in Newcastle (27°46’23.916’’S, 29°54’38.196’’E) Kwazulu-Natal province of South Africa while leaves of all other plants and pods of *M. oleifera* were harvested at the University of Ibadan, Nigeria (7°25’38.952’’N 3853’0.63’’E). Samples were collected from 10 different plants of the same species. The harvested plant materials were authenticated in the herbarium of the Department of Botany, University of Ibadan. Permit for importation of these plant materials was obtained from the Department of Agriculture, Pretoria (P00000353453) and standard procedures as stated in the permit were followed. All plant materials obtained in Ibadan, Nigeria were refrigerated, air lifted same day and immediately stored at -20 °C for further processing on arrival at the

Department of Animal and Wildlife Sciences, Pretoria. Leaves and pods were freeze-dried for 5 days or until constant weight was achieved.

2.2 Preparation of plant extracts

Dried leaves and pods were milled to pass through 1mm sieve. They were extracted by pure methanol. To 200 g of dried leaves were added 2000 ml of methanol. The mixture was placed on a shaker and allowed to soften for 96 hours. Then, the mixture was sieved through 150 µm aperture (Vickers Sieve). The filtrate was placed in the fume cupboard until dried. The semi-dried extracts were later transferred to freeze-drying machine until constant weight was achieved. The dried extracts of each of the plants were reconstituted by dissolving, 25, 50, 75 and 100 mg in 1000 ml of distilled water separately to give 4 different levels of concentrations. All plant extracts were kept under refrigeration at 4°C until further use.

2.3 Chemical analysis

The feed sample, *Eragrostis curvula* hay, was analysed for dry matter (DM) and total ash using the method of AOAC (2002). Ether extract was determined using ether extraction in the Tecator Soxtec (HT6) system (AOAC 2002). The neutral detergent fibre, acid detergent fibre and acid detergent lignin contents were determined using an ANKOM200/220 fibre analyser (ANKOM Technology, Fairport, NY, USA) by the methods described in Robertson and Van Soest (1981). Nitrogen was analysed by the method described in AOAC (2002) (FP-2000 Nitrogen/Protein Analyser, Leco Instrumente GmbH, Kirchheim, Germany) and crude protein was obtained by multiplying nitrogen by 6.25.

2.4 *In vitro* gas and methane production

Inoculum and rumen fluid

Buffer mineral solution was prepared following the procedure of Meinke and Steingass (1988). The modification by Mould *et al*. (2005) to replace $MgSO_4.7H_2O$ with $MgCl_2.6H_2O$ was utilised to reduce the level of SO_4 in the media. Reducing solution L-cysteine and $Na_2S_9.H_2O$ were added as

recommended. The solution was bathed at 39 ^{0}C. Rumen fluid was collected from 2 ruminally fistulated Merino sheep placed on alfalfa hay *ad libitum.* Ruminal fluid was collected before morning feeding, approximately 900 ml of fluid was strained from each donor sheep through 4 layers of cheese cloth into pre-heated thermos flask. The rumen fluid was transported to the laboratory within 10 minutes of collection and continuously flushed with CO_2 to minimise changes in microbial population and to avoid O_2 contamination, it was placed in water bath set at 39 °C.

***In vitro* incubation**

In vitro ruminal incubation was done using 150 ml serum bottle. Prior to incubation, 400 mg *Eragrostis curvula* hay was weighed into each bottle, 4 ml of already prepared concentrations of leaf extract of *Piper betle, Aloe vera, Carica papaya, Azadirachta indica, Moringa oleifera, Tithonia diversifolia,Jatropha curcas* and *Moringa oleifera* pods extract were added to vials containing 400 mg of *Eragrostis curvula* hay, 25 ml of prepared media and 15 ml rumen fluid. In each run, each dose level for each plant extract and a control treatment added with 4 ml distilled water were incubated in 4 bottles in a randomised complete block design. The whole process was repeated 5 times by having 5 independent run. Three blanks were always included with each run. After the addition of rumen fluid, the vials with contents were purged with CO_2 gas and immediately closed with rubber stopper, crimp sealed and transferred into incubator set at 39^{0}C with oscillatory motion of 120 rpm. A modified needle syringe taps which can be open and closed was inserted on each vial. The taps were opened for 5 seconds to release any build up gas and to set a starting point for all the vials.

2.5 Total gas, methane, volatile fatty acids and *in vitro* organic matter digestibility

Gas produced at 2, 4, 8, 12, 24 and 48 hours of incubation was measured with a pressure transducer (PX4200-015GI; Omega Engineering Inc.) attached to a digital data logger (Tracker 220 series indicators; Omega Engineering Inc., Laval, QC, Canada) which is a semi-automated system (Theodorou *et al*. 1994). The transducer with a modified tip was placed tightly over the syringe tap already fitted to the vials. The tap on the syringe was opened, build up gas in the vials was released to the transducer and the value on the digital data tracker was recorded in psi unit. Gas pressure readings

taken were added to the previous readings to give a cumulative value. Gas samples were taken using different syringes through the same system for methane production from all replicated bottles at 2, 4, 8, 12, 24 and 48 hours. Methane concentration was corrected with headspace gas volume at different collection times and cumulated to give total CH_4 production at 48 hours. Methane concentration in each sample was analysed by gas chromatography equipped with flame ionisation detector (FID) and has a solenoid column packed with silica gel (8610C Gas Chromatograph (GC) BTU Gas Analyser GC System; SRI Instruments GmbH, Bad Honnef, Germany). Gas samples were injected by pull and push method into the GC already calibrated with standard CH_4 and CO_2. Blanks were also analysed and used for correcting CH_4 produced by the inoculum. The incubation was terminated after 48 hours by placing all the bottles on ice in cold room, then centrifuged at 4500 rpm for 15 minutes, all the supernatant were filtered off and 5 ml samples pipetted and stored at -20 °C for acetic, propionic, butyrate, iso-butyric and valeric acids proportions analyses (Ottenstein and Bartley 1971). *In vitro* organic matter digestibility was carried out by the procedure developed by Tilley and Terry (1963) on the fermented residue by adding HCL-pepsin solution. A 2 stage digestion process modified by Engels and Van der Merwe (1967).

Gas pressure was converted to volume using Boyle's Gas Law relationship as reported by Mauricio *et al*. (1999):

$$Gas\ volume\ (mls) = \frac{\mathrm{Vh}}{\mathrm{P}a} \times \mathrm{Pt}$$

Where Vh is the volume of head space in the incubating vials (ml); Pa is the atmospheric pressure (psi); Pt is the reading from the pressure transducer attached to a data tracker (psi)

Corrected cumulative CH_4 concentration in the headspace captured from GC in ppm was converted to ml by;

$$Methane\ (mls) = Totalgas\ produced\ (mls) x\ \%\ methane\ in\ concentration$$

Partitioning Factor (PF) was calculated according to Blummel *et al.* (1997).

2.6 Statistical Analysis

All statistical analyses were carried out using SAS 9.4 and Microsoft excel. P values ≤ 0.01 were deemed significant. Various parameters (i.e. gas production, CH_4, *in vitro* organic matter digestibility, volatile fatty acids and all the ratios) were measured in response to different plant extract dosages. One way repeated ANOVA was conducted to assess potential differences in responses between the groupings variables (25, 50, 75, 100 mg/L) compared to the control. Where significant differences were identified, subsequent post hoc analyses was then carried out using tukey test. Therefore, each plant extract underwent separate analyses to investigate the parameter changes in response to various concentrations of each plant extract compared to the control. This analysis was carried out for each plant individually**.**

3. Results

3.1 Chemical composition of *Eragrostis curvula*

The chemical composition of *Eragrostis curvula* hay used in this study indicates that DM and ash were 94% and 18.4% respectively. Crude protein stands at 5.89 %, ether extract 1.4 %, while the fibre fractions NDF, ADF and ADL had 74.6, 44.7 and 7.9 % respectively.

3.2 Total gas and methane production

Significant increase in TGP were only recorded for *A. vera* and *P. betle* at 75 and 100 mg/L ($P < 0.01$) dosage levels as shown in Table 1 with no difference in other dosages of *P. betle*, *A. vera* (25 & 50 mg/L) and all doses of *T. diversifolia* compared with the control. In contrast, all dosages of *J. curcas, M. oleifera* pods and *A. indica* significantly ($P < 0.01$) reduced TGP volume. *M. oleifera* 25 and 50 mg/L dose presented the same trend and reduced total gas volume as compared to the control. However TGP was similar between control and 75 and 100 mg/L dosages of *C. papaya* and *M. oleifera*. Extracts of *A. indica*, *C. papaya*, and *T. diversifolia* at 25 and 50 mg/L dosage level reduced CH_4 by up to 15 %. Further reductions in CH_4 volume by up to 30 % were recorded at the same dose for *J. curcas* and *M. oleifera* leaves and pods. Generally, an increased trend in CH_4 volume were noted for plant extracts of *A. indica, C. papaya, J. curcas, M. oleifera* leaf and pods, as the dosages increased from 25 to 100 mg/L, although all had lower values than the control. Significant ($P < 0.01$)

Table 1. Fourty eight hour gas production, methane, and *in vitro* organic matter digestibility of *Eragrostis curvula* hay treated with different plant extracts at different dosages

Parameter	Mean Values and SD								
	Dose	AV	AZ	CP	JA	MO	MOP	PB	TD
TGP (ml)	Control	49.2±.53C	49.3±.53A	49.3±.53A	49.3±.53A	49.3±.53AB	49.3±.53A	49.3±.53BC	49.3±.53A
	25mg/L	50.6±2.2aC	42.9±1.5dC	46.2±2.3bcdB	44.6±2.2cdB	43.2±2.2dC	47.2±1.3abcB	48.5±1.3abC	48.4±2.4abA
	50mg/L	50.8±1.4aC	46.3±1.5bB	46.5±1.9bAB	44.8±1.6bB	45.1±1.2bC	46.2±.97bBC	50.6±1.1aB	49.3±.31aA
	75mg/L	56.9±1.0aA	46.9±.43cdB	46.7±1.2cdAB	45.3±.76eB	47.9±.39cB	46.1±.09deBC	57.6±.73aA	50.1±.42bA
	100mg/L	53.7±1.7bB	46.2±.20eB	48.5±1.1dAB	44.1±.11fB	50.2±.08cA	45.0±.16efC	57.8±.92aA	48.2±.46dA
CH_4 (ml)	Control	17.4±.51C	17.4±.51A	17.4±.51A	17.4±.51A	17.4±.51A	17.4±.51A	17.4±.52C	17.4±.51A
	25mg/L	16.8±.61bC	12.3±1.2cdC	14.6±2.7bcA	10.8±2.8dC	12.3±.27cdB	11.5±.32cdD	24.1±1.56aB	14.5±1.0bcB
	50mg/L	16.9±.55bC	14.7±.51cB	14.8±1.4bcA	12.1±1.4dBC	12.4±2.2dB	11.3±.74dD	25.0±.52aB	14.9±1.3bcB
	75mg/L	21.6±.81bA	16.5±1.8cAB	16.6±2.5cA	13.8±.34dB	16.5±.29cA	15.0±.43cdB	29.8±2.3aA	17.2±.13cA
	100mg/L	19.5±1.4bB	16.7±1.1cAB	16.5±.54cA	13.9±.57dB	16.6±.32cA	13.3±.44dC	22.9±1.9aB	14.9±.24cdB
IVOMD (g/kg)	Control	309.8±3.9D	309.9±3.9C	309.9±3.9D	309.9±3.9B	309.9±3.9B	309.9±3.9C	309.9±3.9C	309.9±3.9C
	25mg/L	415.1±2.9aA	375.3±4.5bA	380.2±.97bB	333.0±4.8dA	359.1±4.2cA	330.9±15.9dB	379.1±8.4bA	360.9±6.6aB
	50mg/L	393.7±6.3aB	380.5±3.6bA	383.3±3.6bAB	334.2±1.7eA	353.3±5.7dA	325.8±3.4eB	379.2±8.4bA	363.3±8.9cAB
	75mg/L	392.2±1.5aB	364.7±4.1bcB	392.4±11.1aA	335.5±1.1dA	356.0±1.3cA	356.7±7.6cA	375.9±8.9bA	367.3±8.7bcA
	100mg/L	366.1±8.7aC	363.4±3.3abB	364.9±5.8aC	339.5±8.7cA	358.0±.02abA	331.9±1.4cB	357.2±5.5abB	352.7±7.4bB

*Upper case letters compares means among all dosages of each plant extracts and the control across the column, lower case letters compares means along the rows of different plant extracts.

*Means with different lower case letters across the rows or upper case letters along the column for each parameter are significantly (P<0.01) different. *AV-*Aloe vera*, AZ-*Azadirachta indica*, CP-*Carica papaya*, JA-*Jatropha curcas*, MO-*Moringa oleifera*, MOP-*Moringa oleifera* pod, PB-*Piper betle*, TD-*Tithonia diversifolia*, TGP-Total gas production, CH_4-Methane, IVOMD-*in vitro* organic matter digestibility, SD-Standard deviation

decrease in CH_4 volume at 25 mg/L and 50 mg/L dose levels were recorded for extracts of *J. curcas*, *M. oleifera*, and *T. diversifolia*. In addition, *M. oleifera* pods reduced CH_4 volume significantly at all dosages while only the lowest dosages (25 and 50 mg/L) of *A. indica* differ significantly ($P < 0.01$) from the control. When compared to the control, the use of plant extracts from *C. papaya* did not produce any significant CH_4 reduction while *P. betle* plant extracts significantly increased CH_4 volume in response to all dose levels.

3.3 Digestibility

All plant extracts improved *in vitro* organic matter digestibility of *E. curvula* hay which ranges from 5% to 25% (Table 1 and Fig. 1). When compared to the control *A. vera, A. indica, T. diversifolia, M. oleifera, P. betle, J. curcas* and *C. papaya* extracts significantly ($P < 0.01$) increased IVOMD when used between 25 to 100 mg/L dosages. *M. oleifera* pod only showed a significant value at 75 mg/L ($P < 0.01$).

3.4 Volatile fatty acids

Individual volatile fatty acids expressed as molar concentration of the total volatile fatty acids as shown in Table 2. When compared to the control the ruminal acetic acid concentrations were highest for *A. indica, A. vera, M. oleifera* and *P. betle* plant extracts. However, a significantly lower concentrations of acetic acid was recorded for extracts of *J. curcas* (75 mg/L), all other dosages of *M. oleifera* pods, *J. curcas, C. papaya, T. diversifolia* and *A. indica* had significantly higher or same as the control. Higher propionic acid concentration was found at dosages of 75 and 100 mg/L for *J. curcas* and 50 and 75 mg/L for *M. oleifera* pods. Significant lower ($P<0.01$) concentrations of propionic acid were obtained for *A. vera* (25, 50, 100 mg/L), *A. indica* (75, 100 mg/L), *M. oleifera pods* (100 mg/L), *P. betle* (25, 50, 75, 100 mg/L) and *T. diversifolia* (50 mg/L). Molar concentrations of butyric acid decreases as the dosages increase from 25 to 75 mg/L in *A. indica, M. oleifera, P. betle, J. curcas* and *M. oleifera* pods while others did not follow a particular pattern. Other volatile fatty acids are iso-butyric and valeric acid, which vary in values based on treatments and dosages. The C2/C3 volatile fatty acid ratio were significantly ($P < 0.01$) lowest at 25, 50 and 75, 100 mg/L

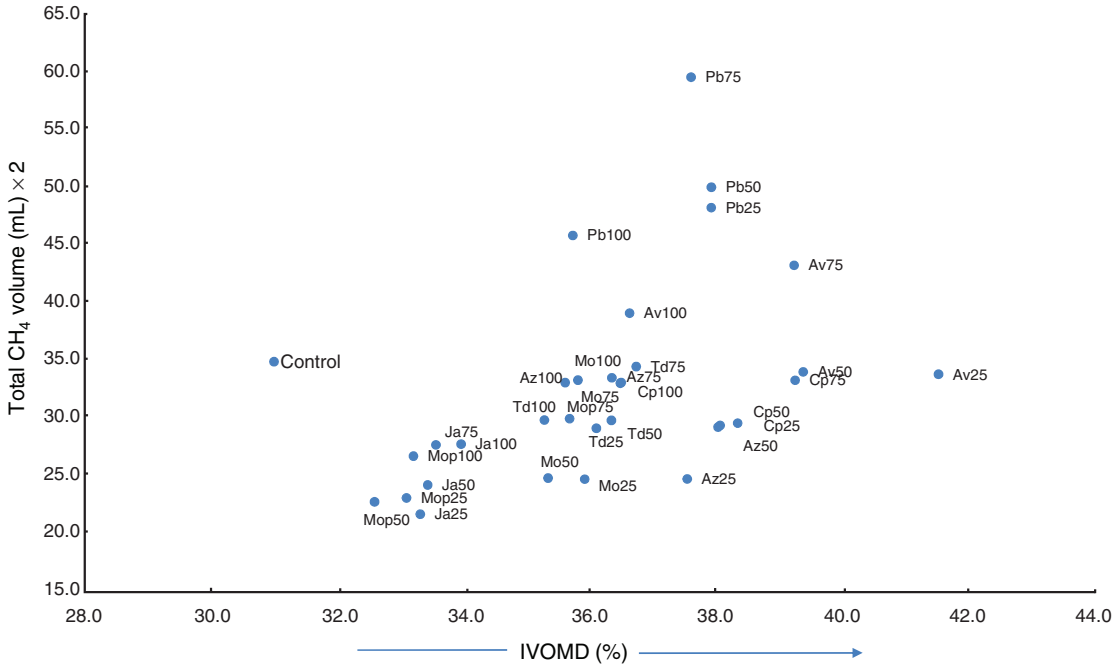

Fig. 1. Scatter diagram showing CH4 volume versus IVOMD when *Eragrostis curvula* hay was treated with different plant extracts at different doses. Plant extracts in the same box are not different significantly ($P < 0.01$). Av – *Aloe vera*, A z – *Azadirachta indica*, Cp – *Carica papaya*, J a – *Jatropha curcas*, M o – *Moringa oleifera*, Mop – *Moringa oleifera pod*, P b – *Piper betle*, and Td – *Tithonia diversifolia* plant extracts. 25, 50, 75, and 100 signifies dosages of 25, 50, 75 and 100 mg/L plant extract dosages.

Table 2. Relative molar proportions of volatile fatty acids expressed as percentage of total volatile fatty (mg/L) acids in supernatant after 48 hours of incubating *Eragrostis curvula* hay sprayed with different plant extracts

Volatile fatty acids (mg/L) (% of TVFA)		Mean ± SD							
	Dosage	**AV**	**AZ**	**CP**	**JA**	**MO**	**MOP**	**PB**	**TD**
Acetic Acid	Control	46.2±1.2C	46.2±1.2D	46.2±1.2B	46.2±1.2AB	46.2±1.2B	46.2±1.2A	46.2±1.2C	46.2±1.2D
	25 mg/L	58.4±.27aA	56.8±.61abC	58.9±1.8abA	47.7±3.0cdAB	50.8±1.6bcB	42.8±.02dA	55.7±3.7abB	52.4±.09abcC
	50mg/L	53.4±1.4cB	61.3±.89bB	57.7±1.1bA	50.1±.73cdA	48.9±1.0dB	41.6±.41eA	57.7±.12bAB	68.6±1.7aA
	75 mg/L	53.5±1.5cdB	70.1±.02aA	59.3±.65abcA	39.9±.27eC	57.1±1.4bcdA	49.4±6.3deA	65.9±1.9abA	52.9±.34cdC
	100 mg/L	61.3±.63bcA	73.7±1.2aA	60.5±1.6bcA	46.2±.34eBC	56.9±.03cA	47.4±1.1dA	62.9±2.0bAB	61.1±.68bcB
Propionic Acid	Control	26.7±.18A	26.7±.18A	26.7±.18A	26.7±.18C	26.7±.18A	26.7±.18B	26.7±.18A	26.7±.18A
	25 mg/L	23.4±.57dB	25.3±.19cdAB	27.4±1.3cdA	28.6±1.8abBC	27.3±.41abcA	29.8±.41aAB	23.5±.14dB	26.2±.52bcdA
	50mg/L	18.8±.32eC	27.3±.58bcA	24.6±.56cdA	29.1±.77bBC	27.6±.39bA	34.8±.06aA	23.9±.10dB	23.3±.23dB
	75 mg/L	23.9±1.5cAB	23.3±.12cBC	24.9±.20bcA	39.6±.73aA	26.7±.71bcA	29.7±3.4bAB	22.9±.93cB	26.8±.07bcA
	100 mg/L	23.8±.56bcdB	21.3±1.2cdC	25.3±1.1bA	35.5±3.5aAB	27.9±.04bA	20.1±.57dC	24.6±.44bcdB	26.2±.22bcA
Butyric Acid	Control	15.7±.64B	15.7±.64A	15.7±.64A	15.7±.64A	15.7±.64A	15.7±.64B	15.7±.64A	15.7±.64A
	25 mg/L	11.6±.03bC	10.3±.20bB	6.8±.35bB	14.3±.52abAB	12.4±.77abB	16.1±.61aAB	13.3±2.8abA	12.7±.05abA
	50mg/L	20.1±.38aA	4.3±.22efC	10.2±.38cdeBC	5.7±.93defBC	13.9±.40bAB	9.2±.50cdC	11.1±.02bcAB	3.1±2.2fB
	75 mg/L	14.6±.26aB	1.9±.28eD	7.8±.27cdBC	4.1±1.6deC	9.5±.44bcC	9.3±1.1bcC	5.9±.67cdB	11.5±.32abA
	100 mg/L	9.1±.67bD	1.8±.10bD	7.7±1.4bC	7.7±4.9bABC	7.8±.04bC	18.9±.50aA	5.5±1.4bB	6.7±.20bB
Others*	Control	11.3±.41A	11.3±.41A	11.3±.41A	11.3±.41BC	11.3±.41A	11.3±.41A	11.3±.41A	11.3±.41A
	25 mg/L	6.6±.02cdC	7.5±.20bcdB	6.8±.10bcd B	9.2±.68bC	9.3±.47bB	11.2±.17aA	7.3±.75cdB	8.5±.37bcB
	50mg/L	7.6±.20bcB	6.9±.53cB	7.4±.19bcB	15.1±.88aAB	9.4±.23bB	14.3±.02aA	7.2±.00cB	5.1±.74dC
	75 mg/L	8.1±.11cdB	4.6±.18eC	7.8±.17cdeB	16.2±.61aA	6.6±.28cdeC	11.4±1.7bA	5.3±.38deC	8.6±.09bcB
	100 mg/L	6.2±.30bC	3.1±.12cD	6.5±.92bB	13.9±1.2aAB	7.3±.12bB	13.6±.01aA	6.9±.02bB	5.9±.25bC
Acetic/Propionic ratio	Control	1.7±.05C	1.7±.05D	1.7±.05B	1.7±.05A	1.7±.05C	1.7±.05AB	1.7±.05B	1.7±.05D
	25 mg/L	2.5±.02aAB	2.3±.06abcC	2.1±.17abA	1.6±.21deAB	1.8±.08cdeABC	1.4±.01eB	2.3±.17abA	1.9±.04bcdC
	50mg/L	2.8±.30abA	2.2±.08cdC	2.3±.10cA	1.7±.07eA	1.7±.06deBC	1.2±.00fB	2.4±.01bcA	2.9±.10aA
	75 mg/L	2.2±.11abcB	3.0±.01aB	2.4±.04abcA	1.0±.01dC	2.1±.11bcA	1.6±.40cdAB	2.9±.20abA	1.9±.00cC
	100 mg/L	2.6±.06bAB	3.4±.21aA	2.4±.17cdAB	1.1±.13eC	2.0±.00dAB	2.3±.12bcdA	2.5±.12bcA	2.3±.04bcdB

*Upper case letters compares means among all dosages of each plant extracts and the control across the column, lower case letters compares means along the rows of different plant extracts.

*Means with different lower case letters across the rows or upper case letters along the column for each parameter are significantly ($P<0.01$) different

*AV-*Aloe vera,* AZ-*Azadirachta indica,* CP-*Carica papaya,* JA-*Jatropha curcas,* MO-*Moringa oleifera,* MOP-*Moringa oleifera* pod*,* PB-*Piper betle,* TD-*Tithonia diversifolia,* SD-Standard deviation, Others*-Iso-butyric and Valeric acids, TVFA-Total volatile fatty acids

dose level for *M. oleifera* pods and *J. curcas* respectively. *A. vera, A. indica, C. papaya, M. oleifera, P. betle and T. diversifolia* all had significant higher C2/C3 VFA ratios.

3.5 Calculated ratios

Percentages of TGP/IVOMD, CH4/TGP, CH4/TVFA, TGP/TVFA and PF (mg/ml) are presented in Table 3. TGP/IVOMD were all significantly lower ($P<0.01$) for all plant extracts and dosages used in this study except 75 and 100 mg/L dosages of *P. betle* which did not differ with the control. CH4/TGP expressed significant lower values ($P<0.001$) at lower doses (25, 50 mg/L) of *A. vera, A. indica, C. papaya, J. curcas, M. oleifera* and *T. diversifolia*. All dosages of *M. oleifera* pods were significantly lower than the control and contrasted by *P. betle* dosages having relatively higher values compared to the control. The calculated values for CH_4/TVFA and TGP/TVFA (Table 3) showed that all extracts at 25 mg/L had significantly lower ($P < 0.001$) values for these parameters than that of the control. PF was significantly higher at all doses of all plant extracts except 75 and 100 mg/L dosages of *P. betle.* Fig. 1 shows a scatter diagram of CH_4 volume plotted against IVOMD, all plant extracts had increased IVOMD with reduced CH_4 production except for *P. betle* and *A. vera* at 75 and 100 mg/L dosages. *P. betle* extracts had the highest significant CH_4/IVOMD value than that of the control. All the other extracts at all dosages had significant lower CH_4/IVOMD values than that of the control at all dosages. However, the lowest significant ($P < 0.01$) values for CH_4/IVOMD were found at a dose of 25 mg/L for extracts of *A. indica, C. papaya, J. curcas, M. oleifera* and *M. oleifera* pods, and as the dosage increases CH_4/IVOMD value leans higher.

4. Discussion

Eragrostis curvula hay used in this study was of low quality, characterized by low crude protein, low fat content and high fibre portions suggesting that it is a poor quality roughage that warrants improvement in terms of its utilisation. In this study, the intent was to test the effectiveness of the plant extracts on methane emission reduction associated with fermentation of such feeds without adversely affecting its digestibility.

Table 3. The ratios of methane to *in vitro* organic matter digestibility, total gas production, total volatile fatty acids, and the ratio of total gas produced to total volatile fatty acids, and partitioning of *Eragrostis curvula* hay treated with different plant extracts at different dosage

Parameters		Mean±SD							
	Dose	AV	AZ	CP	JA	MO	MOP	PB	TD
TGP/IVOMD	Control	15.9±.33A	15.9±.33A	15.9±.33A	15.9±.33A	15.9±.33A	15.9±.33A	15.9±.33A	15.9±.33A
(%)	25mg/L	12.2±.55bcdC	12.2±.52dD	12.2±.60bcdC	13.3±.85abB	12.0±.65cdD	14.3±.90aB	12.8±.61bcB	13.4±.71abB
	50mg/L	12.9±.54bcC	12.2±.33dC	12.1±.40dC	13.4±.54abcB	12.8±.17cdC	14.6±.21aB	13.3±.51bcB	13.5±.38abB
	75mg/L	14.5±.25bB	12.9±.25dB	11.9±.64eC	13.5±.22cdB	13.4±.13cdB	12.9±.25dC	15.3±.54aA	13.6±.33cB
	100mg/L	14.7±.12bB	12.7.16fBC	13.3±.52deB	13.0±.33efB	14.0±.02cB	13.6±.10cdBC	16.2±.39aA	13.7±.41cdB
CH_4/TGP	Control	35.3±1.4AB	35.4±1.4AB	35.4±1.4A	35.4±1.4A	35.4±1.4A	35.4±1.4A	35.4±1.4B	35.4±1.4A
(%)	25mg/L	33.3±1.1bB	28.7±1.9bcC	31.4±4.3bA	24.2±5.8cC	28.6±.91bcB	24.4±1.2cD	49.8±2.1aA	30.0±.72bB
	50mg/L	33.4±.29bB	31.6±1.2bcBC	31.8±2.2bcA	27.0±3.7cdBC	27.5±4.7cdB	24.6±1.3dD	49.5±1.9aA	30.2±2.9bcB
	75mg/L	37.9±1.7bB	35.2±3.8bcAB	35.6±4.7bcA	30.5±.57cAB	34.5±.37bcA	32.5±.97cB	51.7±4.1aA	34.4±.54bcA
	100mg/L	36.3±1.9bA	36.2±2.3bA	34.1±.63bcA	31.4±1.2cdeAB	33.1±.69cdA	29.6±.92eC	39.6±2.8aA	30.9±.44deB
PF (mg/ml)	Control	1.3±.03D	1.3±.03C	1.3±.03C	1.3±.03B	1.3±.03D	1.3±.03C	1.3±.03B	1.3±.03B
	25mg/L	1.6±.08abcA	1.8±.08aA	1.6±.08abcA	1.5±.09cdA	1.7±.09abA	1.4±.09dB	1.6±.08bcA	1.5±.08cdA
	50mg/L	1.5±.07bcB	1.6±.05aB	1.7±.06aA	1.5±.06bcdA	1.6±.02abB	1.4±.02dB	1.5±.06bcdA	1.5±.04cdA
	75mg/L	1.4±.02dC	1.6±.03bB	1.7±.09aA	1.5±.02bcA	1.5±.01bcBC	1.5±.03bcA	1.3±.05dB	1.5±.04cA
	100mg/L	1.4±.01eC	1.6±.02aB	1.5±.06bcB	1.5±.04abA	1.4±.00dC	1.5±.01cdAB	1.2±.03fB	1.5±.04cdA
CH_4/TVFA	Control	2.2±.05A	2.2±.05A	2.2±.05A	2.2±.05B	2.2±.05C	2.2±.05B	2.2±.05B	2.2±.05B
	25mg/L	0.7±.03bD	0.9±.03abC	1.1±.21abB	1.0±.19abC	1.3±.01aD	1.4±.09aC	1.3±.46aC	1.4±.10aD
	50mg/L	1.5±.05cdC	1.2±.10dB	1.3±.01cdB	2.5±.58aB	1.2±.21dD	2.4±.12abB	1.6±.05cdBC	1.9±.15bcC
	75mg/L	1.8±.08cB	0.9±.13dBC	2.4±.56bcA	3.8±.00aA	2.6±.14bcB	3.9±.67aA	3.0±.17bA	1.8±.02cC
	100mg/L	2.1±.20dAB	1.1±.11eBC	2.8±.15bcA	4.3±.59aA	3.0±.00bcA	3.3±.10bA	2.8±.21bcA	2.7±.08cdA
TGP/TVFA	Control	6.2±.13A	6.2±.13A	6.2±.13C	6.2±.13C	6.2±.13C	6.2±.13B	6.2±.13B	6.2±.13B
	25mg/L	2.3±.16dE	3.3±.07cdC	3.5±.23cE	4.7±.61bC	4.6±.21bD	5.9±.49aB	2.5±.82dC	4.7±.18bD
	50mg/L	4.3±.18cD	3.6±.18dB	4.4±.18cD	9.7±.48aB	4.6±.00cD	9.7±.14aA	3.2±.03dC	6.5±.37bB
	75mg/L	4.9±.03cdC	2.8±.05dD	6.8±.29bcB	12.8±.00aA	7.6±.42bB	12.2±.2.5aA	6.0±.27bcB	5.3±.17cC
	100mg/L	5.7±.12eB	3.1±.02fC	8.4±.26cdA	14.2±1.5aA	9.3±.13cA	11.1±.07bA	7.3±.22dA	8.6±.13cdA

*Upper case letters compares means among all dosages of each plant extracts and the control across the column, lower case letters compares means along the rows of different plant extracts.

*Means with different lower case letters across the rows or upper case letters along the column for each parameter are significantly (P<0.01) different

*AV-*Aloe vera,* AZ-*Azadirachta indica,* CP-*Carica papaya,* JA-*Jatropha curcas,* MO-*Moringa oleifera,* MOP-*Moringa oleifera* pod*,* PB-*Piper betle,* TD-*Tithonia diversifolia*, TVFA-Total volatile fatty acids, IVOMD-*In vitro* organic matter digestibility, PF-Partitioning factor, TGP-Total gas produced, CH_4-Methane, SD-Standard deviation

Extracts of *P. betle* and *A. vera* increased TGP, CH_4 volume and IVOMD. The presence of biochemical catalysts such as amylase and lipase (Basak and Guha 2015; Ray *et al.* 2015; Abrahim *et al*. 2012) in their leaves samples could be partly responsible for the high volume of gases recorded at all dosage levels. Higher gas production from *in vitro* fermentation has been attributed to faster rate of substrate degradation in the rumen. Increased digestibility and gas production recorded could be due to the phytochemicals (amylase and lipase) present in the plant extracts used in this study, these phytochemicals have been reported to support fibrolytic microbes in the rumen by increasing the proximity between substrates and microbes (Morgavi *et al.* 2000), causing faster rate of fermentation and subsequent degradation of substrates (Beauchemin *et al.* 2003), and rapid stimulation and reproduction of bacterial activity in the rumen (Beauchemin *et al*. 2003). Higher CH_4 volumes recorded for all dosages of *P. betle* could be due to higher fermentation activities and digestion process stimulated by the phytochemicals present in the leaf sample, which according to Prabhu *et al*. (1995) had a significant stimulatory influence on intestinal lipase and amylase activity when tested in rats. The corresponding increase in TVFA with increasing dosage level in *A. vera* and *P. betle* could be traced to higher concentrations of such biochemical catalysts in the treatment, an indication of faster rate of organic matter degradation from the fibrolytic microbes (Menke *et al*. 1979) which resulted into a reduction in rumen retention time when these extracts were administered. However, feeding higher dosage level could alter the microbial population and can as well become toxic to the animals. Furthermore, the presence of anthraquinones, a phenolic compound present in medicinal plants used in this study might have played a stimulating effects on the bowels and are effective antibiotic agents which could have also generated more gas through their laxative effects. Anthraquinone is employed massively as digester in paper making (Haddad *et al*. 2009), it works as a redox catalyst on *Eragrostis curvula* hay by forming a complex with the reducing end of polysaccharides in cellulose and hemicellulose as well as accelerating the rate of delignification through the cleavage of the β-phenyl ether bond of lignin. *A. vera* and other plant extracts used in this study contains varied quantities of naturally occurring anthraquinone glycosides, these plants have been used traditionally to relieve chronic and serious digestive problems. *P. betle* contains a variety of biologically active enzymes which can speed up digestion, two of which are catalase and diastase.

Diastase breaks down complex starch polymer into its monomers while catalase influences the conversion of hydrogen peroxide to water and hydrogen. The high volume of CH_4 gas recorded for *P. betle* could be due to faster rate of degradation and subsequent production of hydrogen in the rumen which enables the methanogens to convert H_2 to CH_4 according to Dey *et al*. (2014). Although *P. betle* and *A. vera* improved IVOMD at all dosages, the resultant CH_4 produced per unit of IVOMD was higher than the control (Fig. 1) with the exception of *A. vera* at 25 mg/L. Extracts of *P. betle* and *A. vera* have been used in various studies as laxative agents (Nouri *et al*. 2014; Abrahim *et al*. 2012).

The higher IVOMD, lesser TGP and CH_4 volumes for *A. indica, J. curcas, M. oleifera* and *M. oleifera* pods in low dosages (25 and 50 mg/L) could be partly attributed to the presence of *azadirachtin* (Harry-Asobara and Samson 2014), *curcin* (Oskoueian et al. 2014), and *alkaloids* (Gautam et al. 2007; Ojiako 2014) respectively. Non-toxic azadirachtin have been used as a feed inhibitor and pesticides, curcin and protease inhibitors have been reported to be present in *J. curcas* whereas both leaves and pods of *M. oleifera* are rich in alkaloids, which have a bitter taste making it undesirable for some microbes. Total gas produced, which is an indication of forage degradation characteristics and kinetics of fermentation have not been affected negatively by all plant extracts used in this study as evidenced by their digestibility compared with the control. Significant CH_4 reductions recorded for *J. curcas* could be partly attributed to the presence of inhibitory agents. The effectiveness of this plant at reducing CH_4 in the rumen might be related to its purgative, anthelminthic and antiseptic properties (Kumar *et al*. 2016; Liu *et al*. 2015). The extract would have reduced CH_4 production by combating the methanogens. The findings of Oskoueian et al.(2014) showed a significant suppression in rumen microbial population by direct effects of phorbol esters or other metabolites present in *J. curcas*, which might have occurred by damaging membrane proteins or causing an increased membrane permeability, which finally results into leakage of cytoplasmic constituents. *M. oleifera* leaf and pod extracts contains alkaloids, tannins, saponins and flavonoids, which are strong antioxidants capable of inhibiting rumen microbes by bonding with their membranes. Phenolics compounds from this plant were found to be active against bacteria such as *Staphylococcus aureus*, *Eucherichia coli* and *Salmonella tiphy*, which might have been responsible for the suppression of CH_4-producing microbes in the rumen. *A. indica* had been researched for

effectiveness against methanogens (Pal et al. 2015). The results of this study were in agreement with those obtained by Pal et al.(2015). The presence of limonoids azadirachtin, salannin, meliantriol and nimbin were responsible for the antimicrobial properties of *A. indica.*

M. oleifera leaf tested *in vitro* significantly reduced CH_4 with increase in VFAs and organic matter digestibility which also corresponds to the findings of this study (Dey *et al*. 2014). There is a linear relationship between dosage and digestibility until peaked at 75 mg/L, this is true with the results obtained using different plant extracts by Dey *et al*. (2014) and Marhaeniyanto and Susanti (2014). The mechanism of increased *in vitro* digestibility in *A. indica, J. curcas* and *T. diversifolia* may be due to their antimicrobial properties and laxative properties (Harry-Asobara and Samson 2014) by making the rumen more conducive for beneficial organisms to degrade the substrates. According to Oskoueian (2014) the presence of phorbol esters could have contributed to the reduction of rumen methanogens, in addition to the alkaloids, saponins, tannin and saponins glycoside that have been attributed to methane reduction (Bhatta *et al*. 2012), while the improvement in *in vitro* organic matter digestibility shows the dosage used in this study were not toxic and rumen microbes can tolerate better.

Tithonia. diversifolia and C. papaya reduced CH_4 partly due to the presence of alkaloids, flavonoids, phenols sesquiterpenes, monoterpenes and diterpenes in the leaf extracts, a dosage of 25mg/L effectively reduced CH_4 production. Agidigbi *et al.* (2014) confirmed the antibacterial activities of *T. diversifolia* extracts when tested at 20 mg/ml through inhibitory actions against *S. aureaus* and *E. coli*. Improved digestibility, individual and total volatile fatty acids were positively related in this study. *C. papaya* response could be traced to the presence of papain in papaya leaf extract which aided in the breaking down and subsequent digestion of *E. curvula* hay. A higher partitioning factor obtained across the treatments is an indication of huge microbial biomass synthesis which can indicate non-toxicity of plant extracts and increased fermentation of feedstuff.

5. Conclusion

Methanolic extracts of *A. vera, C. papaya, J. curcas, M. oleifera, M. oleifera* pods and *T. diversifolia* significantly reduced CH_4 volume at 25 mg/L and 50 mg/L dosages with simultaneous improvement in *in vitro* organic matter digestibility of 400 mg *E. curvula* hay. The exact mechanism and contribution of various phytochemicals present in these plants responsible for the observed improvement needs to be elucidated in the future. However, some of the promising plant extracts identified in this study can be promoted to the next stage of screening to confirm the repeatability of the results using *in vivo* studies.

Acknowledgement

The authors will like to acknowledge funding received from ANIMALCHANGE, the Department of Science and Technology, and National Research Foundation (NRF) South Africa and bursary support for the first author from the University of Pretoria, South Africa.

References

Abrahim NN, Kanthimathi MS, Abdul-Aziz A (2012) *Piper betle* shows antioxidant activities, inhibits MCF-7 cell proliferation and increases activities of catalase and superoxide dismutase. *BMC Complementary and Alternative Medicine* **12**, 220

Agidigbi TS, Odeyemi AT, Adefemi SO, Fasuan SO (2014) Antibacterial activities of crude extracts of *Tithonia diversifolia* against common environmental pathogenic bacteria. *The Experiment* **20** (4), 1421-1426

AOAC (2002) Official methods of analysis. Association of official analytical chemists. Arlingtion, Virginia, USA

Basak S, Guha P (2015) Modelling the effect of essential oil of betel leaf (*Piper betle* L.) on germination, growth, and apparent lag time of *Penicillium expansum* on semi-synthetic media. *International. Journal of. Food Microbiol*ogy **215**, 171-178

Beauchemin KA, Colombatto D, Morgavi DP, Yang WZ (2003) Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of. Animal. Science* **81**, 37-47

Béné C, Barange M, Subasinghe R, Pinstrup-Andersen P, Merino G, Hemre G, Williams M (2015) Feeding 9 billion by 2050 – Putting fish back on the menu. *Food Sec*urity. **7**, 261-274

Bhatta RM, Saravanan LB, Sampath KT (2012) Nutrient content, in vitro ruminal fermentation characteristics and methane reduction potential of tropical tannin-containing leaves. *Journal of the Science of Food and Agriculture* **92**, 2929-2935

Bodas R, Prieto N, García-González R, Andrés S, Giráldez, FJ, López S (2012) Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Animal Feed Science and Technology* **176**, 78-93

Blummel M, Steingab H, Becker K (1997) The relationship between *in vitro* gs production*, in vitro* microbial biomass yield and 15N incorporation and its implication for the prediction of voluntary feed intake of roughages. *British Journal of Nutrition* **77**, 911-921

Cieslak A, Zmora P, Pers-Kamczyc E, Szumacher-Strabel M (2012) Effects of tannins source (Vaccinium vitis idaea L.) on rumen microbial fermentation in vivo. *Animal Feed Science and Technology* **176**, 102-106

Dey A, Paul SS, Rathore PR (2014) Potential of Moringa oleifera leaves in modulating in vitro methanogenesis and fermentation of wheat straw in buffalo. *Indian Journal of Animal Sciences* **84** (5), 533-538

Engels EAN, Van der Merwe FJ (1967) Application of an *in vitro* technique to South African forages with special reference to the effect to certain factors on the results. *South African Journal of Agricultural Science* **10**, 983-992

Gautam R, Saklani A, Jachak SM (2007) Indian medicinal plants as a source of antimycobacterial agents. *Journal of Ethnopharmacology* **110** (2), 200-234

Gemeda BS, Hassen A (2015) Effect of Tannin and Species Variation on In vitro Digestibility, Gas, and Methane Production of Tropical Browse Plants. *Asian Australasian Journal of Animal Sciences* **28**(2):188-199

Haddad A, Lachenal D, Marechal A, Janin G, Labiod M (2009) Delignification of Aleppo pine wood (*Pinus halepensis* Mill) by soda-anthraquinone process: pulp and paper characteristics. *Cellulose Chemistry and. Technol*ogy., **43** (7-8), 287-294

Harry-Asobara JL, Samson EO (2014) Comparative study of the phytochemical properties of *Jatropha curcas* and *Azadirachta indica* plant extracts. *Journal of Poisonous and Medicinal Plants Research* **2** (2), 20-24

Hassen A, Theart JJF, Van Niekerk WA, Adejoro FA, Gemeda BS (2016) *In vitro* methane and gas production characteristics of *Eragrostis trichopophora* substrate supplemented with different browse foliage. *Animal Production Sciences* **56**:634-640.

Holter JB, Young AJ (1992) Methane production in dry and lactating dairy cows. *Journal of Dairy Sci*ence. **75**, 2165-2175

Kumar P, Srivastava VC, Jha MK (2016) *Jatropha curcas* phytotomy and applications: Development as a potential biofuel plant through biotechnological advancements. *Renewable and Sustainable Energy Reviews* **59**, 818-838

Liu J, Yang Y, Xia J, Li X, Li Z, Zhou L, Qiu M (2015) Cytotoxic diterpenoids from *Jatropha curcas* cv. Nigroviensrugosus CY Yang roots. *Phytochemistry* **117**, 462-468

Lovett PE, Miller PM, Adams V, Kannenberg C (2005) Shea butter export guide. West Africa trade hub. A project of USAID. West Africa Regional programme.

Marhaeniyanto E, Susanti S (2014) Product fermentation and gas production *in vitro* of feed content from *Moringa oleifera,* Lamm and *Paraserianthes falcataria* leaves. *Journal of Agriculture and Veterinary Science* **7** (10), 12-18

Mauricio RM, Mould FL, Dhanoa MS, Own E, Channa S, Theodorou MK (1999) A semi-automated *in vitro* production technique for ruminant feedstuff evaluation. *Animal Feed Science and Technology* **79** (4), 321-330

Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider E (1979) The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor in vitro. *Journal of Agricultural Science* **93**, 217-222

Menke KH, Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* **28**, 7-55

Mould FL, Morgan R, Kliem KE, Krystallidou E (2005) A review and simplification of the *in vitro* incubation medium. *Animal Feed Science and Technology* **123-124**, 155-172

Morgavi DP, Newbold CJ, Beever DE, Wallace RJ (2000) Stability and stabilization of potential feed additive enzymes in rumen fluid. *Enzyme and. Microbial Technology* **26**, 171-177

Nouri L, Nafchi AM, Karim AA (2014) Phytochemical, antioxidant, antibacterial, and α-amylase inhibitory properties of different extracts from betel leaves. *Industrial Crops and Products* **62**, 47-52

Ojiako EN (2014) Phytochemical analysis and antimicrobial screening of *Moringa oleifera* leaves extracts. *The International Journal of Engineering and Science* **3** (3), 32-35

Oskoueian E, Abdullah N, Saad WZ, Omar AR, Ho YW (2014) Dose-response effects of phorbol esters isolated from Jatropha meal on rumen microbial activities. *Asian Journal of Animal and Veterinary Advances* **9** (1), 37-46

Ottenstein DM, Bartley DA (1971) Separation of free acids C_2-C_5 in dluted aqueous solution column technology. *Journal of Chromatography Science* **9**, 673-681

Pal K, Patra AK, Sahoo A, Kumawat PK (2015) Evaluation of several tropical tree leaves for methane production potential, degradability and rumen fermentation in vitro. *Livestock Science* **180**, 98-105

Puchala R, Min BR, Goetsch AL, Sahlu T (2005) The effect of a condensed tannin-containing forage on methane emission by goats. *Journal of Animal Science.* **83**, 182-186

Prabhu MS, Platel K, Saraswathi G, Srinivasan K (1995) Effect of orally administered betel leaf (*Piper betle* Linn.) on digestive enzymes of pancreas and intestinal mucosa and on bile production in rats. *Indian Journal of. Experimental. Biol*ogy. **33** (10), 752-756

Ray A, Ghosh S, Ray A, Aswatha SM (2015) An analysis of the influence of growth on potential functional and biochemical properties and thermal analysis of freeze-dried *Aloe vera* L. gel. *Industrial Crops and Products* **76**, 298-305

Robertson J, Van Soest P (1981) The detergent system of analysis, in: James WP, Theander O (Eds), The analysis of dietary fibre in food. Marcel Dekker, New York.

SAS (2012) SAS Version 9.4. SAS Institute INC. USA

Theart JFF, Hassen A, Van Niekerk WA, Gemeda BS (2015) *In-vitro* screening of Kalahari browse species for rumen methane mitigation. *Scientia Agricola* **72** (6): 478-483.

Theodorou MK, Williams BA, Dhanoa MS, McAllen AB, France J (1994) A simple gas production method using pressure transducers to determine the fermentation kinetics of ruminant feed. *Animal Feed Science and Technology* **48**, 185-197

Tilley JMA, Terry RA (1963) A two-stage technique for the in vitro digestion of forage crops. *Journal of the British Grassland Society* **18**, 104-111

Yan T, Mayne CS, Porter MG (2006) Effects of dietary and animal factors on methane production in dairy cows offered grass silage based diets. *International. Congress. Series.* **1293**, 123–126