Effect of medicinal plant extracts from West Africa on rumen fermentation parameters, enteric methane emission and growth performance in Merino sheep

By

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Declaration

I, **Abiodun Mayowa Akanmu**, declare that this thesis, which I hereby submit for the degree of PhD (Animal Science) at the University of Pretoria, is my own work, except where references have been made, and it has not previously been submitted by me for any degree at this university or any other university or tertiary institution.

Signature _____

Abiodun Mayowa Akanmu

Preface

This dissertation was completed in the Department of Animal and Wildlife Sciences and it is based on the following chapters, which have already been published or are to be submitted for publication in peer-reviewed journals.

Peer-reviewed journals

Akanmu AM, Hassen A. 2018. The use of certain medicinal plant extracts reduced *in vitro* methane production while improving *in vitro* organic matter digestibility. Animal Production Science 58(5): 900-908

Akanmu AM, Hassen A. 2018. Associative effect of plant extracts on anti-methanogenic properties, volatile fatty acids and organic matter digestibility of *Eragrostis curvula* hay. (Revised version to be submitted to PLOS ONE)

Akanmu AM, Hassen A. 2018. Effects of substrate and storage time on the efficacy of plant extracts used as an environmentally friendly alternative additive to monensin to modulate rumen fermentation and reduce enteric methane emission (to be submitted to Journal of Cleaner Production)

Akanmu AM, Hassen A. 2018. Oral dosage of medicinal plant extract as an additive reduced methane emission without negatively affecting feed utilization and performance of SA Mutton Merino sheep (to be submitted to Journal of Cleaner Production)

Akanmu AM, Hassen A. 2018. Effect of extracts of *Moringa oleifera*, *Jatropha curcas* and *Aloe vera* supplementation on haematology, serum biochemistry and overall performance of SA Mutton Merino sheep (to be submitted to Journal of Cleaner Production)

Conference presentations

Akanmu AM, Hassen A. 2015. Potentials of medicinal plant extracts on digestibility, *in vitro* methane gas production of *Eragrostis curvula* forage. Presented at 3rd Global Science Conference on Climate Smart Agriculture 16–18 March, Le Corum, Montpellier, France

Akanmu AM, Hassen A. 2015. Fermentation and digestibility of *Eragrostis curvula* hay treated with *Jatropha curcas* and *Moringa oleifera* pods extracts *in vitro*. An oral presentation at SASAS Congress 48, 21–23 September. Empangeni, South Africa

Akanmu AM, Hassen A. 2017. Associative effect of plant extracts on anti-methanogenic properties, volatile fatty acids and organic matter digestibility of *Eragrostis curvula* hay. Presented at 4th Global Science Conference on Climate Smart Agriculture 28–30 November, Johannesburg, South Africa

These studies were designed to test the effectiveness of medicinal plant extracts as alternative additives to reduce methane without adversely affecting feed digestibility. Certain plant materials from West Africa were selected based on the literature and traditional usage. This dissertation starts with a general introduction in which the need for this study was justified.

Chapter 1 consists mainly of a review of the literature with detailed information about medicinal plants used in this study, and ways in which the phytochemicals could help reduce methane emission from ruminants. This chapter also discusses the process of methanogenesis in the rumen and methods that are already being used to combat enteric methane emissions. Chapter 2 is based on the first study, which dealt with the screening of plant extracts for methane reduction potential and from which the optimum level of inclusion was obtained. Chapter 3 investigated whether two-way cocktails of effective plant extracts (identified in Chapter 2) at the recommended dosage would have any associative effect in the reduction of methane emission without adverse effects on gas production and digestibility. Chapter 4 compares the effect of various plant substrates, which are characterized as poor, average and good quality feed. In addition, the stability of plant extracts after storage for 12 months was assessed in the study. Chapters 5 and 6 test *in vivo* the effects of dosing plant extracts on digestibility, rumen fermentation parameters, methane emission, growth performance, carcass yield and blood profile of SA Mutton Merino sheep. The general conclusions and recommendations based on this research are synthesized in Chapter 7.

This thesis was prepared following the instructions to authors for publication of manuscripts in Animal Production Science, in which the first study was published.

Acknowledgement

My appreciation goes to my promoter, Prof Abubeker Hassen, for his thoroughness, eye for detail, and unrelenting and untiring personal and academic support throughout my study period. I will never forget the contribution, knowledge and support I received from you during my study period.

I would also like to show my gratitude to Animal Change Project EU, Department of Science and Technology (DST) and National Research Foundation (NRF), South Africa, for funding various components of this study. My appreciation goes to the University of Pretoria, South Africa, for the PhD bursary award. I am grateful to Animal Change and NRF for sponsoring presentations of my research findings at Global Science Conference – Climate Smart Agriculture Montpellier, France 2015, and South African Society for Animal Science Congress 48, Empangeni, South Africa.

I would like to thank the head of department and the entire staff, especially Profs van Niekerk, Webb and Erasmus of the Animal and Wildlife Department, University of Pretoria, for their support, and all the laboratory technologists who helped me with various laboratory procedures, as well as Mr Roelf Coertze, Ms Corlia Swanepoel and the staff at Small Stock Division, Proefplaas Experimental Farm, who assisted me during the animal research period and deserve my huge appreciation. Mrs Kate Nkau has played a motherly role since my arrival at the department.

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My big brother, Dr Akinlolu Akanmu, with the help of my younger men, Engineer Femi Akanmu, Barrister Rotimi Akanmu, and my parents, Pa and Dns Adekunle and Olutayo Akanmu, are responsible for the tedious procedure of identification, harvesting, processing and all logistics involved in transportation of fresh plant materials from Nigeria to South Africa. I want specially to thank you for this labour of love, prayer and family bonding.

My wonderful wife, Oluwafunmike Oluwasebebe, has been my pillar of strength. Words alone cannot fully express the extent of my gratitude and appreciation for your support. Thank you for loving me, for your prayers and the peace of mind I enjoyed throughout. I bless the day I found you.

Finally, I would like to thank my Lord and Saviour Jesus Christ for the gift of life and salvation of my soul and for enabling me with strength, courage, wisdom, and knowledge to face everyday challenges intertwined with doctoral research. Where you have placed me today, I never knew I could attain. Thank you, my Lord.

"Finally, brothers and sisters, whatever is true, whatever is noble, whatever is right, whatever is pure, whatever is lovely, whatever is admirable—if anything is excellent or praiseworthy – think about such things (Phil 4:8)."

Dedication

To

Oluwafunmike
Who believes in me

Iremide Ireayo
Whom I am still waiting for

Adekunle and Olutayo Akanmu
Who sacrificed luxury to train all their children

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List of acronyms

ADF	Acid detergent fibre
ADL	Acid detergent lignin
AOAC	Association of Official Analytical Chemists
AV	*Aloe vera*
AZ	*Azadirachta indica*
CH_4	Methane
CP	Crude protein
CP extract	*Carica papaya*
DDM	Digestible dry matter
DM	Dry matter
DOM	Digestible organic matter
DMI	Dry matter intake
EE	Ether extract
FCE	Feed conversion efficiency
g	Gram
GP	Gas production
IVOMD	*In vitro* organic matter digestibility
JA	*Jatropha curcas*
kg	Kilogram
ml	Millilitres
MO	*Moringa oleifera*
N	Nitrogen
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
OM	Organic matter
PB	Piper betle
SA	South Africa
SAMM	South Africa Mutton Merino sheep
SAS	Statistical Analysis Software
TD	*Tithonia diversifolia*
TGP	Total gas production
TMR	Total mixed ration
VFA	Volatile fatty acids
$W^{\wedge 0.75}$	Metabolic body weight

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By

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Degree: PhD Animal Science (Animal Nutrition)

Summary

A series of *in vitro* and *in vivo* experiments was conducted to evaluate the methane-reducing potential of some medicinal plant extracts in order to use them as alternative additives to replace antibiotics such as monensin. A methanolic extraction procedure was used to extract secondary metabolites from plants because these metabolites have been found to have rumen modulation properties, which may help to improve nutrient utilization in ruminants, thereby reducing methane gas production. This study assessed the beneficial effects of *Aloe vera* (AV), *Moringa oleifera* (MO) pods and leaves, *Jatropha curcas* (JA), *Carica papaya* (CP), *Tithonia diversifolia* (TD), *Azadirachta indica* (AZ) *and Piper betel* (PB) extracts and their two-way combination on methane reduction attributes when evaluated in *in vitro* and *in vivo* studies.

Plants extract that were obtained using pure methanol were screened for their effectiveness in methane reduction at graded levels in an *in vitro* production study. The standardized crude plant extracts were added at the rate of 25, 50, 75 and 100 mg plant extract powder per kg of feed dry matter. Gas measurements were taken at regular intervals during the incubation period. Methane emission was determined for each gas sample with the use of gas chromatography. In subsequent *in vitro* studies, combinations of extracted plant metabolites were incubated with feed samples to test methane-reducing abilities, their effect on gas production, feed degradability, and volatile fatty acids production. The last *in vitro* study tested the effects of recommended dosages of plant extracts from previous *in vitro* studies on methane production, digestibility and volatile fatty acids of various feed substrates.

At the end of *in vitro* trials, plant extracts of *Moringa oleifera, Jatropha curcas* and *Aloe vera* were selected from the most promising and effective plant extracts for *in vivo* evaluation of

feed digestibility, rumen fermentation parameters, methane emission, growth performance and blood profile of SA Mutton Merino sheep. Forty (40) Merino lambs were first ranked according to their body weight and divided into four groups with equal weight measurement. The four groups were randomly allocated to various plant extract treatments and the control. The plant extract treatments MO, JA and AV were drenched to the sheep, while animals in the control group were drenched with the same volume of water. All treatments were placed on the same total mixed ration formulated with 42% roughage component. Growth performance lasted 103 days, including adaptation, and the feed digestibility study lasted for 21 days, after which the animals were moved into the open circuit respiratory chambers for methane emission measurements in five batches.

Results obtained from the *in vitro* experiments indicated that plant extracts AV, MO, AZ, TD, CP, and JA had generally reduced ($P<0.05$) methane production without adversely affecting total gas production. All plant extracts significantly ($P<0.05$) increased the organic matter digestibility of *Eragrostis curvula*, while PB at all doses increased methane, total gas, volatile fatty acids and organic matter digestibility. Although the cocktails of promising plant extracts (MO, AZ, JA, AV, TD, and CP), which were effective against methane reduction, showed potential to increase propionic acid concentration, the effect was insufficient compared with the magnitude in which single plant extracts had reduced methane and increased digestibility. It was also recorded through the *in vitro* study that plant extracts are mostly effective in terms of methane reduction and increased organic matter digestibility when used on poor-quality forage.

Plant extracts MO and JA generally increased DM, NDF, ADF, CP and starch digestibility with significant effect on ($P<0.05$) on DM and CP digestibility compared with the control. Higher average daily gain and total weight gain were recorded for MO and JA. Better feed conversion efficiency was recorded for MO and JA, while feed dry matter (DM) intake was not significant across these treatments. Plant extracts MO, JA and AV significantly ($P<0.05$) reduced methane production from SA Mutton Merino (SAMM) sheep by about 20%, 35% and 28%, respectively, per kg of DM intake. Plant extracts MO, JA and AV reduced significantly ($P<0.05$) ammonia nitrogen (NH_3-N) in the rumen with a corresponding increase in ruminal propionic acid concentration for MO and JA plant extracts. Significant reduction ($P<0.05$) by MO and JA was recorded only for white blood cells and lymphocytes in blood haematology. Thus, the findings of the *in vivo* study confirmed the *in vitro* results that plant extracts MO, JA and AV are able to reduce methane emission from ruminants with significant improvement in

feed digestibility. This study also confirmed that supplementation of these plant extracts to animals is not toxic at inclusion level of 50 mg/kg DM feed. In addition, plant extracts of MO and JA can be used as additives to ruminants at the rate of 50 mg/kg DM feed to provide co-benefit in terms of improved performance. Further studies need to be conducted to encapsulate the plant extracts for better delivery and test the response of animals at 75 mg/kg DM feed for better improvement.

General Introduction

Background

The human population is expected to increase to 9 billion people by 2050 (Food and Agriculture Organization of the United Nations (FAO) 2013). This increase will demand up to 70% greater food production (FAO 2013). Scientists are faced with the challenge of meeting the rising demands for food for human beings by improving productivity, while at the same time being conscious of the likely impact this increase in production will have on environmental pollution. Ruminants are one of the most valuable and renewable resources for humankind. Their unique ability to utilize non-competitive foods such as grass, crop residue and agro-industrial wastes make them indispensable to human beings as they are capable of meeting meat and milk demands. But a rapid increase in ruminant animal populations is also mentioned as a major challenge, as ruminants are responsible for up to one third of methane emission worldwide (Storm *et al.* 2012).

Justification

The use of concentrates has been recommended as a strategy for reducing methane production from ruminants (Holter & Young 1992; Duan *et al.* 2006)**.** Lovett *et al.* (2003) demonstrated that increased concentrate use, compared with pasture, reduced the enteric methane per kg of animal product. The findings of Puchala *et al.* (2005) also suggested the use of forages rich in condensed tannins *(Lespedeza cuneata)* to supplement low-quality grasses in ruminant diets in order to reduce methane production. Generally, a reduction in methane production is expected when the residence time of feed (concentrates and forage) in the rumen is reduced, since ruminal degradation decreases, and methanogenic bacteria are less able to compete in such conditions. Furthermore, a rapid passage rate favours propionate production and the relevant hydrogen (H_2) use. According to Kennedy and Milligan (1978) and Okine *et al.* (1989), a 30% decline in methane production was observed when the ruminal passage rate of liquid and solid phases was increased by 54% to 68%. Feeding concentrate diets reduces the residence time of the feed as the digesta will be digested in the subsequent in the hindgut, while fibre digestion relies mainly on rumen fermentation.

The impact of ionophore administration on enteric methane emission was evaluated by Guan *et al.* (2006). Daily enteric methane emissions, as measured with a sulphur hexafluoride tracer gas technique, ranged from 55 to 370 L/steer daily. Ionophore supplementation resulted in decreased emissions by 30% for cattle that received a high-concentrate diet for two days and by 27% for cattle that received a low-concentrate diet for four days. Other attempts to lower methane production from ruminants included the use of dicarboxylic organic acids such as malate, which may alter rumen fermentation in a manner similar to ionophores. Finding alternate pathways for substrate meant for methane production is also an option. The removal of protozoa from the rumen has also been used to investigate the role of protozoa in rumen function, and to study their effect on methane production (Hart *et al.* 2009). These authors indicated that protozoa-free lambs produced 26% less methane than the control group. Some of the methods already proposed still require further refinement or are expensive to implement. Moreover, cleaner production with the use of natural products is now gaining popularity owing to consumer preference standing against the use of antibiotics and other synthetic products.

The development and implementation of natural feeding and management strategies aimed at reducing methane emissions would potentially increase the efficiency of dietary energy use. This means that the use of natural products as additives would not only reduce the greenhouse gas contribution of livestock to the atmospheric budget, but also enhance production efficiency. It would decrease over-reliance on antibiotic growth promoters such as monensin, which is currently in use in the feedlot system. The cattle industry is the most dependent on growth promoters, as cattle have high energy requirements that cannot be met easily without the use of concentrate feeds and these antibiotic growth promoters. But antibiotic growth promoters have residual effects in meat and milk products, putting consumers of such products at risk of developing antibiotic resistant syndrome. As such, to maintain improved production efficiency of ruminants, alternative additives must be found. The use of natural feed additives capable of replacing ionophores that have already been banned in the European Union has been the focus of recent studies.

In this regard, extracts from plants rich in bioactive compounds such as *Piper betle, Aloe vera, Jatropha curcas, Carica papaya, Moringa oleifera leaves, Tithonia diversifolia, Azadirachta indica* and *Moringa oleifera* pods need to be evaluated systematically to exploit their potential as alternative feed additives to modulate rumen fermentation and reduce methane emission by ruminants.

General objective

The overall objective of this study is to identify, develop and possibly commercialize effective plant extracts as alternative additives to modulate ruminal fermentation, improve nutrient utilization and reduce methane emission from small ruminants.

Specific objectives

The specific objectives of this study are:

- To identify through *in vitro* studies, plant extracts that are effective in reducing methane emission without negatively affecting the degradability of organic matter
- To determine the optimum inclusion level of (four to six) effective plant extracts that result in maximum methane reduction without negatively affecting the degradability of the feed
- To determine the effectiveness of a combination of screened plant extracts in reducing methane emission through *in vitro* studies without negatively affecting the degradability of organic matter.
- To investigate the impact of storage and extraction time on the effectiveness of plant extracts on methane reduction
- To investigate the impact of effective plant extracts on different substrates, low quality roughage, good quality roughage and total mixed ration
- To monitor methane gas production and total mixed ration digestibility of sheep fed effective plant extracts
- To monitor growth performance and haematological parameters in sheep fed effective plant extracts

CHAPTER ONE

Literature review

1.1 Introduction

Methane production through enteric fermentation is of concern worldwide owing to its contribution to the accumulation of greenhouse gases in the atmosphere. In the past, the earth's climate changed as a result of natural causes in the atmosphere, but the current changes we are witnessing and those that are predicted are due largely to human and animal activities (IPCC 2013). Methane is a potent greenhouse gas and its release into the atmosphere is linked directly with animal agriculture. The data released by the US Environmental Protection Agency (USEPA) (2015) indicated that enteric fermentation accounted for up to 23% of all methane emission in the United States and globally ruminants produced, about 80 million tonnes of methane annually (Patra 2011). Methane ranks third among the worst greenhouse gases, emitted mainly during the production and transportation of natural gas, oil, coal and solid waste landfills, and from agricultural practices, which include organic waste and livestock farming as shown in Figure 1.1 (USEPA 2015). Emissions from these sources cause damage to ozone layer formation. '[The] ozone layer is a chemically controlled stratosphere where molecular oxygen is broken down in the stratosphere by solar radiation to yield atomic oxygen which then combine with molecular oxygen to form ozone' (UNEP 1992). Ozone presence in the stratosphere filters out ultraviolet radiation from the sun, which might be harmful to life on earth. Factors such as the abundance of CO_2 in the atmosphere influence these chemical reactions as they are temperature dependent. An increase in the abundance of methane, which is a source of hydrogen in the atmosphere, also affects the distribution of stratospheric ozone, leading to destruction in ozone formation.

Methane is produced in significant amounts among domesticated ruminant animals (cattle, buffalo, sheep, goats and camels), which produce it as part of their normal rumen fermentation processes. In the rumen of these animals, microbial fermentation converts feed into fermentation products that can be used by animals as major energy sources or for microbial protein synthesis. This microbial fermentation process, referred to as enteric fermentation, produces methane as a by-product, which is exhaled by the animal during eructation (belching). This chapter reviews rumen methanogenesis and enteric methane mitigation options already established or proposed in the literature, including their limitations. This is followed by a discussion of the use of natural products as alternative additives, which includes the

development of extracts from medicinal plants that have antibiotic and antiprotozoal properties that are effective against methanogens in the rumen.

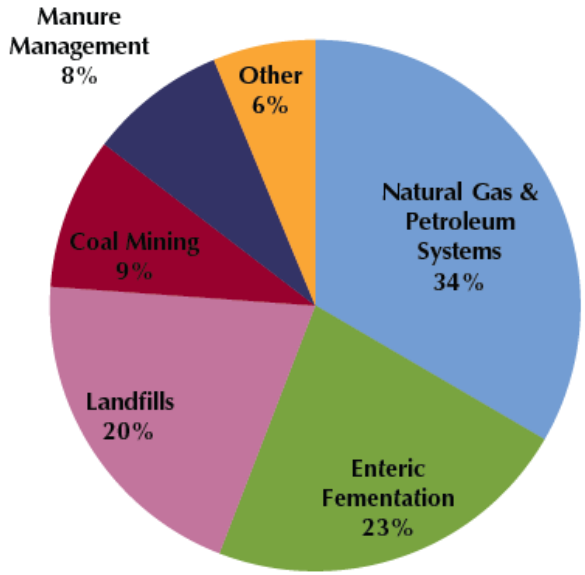


Figure 1.1. Inventory of methane emission sources in the US 2015

Source: United States Environmental Protection Agency (2015)

1.2 Methanogenesis in the rumen

Ruminants are unique in their ability to use forage as an energy source for maintenance, growth and milk production. Plant carbohydrates are broken down by bacteria in the rumen, producing volatile fatty acids (VFA), the major energy source for the animal. The main VFAs are acetate, propionate, and butyrate. The proportion of each depends on the type of feed. Generally, ruminal digestion generates hydrogen (H_2) as an end product. However, the amount of H_2 in rumen depends on the abundance and types of VFA produced. For example, the formation of acetate generates twice the amount of H_2 compared with the formation of butyrate, whereas the formation of propionate uses H_2. The accumulation of H_2 beyond a certain level in the rumen inhibits feed digestion.

Microorganisms in the rumen that produce methane are referred to as methanogens, and they convert H_2 and CO_2 into CH_4 and water. This process lowers the amount of H_2 in the rumen (Henderson *et al.* 2010). Methane production is the main way that H_2 is used up in the rumen.

Thus, strategies to lower enteric CH_4 production should involve reducing the production of H_2 in the rumen to inhibit the formation of CH_4, or should redirect the H_2 into products such as propionate or butyrate, as shown in Figure1.2, or other harmless compounds in the rumen that serve as a hydrogen sink (Henderson *et al.* 2010).

Enteric methane is produced as a result of microbial fermentation of feed components (cellulose, starch and sugars). Methane, a colourless, odourless gas, is produced predominantly in the rumen (87%) and to a small extent (13%) in the large intestines (Murray *et al.* 1976)). Methanogens are microorganisms that produce methane as a metabolic by-product in anaerobic conditions. Methanogens are also found in the wetlands, other than in the digestive tracts of animals such as ruminants and human beings. They are responsible for methane belched by ruminants and flatulence in humans (Lengeler *et al.* 1999). The conversion of feed materials to methane in the rumen involves the integrated activities of various microbial species, with the final step being carried out by methanogenic bacteria (Moss *et al.* 1994; McAllister *et al.* 1996).

Primary digestive microorganisms (bacteria, protozoa and fungi) hydrolyse proteins, starch and plant cell wall polymers into amino acids and sugars. These simple products are then fermented to volatile fatty acids (VFAs), hydrogen (H_2), and CO_2 by both primary and secondary digestive microorganisms. Acetate, propionate, and butyrate, which are the major VFAs, are then absorbed and utilized by the host animal. The major producers of H_2 are microorganisms that produce acetic acid as one of their products in the fermentation pathway. These two by-products of the rumen (H_2 and CO_2) form the substrate for the production of methane in the rumen. But methane is classified as a greenhouse gas capable of damaging the ozone layer and contributing to global warming. The methanogenic pathway also indicates that enteric methane is a form of energy loss to the animal and the reduction of these emissions from ruminants would increase their feed use efficiency.

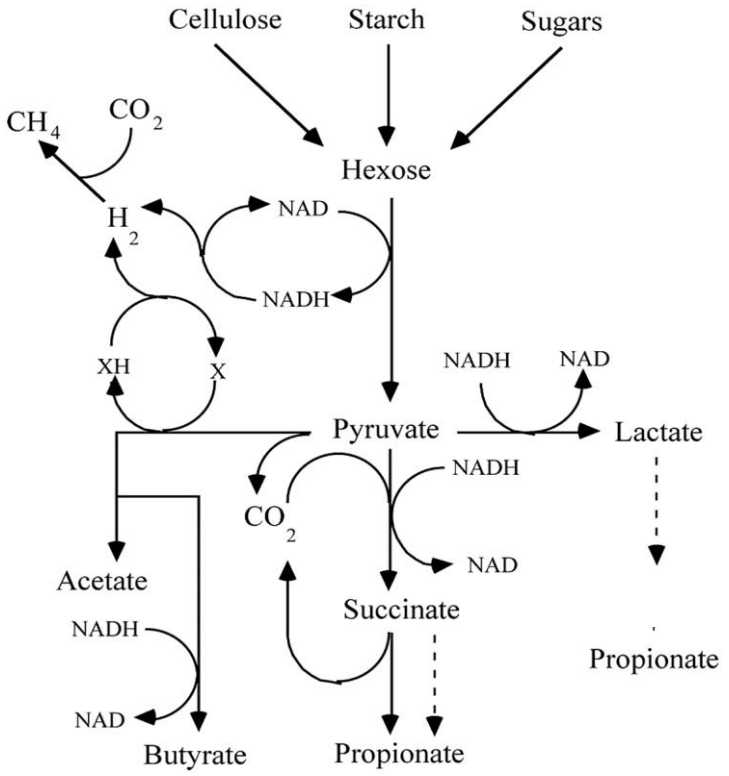


Figure 1.2. Schematic diagram showing the major pathways of carbohydrate fermentation by ruminal bacteria where the formation of acetate leads to production of hydrogen while butyrate and propionate use up hydrogen in the rumen

**X denotes alternative electron carrier (e.g. ferredoxin)*

Source: Russell & Rychlik (2001)

1.2.1 Methane contribution of ruminants to atmospheric budget

Livestock (mainly ruminants) account for up to one third of the methane emitted worldwide, and methane has a greenhouse potential of 25 times that of CO_2 (IPCC 2007). Methane accounts for a great part of the emitted CO_2 equivalents from agriculture. The most important gases other than methane that cause climate change are CO_2, nitrous dioxide (NO_2) and chlorofluorocarbons (CFCs).

1.2.2 Methane emission as energy loss to animals

Several scientific reports have highlighted the effect of methane emission on the performance of ruminant animals. Enteric methane emission by ruminants has been accounted to represent 5% to 15% of their gross energy loss (Wanapat *et al*. 2015; Carvalho *et al*. 2016). The level of gross energy loss depends on the quality of forage fed to the animals and the breed of the

animal, which causes a genetic effect. Poor-quality forages have been found to generate more acetate as end products of rumen fermentation, which in turn increases the volume of H_2 in the rumen and ultimately leads to the production of more methane gas that the animals eject from the rumen through belching (Wanapat *et al*. 2015; Carvalho *et al.* 2016). Over the years, scientist have researched enteric methane mitigation options and proposed these mitigation strategies.

1.3 Mitigation strategies for methane emission from ruminants

Kumar *et al*. (2014) classified various mitigation strategies for methane emission from ruminants into three major groups. The first group is the use of rumen controls, which includes immunization, defaunation and application of phage therapy. The second group consists of systematic changes in animal development. This has to do with breed selection and intensiveness of production, among others. The third group is about dietary manipulation, which involves feeding additives, the use of secondary compounds of plants, and halogenated compounds.

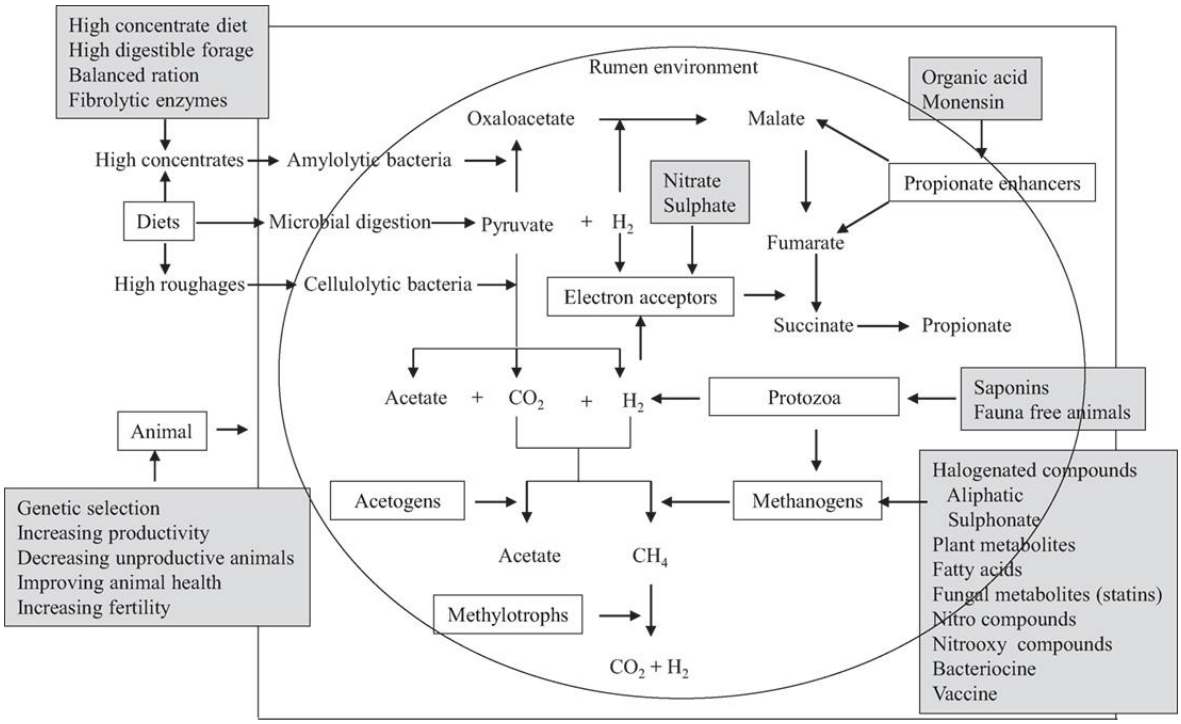


Figure 1.3: Potential target for reducing enteric methane production in the rumen

White boxes could be targets for suppressing CH_4 emissions. Dark boxes are options that have been studied *in vitro* or *in vivo* to decrease CH_4 production.

Source: Patra (2016)

1.3.1 Rumen controls

Rumen controls have been included among recent methane mitigation option studies, and employ the development of vaccines for immunization (Kumar *et al*. 2014). These vaccines activate the immune response of the animal against methanogens, thereby eliminating methanogens in the rumen. This method is still under development, and requires a large pool of genomic data to identify immunization targets. Moreover, differences in dietary regimen pose the difficulty of inadequate targets of vaccines (Kumar *et al*. 2014). Phage therapy is also part of the rumen control mechanism, which involves targeting methanogens with appropriate phages. This method inhibits archaea methanogens and redirects H_2 to reductive rumen bacteria, which may be propiogenic or acetogenic (McAllister *et al*. 2008). Phage therapy has the ability to penetrate and consequently cause lysis in the host cell. This is a potential strategy to mitigate methane production, but it has been reported by Buddle *et al*. (2011) that quick adaptation of microorganisms to bacteriophages challenges the use of the strategy and as a result bacteriophages have to be identified, sequenced and characterized. Others are defaunation mechanisms with the use of chemical inhibitors, plant extracts and vaccines against protozoa. This method reduces hydrogen in the rumen by the removal of protozoa. Hook *et al*. (2010) reported a symbiotic relationship between rumen protozoa and methanogens. This relationship results in interspecies hydrogen transfer, which provides methanogens with a regular supply of hydrogen required to reduce CO_2 to CH_4. The disadvantage of this method is that some of the chemicals or plant extracts might be toxic and cause severe disease for the animal.

1.3.2 Systemic changes

Knapp *et al*. (2014) described systemic changes in animal development as increasing animal production through genetics and other management approaches. Goals of this method are to improve nutrient utilization for productive purposes, to increase feed efficiency and to decrease methane production per unit of product, whether meat or milk. Total emissions will also be reduced if the annual production of meat and milk remains constant, but with fewer ruminant animals being needed to produce the same volume of ruminant products. Animal selection and

breeding for low methane producers is a long-term strategy that could increase digestibility and reduce methane emission, but requires time and resources to be accomplished (Kumar *et al*. 2014).

1.3.3 Dietary manipulation

(i) Increasing concentrate to roughage ratio

Kumar *et al*. (2014) listed the various components of animal dietary manipulation, which include manipulation of diet composition, which tends to shift towards feeding more concentrate diets and using fewer roughages. This method improves the passage rate of feed, and improves digestibility with reduced rumen pH and consequent reduction in protozoa counts. It favours the production of more propionate than acetic acid because of the shorter retention time, which promotes the production of more butyrate and propionate at the expense of acetate. A feed ration that is high in concentrate has also been found to increase the concentration of total protein and high density lipoprotein in small ruminants (Chen *et al*. 2015). In practice, feeding more concentrate to ruminants is not achievable in developing countries, especially in sub-Saharan Africa, which have limited grain production per year. This would create an unhealthy grain competition between humans and ruminant animals raised for commercial purposes, since poultry production already relies heavily on grain diets. In addition, feeding a concentrate-based diet increases the chances of acidosis in ruminants, and may not be cheap and profitable for farmers in the developing world.

(ii) Organic and halogenated compounds

The addition of organic acids such as fumaric and malic acids to ruminant diets has acted as hydrogen sinks, which shifts the fermentation process of rumen towards propionate formation (Carro & Ungerfeld 2015). There are indications that the effectiveness of this method might be influenced by the type of diet, and could be constrained by the risk of rumen acidity (Carro & Ungerfeld 2015). Halogenated compounds such as bromo-alkyl sulfonates, lumazine, ethyl 2-butyonate, anthraquinone and amichloral have been used to inhibit protozoa and methanogens (Patra *et al*. 2017). These have resulted in a decrease of substrates used by methanogens in the rumen. This method still needs development and *in vivo* confirmation. Some risk is associated with the use of some of these compounds, as they might cause toxicity in animals owing to their toxic nature if their usage is not controlled.

(iii) Use of antibiotic additives

Since the introduction of the therapeutic use of antibiotics, several antibiotics have been in use as growth promoters of farm animals (Butaye *et al*. 2003). Most ionophore antibiotics are produced by *Streptomyces, Streptoverticillium, Nocardiopsis, Nocardia* and *Actinomadura* spp (Benno *et al*. 1988). Ionophores work by inhibiting protozoa and gram-positive bacteria, thereby decreasing the number of methanogens in the rumen. Ionophores are feed additives that alter rumen microbial population through ion transfer across cell membranes. Ionophores have been used extensively in the beef industry in US and Canada since 1977 (Duffield & Bagg 2000).

(iv) Plant secondary compounds

An important alternative strategy that is included as part of the dietary mitigation strategy is the use of natural products, which involves feeding additives that are based on plant secondary compounds. Medicinal plants have been found to be rich sources of condensed tannins, phenolic monomers, saponins and other phytochemicals that have antimicrobial and antifungal properties (Cragg & Newman 2013). Several *in vitro* studies have indicated that plant secondary compounds are an effective means of inhibiting protozoa activities in the rumen, while some are capable of digesting complex polysaccharide chains (Demeyer 1981; Busquet *et al.* 2006a; Patra & Saxena 2010; Sirohi *et al.* 2012; Lee *et al.* 2015; Kim *et al.* 2015). *In vitro* and *in vivo* results have shown that temperate legumes, which are rich in secondary compounds such as condensed tannins, are toxic to some rumen microbes, especially the ciliate protozoa and methanogen archaea, and as a result methanogenesis in the rumen can be reduced (Lascano & Cárdenas 2010).

Plant secondary compounds are effective at reducing methanogens and hydrogen in the rumen (Kamra *et al.* 2012). But plants rich in tannins have been found to lower fibre digestibility (Lascano & Cárdenas 2010), and reduce the palatability of feeds, which in turn affects the performance of the animal. Screening medicinal plants that might have lower concentrations of tannins, but have active phytochemicals that are capable of influencing methanogenesis, could be explored as an alternative option to reducing methane emission without negatively affecting the digestibility of feed and the performance of animals.

1.4 Potential use of medicinal plants to mitigate enteric methane production

Since the discovery and application of penicillin in 1940s, antibiotics have played unparalleled roles in the prevention, control, and treatment of infectious diseases for humans and animals. It is also proved that the use of antibiotics in animal feeds is an important way to enhance feed efficiency, promote animal growth, and improve the quality of the animal products (Cheng *et al*. 2014).

Commercial animal production has relied heavily on the use of antibiotics, and unreasonable inclusion of these products in animal rations has given rise to the development of resistant bacteria that are transmittable from animals to humans (Stanton 2013). Screening plants with secondary metabolites as replacements for antibiotic growth promoters is already in focus. Studies have documented the replacement value of antibiotics, especially with medicinal plant extracts. Medicinal plants have been tested *in vitro* and have been found to decrease the population of protozoans in the rumen, to act against disease-causing bacteria, and to be effective in improving feed digestibility and the welfare of animals (Gautam *et al.* 2007; García-González *et al.* 2008; Bhatta *et al.* 2013; Sharifi *et al.* 2013). Section 1.4.1 reviews the literature with focus on the benefits and risks associated with the use of medicinal plants as alternative feed additives for ruminant animals.

1.4.1 Co-benefit and risks of using medicinal plants for methane mitigation in ruminants, feed digestibility, performance and welfare of animals

(i) Co-benefits

Secondary compounds that are present in medicinal plants are capable of reducing methane emission without affecting feed digestibility adversely. Essentials oils (EOs) derived from oregano and rosemary reduced *in vitro* methane production by up to 71% when applied at a dosage rate of 2.0 g/L. Ruminal ammonia production was also significantly reduced by up to 78% with essential oil supplementation (Cobellis *et al*. 2015). In a study by Chaturvedi *et al*. (2015), herbal additives of *Ocimum sanctum, Curcuma longa, Emblica officinalis, Azadirachta indica,* and *Clerodendrum phlomidis* did not influence total gas production and in vitro dry matter digestibility (IVDMD) negatively, but reduced methane and ammonia nitrogen production significantly. Some of these compounds are beneficial and serve as alternatives to the use of antibiotics to mitigate methane emission and improve animal performance.

Jeronimo *et al*. (2016) reported that 'tannins may prevent bloat, enhance protein utilization during digestion, act to control internal parasites, and induce improvements in growth performance, wool growth, and milk production' (Waghorn 2008). These benefits are brought about by the antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects of tannins (Westendarp 2006). Tannins act by iron deprivation and interactions with vital proteins such as enzyme. An alkaloid is a DNA intercalator and an inhibitor of topoisomerase, while saponins form complexes with sterols that are present in the membrane of microorganisms, thereby causing membrane damage and a consequent collapse of cells (Cheng *et al*. 2014).

Studies have also shown that the addition of various medicinal plant extracts improved the haematology and serum biochemical parameters and boosted the immunity of various animals (Oleforuh-Okoleh *et al*. 2015; Mwale *et al*. 2014).

(ii) Risks

Risks involved in the use of medicinal plant extracts to reduce methane include reduction in total volatile fatty acids (TVFA), DM and neutral detergent fibre (NDF) degradability (Cobellis *et al*. 2015). Plant extracts also have a complex blend of bioactive components with many variations in their composition owing to biological factors, manufacturing and storage conditions (Baert *et al*. 2011). Parameters that affect the efficacy of plant extracts are genetic variations of the plant, the age of the plant, dosage, extraction method, harvest time and compatibility with other ingredients (Yang *et al*. 2009). All these conditions can have adverse effects on animal performance and can become toxic with uncontrolled usage.

1.4.2 Modes of action of medicinal plants on mitigation of enteric methane

Antioxidants can act in cell membranes or food products by scavenging free radicals, which initiate oxidation, removing reactive oxygen species such as oxygen radicals, breaking the initiated chain of reaction, and quenching or scavenging singlet oxygen (Lobo *et al.* 2010), destroying peroxides to prevent radical formation, and removing oxygen or decreasing local oxygen concentration/pressure (Eskin & Robinson 2001). The antioxidant property of many phytogenic compounds can contribute to the protection of feed lipids from oxidative damage. Their anti-oxidative activity arises from phenolic terpenes, such as ginger, scent leaf, garlic and other plants rich in flavonoids, which have been described as exerting anti-oxidative properties (Nakatani 2000).

Tannins are complex polyphenolics that are found widely in the plant kingdom. They are usually divided into two major groups, hydrolysable tannins and condensed tannins. Diets rich in tannin content have been found to reduce the growth rate of animals. This is due to a reduction in feed intake caused by phenolic contents, which have a bitter taste. It may also be due to the unavailability of nutrients, especially nitrogen, in the diet (Woodward & Reed 1989).

Methane emission is decreased when ruminants are fed tannin-rich forages, such as sainfoin, sulla, bird's-foot trefoil and other tropical legumes (Ramírez-Restrepo & Barry 2005; Waghorn 2008). Therefore, the antimethanogenic activity of hydrolysable and condensed tannins has been extensively demonstrated in several *in vitro* and *in vivo* studies (Hess *et al.* 2006; Goel *et al.* 2008). Condensed and hydrolysable tannins extracted from a diverse array of plant materials reduced CH_4 production *in vitro*, although with variable efficacy, depending on the tannin source (Pellikaan *et al.* 2011).

Plant metabolites may be antimicrobial compounds that inhibit some ruminal microorganisms. In particular, studies have shown that phytochemicals may inhibit, through bactericidal or bacteriostatic activities, the growth or activity of rumen methanogens (Tavendale *et al.* 2005; Liu *et al.* 2011), probably by binding proteins and enzymes of microbial cells. Tannins also inhibit some ruminal protozoa, and indirectly affect the associated methanogens. These secondary compounds may also adversely affect cellulolytic bacteria (Waghorn 2008; Patra & Saxena 2010) and, consequently, the anaerobic fermentation of carbohydrates to short-chain fatty acids, in particular acetate, thereby reducing CO_2 and H_2 formation required for methanogenesis.

1.5 Chemical compounds and methane reduction potential of medicinal plants used in this study

1.5.1 Jatropha curcas

In a study conducted by Santra *et al*. (2012), large numbers of plants were screened for potential methane reduction properties. Ethanolic extracts of *Piper betle* and *Jatropha gossypifolia* reduced methane production by 66% and 31%, respectively. An anticiliate protozoal effect of *Piper betle* leaf extract was also reported by Santra *et al.* (2012). Extracts of *Jatropha gossypifolia* inhibited the growth of rumen protozoal population *in vitro* due to presence of phorbol esters, tannins and saponins in their extracts, whereas decreased rumen protozoal counts with supplementation with saponins rich extracts were reported by Hristov *et al*. (1999).

(A full list of secondary compounds in *Jatropha curcas* is presented in Table 1.1.) *Jatropha curcas* is rich in phorbol esters and diterpene, which are antinutritional factors, and may become toxic if used excessively, which is part of the reason that the plant is used widely as a fence to protect food crops in some parts in Africa (Katole *et al*. 2011). Studies by Adam and Magzoub (1975) reported toxicity to animals, whereas Stripe *et al*. (1976) confirmed the presence of curcin, which acts to inhibit protein synthesis. The seed of Jatropha is the most toxic, as there are reports of severe vomiting, dehydration and restlessness as a result of accidental ingestion of Jatropha seed by children between the ages of three and five (Levis *et al*. 2000; Abud-Aguye *et al*. 1986). Therefore, the use of Jatropha both *in vitro* and *in vivo* in this study was approached with caution and as much as possible with minimum dosage.

Jatropha curcas

Source: http://troop75.typepad.com/photos/common_poisonous_plants_o/physic-nut-jatropha-curcas-flower-1.html

Table 1.1. Phytochemicals present in the leaf, seed and roots of *Jatropha curcas*

Compound class, name and derivatives	Biological activity	Part
Diterpenes		
Phorbol esters		
12-Deoxy-16-hydroxyphorbol (DHPB)	Tumor promoter	Seed
Jatrophol	Cytotoxic activity	Roots
Jatropha factor C1	Antimicrobial, antitumor, molluscicidal, insecticidal and cytotoxic activity	Oil
Jatropholones A	Antiplasmodial, gastroprotective	
Jatropholones B	Cytotoxic and molluscicidal activities	
Riolozatrione	NA	Roots
Acetoxyjatropholone	Cytotoxic activity	Roots
1.2 Rhamnofolane diterpenes		
Caniojane	Antiplasmodial and cytotoxic activities	Roots
1.3 Lathyrane diterpenes		
Jatrogrossidione	Leshmanicidal, trypanocidal	Roots
Heudolotione	Cytotoxic activity	Aerial
Alkaloids		
Pyrrolidine (5-Hydroxypyrrolidin-2-one)	Anticancer	Leaf
Pyrimidine-2,4-dione	Anticancer	Leaf
Diamide (curcamide)	Anticancer	Seed
Flavonoids		
Flavonoid glycoside I	Anticancer	
Flavonoid glycoside II	Anticancer	Root
Nobiletin	Anticancer	Root
Tomentin	Anticancer	Aerial

Extracted from Abdelgadir & Van Staden (2013)

1.5.2 Piper betle

Piper betel (betle) is an evergreen perennial creeper that has been used by human beings since time immemorial (Uddin *et al*. 2015). Traditional healers used betel leaf to treat halitosis, boils and abscesses, constipation, swelling of gums, cut and injuries. The essential oil found in betel leaf have anti-bacterial, anti-protozoan and anti-fungal properties. 'Antioxidants are type of molecules that neutralise harmful free radicals in the body, these radicals are produced through a chain of reactions that can damage living cells, and degrade materials such as rubber, gasoline and lubricating oils' (Uddin *et al*. 2015).

Although betel leaf has not been tested *in vivo* for methane reduction, the characteristics and properties of this plant make it a unique medicinal plant to investigate. Any plant with the antioxidant property capable of degrading materials such as rubber will be effective in degrading the highly lignified cell wall found in poor-quality roughages in Africa.

Piper betel

Source: http://www.chhajedgarden.com/piper-betle.html

1.5.3 Moringa oleifera

Moringa oleifera is a multipurpose tree of significant economic importance, because it can be used for several industrial and medicinal applications, and various products that are used as food and feed can be derived from its leaves and fruits (Ganatra Tejas *et al.* 2012). When

Moringa was tested *in vitro* by substituting it for sugarcane in a ruminant feeding system (Moreira *et al*. 2016), a significant reduction in CH_4 was recorded compared with the control treatment, thereby decreasing energy loss by the animal through reduction in acetate production and increase in the molar concentration of propionate and butyric VFA. Bhatta (2013) reported the effectiveness of *Moringa oleifera* and *Jatropha curcas* on methane reduction per ml of total gas production. Jatropha and Moringa were found to reduce methane without the addition of poly-ethyl-glycol (PEG), whereas there was an increase when PEG was added. It was also discovered that *Jatropha curcas, Ficus religiosa* and *Autocarpus integrifolis* effectively reduced methane production per unit of total gas. But for the purposes of this study and due to availability, *Jatropha curcas* was the only one that was studied of the three that are listed.

Moringa oleifera pods and leaves

Source: http://bjournal.co/new-year-new-superfood/moringa-oleifera-leaves-and-pods/

Table 1.2 Bioactive compounds in *Moringa oleifera* seeds

Class of compounds	Compounds
Alkaloids	Glycosides
Moringine	Strophantidin
	4-(-L-rhamnosyloxy)benzyl isothiocyanate
Flavonoids	4-(4'-O-acetyl- -L-rhamnosyloxy)benzyl isothioyanate
Catechin	4-(-D-glucopyranosyl-1!4- -L-rhamnopyranosyloxy)
	benzyl thiocarboxamide
Epicatechin	4-O-(-L-rhamnosyloxy)benzyl glucosinolate
Quercetin	4-(-L-rhamnopyranosyloxy)-benzylglucosinolate
Kaempferol	Niazimicin
	4-(-L-rhamnosyloxy)benzyl acetonitrile (niazirin)
Phenolic acids	O-ethyl-4-(-L-rhamnosyloxy)benzyl carmate
Gallic acid	Glycerol-1-1-(9-octadecanoate)
p-Coumaric acid	3-O-(6'-O-oleoyl- -D-glucopyranosyl)- -sitosterol
Ferulic acid	-sitosterol-3-O- -D-glucopyranoside
Caffeic acid	3-Hydroxy-4-(-L-rhamnopyranosyloxy)benzyl glucosinolate
Protocatechuic acid	4-(2/3/4^0-O-acetyl- -L-rhamnopyranosyloxy)benzyl glucosinolate
Cinnamic acid	Glucosinalbin
Ellagic acid	Glucoraphanin
	Glucoiberin

Source: Leone *et al*. (2016)

Table 1.3 Phyto-components identified in *Moringa oleifera* leaves

Compound name	RT	Peak area (%)	Molecular formula	Molecular weight	Compound nature
L(+) Milchsaure	8.017	21.07	$C_3H_6O_3$	90	Alcohol
2-Propanol	8.017	21.07	C_3H_8O	60	Alcohol
Methyl lactate	8.017	21.07	$C_4H_8O_3$	104	fatty acid ester
Ethyl alcohol	8.017	21.07	C_2H_6O	46	Alcohol
Propylene glycol	8.075	3.58	$C_3H_8O_2$	76	Alcohol
Lactic acid	8.142	3.23	$C_3H_6O_3$	90	acid compound
Ethyl 2-hydroxypropanoate (lactate)	8.182	3.66	$C_5H_{10}O_3$	118	lactic acid ethyl ester
Methyl butyl ether	8.242	2.2	$C_5H_{12}O$	88	ether compound
Ethyl 2-hydroxypropanoate (lactate)	8.292	7.97	$C_5H_{10}O_3$	118	lactic acid ethyl ester
2-Pyrrolidinone	13.568	2.51	C_4H_7NO	85	organic compound
Phenethyl alcohol	14.9	1.03	$C_8H_{10}O$	122	alcoholic compound
Pyrocatechol	17.509	1.25	$C_6H_6O_2$	110	organic compound
Quinhydrone	17.509	1.25	$C_{12}H_{10}O_4$	218	ketone compound
2,3-Butanedione	19.53	1.18	$C_4H_6O_2$	86	vicinal diketone
2-[(2-acetoxyethyl)-sufinylaniline	19.53	1.18	$C_4H_6O_2$	86	nitro compound
Linalool oxide (2)	22.979	1.08	$C_{10}H_{18}O_2$	170	terpene alcohol

Extracted from Karthika *et al*. (2013)

1.5.4 Carica papaya

Little or no information was available about the screening of *Carica papaya* leaf extracts for possible methane reduction ability. *C. papaya* was selected for screening because its extracts possess antibacterial and anti-inflammatory activity. Leaf extracts of *C. papaya* tested by Ifesan *et al*. (2013) showed that the ethanolic extract of the plant has free radical scavenging activity of about 45%. This characteristic makes it capable of decreasing oxygen concentration, intercepting singlet oxygen, preventing first chain initiation by scavenging initial radicals, such as hydroxyl radicals, chelating metal ion catalyst, decomposing primary product of oxidation to non-radical specie, and breaking chains to prevent continued hydrogen abstraction from substance. Ethanolic extracts of *C. papaya* also showed antimicrobial activity against five microorganisms. *Carica papaya* contains many phenolic compounds, which accounts for its antibacterial and digestive properties (Ifesan *et al*. 2013).

Carica papaya

Source: https://www.flickr.com/photos/hgcharing/3690603958

1.5.5 Aloe vera

Aloe vera leaf is rich in anthraquinone, a phenolic compound that has stimulating effects on the bowels and antibiotic properties. It contains saponins, which are soapy substances found in the gel, which are capable of cleansing. It performs strongly as an antimicrobial agent against bacteria, viruses and yeasts. Other phytochemicals present are tannins, resins, mannins and proteins such as lectins, mono-sulfuric acid and gibberlin. These are used as digestive aids and test positive to tannins, saponins and flavonoids (Kedarnath *et al*. 2012). Earlier findings revealed that acetone and methanolic extraction of aloe extract reduced methane production *in vitro* (Sirohi *et al.* 2009).

Aloe vera

Source: https://www.indiamart.com/proddetail/aloevera-plants-7683517733.html

Table 1.4 Summary of the chemical composition of *Aloe vera* leaf pulp and exudate

Anthraquinones/ Anthrones	Aloe-emodin, aloetic acid, aloin A and B (or collectively known as barbaloin), iso-barbaloin, emodin, ester of cinnamic acid
Carbohydrates	Pure mannan, acetylated manna, acetylated glucomannan, glucogalactomannan, arabinogalactan, galactoglucoarabinomannan, pectic substance, xylan, cellulose
Chromones	8-C-glucosyl-(2'-O-cinnamoyl)-7-O-methylaloediol A, 8-C-glucosyl-(S)-aloesol, 8-C-glucosyl-7-O-methyl-(S)-aloesol, 8-C-glucosyl-7-O-methyl-aloediol, 8-C-glucosyl-noreugenin, isoaloeresin D, isorabaichromone,
Enzymes	Alkaline phosphatase, amylase, carboxypeptidase, catalase, cyclooxidase, cyclooxygenase, lipase, oxidase, phosphoenolpyruvate carboxylase,
Organic compounds and lipids	superoxide dismutase, triglycerides, triterpenoids, gibberillin, lignins, potassium sorbate, salicylic acid, uric acid
Non-essential and essential amino acids	Alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, valine
Proteins	Lectins, lectin-like substance
Vitamins	B1, B2, B6, C, β-carotene, choline, folic acid, α-tocopherol

Source: Hamman (2008)

1.5.6 Azadirachta indica

Azadirachta indica, commonly known as neem, is classified as a multi-purpose tree as it has more than one output. There are so many traditional usages of this plant. In Nigeria, locals use the leaves, bark and nuts in the treatment of malaria by making some form of concoction from the leaves and barks. It can also be used as an insect repellent, nitrogen fixer for the soil, and pesticide. The seed of the neem plant is highly toxic and has to be detoxified before it can be fed to animals. In a previous study by Akanmu and Adeyemo (2012), neem leaf meal fed to broilers showed reduction in the feed intake of broilers, which consequently led to lower final weight, although the health condition of the animals was good compared with the control when

the blood profile was tested. Active ingredients found in neem, as reported by NRC (1992), are azadirachtin, meliacin, gedunin, salanin, nimbin, valassin and a host of others. The presence of these compounds leads to anti-inflammatory, anti-hyperglycaemic, anti-ulcer, anti-malaria, anti-fungal, anti-bacterial, anti-oxidant and other properties. Due to the presence of phytochemicals as listed by NRC (1992) *Azadirachta indica* potency might be useful in combating the methanogens that are present in the rumen. It can also be effective against protozoa population in the rumen which enhances methane and acetate formation in the rumen.

Azadirachta indica

Source: https://keyserver.lucidcentral.org/weeds/data/media/Html/azadirachta_indica.htm

1.5.7 Tithonia diversifolia

Tithonia diversifolia has not been tested for methane reduction attributes. This plant is found in the humid and sub-humid tropics of Africa and other parts of the world. *T. diversifolia* was included in this study because of its reported use as a pesticidal plant. It contains compounds such as sesquiterpene lactones and diterpenoids, which have biological activities against insects. Infusion of these plants has been reported to relieve constipation, stomach pains, indigestion, sore throat, liver pains and malaria (Kandungu *et al*. 2013). Reports also suggested anti-inflammatory, analgesic, antiviral, antidiabetic, anti-diarrhoeal, antimicrobial, antispasmodic, and vasorelaxant activities. The fodder leaves are soft and have good nutritive quality.

Tithonia diversifolia

Source: https://keyserver.lucidcentral.org/weeds/data/media/Html/tithonia_diversifolia.htm

1.6 Conclusions from the review of literature

Many methods have been used in the mitigation of enteric methane from ruminants. But a crucial and practicable option for farmers is dietary manipulation. The use of natural alternatives instead of common antibiotic growth promoters would improve animal welfare and provide a resultant increase in production. Some medicinal plants already listed have the attributes of reducing methane emission from ruminants without adverse effects on health and do not leave chemical traces in meat and milk products, which leads to the antibiotic resistant syndrome in humans that consume these animal products. This study explores the effect of medicinal plant extracts of *Aloe vera, Azadirachta indica, Moringa oleifera, Carica papaya, Tithonia diversifolia*, *Jatropha curcas* and *Piper betel* as additives on methane emission, digestibility, growth performance, rumen fermentation parameters and the blood profile of SAMM sheep.

1.7 Hypotheses

H_0: There is no significant difference in *in vitro* organic matter degradation and methane reduction properties of different plant extracts when used as an additive.

H_1: There are significant differences among plant extracts in terms of their effect on *in vitro* organic matter degradation and methane reduction properties when used as an additive.

H_0: Storage of plant extracts has no significant effect on efficient plant extracts at reducing methane emission when the plants are used as an additive.

H_1: Storage of plant extracts has significant effect on the efficient plant extract at reducing methane emission when the plant extracts are used as an additive.

H_0: There is no significant difference in *in vitro* organic matter degradation and methane reduction properties of different combinations of plant extracts.

H_1: There is significant difference among the various combinations of plant extracts in terms of their effect o*n in vitro* organic matter degradation and methane reduction properties when used as an additive.

H_0: There are no significant differences in the performance of effective plant extracts when tested on *Eragrostis curvula*, lucerne and total mixed ration.

H_1: There are significant differences in the performance of effective plant extracts when tested on *Eragrostis curvula*, lucerne and total mixed ration.

H_0: Inclusion of selected plant extracts or their combinations in the diet of animals has no significant effect on *in vivo* methane production, growth performance and organic matter degradability in SA Mutton Merino sheep.

H_1: Inclusion of selected plant extracts in the diet of animals has significant effect on *in vivo* methane production, growth performance and organic matter degradability in SA Mutton Merino sheep.

H_0: Inclusion of promising plant extracts and their combinations in the diet of animals has no significant effect on the performance and haematological parameters of SA Mutton Merino sheep.

$H_{1:}$ Inclusion of promising plant extracts and their combinations in the diet of animals has significant effect on the performance and haematological parameters of SA Mutton Merino sheep.

CHAPTER TWO

Effect of certain medicinal plant extracts and dose rate on *in vitro* organic matter digestibility, gas and methane production of *Eragrostis curvula* hay

Abstract

Some medicinal plants have a tendency to manipulate rumen microbial ecosystem, which in turn might reduce methane emissions. The anti-methanogenic activities of leaf fraction of *Piper betle, Aloe vera*, *Carica papaya*, *Azadirachta indica*, *Moringa oleifera*, *Tithonia diversifolia*, *Jatropha curcas* and *Moringa oleifera* pods were studied at different doses. Plant materials were extracted with pure methanol and subsequently reconstituted at the rate of 2.5, 5.0, 7.5 and 10.0 mg in 1000 mL distilled water. Four mL of each plant extract 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 lower 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 methane volume at dosages of 25 and 50 mg/kg dry matter feed owing 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 was significantly lower in extracts of *T. diversifolia*, *M. oleifera*, *A. indica*, *M. oleifera* pods and higher in *P. betle* and *A. vera*. It can be concluded that methanolic extracts of *A. indica*, *C. papaya*, *J. curcas*, *M. oleifera*, *M. oleifera* pods and *T. diversifolia* resulted in reduced methane production, and thus could be used to manipulate rumen condition, improve feed digestibility and reduce enteric methane emission from ruminants. However, the *in vitro* results need 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: *Eragrostis curvula* hay, methane, organic matter digestibility, volatile fatty acids

2.1 Introduction

The need to produce enough food for the population boom by 2050 (Béné *et al*. 2015) and beyond has gathered momentum. A huge world population requires not only space, which is already evidenced by the current rapid deforestation, but increased production of meat and milk to meet its bodily needs. Scientists, therefore, need to find ways of improving animal feed without having to compete for limited available food for humans. Ruminants for many years have been listed as one of the most valuable and renewable resources in terms of utilization of non-competitive food for meat and milk production. However, enteric methane (CH_4) production from ruminants poses a major challenge, as it causes energy loss, and a consequent reduction in performance. This emission from ruminants also contributes to greenhouse gases, which cause global warming.

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

The development and implementation of natural feeding and management strategies aimed at reducing CH_4 emissions and subsequent increase of dietary energy efficiency are therefore needed. This feeding strategy should not only reduce greenhouse gas contribution of livestock to the atmospheric budget, but enhance production efficiency. This would decrease over-reliance on antibiotic growth promoters such as monensin, which is still in use. Renewed interest in the use of natural feed additives to replace antibiotic ionophores that have been banned in the European Union is a result of the effects on humans of consuming such meat products, such as antibiotic resistant syndrome in humans.

Several studies (Bodas *et al*. 2012; Cieslak *et al*. 2012; Gemeda & Hassen 2015; Pal *et al*. 2015; Theart *et al*. 2015; Hassen *et al*. 2016) have reported the reducing effect of plant secondary compounds on CH_4 emission, improvement in performance and reduction in protein degradation in the rumen. *Piper 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, the presence of phytochemicals, which aids digestion, antimicrobial/antiprotozoal properties, and laxative effects. These plants were

evaluated in order to exploit their potential as feed additives with the aim of identifying plant extracts that are capable of reducing CH_4 production without reducing the *in vitro* gas production and organic matter digestibility of the test substrate. Extracts of all plants used in this study are not toxic to animals.

2.2 Materials and methods

2.2.1 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, South Africa, and 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 ten plants of the same species. The harvested plant materials were authenticated in the herbarium of the Department of Botany, University of Ibadan. A 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 the 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 five days or until constant weight was achieved.

2.2.2 Preparation of plant extracts

Dried leaves and pods were milled to pass through a 1-mm sieve. They were extracted by pure methanol. To 200 g of dried leaves was 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 a 150-µm aperture (Vickers sieve, Durban, South Africa). The filtrate was placed in the fume cupboard until dried. Semi-dried extracts were later transferred to a freeze-drying machine until constant weight was achieved. The dried extract of each plant was reconstituted by dissolving, 2.5, 5.0, 7.5 and 10.0 mg in 1000 mL of distilled water separately to give four levels of concentration. All plant extracts were kept under refrigeration at 4°C until further use.

2.2.3 Chemical analyses

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). Neutral detergent fibre (NDF), acid detergent fibre (ADF)

and acid detergent lignin (ADL) contents were determined using an ANKOM200/220 fibre analyser (ANKOM Technology, Fairport, NY, USA) by the methods described by 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.

2.2.4 In vitro gas and methane production

Inoculum and rumen fluid

Buffer mineral solution was prepared following the procedure of Menke and Steingass (1988). The modification by Mould *et al*. (2005) to replace $MgSO_4 \cdot 7H_2O$ with $MgCl_2 \cdot 6H_2O$ was utilized 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 °C. Rumen fluid was collected from two ruminally fistulated SA Mutton Merino (SAMM) sheep placed on alfalfa hay *ad libitum.* Ruminal fluid was collected before morning feeding, and ~900 mL of fluid was strained from each donor sheep through four layers of cheesecloth into pre-heated thermos flasks. The rumen fluid was transported to the laboratory within 10 min of collection and continuously flushed with CO_2 to minimize changes in microbial population. To avoid O_2 contamination, it was placed in a water bath set at 39 °C.

In vitro incubation

In vitro ruminal incubation was done using a 150-mL serum bottle. Prior to incubation, 400 mg *E. curvula* hay was weighed into each bottle, and 4 mL of already prepared concentrations of leaf extracts of *P. betle, A. vera, C. papaya, A. indica, M. oleifera, T. diversifolia, J. curcas* and *M. oleifera* pod extracts were added to vials containing 400 mg of *E. 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 with 4 mL distilled water added was incubated in four bottles in a randomized complete block design. The whole process was repeated five times in five independent runs. Three blanks were always included with each run. After the addition of rumen fluid, vials with contents were purged with CO_2 gas and immediately closed with a rubber stopper, crimp sealed, and transferred into an incubator set at 39 °C with oscillatory motion of 120 rpm. A modified needle syringe tap, which can be opened and closed, was inserted on each vial. Taps were opened for five seconds to release built-up gas and to set a starting point for all the vials.

2.2.5 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., Laval, QC, Canada) attached to a digital data logger (Tracker 220 series indicators; Omega Engineering Inc.), which is a semi-automated system (Theodorou *et al*. 1994). The transducer with a modified tip was placed tightly over the syringe tap, which was already fitted to the vials. The tap on the syringe was opened, built-up gas in vials was released to the transducer, and the value on the digital data tracker was recorded in psi units. Gas pressure readings were added to the previous readings to give a cumulative value. Gas samples were taken using different syringes through the same system for CH_4 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 a flame ionisation detector and 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 the pull and push method into the GC, which was 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 a cold room, then centrifuged at 4500 *g* for 15 min at 20 °C. All the supernatant were filtered off and 5-mL samples were pipetted and stored at –20°C for acetic, propionic, butyrate, iso-butyric and valeric acid proportion analyses (Ottenstein & 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 two-stage digestion process as modified by Engels and Van der Merwe (1967) was utilized.

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

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

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;

$$\text{CH4}\ (mls) = \text{total gas produced}\ (mls) \times \%\ \text{CH4 in concentration}$$

Partitioning factor was calculated according to Blümmel *et al*. (1997).

2.2.6 Statistical analyses

All statistical analyses were carried out using SAS 9.4 (SAS Institute Inc. NC) and Microsoft Excel. *P*-values < 0.01 were deemed significant for screening purposes. 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 various plant extract dosages. One-way repeated ANOVA was conducted to assess potential differences in response among the grouping variables (25, 50, 75, 100 mg/kg DM feed) compared with the control. Where significant differences were identified, subsequent post hoc analyses was then carried out using Tukey's test. Therefore, each plant extract underwent separate analyses to investigate parameter changes in response to various concentrations of each plant extract compared with the control. This analysis was carried out for each plant individually.

Incubating vials with modified tap with butyl stoppers

2.3 Results and discussion

2.3.1 Chemical composition of Eragrostis curvula

The chemical composition of *E. curvula* hay indicates that amounts of DM and ash were 94% and 18.4%, respectively. Crude protein stands at 5.89%, ether extract at 1.4%, and the fibre

fractions of neutral detergent fibre, acid detergent fibre and acid detergent lignin are 74.6%, 44.7% and 7.9%, respectively.

2.3.2 Total gas and methane production

Significant increases in total gas produced (TGP) were recorded only for *A. vera* and *P. betle* at 75 and 100 mg/kg DM feed ($P < 0.01$) dosage levels as shown in Table 2.1 with no difference in other dosages of *P. betle*, *A. vera* (25 and 50 mg/kg DM feed) 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/kg DM feed dose presented the same trend and reduced total gas volume compared with the control. However, TGP was similar between the control and 75 and 100 mg/kg DM feed dosages of *C. papaya* and *M. oleifera*. Extracts of *A. indica*, *C. papaya*, and *T. diversifolia* at 25 and 50 mg/kg DM feed 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 was 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/kg DM feed, although all had lower values than the control. Significant ($P < 0.01$) decreases in CH_4 volume at 25 and 50 mg/kg DM feed 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, whereas only the lowest dosages (25 and 50 mg/kg DM feed) of *A. indica* differed significantly ($P < 0.01$) from the control. When compared with the control, the use of plant extracts from *C. papaya* did not produce significant CH_4 reduction, whereas *P. betle* plant extracts significantly increased CH_4 volume in response to all dose levels.

Table 2.1. Forty-eight-hour gas production, methane, and *in vitro* organic matter digestibility of *Eragrostis curvula* hay treated with various 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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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

Uppercase letters compare means among all dosages of each plant extracts and the control across the column; lowercase letters compare means along the rows of different plant extracts. Means with different lowercase letters across the rows or uppercase 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; CH4: methane; IVOMD: in vitro organic matter digestibility; s.d.: standard deviation

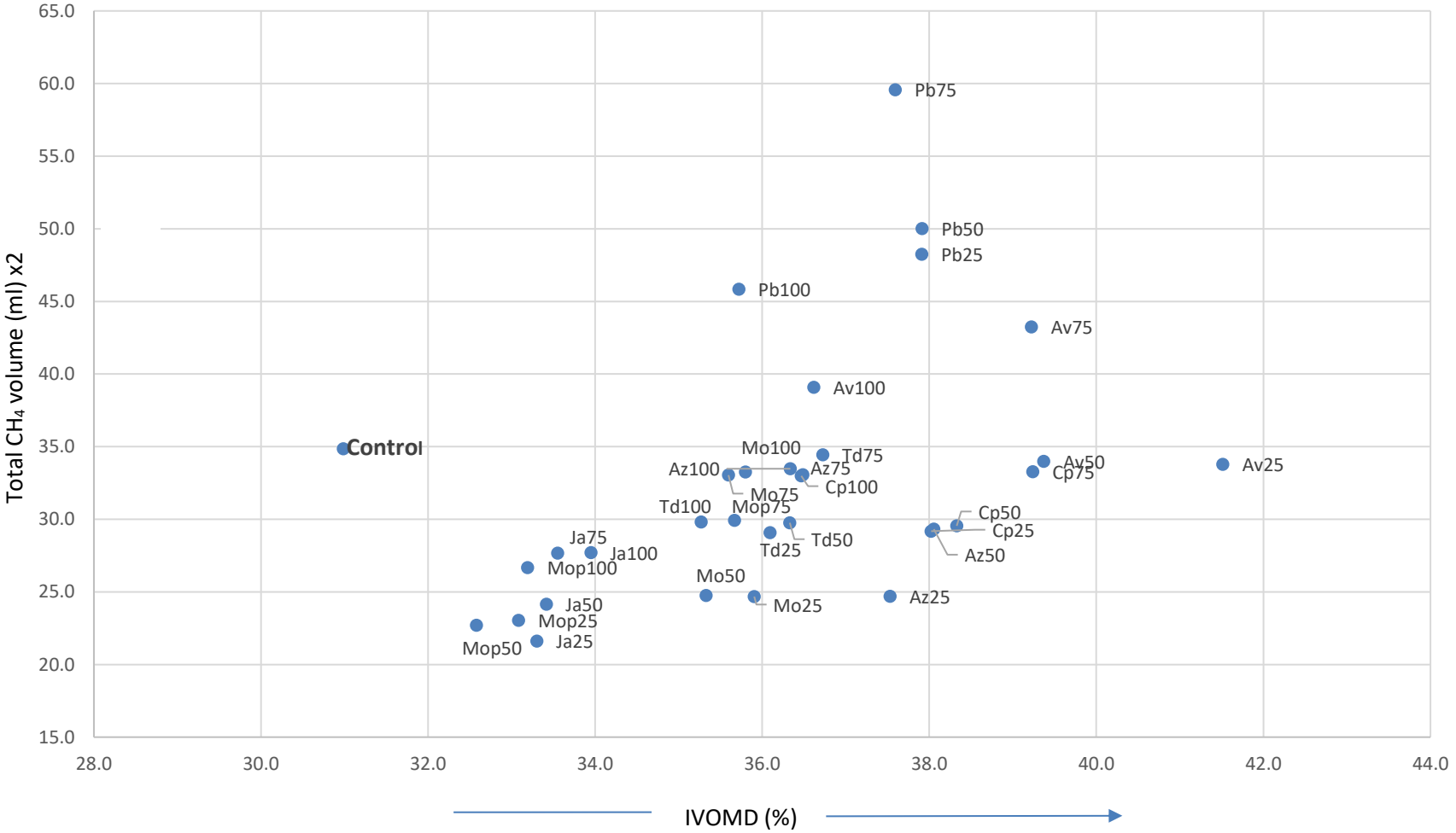


Figure 2.1. Scatter diagram showing CH_4 volume versus IVOMD when *Eragrostis curvula* hay was treated with different plant extracts at diferent doses [no abbrevations in caption]

Plant extracts in the same box are not different significantly (P<0.01). Av: *Aloe vera*, Az: A*zadirachta indica*, Cp:*Carica papaya*, Ja-*Jatropha curcas*, Mo:*Moringa oleifera*, Mop:*Moringa oleifera pod*, Pb:*Piper betle*, and Td:*Tithonia diversifolia* plant extracts. 25,50,75, and 100 signifies dosages of 25, 50, 75 and 100 mg/kg plant extract dosages

2.3.3 Digestibility

All plant extracts improved *in vitro* organic matter digestibility of *E. curvula* hay, which ranges from 5% to 25% (Table 2.1 and Figure 2.1). When compared with the control, *A. vera, A. indica, T. diversifolia, M. oleifera, P. betle, J. curcas* and *C. papaya* extracts significantly ($P < 0.01$) increased in vitro organic matter digestibility (IVOMD) when used in between 25 and 100 mg/kg DM feed dosages. The *M. oleifera* pod showed a significant value only at 75 mg/kg DM feed ($P < 0.01$).

2.3.4 Volatile fatty acids

Individual volatile fatty acids (VFA) were expressed as molar concentration of total volatile fatty acids (TVFA) as shown in Table 2.2. When compared with the control, ruminal acetic acid concentrations were highest for *A. indica, A. vera, M. oleifera* and *P. betle* plant extracts. However, a significantly lower concentration of acetic acid was recorded for extracts of *J. curcas* (75 mg/kg DM feed). All other dosages of *M. oleifera* pods, *J. curcas*, *C. papaya*, *T. diversifolia* and *A. indica* had significantly higher TVFA than or the same as the control. Higher propionic acid concentration was found at dosages of 75 and 100 mg/kg DM feed for *J. curcas* and 50 and 75 mg/kg DM feed for *M. oleifera* pods. Significant lower ($P < 0.01$) concentrations of propionic acid were obtained for *A. vera* (25, 50, 100 mg/kg DM feed), *A. indica* (75, 100 mg/kg DM feed), *M. oleifera* pods (100 mg/kg DM feed), *P. betle* (25, 50, 75, 100 mg/kg DM feed) and *T. diversifolia* (50 mg/kg DM feed). Molar concentrations of butyric acid decreased as the dosages increased from 25 to 75 mg/kg DM feed in *A. indica*, *M. oleifera*, *P. betle*, *J. curcas* and *M. oleifera* pods whereas others did not follow a particular pattern. Other VFA are iso-butyric and valeric acid, which vary in values based on treatments and dosages. The C2 : C3 VFA ratio were significantly ($P < 0.01$) lowest at 25, 50 and 75, 100 mg/kg DM feed 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 volatile fatty acid ratios.

2.3.5 Calculated ratios

Percentages of TGP/IVOMD, CH_4/TGP, CH_4/TVFA, TGP/total volatile fatty acids and PF (mg/mL) are presented in Table 2.3. TGP/IVOMD were all significantly lower ($P < 0.01$) for all plant extracts and dosages used in this study except 75 and 100 mg/kg DM feed dosages of *P. betle*, which did not differ from the control. The methane/TGP expressed significant lower values ($P < 0.001$) at lower doses (25, 50 mg/kg DM feed) 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 with *P. betle* dosages, which had relatively higher values compared with the control. The calculated values for CH_4/TVFA and TGP/TVFA (Table 2.3) showed that all extracts at 25 mg/kg DM feed 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/kg DM feed dosages of *P. betle.* Figure 2.1 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/kg DM feed dosages. *P. betle* extracts had a higher significant CH_4/IVOMD value than those of the control. All the other extracts at all dosages had significant lower CH_4/IVOMD values than those 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/kg DM feed for extracts of *A. indica, C. papaya, J. curcas, M. oleifera* and *M. oleifera* pods. As the dosage increases, CH_4/IVOMD values tend to become higher.

Table 2.2. 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 various plant extracts

VFAs		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
	25mg/kg	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/ kg	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
	75mg/ kg	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
	100mg/ kg	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
	25mg/kg	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/ kg	18.8±.32eC	27.3±.58bcA	24.6±.56cdA	29.1±.77bBC	27.6±.39bA	34.8±.06aA	23.9±.10dB	23.3±.23dB
	75mg/ kg	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
	100mg/ kg	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
	25mg/kg	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/ kg	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
	75mg/ kg	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
	100mg/ kg	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[A]	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
	25mg/kg	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/ kg	7.6±.20bcB	6.9±.53cB	7.4±.19bcB	15.1±.88aAB	9.4±.23bB	14.3±.02aA	7.2±.00cB	5.1±.74dC
	75mg/ kg	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
	100mg/ kg	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
	25mg/kg	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/ kg	2.8±.30abA	2.2±.08cdC	2.3±.10cA	1.7±.07eA	1.7±.06deBC	1.2±.00fB	2.4±.01bcA	2.9±.10aA
	75mg/ kg	2.2±.11abcB	3.0±.01aB	2.4±.04abcA	1.0±.01dC	2.1±.11bcA	1.6±.40cdAB	2.9±.20abA	1.9±.00cC
	100mg/ kg	2.6±.06bAB	3.4±.21aA	2.4±.17cdAB	1.1±.13eC	2.0±.00dAB	2.3±.12bcdA	2.5±.12bcA	2.3±.04bcdB

Uppercase letters compares means among all dosages of each plant extracts and the control across the column: lowercase letters compares means along the rows of different plant extracts. Means with different lowercase letters across the rows or uppercase 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*; s.d.: standard deviation

[A]Iso-butyric and Valeric acids, TVFA – total volatile fatty acids.

Table 2.3. 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 various plant extracts at different dosages

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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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/kg	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/ kg	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/ kg	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/ kg	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

Uppercase letters compares means among all dosages of each plant extracts and the control across the column, lowercase letters compares means along the rows of different plant extracts. Means with different lowercase letters across the rows or uppercase 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; s.d.: standard deviation

The *E. curvula* hay that was 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 utilization. In this study, the intent was to test the effectiveness of the plant extracts on CH_4 emission reduction associated with fermentation of such feeds without adversely affecting digestibility.

Extracts of *P. betle* and *A. vera* increased TGP, CH_4 volume and IVOMD. The presence of biochemical catalysts such as amylase and lipase (Abrahim *et al*. 2012; Basak & Guha 2015; Ray *et al*. 2015) in their leaf 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 a faster rate of substrate degradation in the rumen. Increased digestibility and gas production 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 a 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 owing to higher fermentation activities and the 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 in a reduction in rumen retention time when these extracts were administered. However, feeding a higher dosage level could alter the microbial population and could become toxic to the animals. Furthermore, the presence of anthraquinone, a phenolic compound present in the medicinal plants used in this study, might have played a stimulating effect on the bowels and served as an effective antibiotic agent, which could have generated more gas through its laxative effects. Anthraquinone is employed massively as a digester in paper making (Haddad *et al*. 2009). It works as a redox catalyst on *E. curvula* hay by forming a complex with the reducing end of polysaccharides in cellulose and hemicellulose and 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 contain 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 that can speed up digestion, two of which are catalase and diastase. Diastase breaks down complex starch polymer into its monomers, whereas 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 a 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 (Figure 2.1) with the exception of *A. vera* at 25 mg/kg DM feed. Extracts of *P. betle* and *A. vera* have been used in various studies as laxative agents (Abrahim *et al*. 2012; Nouri *et al*. 2014). The higher IVOMD and lower TGP and CH_4 volumes for *A. indica, J. curcas*, *M. oleifera* and *M. oleifera* pods in low dosages (25 and 50 mg/kg DM feed) could be attributed partly to the presence of *azadirachtin* (Harry-Asobara & Samson 2014), *curcin* (Oskoueian *et al*. 2014), and *alkaloids* (Gautam *et al*. 2007; Ojiako 2014), respectively. Non-toxic azadirachtin has been used as a feed inhibitor and pesticide. Curcin and protease inhibitors have been reported to be present in *J. curcas,* whereas leaves and pods of *M. oleifera* are both rich in alkaloids, which have a bitter taste, making them undesirable for some microbes. Total gas produced, which is an indication of forage degradation characteristics and kinetics of fermentation, has 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 attributed partly 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 (Liu *et al*. 2015; Kumar *et al*. 2016). 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 by causing increased membrane permeability, which finally results in leakage of cytoplasmic constituents. *M. oleifera* leaf and pod extracts contain alkaloids, tannins, saponins and flavonoids, which are strong antioxidants that are capable of inhibiting rumen microbes by bonding with their membranes. Phenolic compounds from this plant were

found to be active against bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*, 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 such as azadirachtin, salannin, meliantriol and nimbin was responsible for the antimicrobial properties of *A. indica*.

The *M. oleifera* leaf tested *in vitro* significantly reduced CH_4, with an increase in VFA and organic matter digestibility, which corresponds with findings of Dey *et al*. (2014). There was a linear relationship between dosage and digestibility until it peaked at 75 mg/kg DM feed. This was true of the results obtained from using various 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 & Samson 2014) by making the rumen more conducive to beneficial organisms to degrade the substrates. According to Oskoueian *et al*. (2014), the presence of phorbol esters could have contributed to the reduction of rumen methanogens, in addition to the alkaloids, saponins, tannin and saponin glycosides that have been attributed to CH_4 reduction (Bhatta *et al*. 2012), whereas the improvement in IVOMD shows the dosage used in this study was not toxic to rumen microbes.

Tithonia diversifolia and *C. papaya* reduced CH_4 partly because of the presence of alkaloids, flavonoids, phenol sesquiterpenes, monoterpenes and diterpenes in the leaf extracts. A dosage of 25 mg/kg DM feed effectively reduced CH4 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, and individual VFA and TVFA were positively related in this study. The response of *C. papaya* 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 could indicate non-toxicity of plant extracts and increased fermentation of feedstuff.

2.4 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 and 50 mg/kg DM feed dosages with simultaneous improvement in IVOMD 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 could be promoted to the next stage of screening to confirm the repeatability of the results using *in vivo* studies.

CHAPTER THREE

Associative effect of plant extracts on anti-methanogenic properties, volatile fatty acids and organic matter digestibility of *Eragrostis curvula* hay

Abstract

Previous studies had revealed beneficial effect of certain medicinal plants in mitigating enteric methane production from ruminant animals. In this study the associative effects of six medicinal plant extracts that are known to reduce *in vitro* methane production were tested in a two-way combination. A 50 : 50 mixture of the extracts from *Aloe vera* (AV), *Carica papaya* (CP), *Azadirachta indica* (AZ), *Tithonia diversifolia* (TD), *Jatropha curcas* (JA) and *Moringa oleifera* (MO) leaves were used to form various cocktails. These plants were harvested fresh and freeze-dried before extracting with pure methanol. Four ml of each reconstituted extract of 50 mg/kg dry matter (DM) feed concentration cocktail was sprayed on 400 mg *Eragrostis curvula* hay and incubated for 48 hours. *In vitro* organic matter digestibility, total gas and methane production, ammonia nitrogen and volatile fatty acids were determined after 48 hours' incubation from the residue, using standard procedures. Cocktails reduced CH_4 production up to 74% without affecting cumulative total gas volume at 48 hours, while individual plant extracts generally improved IVOMD better than cocktails and reduced CH_4 by up to 71%. Cocktails showed positive associative effects on TVFA, CH_4/IVOMD, CH_4/TGP and CH_4/TVFA production. The control group produced the highest concentration of ammonia N and CH_4, while cocktails of AZ+AV, AV+JA, AV+CP, AV+MO, AV+TD, AZ+JA, AZ+CP, AZ+M0, AZ+TD, JA+CP, CP+MO, CP+TD all significantly ($P<0.05$) improved the propionic acid concentration compared with other cocktails, monensin, control or individual plant extract treatments. The study showed all cocktails of plant extracts used in this study proved effective at increasing the propionic acid concentration and total volatile fatty acids. However, in terms of CH_4 production, additive effects were recorded only for cocktail plant extracts except JA+MO compared with the use of individual plant extracts. AZ+AV and AV+JA could be used to lower CH_4 production while increasing animal performance through increased production of VFA.

Keywords: medicinal plants; methane, volatile fatty acids, organic matter digestibility, lucerne, *Eragrostis curvula*, associative effect.

3.1 Introduction

The current competition for feedstuffs between humankind and animals, especially monogastric ones, has kindled renewed interest in the development of ruminant animals as an important source of animal protein. Ruminants' unique ability to convert dietary fibre into milk, meat and wool has made them important components of agro-ecosystems (Thornton 2010; Nathani *et al.* 2015). Over recent years, they have been managed intensively to increase animal performance and production efficiency. The use of antibiotic growth promoters and rumen modulators has enabled individual animals to decrease energy loss through eructation, to increase bowel movements, to suppress the actions and activities of protozoans in the rumen, and to reduce rumen methanogens or render them inactive (Hristov *et al.* 2003; Thornton 2010), which converts surplus H_2 in the rumen to CH_4. Methane is a greenhouse gas that is 23-25 times more potent than CO_2 in ozone layer depletion (IPCC 2007; Kim *et al.* 2013).

However, the recent ban on antibiotic growth promoters in livestock feeding by the European Union (Cieslak *et al.* 2012) has necessitated the search for alternative products, preferably from natural sources. Medicinal plants have been used for various purposes. In traditional medicine, some plants have been identified for the treatment of various diseases (Ke *et al.* 2012). Scientific research of these plants has revealed the presence of certain secondary plant metabolites that are responsible for their activities and ability to cure certain conditions. These secondary metabolites have been developed over time by plants as defensive mechanisms to ensure their survival against grazing, drought and endemic or new disease conditions (De-la-Cruz Chacón *et al.* 2013), as the case may be. In ruminant nutrition, various plant secondary metabolites have been assessed and researched to determine their potential in terms of reducing CH_4, combating disease, and improving digestibility without adversely affecting the animal (Barahona *et al.* 2006; Busquet *et al.* 2006a; Alexander *et al.* 2008).

Akanmu and Hassen (2017) reported that plant extracts of *Aloe vera* (AV), *Azadirachta indica* (AZ), *Carica papaya* (CP), *Jatropha curcas* (JA), *Moringa oleifera* (MO), and *Tithonia diversifolia* (TD) have been shown to reduce *in vitro* methane production of low-quality roughage feed when applied at a 50 mg/kg DM feed dose. These medicinal plants are rich sources of phytochemicals such as flavonoids, saponins, anthraquinones, phenols, azadirachtin, papain and curcin (Devant *et al.* 2007; Cieslak *et al.* 2013; Kim *et al.* 2013; Dey *et al.* 2014; Kumar *et al.*

2016). Various digestive enzymes have been reported in some of these plants, and are important sources of antimicrobial agents (Patra & Saxena 2010). Some of these phytochemicals have shown effectiveness in methane reduction, which can be attributed to their direct effect on rumen protozoa (Santra *et al.* 2012), and ability to boost the immune systems of animals because of their antibacterial properties. Several of these plants are known to contain more than one type of secondary plant metabolite at various concentrations. Thus some of the secondary compounds might be effective in mitigating the activities of methanogens, and others might combat protozoans in the rumen or work to break down cell walls in roughages, suggesting the possibility of having an associative effect from secondary compounds in a cocktail of these plant extracts. This study was designed to further assess the effectiveness of promising plant extracts and investigate the associative effect of two-way combinations (cocktails) of six plant extracts of AV, AZ, CP, JA, MO and TD on gas and CH_4 production, organic matter digestibility and volatile fatty acids concentrations *in vitro*.

3.2 Materials and Methods

3.2.1 Collection and preparation of plant extracts

Plant materials were collected, authenticated and transported as reported in Section 2.2.1. Fresh samples of 4 kg each of AV, AZ, TD, CP, JA and MO were harvested from growing trees, washed and freeze-dried until constant weight was achieved. Dried samples were milled through 1-mm sieve and extracted by dissolving 200 g dried plant materials in flasks containing 2000 ml methanol. The mixture was placed on a shaker at 20 °C for 96 hours, and the contents of the flask were filtered by squeezing through a 150 µm aperture. The excess methanol in the filtrate was evaporated in the fume chambers and then transferred to the freeze dryer for complete dryness. All extracts recovered were in powder form except those from AV, which yielded a stiffened jelly-like substance. The crude extracts were stored at 4 °C for further use.

3.2.2 Experimental design and treatments

Extracts of AV, AZ, TD, CP, JA and MO were reconstituted by dissolving 5.0 mg of each extract in 1000 ml of distilled water. Equal proportions of each plant extract solution were combined to form two-way cocktails and 4 ml was administered as various treatments to 400 mg *Eragrostis curvula* hay in the following order: AZ+AV, AV+JA, AV+CP, AV+MO, AV+TD, AZ+JA,

AZ+CP, AZ+MO, AZ+TD, JA+CP, JA+MO, JA+TD, CP+MO, CP+TD, and MO+TD. Monensin sodium at a recommendation of 5.0 mg/kg was dissolved in distilled water and used as a positive control, while 4 ml distilled water was added as a placebo to the second control group. Individual plant extracts were included in these treatments to test the difference between expected vs observed values using a chi square test. A total of 23 treatments (2 control, 6 single plant extracts, and 15 cocktails) were tested and in all cases 4 ml of plant extract/monensin/water were administered.

3.2.3 Preparation of rumen fluid and buffer mineral solution

Two ruminally fistulated SA Mutton Merino (SAMM) sheep fed alfalfa hay *ad libitum* were used as rumen fluid donors throughout the experiment. Ruminal fluid was collected before morning feeding. Approximately 1000 ml of fluid was strained from each donor sheep through four layers of cheesecloth into pre-heated thermos flasks. The rumen fluid was transported to the laboratory within 10 minutes of collection. It was placed in water bath set at 39 °C, mixed together, and continuously flushed with CO_2 to sustain anaerobic environment and reduce O_2 contamination. A buffer mineral solution was prepared according the procedure of Meinke and Steingass (Menke & Steingass 1988). The replacement of $MgSO_4.7H_2O$ with $MgCl_2.6H_2O$ in the buffer media was done to reduce the level of SO_4 in the media (Mould *et al.* 2005). Reducing solution L-cysteine and $Na_2S_9.H_2O$ was added as recommended. The solution was bathed at 39 $^{\circ}$C.

3.2.4 In vitro incubation for ruminal gas production

In vitro ruminal incubation was done using 100 ml serum bottles. Prior to incubation, 400 mg *Eragrostis curvula* hay was weighed into each bottle. Then 4 ml of already prepared single and two-way cocktails of AV, CP, AZ, MO, TD and JA was added to vials containing 400 mg of *E. curvula* hay, 25 ml of prepared media and 15 ml rumen fluid. In each run, all treatments and the control groups had three replicates in a randomized complete block design. The whole process was repeated five times by having five independent runs. All replicates in a run were used to calculate average values, and these runs were used as blocks. Three blanks were always included in each run. After adding rumen fluid, vials with contents were purged with CO_2 gas and immediately closed with a rubber stopper, crimp sealed, and transferred to an incubator set at 39 $^{\circ}$C with an oscillatory motion of 120 rpm. A modified needle syringe tap, which can be opened and closed,

was inserted in each vial. These taps were opened for five seconds to release built-up gas and to set a starting point for all the vials.

3.2.5 Chemical analysis, total gas, methane, volatile fatty acids and in vitro organic matter digestibility

The chemical analysis of the test feed sample *E. curvula* was carried out (AOAC 2000). Nitrogen was analysed with C/N-Analyser, (Leco-Analysator Type FP-2000 (Leco Instrumente GmbH, Kirchheim, Germany) and crude protein was obtained by multiplying N by 6.25. Ether extract content was analysed for using Tecator Soxtec (HT6) system. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were all analysed using ANKOM200/220 fibre analyser (ANKOM Technology, Fairport, NY), as described by Van Soest *et al*. (1991). Gas produced at 3, 6, 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 (Mauricio *et al.* 1999). The transducer with a modified tip was placed tightly over the syringe tap that had already been fitted to the vial. The tap on the syringe was opened, built-up gas in the vials was released to the transducer, and the value on the digital data tracker was recorded in psi units. Gas pressure readings taken at each time interval were added to previous readings to give a cumulative value. Gas samples were taken from all vials at 3, 6, 12, 24 and 48 hours for methane gas production from all replicated bottles.

Methane concentration in each sample was analysed by gas chromatography equipped with flame ionization detector (FID) and 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 the pull and push method into the GC, which was already calibrated with standard CH_4 and CO_2. Blanks were analysed and used to correct the CH_4 produced by the inoculum. Fermentation was terminated at 48 hours by placing all these bottles on ice in the cold room, and centrifuging at 4500 g to take 5 ml supernatant samples to determine acetic, propionic, butyrate, iso-butyric, iso-valeric and valeric acids (Ottenstein & Bartley 1971). Two-stage *in vitro* organic matter digestibility of Tilley and Terry (1963) was modified by incubating the residue from fermentation vials with HCL-pepsin solution for 48 hours.

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

$$\text{Gas volume (mls)} = \frac{\text{Vh}}{\text{Pa}} \times \text{Pt}$$

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)

Methane concentration captured from GC in ppm was converted to ml using the formula;

$$\text{Methane (mls)} = \text{Total gas produced (mls) x\% methane concentration of a gas sample}$$

3.2.6 Statistical Analysis

All data were subjected to analysis of variance and analysed with SAS 9.3. Treatment means were compared using Tukey's test and differences were considered statistically significant if $P<0.05$. Principal component analysis was carried out with PAST 3 software. Associative effects were analysed using a chi square test (SAS 2011). Lack of significant differences between expected and observed values of a parameter indicated an additive effect, while the presence of significant differences between expected and observed values indicated the presence of positive or negative associative effect between the two plant extracts when used in the form of a cocktail. The associative effect from the combination of plant extracts was calculated as stated below:

$$Associative\ effect\ = (\text{observed}\ value - expected\ value)\ \text{x}\ 100/\text{expected value}$$

Chi square was calculated using the formula below. Significance was tested by comparing chi square values with critical values on the chi square distribution table.

$$Chi\ square = \sum (\text{observed}\ value - expected\ value)\text{^}2/\text{expected value}$$

The expected value for each cocktail was calculated from the two single plant extracts that form the cocktail as follows

$$The\ expected\ value\ = \sum (\text{observed}\ value\ plant\ \frac{plant\ extract1}{2} + \frac{plant\ extract\ 2}{2}\ ).$$

3.3 Results and Discussion

Principal component analysis (PCA) carried out on all fermentation parameters gave three major principal components (PC) responsible for about 97% of the total variation. PC1 alone explained 70% of total variation and is positively correlated with TGP, TGP/IVOMD, CH_4/IVOMD, TVFA, CH_4/TGP and negatively correlated with IVOMD. It also showed that the control and monensin each had more than 50% positive contribution, while AV+CP, AV+MO, and AZ+JA have more than 25% contribution. MO+TD and MO have 35% negative contribution. All the single plant extracts have negative values for PC1. Table 3.1 presents the principal component loadings responsible for the variations in the principal component analysis. The main parameters that are responsible for PC1 are TGP (43%), CH_4/IVOMD (42%), and TGP/IVOMD (81%), while the main parameter for PC2 is TVFA (82%). PC3 is composed of CH_4/TVFA (43%), CH_4/IVOMD (47%), CH_4/TGP (39%), TGP (30%) and CH_4 (25%). PC3 is positively correlated with CH4/IVOMD, CH_4/TVFA, and CH_4/TGP and negatively correlated with TVFA. This means the control, CP+TD and JA+MO had higher CH_4/IVOMD, CH_4/TVFA and CH_4/TGP values when compared with the cocktails of AZ+AV, AV+JA, AV+CP and AV+MO. The former group had lower TVFA production, while the latter group has relatively higher TVFA.

Figure 3.1 shows a distribution of all single extracts and cocktails across PC1 and PC2. MO+TD had lower TGP, TGP/IVOMD, and CH_4/IVOMD when compared with the control, monensin, AV+MO, AV+CP and AZ+JA. Among the latter group, AV+MO had lower TVFA production and higher TGP/IVOMD. Cocktails AV+CP, AZ+JA, and AV+MO had higher TGP per unit of IVOMD when compared with their individual plant extracts, which suggests the presence of associative effect. The combination of AZ+CP, monensin and control had increased CH_4 and CH_4 per unit IVOMD, with opposite reductive effect of CH_4 per unit IVOMD from AZ+TD, JA+MO, JA +TD, JA+CP, AZ+MO, CP+TD, CP+MO. Cocktails AV+JA, AV+TD and AZ+AV had increased acetic acid and TVFA, which may be traced to phytochemicals present in AV, as reported in our previous study (Akanmu & Hassen 2017). Broadly, Figure 3.2 shows the tendency for associative effect in IVOMD for CP+TD, JA+MO, JA+TD, AZ+TD, JA+TD, and AZ+MO, which had improved values compared with the individual plant extracts, while AV+JA had negative associative effect in IVOMD. AZ+AV, CP+MO and AV+JA had positive associative effect in terms of TVFA in Figure 3.3. Figure 3.4 is a three-dimensional plot showing the

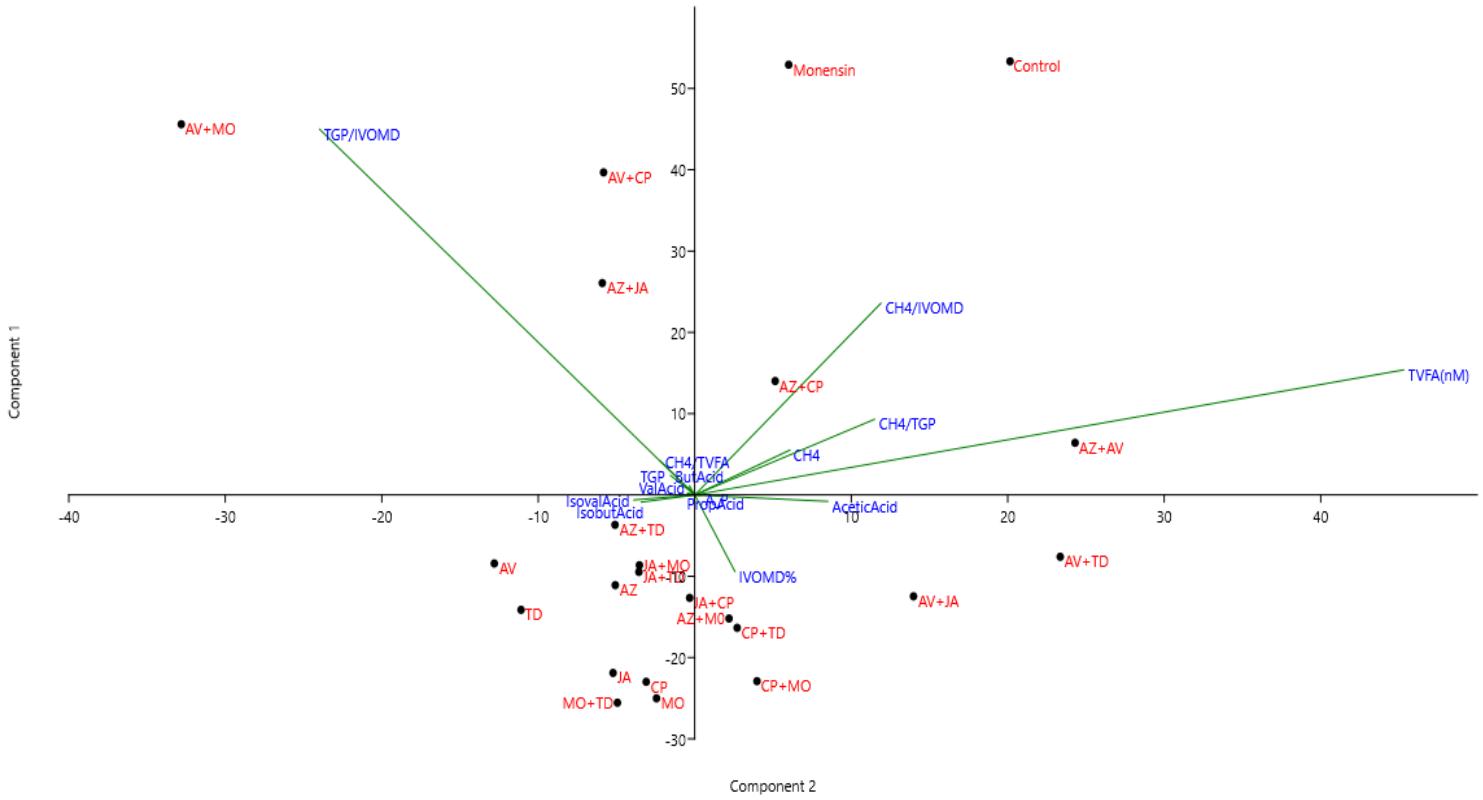
relationship of TVFA with CH4/IVOMD and TGP/IVOMD. The cocktail that resulted in higher TGP/IVOMD, lower CH4/IVOMD and higher TVFA is AZ+AV. The dendrogram that showed percentage similarity is presented in Figure 3.5. Three major groupings were identified. Monensin and control showed around 95% similarities for all parameters. AZ+AV and AV+JA shared the same tree cluster, while all other cocktails and individual plant extracts occupied the third cluster as response to all fermentation parameters. The results indicated close similarities in the third cluster among the cocktails and the individual plant extracts.

Like monensin and the control treatment, cocktails of AV+MO, AV+CP, AZ+JA, and AZ+CP plant extracts generally resulted in higher TGP/IVOMD and TGP, but cocktails AV+MO, AV+CP and AZ+JA had relatively lower TVFA, CH_4/IVOMD, CH4/TGP, and acetic acid values when compared with the control, monensin and cocktail of AZ+CP. The higher TGP/IVOMD value observed for AV+MO is associated with the low IVOMD and high TGP values, whereas the higher CH_4/IVOMD and CH_4/TGP observed for the control, monensin and AZ+CP is associated partly to the relatively higher CH_4 and lower TGP values. On the other hand, all the individual plant extracts and cocktails of MO+TD, CP+MO, AZ+MO and JA+CP resulted in higher IVOMD values. However, like monensin and the control, cocktails of AZ+AV, AV+TD and AV+JA had lower TGP/IVOMD values, but higher TVFA, CH_4/IVOMD, CH_4/TGP and CH_4 values. The high TVFA is closely related to higher acetic acid and lower butyric acid values. In contrast, most of the individual plant extracts and cocktails of AZ+TD and MO+TD, reduced TVFA, CH_4/IVOMD, CH_4/TGP, acetic acid, while increasing TGP/IVOMD and iso-butyric acid values.

Table 3.1. Principal component loadings of fermentation parameters of *Eragrostis curvula* hay fermented with individual and combinations of plant extracts

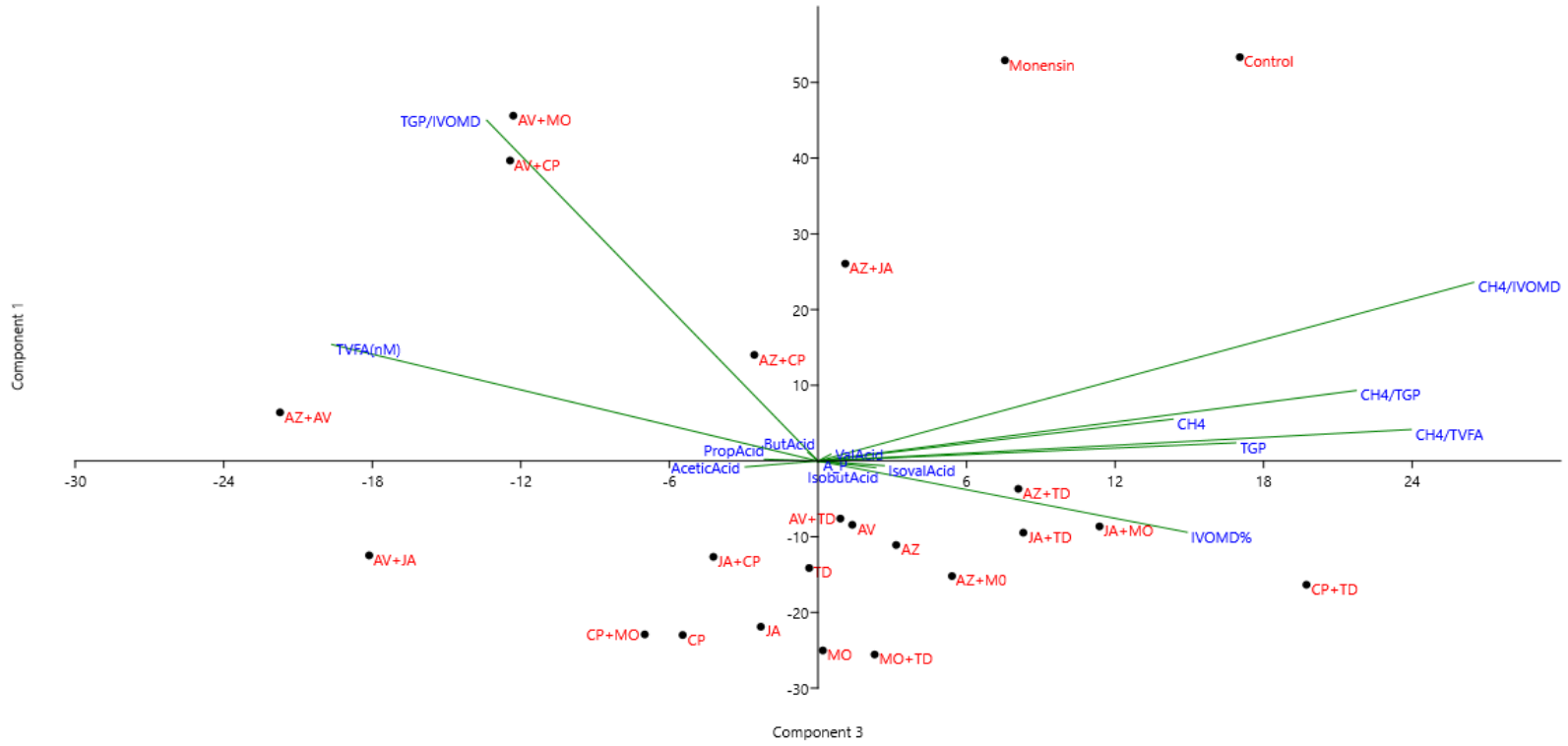
Parameters	PC 1 x100	PC 2 x100	PC 3 x100
TGP	43.53	-2.81	30.54
CH_4	9.99	10.99	25.95
IVOMD	-17.03	4.63	26.99
CH_4/TGP	16.85	20.79	39.35
CH_4/IVOMD	42.70	21.53	47.93
TGP/IVOMD	81.47	-43.40	-24.22
Acetic acid	-1.41	15.38	-5.31
Propionic acid	0.43	-0.84	-3.92
Iso-butyric acid	-1.58	-6.18	4.21
Butyric acid	2.06	-0.62	-0.73
Iso-valeric acid	-1.09	-7.03	4.83
Valeric acid	1.59	-0.68	0.92
Acetic/Propionic	-0.07	0.72	0.05
TVFA	27.85	82.01	-35.55
CH_4/TVFA	7.53	-3.96	43.37
NH_3-N	0.53	0.52	0.95
Eigenvalue	657.90	154.76	102.91
% variance	69.82	16.42	10.92

PC: principal component, TGP: total gas produced, CH_4: methane, IVOMD: *in vitro* organic matter digestibility, TVFA: total volatile fatty acids, NH_3-N: ammonia nitrogen.



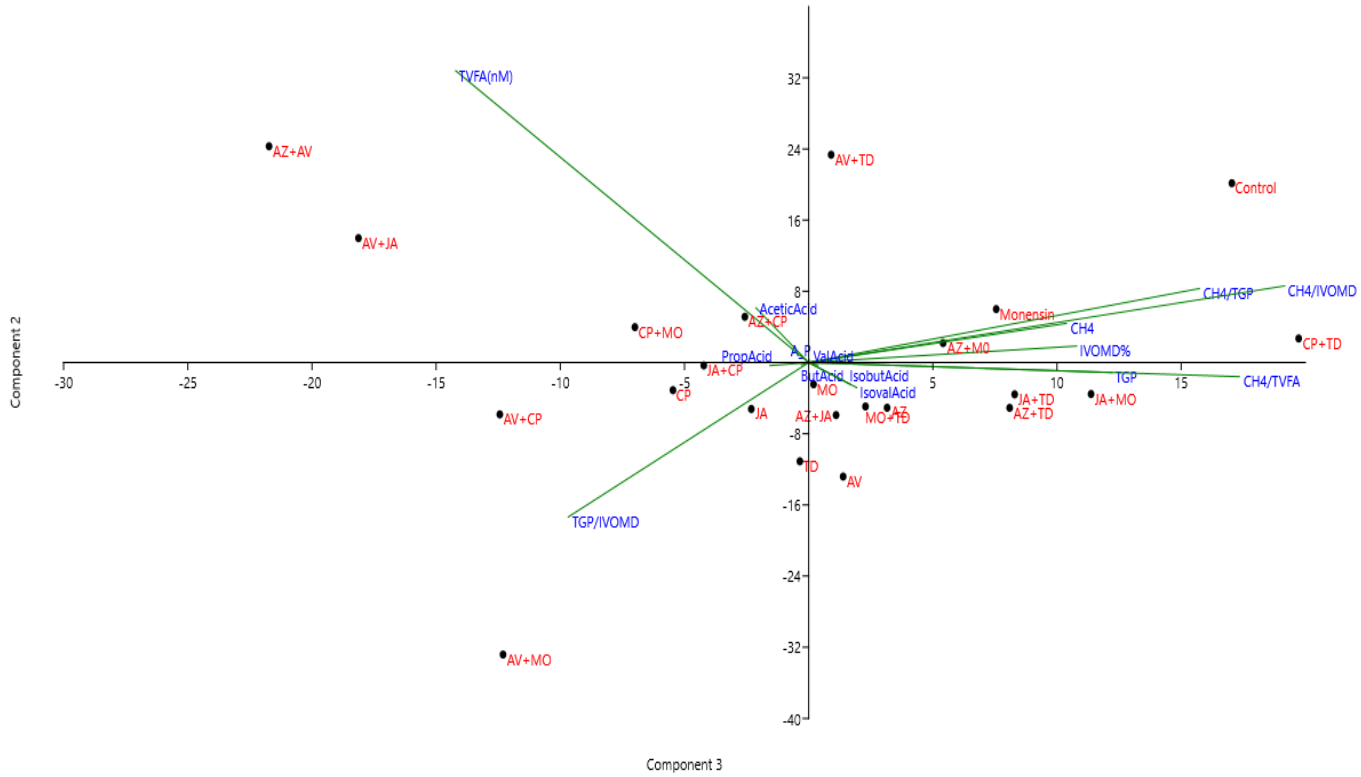
AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia*, TGP: total gas produced, IVOMD: *in vitro* organic matter digestibility, TVFA: total volatile fatty acids

Figure 3.1. Principal component analysis Plot 1 vs Plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with individual and cocktail plant extracts.



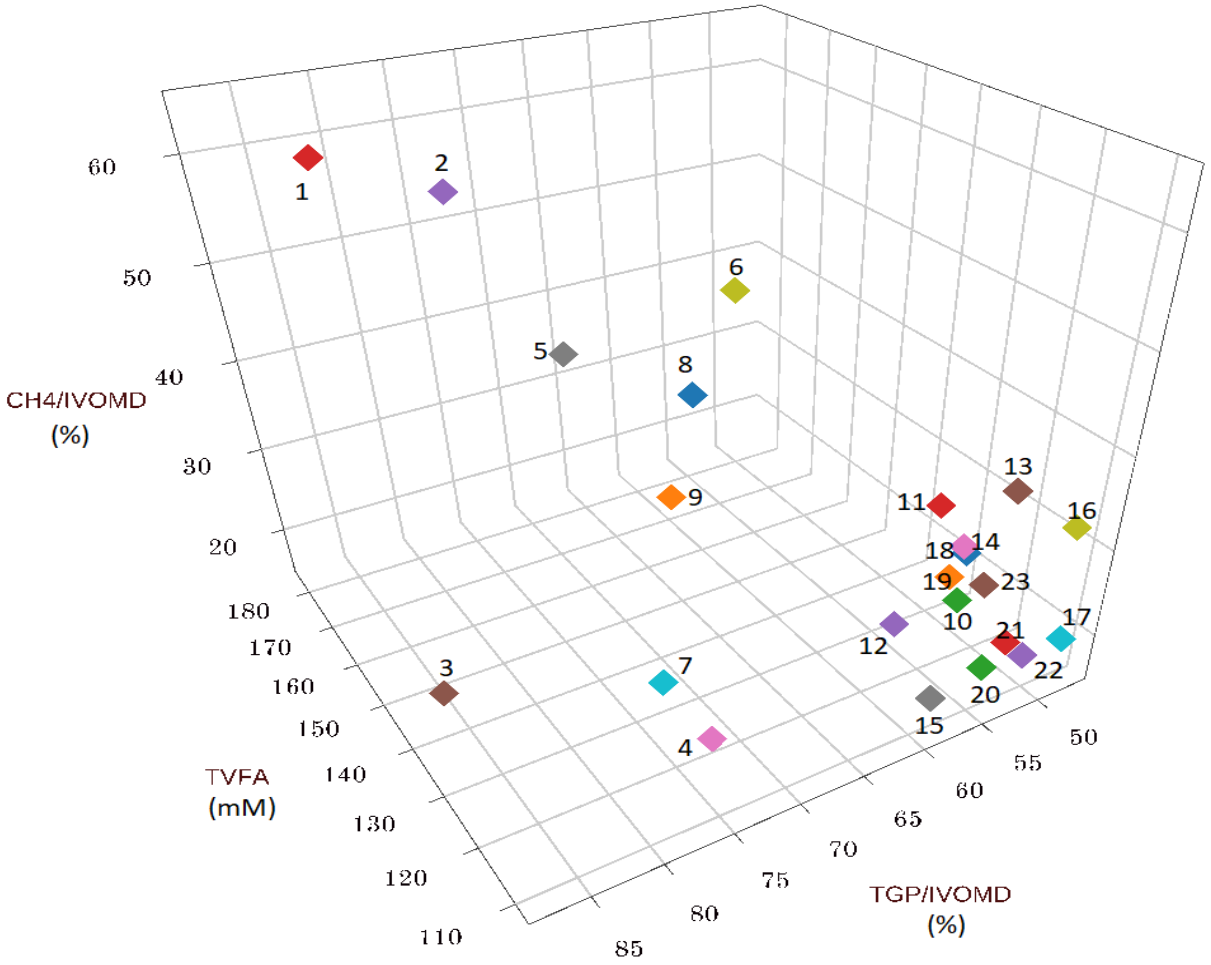
AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia*, TGP: total gas produced, IVOMD: *in vitro* organic matter digestibility, TVFA: total volatile fatty acids

Figure 3.2. Principal component analysis Plot 1 vs Plot 3 of all fermentation parameters of *Eragrostis curvula* hay fermented with individual and cocktail plant extracts.



AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia*, TGP: total gas produced, IVOMD: *in vitro* organic matter digestibility, TVFA: total volatile fatty acids

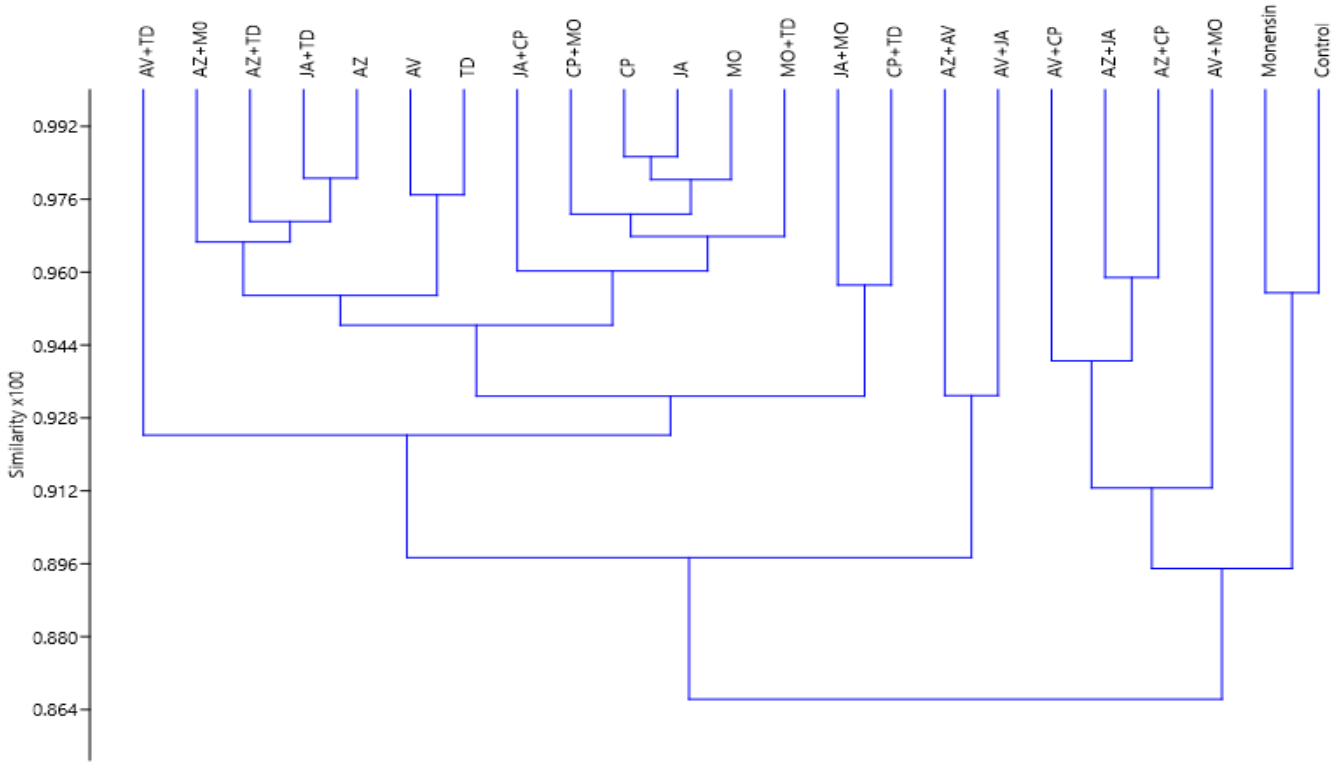
Figure 3.3. Principal component analysis Plot 2 vs Plot 3 of all fermentation parameters of *Eragrostis curvula* hay fermented with individual and cocktails of plant extracts.



KEY:

1	Control
2	Monensin
3	AZ+AV
4	AV+JA
5	AV+CP
6	AV+MO
7	AV+TD
8	AZ+JA
9	AZ+CP
10	AZ+M0
11	AZ+TD
12	JA+CP
13	JA+MO
14	JA+TD
15	CP+MO
16	CP+TD
17	MO+TD
18	AV
19	AZ
20	CP
21	JA
22	MO
23	TD

Figure 3.4. Three-dimensional plot showing the relationship of total volatile fatty acids with methane and total gas production per unit organic matter digestibility when *Eragrostis curvula* hay was fermented with individual and cocktails of plant extracts.



AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia*

Figure 3.5. Dendrogram of total gas produced, methane, *in vitro* organic matter digestibility, total volatile fatty acids and other fermentation parameters when 400 mg *Eragrostis curvula* hay was fermented with individual and cocktails of plant extracts.

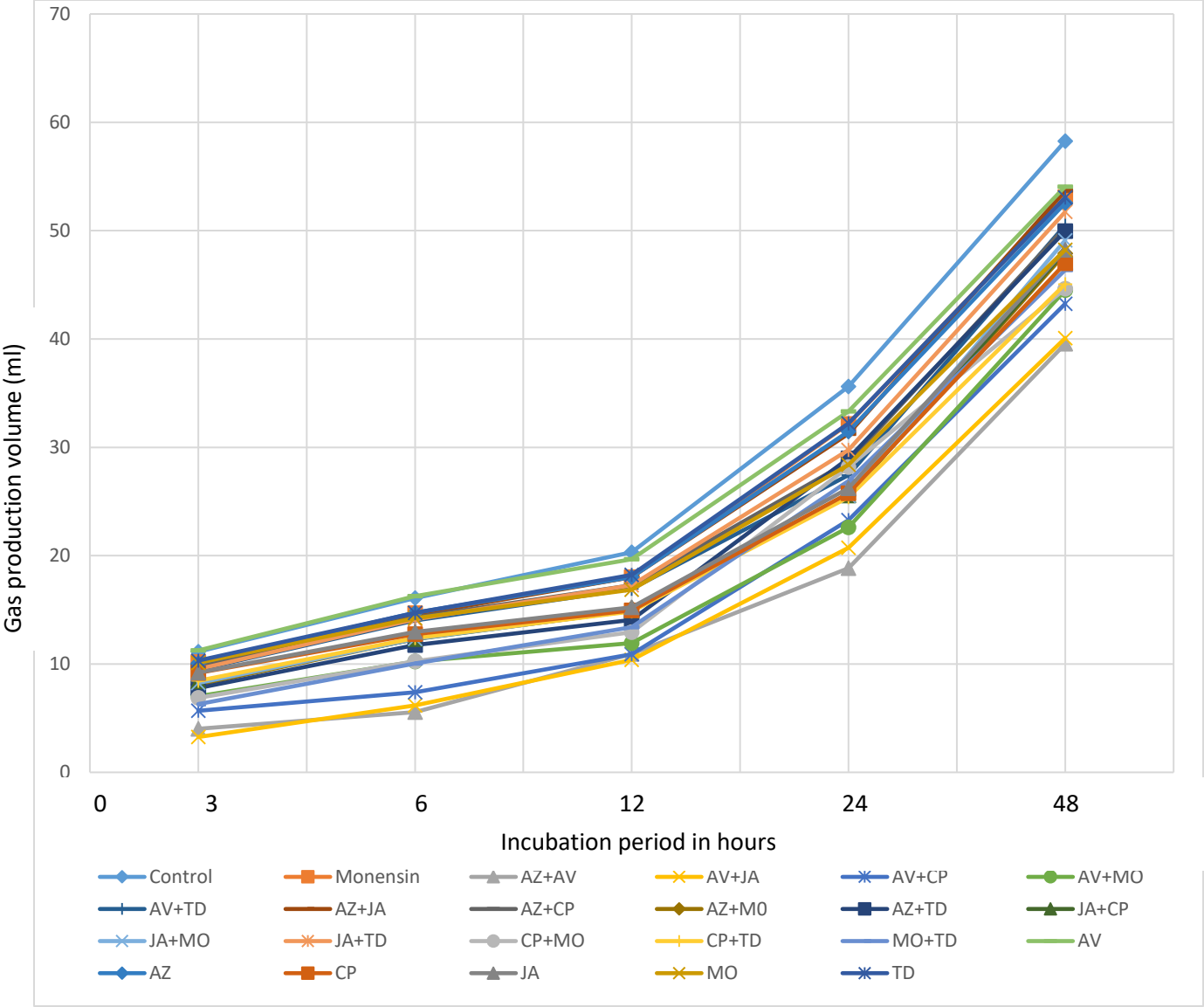
The substrate, *E. curvula* hay used in this study had 5.12% crude protein (CP) and 92% DM. Ash, ether extract, NDF, ADF and ADL were 9.1, 1.3, 75.5, 44.5, and 8.1%, respectively. The substrate's low quality offers an opportunity to detect effectiveness of individual and cocktails plant extracts in reducing methane production. Gas production patterns and average values of TGP for the control, and all treatments are shown in Figure 3.6 and Table 3.2, respectively. Cumulative gas produced after 48 hours revealed that AV+JA and AZ+AV significantly ($P<0.05$) reduced TGP volume compared with the control. However, TGP differences between the control, other plant extracts and cocktails and monensin were not significant ($P>0.05$), although plant extracts showed a tendency to reduce TGP after three hours of incubation (Figure 3.6). Cocktails of plant extract did not show any difference in terms of TGP ($P>0.05$) compared with the individual plant extracts. The initial higher TGP at three hours' incubation by the control (Figure 3.6) was sustained throughout the incubation period, probably because there was no treatment effect, whereas, similar to other studies, the antibiotic growth promoters and plant extracts were reported to have depressing effects on gas production (Akanmu & Hassen 2017).

Methane production and *in vitro* organic matter digestibility are presented in Table 3.2. Leaf extracts of AV, AZ, TD, CP, JA and MO significantly ($P<0.05$) reduced CH_4 volume, with the lowest and highest reductions of 57% and 71% recorded in leaf extracts of AZ and CP, respectively. Generally, the cocktails used in this study reduced CH_4 volume compared with the control treatment. Between 50% and 60% CH_4 volume reductions were recorded for cocktails of AV+CP, AZ+CP, AZ+MO, AZ+TD, JA+TD, while between 60% and 74% reductions in CH_4 volume were recorded for cocktails of AZ+AV, AV+JA, AV+MO, JA+CP, CP+MO and MO+TD. Although the monensin group appeared to have shown a tendency to lower CH_4 volume when compared with control, the difference was not significant ($P>0.05$). Leaf extracts of CP and JA and cocktails of AV+JA, and CP+MO were most effective in reducing methane production compared with monensin.

Monensin showed a tendency to reduce CH_4, perhaps through relative reduction in TGP. It can therefore, be postulated that the plant extract activities are partly similar to those reported for antibiotic growth promoters, which work by inhibiting TGP and CH_4 because of the presence of polyether ionophores. This is a broad-spectrum antibiotic that has huge anti-parasitic action and works by blocking the intracellular transport of Golgi apparatus proteins of selected cells (Łowicki

& Huczyński 2013) and catalyzes the exchange of Na^+ for H^+ across cellular membranes, thereby reducing the actions of protozoans and methanogens. Most of the protozoans have proteolytic activities, and their activities were reduced owing to the direct effect of plant extracts as judged by lower NH_3-N values recorded for plant extracts compared with the control treatment. This reduction of CH_4 by medicinal plants is in agreement with other studies (Busquet *et al.* 2006b; Kim *et al.* 2012; Patra and Yu 2013). There is evidence that antimicrobial properties in these plants might have significantly influenced methanogenesis by affecting the protozoan population indirectly.

Methane production was generally reduced significantly ($P<0.05$) by all plant extracts and their cocktails. However, the lack of significant differences witnessed between individual and cocktail plant extracts suggests minimal or no associative effect for cocktails to reduce CH_4 production. Significant CH_4 reductions of more than 70% by CP, JA, CP+MO and AV+JA would generally have been due to the antimicrobial properties of and the phytochemicals in the individual plant extracts. Phytochemical screening (Ayoola and Adeyeye 2010) indicated the presence of saponins, cardiac glycoside and alkaloids in the green leaf of CP. Cardiac glycosides have been reported to work (Fozzard & Sheets 1985) in the form of polyether ionophores, which, as discussed earlier, could be responsible for the significant reduction in CH_4. Lower CH_4 values without adversely affecting TGP indicates a reduction of energy loss by the animal as the methanogen activities are reduced selectively without negative effects on fibrolytic bacteria.



Plant extracts in the same box are not significantly different

Figure 3.6. Gas production pattern of 400 mg *Eragrostis curvula* hay treated with individual and combinations of plant extracts.

Table 3.2. Total gas produced, methane, *in vitro* organic matter digestibility and total volatile fatty acids of 400 mg *Eragrostis curvula* hay sprayed with individual and a combination of plant extracts

Treatments	TGP (ml)	CH_4 (ml)	IVOMD (%)	TVFA (mM)	TGP/ IVOMD (%)	TGP/ TVFA	CH_4/TGP (%)	CH_4/ IVOMD (%)	CH_4/ TVFA
Control	58.2a	23.1a	36.1a-d*	84.7ab	161ab	0.69abc	39.5a	63.8a	27.3a
Monensin	53.1ab	18.0ab	31.4c-f	75.6abc	169ab	0.72abc	33.7ab	57.1ab	24.2ab
AZ+AV	40.5b	7.97bc	30.4def	87.8a	130ab	0.45c	20.1ab	26.1bc	9.00b
AV+JA	40.1b	6.36c	34.1b-f	73.2abc	117b	0.54bc	15.2b	18.6c	8.30b
AV+CP	43.2ab	10.1bc	26.2ef	68.9abc	167ab	0.63abc	23.5ab	39.6abc	14.9ab
AV+MO	44.5ab	8.43bc	24.5f	48.7c	184a	0.92abc	18.8b	35.6abc	17.4ab
AV+TD	50.5ab	12.6abc	44.2a	77.1abc	115b	0.65abc	25.1ab	28.8bc	16.5ab
AZ+JA	53.6ab	12.8abc	34.8a-e	62.4abc	154ab	0.89abc	24.2ab	36.9abc	21.7ab
AZ+CP	50.3ab	11.9bc	36.1a-d	68.6abc	140ab	0.74abc	23.9ab	33.2abc	17.5ab
AZ+M0	47.8ab	10.5bc	41.5ab	54.6abc	115b	0.89abc	21.9ab	25.3bc	19.6ab
AZ+TD	49.9ab	11.5bc	39.4a-d	51.1bc	127ab	1.00ab	22.4ab	29.5bc	23.6ab
JA+CP	48.1ab	8.25bc	39.7a-d	57.1abc	121ab	0.84abc	17.2b	20.9bc	14.4ab
JA+MO	49.1ab	13.2abc	40.6abc	47.8c	120ab	1.03ab	26.7ab	32.4abc	15.8ab
JA+TD	51.7ab	11.2bc	42.2ab	51.0bc	122ab	1.02ab	21.8ab	26.7abc	22.1ab
CP+MO	44.6ab	6.96c	40.1abc	57.9abc	111b	0.77abc	15.5b	17.2c	11.8ab
CP+TD	45.1ab	13.4abc	41.1abc	48.1c	109b	0.94abc	30.3ab	32.9abc	27.9a
MO+TD	46.3ab	7.87bc	42.1ab	46.8c	110b	0.99ab	17.2b	18.7c	16.8ab
AV	54.1ab	8.72bc	41.6ab	47.0c	129ab	1.15a	16.1b	20.9c	18.7ab
AZ	52.5ab	9.72bc	42.5ab	51.5bc	123ab	1.02ab	18.5b	22.8c	1839ab
CP	46.9ab	6.56c	41.1abc	51.9bc	114b	0.91abc	13.9b	15.9c	12.7ab
JA	48.2ab	7.18c	41.7ab	49.4c	115b	0.97abc	14.8b	17.2c	14.5ab
MO	48.2ab	7.72bc	43.4ab	49.8c	111b	0.97abc	15.9b	17.8c	15.5ab
TD	53.1ab	7.87bc	42.4ab	46.9c	125ab	1.13a	14.8b	18.5c	16.8ab
SEM	3.74	2.65	2.39	8.40	16.3	0.12	5.10	7.9	4.31

Means with different letters across the column are significantly different ($P \leq 0.05$). *a-d equals a,b,c and d. AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia.*

Table 3.3. Ammonia-nitrogen and molar proportions of volatile fatty acids expressed as the percentage of total volatile fatty acids of *Eragrostis curvula* hay sprayed with individual and combination of plant extracts

Parameters	NH_3-N (mg/100ml)	Acetic (mM)	Propionic (mM)	Butyric (mM)	Iso-butyric (mM)	Valeric (mM)	Iso-valeric (mM)	Acetic/ Propionic
Control	5.12a	39.1ab	20.5g	12.9a-d*	7.69fgh	11.4abc	8.32cd	1.89a
Monensin	5.08b	37.5ab	22.0d-g	12.1a-d	7.88d-h	11.7ab	8.64cd	1.72ab
AZ+AV	4.40m	42.5a	23.5a-d	10.8de	6.74h	10.1cde	6.08e	1.80ab
AV+JA	4.51i	37.0b	22.9a-f	13.7ab	7.45gh	10.4b-e	8.43cd	1.62abc
AV+CP	4.69e	33.8cd	23.2a-e	14.1a	8.10c-h	10.5a-e	10.0a-d	1.46abc
AV+MO	4.21p	29.6d	24.0a	14.2a	9.69a-e	11.6a	10.3abc	1.23c
AV+TD	4.49ij	38.4ab	22.6a-f	12.2a-d	7.76e-h	11.0a-e	7.79de	1.70ab
AZ+JA	4.79c	34.4bcd	22.6a-f	12.9a-d	8.89b-g	11.1a-d	9.87a-d	1.53abc
AZ+CP	4.54h	36.6bc	23.9ab	12.4a-d	7.47gh	10.5a-e	8.89bcd	1.53abc
AZ+M0	4.14q	34.7bc	23.5a-d	13.0abc	8.99b-g	10.0cde	9.57a-d	1.48abc
AZ+TD	4.42l	34.8bc	23.9ab	12.2a-d	8.98b-g	9.61e	10.3abc	1.45abc
JA+CP	4.47j	35.1bc	23.8abc	12.4a-d	8.85b-g	10.5a-e	9.08a-d	1.47abc
JA+MO	4.44k	31.5cd	22.3b-g	12.8a-d	10.7ab	11.5abc	11.1ab	1.41bc
JA+TD	4.43kl	35.9bc	22.0c-g	11.5cde	9.89abc	10.2b-e	10.1abc	1.63abc
CP+MO	4.18p	38.1ab	22.3a-f	11.5cde	8.67b-g	9.91de	9.38a-d	1.71ab
CP+TD	4.64f	34.2bcd	22.3a-f	12.6a-d	9.80a-d	10.3b-e	10.5abc	1.53abc
MO+TD	4.57h	34.0bcd	22.1c-g	11.7b-e	9.49a-f	11.2a-d	11.2a	1.54abc
AV	4.79c	36.1bc	21.7efg	9.99e	11.3a	11.1a-d	9.77a-d	1.65abc
AZ	4.75d	36.9b	22.0c-g	11.7b-e	9.08b-g	9.81de	10.3abc	1.67abc
CP	4.50h	37.6ab	21.4fg	11.7b-e	9.58a-f	9.87de	9.64a-d	1.75ab
JA	4.33n	37.2b	21.3fg	11.4cde	9.66a-e	10.2b-e	10.3a-d	1.76ab
MO	4.54gh	37.9ab	22.2b-g	11.2cde	8.9b-g	10.1cde	9.58a-d	1.70ab
TD	4.23o	37.9ab	21.7efg	11.0cde	9.18b-g	10.0cde	10.0a-d	1.74ab
SEM	0.02	7.06	0.79	1.09	0.99	0.58	1.32	0.05

Means with different letters across each column are significantly different ($P \leq 0.05$). *a-d equals a,b,c and d. AV: *Aloe vera,* CP: *Carica papaya,* AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia.* SEM: standard error of mean

Compared with individual plant extracts, cocktails of AZ+AV, AV+JA, AV+CP, AV+MO and AZ+JA had negative associative effect on IVOMD (Table 3.4). However, with the exception of AV+CP and AV+MO, all other individual and cocktails plant extracts had higher or equivalent IVOMD level compared with the control and monensin group. The CH_4 per unit of TGP values recorded for AV, AZ, CP, JA, MO, TD and cocktails of AV+JA, AV+MO, JA+CP, CP+MO and MO+TD were lower ($P<0.05$) than values recorded for the control (Table 3.2). At the application rate used in this study, monensin did not reduce CH_4 per unit of TGP when compared with the control ($P>0.05$). Plant extract cocktails of AZ+AV, AV+JA, AV+TD, AZ+MO, AZ+TD, JA+CP, CP+MO, MO+TD and all individual plant extracts used in this study significantly reduced CH_4 per unit IVOMD when compared with the control and monensin. However, only cocktails of AV+JA and CP+MO were more effective than other cocktails in terms of reducing CH_4 per unit of IVOMD.

The lack of significant differences between most of the cocktails and individual plant extracts in terms of IVOMD suggests their similarity in their mode of action, whereas the observed significant reductions of IVOMD by cocktails of AV+CP and AV+MO compared with individual plant extracts indicated the presence of negative associative effects on digestibility. Thus, these two plant extracts should not be used together as an additive in the current form. Some authors have reported the effectiveness of plant extracts in modulating rumen fermentation without adversely affecting ruminal fermentation parameters (Kim *et al.* 2012, 2015). This is in line with the current study. Molar proportions of VFA, acetic to propionic acid ratio, and NH_3-N are shown in Table 3.3. Ammonia N concentration (mg/100 ml), which is regarded as fermentation waste by protozoans, was highest ($P<0.05$) for the control (5.12), followed by monensin (5.08), which had values higher than individual plant extracts and their cocktails. This could partly be due to more depressing effect of the plant extracts on the protozoa growth. Cocktails of AV+CP, AV+MO and JA+MO had lower acetic acid concentration ($P<0.05$) compared with the control and monensin.

All cocktails except JA+MO, JA+TD and MO+TD had slightly higher ($P<0.05$) propionic acid concentration when compared with the control. However, individual plant extracts did not increase propionic acid concentration when compared with the control or monensin. Positive associative effect witnessed on the molar proportions of propionic acid and reduction of total volatile fatty acids in cocktails and single plant extracts could be attributed to the increased antimicrobial effect

on ruminal fibrolytic microorganisms (Flythe & Aiken 2010; Tekeli *et al.* 2015) that cause reduction in total VFA and a subsequent increase in the relative concentration of propionic acid. Similarly, Flythe and Aiken (2010) reported that at higher concentrations of inclusion of extract of *Humulus lupulus,* increased propionic acid and a decreased C2/C3 ratio were observed. A decrease in CH_4 production by more than 40% when the substrate was treated with plant extracts has already been reported (Kim *et al.* 2015). This was associated with reductions in ciliate population by more than 60% and increased diversity of fibrolytic bacteria. The test feed ingredient had a lot of fibre and cellulose, which is the main source of fibre fragments, and would have resulted in the production of a high volume of acetic acids through gradual degradation during fermentation.

Two scenarios might explain the higher proportion of propionic acid that was recorded for the two-way plant extract cocktail treatments. The first is faster degradation of the fibre component through the actions of phytochemicals in the extracts. This would have increased the passage rate of substrate. The other case could be the suppressive effect of the antimicrobial and antiprotozoal properties of the plant extracts on methanogens, which reduced the usage rate of H_2 in the rumen by the methanogens. Various reports have shown that polyhalogens, for example chloroform, change the fermentation pattern in the rumen by decreasing the production of acetic acid and propionic acid. Ammonia nitrogen concentration, which is often regarded as waste (Sirohi *et al.* 2012), was reduced by plant extracts, probably because of reduction in CP degradability in the rumen by proteolytic protozoans (Alexander *et al.* 2008). MO and some other plants used in this study have been reported to have activity against rumen protozoans, but most cocktails had no associative effect on rumen NH_3-N. However, compared with individual plant extracts, cocktails of AZ+AV, AV+MO, AZ+MO and CP+MO showed negative associative effect in terms of NH_3-N concentration, perhaps because of their selective effect on rumen protozoans.

Table 3.4. Associative effect of cocktails plant extract on total gas produced, methane, organic matter digestibility and volatile fatty acids of 400 mg *Eragrostis curvula* hay

Treatments	TGP	CH_4	IVOMD	TVFA	Propionic acid	Acetic acid	Acetic/ Propionic	CH_4/ TGP	CH_4/ IVOMD	TGP/ IVOMD	CH_4/ TVFA
AZ+AV	-25.81	-12.11	-27.65	78.04*	7.43	16.55	8.58	18.29	20.77	2.59	-51.15*
AV+JA	-21.64	-21.74	-18.27	52.25*	6.58	1.07	-4.83	-3.04	-3.15*	-3.88	-51.41*
AV+CP	-14.39	33.39	-36.69	39.94*	7.89	-8.22	-14.58	56.52*	113.4*	36.91*	-4.83
AV+MO	-13.07	3.45	-42.32	-1.38	9.51	-19.66	-26.58	17.43	83.65*	51.89*	3.87
AV+TD	-5.68	52.64	5.34	64.04*	4.19	4.16	-0.02	61.94*	46.33*	-9.79	-7.29
AZ+JA	6.46	52.24	-17.31	23.43*	4.52	-6.55	-9.82	43.99	84.19*	28.90*	30.67
AZ+CP	1.15	46.93	-13.78	32.71*	10.14	-1.64	-10.85	47.14*	71.02*	18.15*	11.31
AZ+MO	-4.75	20.83	-3.46	7.83	6.14	-6.99	-12.36	27.34	24.91	-1.39	15.18
AZ+TD	-5.55	29.22	-7.23	3.98	9.36	-6.85	-14.87	34.07	40.51*	2.27	29.51*
JA+CP	1.07	21.02	-4.14	12.80	11.6	-6.01	-15.77	19.91	27.12	5.45	6.56
JA+MO	1.88	85.14*	-4.46	-3.54	2.45	-15.72	-17.78	79.11*	97.66*	6.87	6.44
JA+TD	2.13	51.45	0.15	6.01	2.71	-4.04	-6.12	49.43*	52.25*	1.87	42.32*
CP+MO	-6.17	-0.35	-5.22	13.87	2.32	0.90	-1.17	5.15	4.37	-1.25	-14.22
CP+TD	-9.90	90.32*	-1.51	-2.51	3.64	-9.26	-12.48	115.3*	93.68*	-8.49	93.87*
MO+TD	-8.54	1.76	-1.99	-3.12	0.54	-10.16	-10.62	11.95	3.66	-6.62	117.87*

*Indicates significance P<0.05. AV: *Aloe vera*, CP: *Carica papaya*, AZ: *Azadirachta indica*, JA: *Jatropha curcas*, MO: *Moringa oleifera*, TD: *Tithonia diversifolia.* TGP: total gas produced, CH_4: methane, TVFA: total volatile fatty acids, IVOMD: *in vitro* organic matter digestibility

CH_4 per unit of TGP, IVOMD, TVFA and TGP per unit of IVOMD and TVFA all showed that single plant extracts and their cocktails performed the same as or better than the control and monensin group. Plant extract of AV had lower butyric acid values compared with the control or monensin. Cocktails of AV+MO, JA+MO, JA+TD and CP+TD had higher iso-butyric acid values compared with the control. Cocktails of AZ+TD and CP+MO resulted in lower valeric acid values compared with the control. In contrast, cocktails of JA+MO and MO+TD resulted in higher iso-valeric acid values compared with the control. The lowest acetate to propionate ratio was observed for cocktails of AV+MO and JA+MO. Cocktails of plant extract AV+MO, CP+TD, MO+TD, and JA+MO and individual plant extracts AV, JA, MO and TD reduced the TVFA production compared with the control. The volume of CH_4 per unit of TVFA were significantly reduced by AZ+AV and AV+JA cocktails compared with the control. The difference between the control and plant extracts and monensin was not significant in terms of TGP per unit of TVFA.

Table 3.4 presents the associative effect of various plant extract cocktails on *in vitro* gas production, CH_4 and fermentation products. The associative differences recorded in TGP, IVOMD, propionic acid, acetic acid, and acetic per unit of propionic acid as shown in the principal component analysis, dendrogram and cluster diagrams were not significant ($P>0.05$) statistically. JA+MO had positive associative effect in terms of increasing CH_4, showing that this combination is not beneficial in terms of the objective of this study. Significant TVFA ($P<0.05$) positive associative effects were recorded for AZ+AV, AV+JA, AV+CP, AV+TD, AZ+JA and AZ+CP. It was discovered that this synergistic effect was dominated by cocktails with AV or CP as part of their combinations. This could be because of a higher rate of fermentation, as previously reported (Akanmu & Hassen 2017). Plant extract cocktails AV+CP, AV+TD, AZ+CP, JA+MO, JA+TD and CP+MO had positive associative effect in terms of CH_4 per units of TGP and IVOMD. The particular reason for this is not well understood, but individual plant extracts performed better than the cocktails. AZ+AV and AV+JA, apart from increasing TVFA, significantly reduced CH_4/TVFA. This is an indication of synergistic effect in three plant extracts that made up the cocktails.

3.4 Conclusion

Plant extracts of AV, AZ, CP, JA, MO and TD reduced TGP and CH_4 emission and improved digestibility better than their cocktails. All cocktails of plant extract increased propionic acid concentration. Positive associative effects were observed for AZ+AV, AV+CP, AV+TD, AZ+JA, AZ+CP and AV+JA cocktail plant extracts compared with individual plant extracts in terms of TVFA production. The combinations of AZ+AV and AV+JA have the potential to reduce CH_4 production by increasing TVFA and reducing CH_4 per unit of TVFA produced. Most of the associative effect recorded involved combinations with AV and JA. However, among the two-way cocktails, AZ+AV is unique in that it increased TGP/IVOMD, TVFA and reduced CH_4/IVOMD. Thus, there is a need to evaluate different proportions of their plant extracts in a cocktail using *in vitro* studies to identify optimum proportion and confirming the results with *in vivo* studies.

CHAPTER FOUR

Effects of substrate and storage time on the efficacy of plant extracts used as an environmentally friendly alternative additive to monensin to modulate rumen fermentation and reduce enteric methane emission

Abstract

Different plant extracts have different concentrations of numerous phytochemicals, which in turn influences the extent of ruminal modulation functions. Previous studies at the University of Pretoria revealed that some of these plant extracts are effective in reducing methane from low-quality roughage. This study compared the effectiveness of old and freshly extracted *Aloe vera* (AV), *Azadirachta indica* (AZ), *Moringa oleifera* (MO), *Jatropha curcas* (JA), *Tithonia diversifolia* (TD) and *Carica papaya* (CP) plant extracts on *in vitro* gas and methane production, organic matter digestibility (IVOMD) and volatile fatty acids (VFA) when applied to total mixed ration (TMR), lucerne or *Eragrostis curvula* substrates after storage. The fresh extracts were processed from the same batch of material a few days before the test, while the old plant extracts were extracted and stored at 4°C 12 months before the study. Four ml reconstituted 5.0 mg crude extract per 1000 ml distilled water was added to incubation vials that already contained 400 mg TMR, lucerne or Eragrostis hay. Results indicated that storing plant extracts for 12 months had no significant effect ($P<0.05$) on all substrates used in terms of total gas produced (TGP) and CH_4. Generally, increased IVOMD was witnessed from all plant extract groups used on TMR, but no significant difference was witnessed in terms of TGP and CH_4. Generally, plant extracts used in this study performed better than group treated with antibiotic in terms of increasing total volatile fatty acid (TVFA), digestibility (except for lucerne), and reducing methane. Storing the plant extracts or plant material for up to a year did not compromise the efficacy of these plant extracts. In addition, the result of this study confirmed the previous findings that the use of 50 mg/kg of plant extracts of AV, AZ, MO, JA, TD and CP in a forage-based diet would reduce methane production while improving digestibility.

Keywords: medicinal plants; methane, volatile fatty acids, organic matter digestibility, lucerne, *Eragrostis curvula*

4.1 Introduction

Several scientific studies have documented the potential benefits of medicinal plants in solving health issues (Menale *et al.* 2016; Shrestha *et al.* 2016; Subba *et al.* 2016). When used correctly, medicinal plant-based additives in the diet of farm animals pose little or no threat to human beings who consume such products. Previous studies by Akanmu and Hassen (2017); Jahromi *et al*. (2016); Lee *et al*. (2015); and Ramiah *et al*. (2014) revealed the beneficial effects of including medicinal plants for animals as opposed to antibiotics. These plants are capable of combating disease, improving the general welfare of animals, and increasing digestibility. Antibiotics commonly used for treatment of disease and as growth promoters in animal agriculture have notorious attributes of lengthy residence in animal products. This long resident time has a devastating effect on human beings who consume these meat and milk product as individuals become resistant to antibiotic treatments. With limited food resources, improvement in feed conversion efficiency of ruminant animals could be the solution to any meat crisis that may arise in the future owing to rapid increases in human population, economic growth and income of people in developing countries. But ruminants lose about 6% to 15% of ingested energy meant for improvement in performance and production to eructation (Johnson *et al.* 1995; Broucek 2014; Wanapat *et al.* 2015). Although eructation and the release of methane gas is part of normal rumen function, significant energy losses through eructation have been attributed to poor roughage values, especially in developing countries. This energy loss through eructation is a danger to the ozone layer, because agriculture contributes towards greenhouse gases worldwide. Ruminants are alleged to be the principal contributors of 80% of this total greenhouse gas to the ozone layer depletion (Swain *et al.*). There is a need to reduce methane emission from ruminants in order to improve feed efficiency, reduce production time and cost, and slow down the rate of global warming.

Aloe vera (AV), *Azadirachta indica* (AZ), *Moringa oleifera* (MO), *Tithonia diversifolia* (TD), *Jatropha curcas* (JA) and *Carica papaya* (CP) have been reported to possess various phytochemicals such as anthraquinones, saponins, essential oils, catalase, azadirachtin, diastase and various digestive enzymes that are capable of influencing ruminal fermentation (Alexander *et al.* 2008; Ayoola and Adeyeye 2010; Kedarnath N K *et al.* 2012; Cieslak *et al.* 2013). A previous study by Akanmu and Hassen (2017) reported that extracts of these plants at the recommended

dosage of 50 mg/kg feed reduced methane (CH_4) production and increased feed digestibility when tested on low-quality roughage (*E. curvula* hay). Reducing methane production from ruminants without adverse effects on feed digestibility is a win-win situation for ruminant industry. To fully exploit the beneficial effect of these plant materials, it is important to test their effectiveness when applied to a wide variety of feeds that are regarded as high quality (total mixed ration (TMR)), good quality forage (lucerne hay) and low-quality hay (*E. curvula*) available in South Africa. Moreover, it is important to study the effect of prolonged storage of plant extracts and plant material additives prepared from old plant material versus fresh plant material on the effectiveness of these plant extracts by extracting fresh plant materials and storing for 12 months. Thus, in this study newly extracted and stored plant extracts were tested *in vitro* on gas and methane production, volatile fatty acids and organic matter digestibility of TMR, lucerne and *E. curvula*.

4.2 Materials and methods

4.2.1 Collection and preparation of plant extracts

Aloe vera (AV), AZ, MO, TD, JA and CP plant materials were harvested fresh from ten trees in Nigeria in the same area. These plants were rinsed with water to remove impurities, pests and parasites before being transferred to a waiting refrigerated van. All procedures that were involved during plant sample collection, handling and transport of all fresh plant materials to the Department of Animal and Wildlife Sciences, University of Pretoria, have been fully described (Akanmu & Hassen 2017). Frozen plant leaves were freeze dried until constant weight was achieved and were milled through 1-mm sieve. Extraction was carried out by adding 1500 mL pure concentration of methanol to 150 g of each dried plant material. The mixture was then left for about four days and agitated twice a day to allow easy penetration of the solvent. The mixture was sieved through a 150-µm sieve and the filtrate was placed in a fume cupboard to evaporate excess methanol. To achieve complete dryness, the semi-dried plant extracts were later transferred once more to the freeze-drying machine and plant extracts were recovered after constant weight was achieved. Plant extract solutions of AV, AZ, MO, TD, JA and CP were prepared by reconstituting 5.0 mg of each plant extract in 1000 mL distilled water to achieve the recommended dosage (Akanmu & Hassen 2017). This solution was stored at 4 °C for 12 months (old plant extracts). After 12 months, a fresh plant extract was prepared and reconstituted from the same batch of AV, AZ, MO, TD, JA and CP that had been harvested and stored at -20 °C.

4.2.2 Substrates and chemical analyses

The substrates used in this study were total mixed ration (TMR), lucerne hay and *Eragrostis curvula*. These substrates were subjected to chemical analysis to determine dry matter (DM), total ash, and ether extract using the AOAC procedure (AOAC 2000). Fibre fractions of acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) of these substrates were determined using ANKOM200/220 technology (Fairport, USA). Crude protein was obtained by analysing samples for nitrogen content with Leco nitrogen/protein analyser (Leco Instrumente GmbH, Kirchheim, Germany) and multiplying by 6.25.

4.2.3 In vitro gas, methane and organic matter digestibility

Two ruminally fistulated SA Mutton Merino (SAMM) sheep fed *ad libitum* on lucerne hay were used as donor sheep throughout the experiment. Prior to rumen fluid collection, these animals and their rumen were checked closely for three days consecutively. Ruminal fluid of about 900 mL was collected from each donor sheep early in the morning before morning feeding by straining through four layers of cheesecloth into a thermos flask previously filled with hot water. Rumen fluid was transported into the laboratory within 10 minutes of collection and placed in water bath already heated to 39 °C and continuously flushed with CO_2. The buffer mineral solution was prepared by the method described by Menke and Steingass (1988) and modified by Mould *et al*. (2005) as reported by Akanmu and Hassen (2017). Prior to incubation, 400 mg TMR, lucerne, and *E. curvula* were weighed into 120-mL separate serum bottles, and 4 ml each of old or freshly extracted plant extract solutions of AV, AZ, MO, TD, JA and CP had been added the previous day. Monensin was included as a positive control and used as suggested by the manufacturer. All old and newly extracted groups for each plant extract, monensin and negative control (with 4 ml distilled water) groups were replicated four times and the whole experiment was repeated five times. Three blanks and three standard test substrates were always included in each run. All other procedures during *in vitro* incubation were reported previously (Akanmu & Hassen 2017).

4.2.4 Measurement of total gas, methane, volatile fatty acids and in vitro organic matter digestibility

Gas pressure readings in the incubated serum bottles were measured at 3, 6, 12, 24 and 48 hours of incubation using a pressure transducer PX4200-015GI (Omega Engineering Inc. Laval,

Canada), which is attached to a digital data display (Tracker 220 series indicator, Omega Engineering Inc.). The pressure transducer tip was modified to fit into taps on the serum bottles. All gas pressure readings are recorded in psi. Gas pressure readings were added to the previous readings to give cumulative value. Gas samples were also taken at each measurement time (3, 6, 12, 24, 48 hours) for CH_4 analysis. Methane concentration in each sample was obtained by pushing gas through the syringe into gas chromatograph equipped with a flame ionisation detector calibrated with standard CH_4 (8610C, SRI Instruments GmbH, Bad Honnef, Germany). Methane concentration was corrected with each headspace gas volume at various collection times to obtain ml of methane produced and subsequently to estimate the cumulative value at 48 hours. The incubation was terminated at 48 hours by placing all bottles in the cold room. These bottles were centrifuged at 4500 g for 15 minutes at 20 °C. Supernatants were filtered off and 5 mL from each serum bottle was stored at 20 °C for volatile fatty acid analysis (Ottenstein & Bartley 1971). *In vitro* organic matter digestibility was carried out on the residue using two-stage digestion as described by Tilley and Terry (1963) and modified by Engels and Van der Merwe (1967). About 40 mL HCl pepsin was added to the residue in each serum bottle and incubated for another 48 hours. Gas pressure in psi was converted to ml using Boyle's law and cumulative CH_4 was obtained as previously described by Akanmu and Hassen (2017).

4.2.5 Statistical analyses

All data were analysed using general linear model of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Differences among means were compared using the Tukey test of SAS.

4.3 Results and discussion

Medicinal plant extracts used in this study were tested on three substrates with varying degrees of quality as shown in Table 4.1. The TMR formulated to meet the energy and nutrient requirements of growing sheep had crude protein (CP) of 19.2%. The lucerne and *E. curvula* used in this study were of medium quality. The CP of lucerne was 18.5%, while that of *E. curvula* was 5.61%. Chemical analysis (Table 4.1) showed the highest and lowest values recorded for NDF, ADF, and ADL were found in *E. curvula*, lucerne and TMR, respectively. Table 4.2 presents the total gas produced (TGP), methane, *in vitro* organic matter digestibility (IVOMD) and total volatile fatty acids (TVFA) from the three substrates treated with various plant extracts. Old plant extracts of

AV, AZ, MO, TD, JA and CP were as effective (P>0.05) as the new ones in TGP and CH_4 production for all. This could be because of the method of extraction, storage conditions and temperature, which halted the degeneration of biochemical value of active compounds effective on substrate degradation. Mediani *et al*. (2014) reported consistent scavenging activity of air-dried and stored *Cosmos caudatus* when compared with fresh material of the same plant. Part of the reasons Mediani *et al*. (2014) offered for the consistency was the low temperature (-20 °C) used for storage as this could have terminated enzymatic reactions that reduce the effectiveness of phytochemicals in the plant. In a similar study, Stafford *et al*. (2005) discovered that the antibacterial activity of various medicinal plants found in South Africa was retained in most of the species after 12 months of storage. Longer-term storage of various medicinal plants extracts has also shown that total phenolic content found in plant extracts are stable. This is confirmed by Amoo *et al*. (2012) which showed an inhibition of acetylcholinesterase by both fresh and a 12 year old stored plants were not significant.

Table 4.1. Chemical composition of *Eragrostis curvula* hay, lucerne hay and total mixed ratio fermented with different plant extracts

Composition in DM (%)	TMR	Lucerne	*E. curvula*
Crude protein	19.15	18.45	5.61
NDF	30.10	40.58	78.41
ADF	21.35	32.11	49.22
ADL	0.48	0.55	0.78
Ether extract	0.59	0.19	0.12
Ash	0.78	0.76	0.45

Key: DM: dry matter, TMR: total mixed ration, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin.

TGP and CH_4 values recorded on TMR for both old and new plant extracts were not different among plant extracts and when compared with monensin and the control groups. The reason for the minimal or zero effect of medicinal plant extracts on TMR could be the quality of the feed ingredients used in the TMR. These ingredients are highly digestible and could be easily broken down by microbes in the rumen. A report by Martinez *et al*. (2009) indicated that minimal effect

was obtained in animal performance from the monensin-treated group when cows were fed a higher-quality forage diet. The non-significant effect recorded by authors could be due to the high quality of forage offered to lactating animals because a higher forage diet in the study had already increased NDF digestibility. This could also be true of the present study, as TMR are formulated with good-quality ingredients, which would normally be easily digestible by a ruminant. Although plant extracts showed a tendency to reduce CH_4 production from TMR, the effect was not statistically ($P>0.05$) significant. Forabosco *et al*. (2017) and Wanapat *et al*. (2015) reported that high CH_4 emissions are reported mostly from ruminants consuming poor-quality forage. Phytochemicals in plant extracts used in this study therefore had little effect on reducing CH_4 produced by TMR owing to the confounding effect of the substrate on plant extracts.

In contrast, TGP and CH_4 production from *E. curvula* hay were affected significantly ($P<0.05$) by the plant extracts. The observed values were within the range reported by Akanmu and Hassen (2017). The TGP for *E. curvula* hay for the control was highest and was closely followed by monensin and AV, while all other plant extracts had significantly lowered TGP values. *Eragrostis curvula* is a drought-resistant grass with 'midribs that exhibit a robust, lignified region of collenchyma cells (sclerenchyma) above the central vascular bundle and phloem fibres below' (Balsamo *et al.* 2006). These properties give increased tensile strength to *E. curvula*, which in turn yields higher lignification and consequent lower digestibility by ruminants. Methane was significantly reduced by more than 50% by both old and new plant extracts of AV, AZ, MO, TD, JA and CP when compared with the control and monensin group tested on *E. curvula* hay. Monensin tended to reduce CH_4 from *E. curvula* by 19% when used at the recommended dose. Monensin, which is an antibiotic growth promoter, is mostly effective with grazing animals, as it improves forage utilization and feed efficiency. Similarly, plant extracts of AV, AZ, JA, MO, TD and CP were effective when used on low-quality *E. curvula*. The observed significant improvements in CH_4 reduction could be attributed to various phytochemicals in the plant extracts that are effective against methanogens, as discussed (Akanmu & Hassen 2017).

Table 4.2. Total gas produced, methane, *in vitro* organic matter digestibility and total volatile fatty acid response of different substrates treated with various plant extracts prepared at 12 month intervals

Parameter	Treatment	Total mixed ration		Lucerne hay		*Eragrostis curvula*	
		Old	New	Old	New	Old	New
TGP	Control	89.6	89.6	63.8a	63.8a	55.3a	55.3a
(ml)	Monensin	86.2	86.2	56.2c	56.2bc	53.6abc	53.6a
	AV	85.6	83.4	60.1abc	60.4ab	54.2ab	54.4a
	AZ	85.1	81.5	57.3bc	55.1c	49.7d	47.9b
	MO	83.4	82.6	56.2c	59.6ab	50.8bcd	47.7b
	TD	80.4	82.3	58.2bc	57.8bc	49.2d	48.5b
	JA	82.1	82.0	62.8a	59.4b	47.3d	45.7b
	CP	85.6	85.9	61.1ab	60.8ab	50.1cd	48.7b
	SEM	3.15	2.49	1.71	1.75	1.57	1.27
CH_4	Control	30.6	30.6	22.7a	22.7a	17.9a	17.9a
(ml)	Monensin	25.4	25.4	21.1ab	21.1abc	14.5a	14.5b
	AV	22.7	24.1	23.4a	21.8ab	8.72b	8.88c
	AZ	26.7	24.2	18.0bc	19.8abc	9.22b	7.91c
	MO	24.8	24.4	17.8bc	20.1abc	7.72b	7.25c
	TD	22.3	25.3	15.8c	18.2c	7.87b	5.81c
	JA	25.1	25.4	20.1ab	18.3c	7.18b	5.87c
	CP	22.6	22.1	18.6bc	19.2bc	6.56b	7.63c
	SEM	2.03	2.21	1.49	1.29	1.55	1.29
IVOMD	Control	71.1d	71.1d	61.6bc	61.6b	33.6c	33.6d
(%)	Monensin	76.1bc	76.1c	68.6a	68.6a	35.4c	35.4d
	AV	80.1aB	88.2aA	63.4bc	62.5ab	44.6a	45.8a
	AZ	77.2abB	83.1bA	63.7abc	63.1ab	41.5ab	41.8c
	MO	77.5ab	80.3bc	59.6cB	63.7abA	44.4a	45.1ab
	TD	76.1bc	79.8bc	64.4abc	62.7ab	42.5ab	42.6bc
	JA	73.4cB	77.3cA	62.5bc	61.9ab	40.2bB	41.5cA
	CP	76.4bc	77.7c	64.7ab	64.2ab	42.8ab	42.3bc
	SEM	0.92	1.10	1.98	11.5	1.40	1.19
TVFA	Control	121b	121b	95.9bc	95.9abc	82.0a	82.0a
	Monensin	115f	115d	91.8e	91.5e	76.1d	76.1c
	AV	129a	127a	96.9ab	96.9ab	78.8bc	78.7bc
	AZ	117de	117c	93.9d	93.1de	77.1cd	77.8bc
	MO	120bcA	119cB	94.7cd	94.8bcd	80.1b	78.1bc
	TD	118cde	117c	96.4b	97.2a	78.9bc	78.5bc
	JA	119cd	118c	97.9a	97.5a	78.9bc	78.0bc
	CP	117eB	119cA	94.2d	94.5cd	79b	80.1ab
	SEM	0.79	0.84	0.61	0.91	0.68	1.11

*Uppercase letters compare means across rows between old and fresh extracts. Lowercase letters compares mean along the column
*Means with different lower case letters across the columns or upper case letters along the rows for each parameter are significantly ($P<0.05$) different. *AV:*Aloe vera*, AZ:*Azadirachta indica*, CP:*Carica papaya*, JA:*Jatropha curcas*, MO:*Moringa oleifera*, TD:*Tithonia diversifolia*, TGP:Total gas production, CH_4:Methane, IVOMD:*in vitro* organic matter digestibility

When plant extracts were tested on lucerne, the control group, AV, JA and CP had the highest ($P<0.05$) TGP compared with the rest of the treatments, whereas the lowest TGP was recorded for the monensin, MO, AZ and TD groups, as compared with the control. Old plant extracts of CP, JA, TD, MO and AZ significantly reduced ($P<0.05$) CH_4 production from lucerne as compared with the control. However, monensin did not differ significantly from the control, but differed when compared with TD. New plant extracts of TD, JA, and CP effectively reduced CH_4 production from lucerne when used at the recommended dosage of 50 mg/kg, while the newly extracted AV, AZ and MO did not differ from the control or monensin groups. The mixed effect obtained from lucerne hay treated with various medicinal plants in this study could be due to the confounding effect of its chemical compounds. Lucerne has been reported (Gaweł 2012) to contain phytochemicals such as alkaloids, coumarins, saponins, amylase, protease and other compounds. Saponins and alkaloids have been found to reduce methane (Kumar *et al.* 2014). Other anti-nutritional components such as phytate, L-canavanine, and other phytochemicals, could have suppressed or counteracted the activities of secondary compounds in the plant extracts, thus explaining the different responses. Furthermore, IVOMD values recorded for lucerne were highest in the monensin-treated group when compared with plant extracts. This explains the confounding effect discussed earlier as monensin was able to increase lucerne digestibility *in vitro,* possibly because of its chemical origin.

Table 4.3. The ratios of total gas produced, methane and *in vitro* organic matter digestibility response of different substrates treated with various plant extracts prepared at 12 month intervals

Parameter (%)	Treatment	Total mixed ration		Lucerne hay		*Eragrostis curvula*	
		Old	New	Old	New	Old	New
CH_4/TGP	Control	34.1a	34.1a	35.5ab	35.5	28.9a	28.9a
	Monensin	29.5bc	29.5ab	34.7ab	34.7	33.5a	33.5a
	AV	26.5c	28.6ab	38.9a	36.0	16.1b	16.3b
	AZ	31.4ab	29.7ab	31.4bcB	36.1A	18.6b	16.5b
	MO	29.5bc	29.4ab	31.2bc	33.6	15.1b	15.3b
	TD	30.7bc	28.5ab	27.3c	31.5	15.9b	12.0b
	JA	30.5abc	30.9ab	32.1bc	30.8	15.2b	12.9b
	CP	26.4c	25.7b	30.4bc	31.7	13.1b	15.7b
	SEM	1.76	2.32	2.22	2.13	2.53	2.91
CH_4/IVOMD	Control	42.9a	42.9a	36.8a	36.8a	47.7a	47.7a
	Monensin	33.4b	33.4b	30.7abc	30.7b	51.0a	51.0a
	AV	28.1b	27.2c	36.9a	34.7b	19.8b	19.4b
	AZ	34.7b	29.1bc	28.3bc	31.6b	22.2b	18.9b
	MO	31.9b	30.3bc	29.9bc	31.5b	17.4b	16.1c
	TD	30.2b	31.6bc	24.6cB	29.1bA	18.5b	13.6c
	JA	34.2b	32.8bc	32.2ab	29.6b	17.9b	14.1c
	CP	29.7b	28.3bc	28.8bc	30.1b	15.4b	18.0b
	SEM	2.67	2.42	2.52	2.18	4.23	2.71
TGP/IVOMD	Control	1.26a	1.26a	1.04a	1.04a	1.65a	1.65a
	Monensin	1.13b	1.13b	0.88c	0.88b	1.52a	1.52a
	AV	1.06bA	0.95eB	0.94abc	0.96b	1.22b	1.19b
	AZ	1.10bA	0.97deB	0.89bc	0.87b	1.19b	1.15b
	MO	1.08b	1.03cde	0.94abc	0.93b	1.14b	1.06b
	TD	1.07b	1.02cde	0.91bc	0.92b	1.16b	1.14b
	JA	1.11b	1.06bcd	1.00ab	0.95b	1.18bA	1.09bB
	CP	1.12b	1.10bc	0.94abc	0.94b	1.17b	1.15b
	SEM	0.04	0.03	0.04	0.73	0.08	0.07
CH_4/TVFA (%)	Control	25.2a	25.2a	23.6ab	23.6a	21.9a	21.9a
	Monensin	22.0ab	22.1ab	23.0abc	23.1a	19.1a	19.1a
	AV	17.5c	18.9b	24.1a	22.5a	11.1b	11.2b
	AZ	22.7ab	20.5b	19.2cde	21.3ab	10.6b	10.1b
	MO	20.7bc	20.4b	18.8de	21.1ab	9.65b	9.26b
	TD	19.3bc	21.5ab	16.4e	18.7b	9.98b	7.41b
	JA	21.1abc	21.4ab	20.5abcd	18.8b	9.11b	7.52b
	CP	19.4bc	18.5b	19.7bcde	20.4ab	8.31b	9.51b
	SEM	1.70	1.87	1.63	1.43	1.99	1.68
TGP/TVFA	Control	73.9ab	73.9ab	66.6a	66.6a	67.3abc	67.3a
	Monensin	74.8a	74.8a	66.4a	66.4a	70.5a	70.5a
	AV	66.1c	66.0c	61.9abc	62.3ab	68.7ab	69.1a
	AZ	72.3abc	68.8bc	61.1bc	59.2b	66.5abc	61.6b
	MO	69.8abc	69.3bc	59.3c	62.8ab	63.5bcd	61.1b
	TD	67.9bc	70.2ab	60.3bc	59.5b	62.3cd	61.7b
	JA	69.0abc	69.3bc	64.2abc	60.9b	60.1d	58.5b
	CP	73.3ab	72.2ab	64.8ab	64.3ab	63.4bcd	60.7b
	SEM	2.61	2.17	2.13	2.07	2.36	1.63

*Upper case letters compares means across rows between old and fresh extracts; lower case letters compares means along the column
*Means with different lower case letters across the columns or upper case letters along the rows for each parameter are significantly ($P<0.05$) different. *AV:*Aloe vera*, AZ:*Azadirachta indica*, CP:*Carica papaya*, JA:*Jatropha curcas*, MO:*Moringa oleifera*, TD:*Tithonia diversifolia*, TGP:Total gas production, CH_4:Methane, IVOMD:*in vitro* organic matter digestibility

Table 4.4. Molar concentrations of volatile fatty acids of *Eragrostis curvula*, lucerne and total mixed ration treated with various plant extracts prepared at 12 month intervals

Volatile fatty acids (mM)	Treatment	Total mixed ration		Lucerne hay		*Eragrostis curvula*	
		Old	New	Old	New	Old	New
Acetic	Control	45.5a	45.5a	43.3a	43.3a	44.4a	44.4a
	Monensin	44.5a	44.5a	41.9a	41.9a	42.2ab	42.0ab
	AV	33.6b	34.1bc	37.3b	35.4b	35.8c	37.6cd
	AZ	33.9b	35.3b	34.1bcd	35.5b	36.2c	36.1cd
	MO	31.3b	30.6de	33.1cd	34.1b	38.2c	39.0bc
	TD	31.5b	29.7e	36.5bc	34.5b	39.1bc	37.9cd
	JA	33.5b	32.5cd	32.6d	34.6b	38.5c	34.5d
	CP	33.2b	32.2cd	36.1bc	35.6b	38.1c	36.8cd
	SEM	1.07	0.88	1.42	1.20	1.37	1.58
Propionic	Control	22.7ab	22.7	20.1	20.1ab	17.9b	17.9b
	Monensin	23.2a	23.2	19.4	20.9a	20.1a	20.1ab
	AV	20.3cd	20.3	20.8	19.4b	21.9a	22.7a
	AZ	20.2cd	19.9	20.3	19.7ab	22.1a	21.7a
	MO	21.3bcd	22.6	19.7	19.4b	21.4a	22.9a
	TD	19.6d	21.8	20.6	20.2ab	21.5a	23.3a
	JA	19.7d	18.9	19.6	20.7ab	22.5a	21.8a
	CP	21.8abc	21.6	20.7	19.6ab	21.6a	22.6a
	SEM	0.71	2.19	0.86	0.56	0.74	1.30
Iso-butyric	Control	2.92c	2.92c	3.0c	3.0c	4.89c	4.89c
	Monensin	3.97c	3.97c	13.1a	12.5a	5.91bc	5.91bc
	AV	7.66ab	5.8bc	8.3b	9.69ab	12.5aA	7.54abcB
	AZ	6.57b	4.72c	9.59b	9.14ab	8.97ab	9.26ab
	MO	6.46b	6.84abc	8.98b	7.39b	9.39ab	10.3a
	TD	7.42ab	8.63ab	7.51b	7.83b	9.19ab	8.42ab
	JA	7.06b	10.2a	9.16b	8.51ab	8.63b	9.96a
	CP	9.26a	9.21ab	8.62b	9.28ab	9.35ab	10.1a
	SEM	0.77	1.57	0.88	1.69	1.34	1.43
Butyric	Control	12.8d	12.8b	15.8a	15.8a	15.9a	15.6a
	Monensin	14.5c	14.6b	10.9d	10.4c	12.1ab	12.1ab
	AV	18.9ab	19.1a	13.5c	14.1b	8.72bB	14.7aA
	AZ	19.7ab	20.6a	14.1cB	14.3bA	12.1ab	11.6ab
	MO	20.3a	20.7a	14.6abc	14.8ab	11.1ab	9.7b
	TD	20.4a	21.4a	14.2bc	14.7ab	11.4ab	11.6ab
	JA	19.4ab	18.8a	15.6ab	14.8ab	11.1ab	11.6ab
	CP	18.5b	19.5a	13.9c	14.1b	11.4ab	13.7ab
	SEM	0.72	1.22	0.58	0.57	1.93	1.66

Table 4.4 continued

Iso-valeric	Control	11.7a	11.7a	12.9a	12.9a	11.9	11.9
	Monensin	9.52b	9.52b	8.16b	12.8a	10.7	10.7
	AV	10.3abB	11.1abA	10.1ab	11.3ab	9.91	9.25
	AZ	10.1ab	10.6ab	10.8ab	10.8ab	10.7	10.9
	MO	11.1ab	11.1ab	12.1aB	12.9aA	9.79	10.8
	TD	11.3ab	11.1ab	10.8ab	11.8a	9.14	11.2
	JA	10.5ab	10.5ab	12.3a	11.2ab	9.43	11.5
	CP	11.1ab	11.1ab	10.3ab	11.1ab	9.81	9.56
	SEM	0.74	0.85	1.51	1.48	1.46	1.46
Valeric	Control	4.06c	4.05b	4.76c	4.76c	5.22b	5.22
	Monensin	4.21c	4.21b	4.72c	4.72c	6.61b	6.61
	AV	9.13a	9.6a	10b	10.0b	11.2aA	8.16B
	AZ	9.34a	8.99a	11.1ab	10.4b	10.0a	10.4
	MO	9.50a	8.13ab	11.7a	11.4a	10.2aA	7.64B
	TD	9.82a	7.51ab	10.2ab	10.9ab	9.65a	7.52
	JA	9.87a	9.14a	10.6ab	10.1b	9.86a	11.3
	CP	6.83b	6.58ab	10.4ab	10.2b	9.81a	7.61
	SEM	0.32	1.71	0.59	0.43	0.90	2.78
A : P ratio	Control	1.99a	1.99a	2.16a	2.16a	2.48a	2.48a
	Monensin	1.92a	1.92ab	2.17a	2.17a	2.02b	2.02b
	AV	1.65bc	1.67abc	1.78bB	1.82bA	1.63c	1.65c
	AZ	1.67bc	1.79abc	1.67b	1.79b	1.64c	1.66c
	MO	1.46d	1.36c	1.68b	1.75b	1.78c	1.70c
	TD	1.60bcd	1.39c	1.76b	1.71b	1.82c	1.63c
	JA	1.69b	1.71abc	1.66b	1.67b	1.71c	1.63c
	CP	1.52cd	1.48bc	1.75b	1.81b	1.76c	1.6c
	SEM	0.07	0.18	0.11	0.06	0.07	0.07

*Uppercase letters compare means across rows between old and fresh extracts. Lowercase letters compare means along the column

*Means with different lowercase letters across the columns or uppercase letters along the rows for each parameter are significantly ($P<0.05$) different. *AV:*Aloe vera*, AZ:*Azadirachta indica*, CP:*Carica papaya*, JA:*Jatropha curcas*, MO:*Moringa oleifera*, TD:*Tithonia diversifolia*, TGP:Total gas production, CH_4:Methane, IVOMD:*in vitro* organic matter digestibility, A:P:Acetic to propionic acid ratio

Although both old and fresh plant extracts of AV, AZ, MO, TD, JA and CP did not reduce CH_4 production from TMR, a significant ($P<0.05$) increase of TMR and *E. curvula in vitro* organic matter digestibility (IVOMD) was recorded for all plant extracts. Generally, new plant extracts recorded higher IVOMD values when compared with the old extracts. Significant effects ($P<0.05$) were recorded only with extracts AV, AZ, JA for TMR and JA for *E. curvula*, respectively. Monensin also increased IVOMD of TMR and *E. curvula*, but not as much as AV, AZ, and MO. All plant extracts significantly ($P<0.05$) increased IVOMD of *E. curvula* when compared with monensin and the control groups. The increase in IVOMD of *E. curvula* by all the plant extracts confirms our previous study (Akanmu & Hassen 2017), while an increase of up to 12% in TMR by AV could be due to phytochemical diastase and amylase in the sap of AV. Furthermore, the breakdown of polysaccharide long chain in substrates could have been affected positively by anthraquinones, which are present in varying proportions in all these medicinal plants.

Both old and new plant extracts of MO, TD, JA, and CP trended to reduce CH_4/TGP, CH_4/IVOMD, TGP/IVOMD, and CH_4/TVFA percentages of TMR, lucerne and *E. curvula* (Table 4.3). Significant CH_4/TGP reductions ($P<0.05$) of more than 50% were recorded for all plant extracts when tested on *E. curvula*. Plant extracts of AV increased CH_4/TGP of lucerne hay when compared with all the other plant extracts, but the differences between AV, the control and monensin group were not significant ($P>0.05$). In contrast, both monensin and AV significantly ($P<0.05$) reduced CH_4/TGP of TMR substrate, but not for lucerne. AV plant extract significantly ($P<0.05$) reduced CH_4/IVOMD, TGP/IVOMD, CH_4/TVFA and TGP/TVFA for TMR, but not for lucerne. Plant extract of AZ also reduced significantly ($P<0.05$) CH_4/IVOMD, and TGP/IVOMD of TMR and lucerne substrates. Patra and Yu, (2014) reported the anti-methanogenic effect of various medicinal plant extracts, which was achieved by significant reduction in the volume of *in vitro* TGP and consequent CH_4 reduction, which is similar to the present study.

Table 4.4 presents the effect of various old and new plant extracts on the concentration of VFA of TMR, lucerne and *E. curvula*. All plant extracts reduced significantly ($P<0.05$) the acetic acid concentration in TVFA compared with the control and monensin group. Old and new plant extracts had no effect on the propionic acid concentration of lucerne substrate compared with the control owing to the presence of plant secondary compounds in it. In contrast, statistically significant ($P<0.05$) higher propionic acid concentration values were recorded by all plant extracts for *E. curvula*. Valeric acid molar concentrations for TMR and lucerne for all plant

extracts were significantly higher compared with the control and monensin group. There were no significant differences between the old and new plant extracts. The acetate per unit of propionate was significantly ($P<0.05$) reduced by all plant extracts used in this study. Monensin, however, did not differ from the control in terms of acetate propionate ratio, except for *E. curvula* substrate, which was significantly lower than the control, but higher than all plant extracts.

Plant extract of AV resulted in higher TVFA for TMR and lucerne substrates, while for *E. curvula* the TVFA was reduced by the use of all other plant extracts compared with the control. Significant ($P<0.05$) levels of acetic acid reduction for old and new plant extracts were revealed in TMR, followed by lucerne and *E. curvula*. The monensin group also tended towards reduction in acetic acid, but the difference was not significant ($P>0.05$) when compared with the control. Compared with the control and monensin, old plant extracts of AV, AZ, TD, and JA had significantly lower ($P<0.05$) propionic acid concentration of TMR. However, the difference was not significant when the new extracts were tested on TMR.

Lower acetic acid concentrations recorded for all the substrates treated with plant extracts could be owing to the rapid fermentation of all the substrates, while TMR benefited more because it consists mainly of water-soluble carbohydrates, which means its degradation is enhanced by phytochemicals in plant extracts. This is evidenced by the increase recorded in butyric and iso-butyric acid concentrations. Rapid fermentation in the rumen has been known to result in more butyric acid production at the expense of acetic acid, while feed containing a great deal of fibre ferments slowly, thus resulting in a lot of acetic acid. From these results, the addition of plant extracts of AZ, AV, MO, TD, JA and CP tended to improve the rate of fermentation of all substrates.

4.4 Conclusion

Generally, all plant extracts performed better than the control and monensin-treated groups. It can also be established that plant extracts of *A. vera, A. indica, C. papaya, M. oleifera, J. curcas* and *T. diversifolia* were potent methane-reducing agents after 12 months of storage. Furthermore, the effectiveness of these plant extracts is substrate dependent, as all plant extracts effectively reduced methane emission from poor-quality roughage (*E. curvula*), compared with lucerne and TMR rations. The addition of 50 mg/kg DM feed of plant extracts AV, AZ, CP, MO, JA and TD would increase the feed digestibility of TMR ration formulated with high roughage content, while at the same time reducing methane production from such feeds.

CHAPTER FIVE

Oral dosage of medicinal plant extract as an additive reduced methane emission without negatively affecting feed utilization and performance of SA Mutton Merino sheep

Abstract

This study evaluated the effectiveness of selected medicinal plant extracts, namely *Moringa oleifera* (MO), *Jatropha curcas* (JA) and *Aloe vera* (AV), on feed digestibility, methane emission, growth performance and ruminal fermentation parameters of SA Mutton Merino (SAMM) sheep. Forty lambs born in the same season were arranged according to their body weight (light to heavy). Four uniform animals were taken at a time and randomly allocated to one of the four treatment groups. A total of 10 animals were allocated to each treatment group. The four treatments consisted of the control (TMR only), MO (TMR+MO plant extract), JA (TMR+JA plant extract) and AV (TMR+AV plant extract). Methanolic plant extracts of similar mass to MO, JA and AV were reconstituted by adding 96 g of each to 120 L of distilled water and stored at 4 °C. Animals on each treatment were dosed plant extracts with the aid of drench gun at the recommended application rate of 50 mg/kg of feed. Oral drenching of MO, JA and AV plant extracts as an additive significantly ($P<0.05$) increased dry matter (DM) and crude protein (CP) digestibility when compared with the control. Lambs on MO, JA and AV treatments significantly ($P<0.05$, $P=0.002$) reduced CH_4 emission per day by 21%, 35% and 28%, respectively. There were no significant ($P>0.05$) differences in DM and OM intake per day across the treatments. Average daily gain (ADG) for MO and JA lambs was significantly ($P<0.05$) higher when compared with the control and AV groups. Higher rumen ammonia nitrogen ($P<0.05$) were recorded for lambs on the control treatments (23.6 mg/100 ml), compared with MO (15.1mg/100 ml), JA (18.1mg/100 ml) and AV (16.4mg/100 ml) treatments. The results showed that plant extracts of MO, JA and AV could effectively reduce methane emission from ruminants without negative effects on feed digestibility and fermentation parameters. Application of 50 mg of MO, JA and AV per kg DM feed could be used as an alternative additive in organic farming systems to reduce methane emission while improving the performance of lambs. Further *in vivo* studies are recommended to refine the application dose by encapsulating the plant extracts and including 75 mg/kg DM feed of these plants for further CH_4 reduction in ruminants.

Keywords: methane emission, digestibility, growth performance, SA Mutton Merino sheep

5.1 Introduction

Medicinal plants have been studied extensively. Several authors have reported the beneficial effects of supplementing medicinal plants in terms of methane mitigation and increased feed utilization during *in vitro* and *in vivo* production studies (Patra *et al.* 2006a; García-González *et al.* 2008; Patra and Yu 2012; Bhatta *et al.* 2013; Lee *et al.* 2015; Akanmu and Hassen 2017). The methane-reducing potential of these medicinal plants, often coupled with increased animal performance, has been linked to the presence of phytochemicals that have been developed over the years as a form of defence mechanism to wade off invaders, grazers and parasites. The extracted phytochemicals, especially from *Moringa oleifera* (MO), *Jatropha curcas* (JA) and *Aloe vera* (AV), are effective against pathogenic bacteria, diseases and methanogens, which impede growth and animal performance (Oskoueian *et al.* 2011; Kedarnath *et al.* 2012; Meale *et al.* 2012). These medicinal plant extracts have been shown to be beneficial to animal agriculture. Various substitutes have been sourced to replace antibiotic rumen modulators in ruminants, but the most reliable and renewable replacements with long-term advantages can only be sourced naturally. Plant extracts of MO, JA and AV possess various phytochemicals that are capable of improving feed digestibility and reducing methane production, which may promote improved animal performance, as postulated by Akanmu and Hassen (2017). According to these authors, the use of plant extracts of *Azadirachta indica* (AZ), AV, MO, JA, *Tithonia diversifolia* (TD) and *Carica papaya* (CP) were effective in reducing methane production, and improving the digestibility of organic matter in the feed at supplementation rates of 25–50 mg plant extract per kg of feed. Patra *et al.* (2006b) also recorded a reduction in methane emission *in vitro* when AZ plant extract was fermented with feed substrate. The results of the *in vitro* study seem promising, but need to be validated in an *in vivo* study. From the literature, it is hard to find an *in vivo* study on the use of methanolic plant extracts of MO, JA and AV as rumen modulators. Therefore, the objective of this study was to evaluate the effect of methanolic plant extracts of MO, JA and AV on feed digestibility, growth performance characteristics and carcass yield, rumen fermentation parameters and methane emission using SAMM sheep fed high roughage total mixed ration (TMR).

5.2 Materials and methods

5.2.1 Study area

The study was conducted at the University of Pretoria Experimental Farm, Hatfield, South Africa. The annual rainfall in Pretoria is about 573 mm and the city is located at 1700 m above

sea level. The study was conducted between December 2016 and May 2017 and was approved by the Animal Ethics Committee of the University of Pretoria (ECO-030-14).

5.2.2 Collection, preparation and administration of plant extracts

The Department of Agriculture, Pretoria, South Africa, approved the importation of fresh plant materials of MO, JA and AV. Leaves of these plants were harvested in November 2016 at the Botanical Gardens, University of Ibadan, Nigeria. These plant materials were placed in a refrigerated van immediately after harvest, and transported to the airport, where they were air lifted to South Africa the same day under refrigeration. These plant materials were immediately stored at -20 °C on arrival at the department. Plant extracts from MO, JA and AV were prepared, reconstituted and stored according to the method fully described by Akanmu and Hassen (2017). Plant extracts were prepared from equal mass of each plant material. Solutions of plant extract were administered at a dosage of 50 mg/kg of feed DM, as recommended earlier during the *in vitro* study (Akanmu & Hassen 2017) by dissolving 50 mg of plant extracts in 50 ml of distilled water. The actual dosages given to lambs were adjusted once in a week depending on their feed DM consumption record of the previous week. The required dosages were drenched to lambs in the morning before feeding at 07:00 and evening at 16:00 using a 20 ml metal drencher (NJ Philips, Somersby Australia). A lamb consuming 1 kg DM TMR was drenched a total of 50 ml plant extract solution per day.

5.2.3 Experimental design, experimental feed and growth performance

Forty (40) 4-month-old male SAMM sheep with average live weight of 28.8 ± 0.4 kg were used for the experiment. Lambs were first ranked according to their body weight and then blocked into four groups of animals. Subsequently, one animal from each weight block was randomly allocated to one of the four treatments. These treatments were randomly allocated to three plant extracts and a control treatment. All experimental animals were fed a formulated TMR containing approximately 42% roughage. The ingredients and chemical composition of the TMR are shown in Table 5.1. The four treatments included T1 (TMR plus distilled water), T2 (TMR plus MO plant extract at 50 mg/kg DM of feed), T3 (TMR plus JA plant extract at 50 mg/kg DM of feed) and T4 (TMR plus AV plant extract at 50 mg/kg DM of feed).

Lambs were initially adapted to the experimental feed for 14 days. Later they were gradually dosed with the assigned plant extract solutions from low dosages to the recommended level for another 14 days. During this period, lambs were monitored closely to see whether there were

changes in feed consumption or signs of emaciation. Two animals were kept in a pen, and animals in each pen received a different volume of the plant extract solution of MO, JA or AV, which was estimated based on the previous week's DM intake. The growth study lasted for 103 days, including the adaptation time. Prior to the start of the experiment, animals were vaccinated, dewormed, and had their wool shorn. Part of the daily feed allowance of lambs in each pen was given in the morning, while the remaining portion was given in the afternoon. The ort, however, was recorded once every 24 hours before the morning feeding. Corresponding dosages of plant extracts solution were also administered in the morning and the afternoon. Water was provided *ad libitum* throughout the study.

5.2.4 Determination of feed intake and digestibility

All feed offered to the animals and ort mass were weighed daily. Samples of fresh feed and orts were collected every day to calculate dry matter (DM) percentage and subsequently DM and nutrient intake during the growth and digestibility trials. After the growth study has been completed, one sheep from each pen in the same block were transferred to the metabolic cages. A total of 20 sheep (five per treatment) were used for the digestibility trial. Animals in the metabolic cages were allowed 14 days' adaptation, the first eight days to adapt to the metabolic cages, and the last six to adapt to the faecal bags. All animals were given a fixed amount of feed (1.8 kg per day), which was based on the previous feed intake during the growth trial, of which 1.2 kg was given in the morning at 07:00 and 0.6 kg at 16:00. At the same times, lambs were drenched approximately with 60 ml and 30 ml plant extract solution at 07:00 and 16:00, respectively.

Data collection continued for six consecutive days. Every day, the feed, orts, faeces and urine from each lamb were weighed and recorded. After weighing, sub-samples of the feed, orts, faeces and urine were taken daily for analysis (Olson *et al*. 1999). Daily DM of faeces was determined first by transferring 100 g of sub-sampled faeces to a 105 °C oven for 24 hours. Later, a second DM was obtained after milling the dried faeces sample and drying it at the same temperature for 24 hrs. Urine was collected through urine pans into containers in which 20 ml of 72% sulphuric acid was diluted with water up to 4 L, and samples were frozen at -20°C until analysed for nitrogen.

The remaining 20 lambs, representing five from each treatment group, were slaughtered to determine carcass yield. Rumen fluid was collected from the slaughtered sheep at about seven minutes after slaughtering by emptying all the rumen contents into a bucket. This was mixed

thoroughly before a representative sample was taken. Rumen fluid was filtered through four layers of cheesecloth to remove feed particles. About 20 ml of rumen fluid sample was filled into a container with 5 ml 50% H_2SO_4 for NH_3-N analysis (Broderick & Kang 1980) and another 20 ml rumen fluid was preserved with 25% ortho-phosphoric acid for volatile fatty acid analysis (Ottenstein & Bartley 1971).

5.2.5 *Chemical composition of experimental feed*

The organic matter (OM), ash and DM of the TMR used in this study, faeces and feed were determined according to the standard procedure described in AOAC (2002). Acid detergent fibre (ADF), neutral detergent fibre (NDF), and lignin were determined using Ankom technology 200/220 (Ankom Technology, Fairport, NY, USA) as described by Robertson and Van Soest (1981). Nitrogen was analysed with Leco Instrumente GmbH, Kirchheim, Germany, nitrogen/protein analyser. Ether extract was determined by extracting the sample with ether following the Tecator Soxtec (HT6) system (AOAC, 2002).

5.2.6 Methane emission measurement

After measurements of feed intake and digestibility were completed, 20 animals were transferred in a batch of four animals to the open circuit respiration chamber for measurement of methane. In each batch, four lambs were selected from the same block and represented the four treatments. Details of the set-up of open circuit respiratory chambers were described earlier by Gemeda and Hassen (2015). The chambers were constructed using 25 x 25 mm powder-coated steel frames, which gave an approximate volume of 5 m^3. The chamber boxes consisted of a roof, three sides and a bisectional front door. The sides were covered with 1.0 mm thick UV-resistant clear flexible polyvinyl chloride (PVC) sheet. The bottoms of the two doors had air gaps of 330 mm to allow air into the chamber. The air gap facilitated the passage of air into and out of the chamber and acted as a safety mechanism in the event of a power outage when the extractor fans fitted on top of the chamber failed to extract air. Air exiting the chamber through the extractor fan was connected to a pipe containing two 90-degree bends before being vented to the outside of the building through plastic tubing. Air flow speed was taken and recorded on the computer automatically with a fixed hot wire anemometer, which had been pre-set to regular intervals. In addition, every day a manual air flow measurement was taken at regular intervals with portable vane and hot wire anemometers. Data generated by the manual anemometer was used to adjust the airflow, which was measured continuously by

the fixed hot wire anemometer to overcome a potential gradual reduction in its sensitivity as a result of continuous exposure of the sensor to dust.

For all chambers, a sample of air from the duct was collected into a balloon for one hour during the recovery test and for 23 hours per day to determine the concentration of methane during the recovery test and animal measurement. A sub-sample of air leaving the chambers was continually withdrawn after the extraction fan through a 6 mm diameter plastic pipe. The lengths of sampling tube for the chambers were kept at equal lengths and were connected to a peristaltic pump (Model No. 07522-30 Cole-Parmer Instrument Company, USA) fitted with an eight-channel easy-load (Model 77292-50, Masterflex, USA), which sucks samples of the air passing the duct continuously for 23 hours. The sucked air was channelled into pre-emptied collection bags. Ambient samples were also collected from each of the chamber by directly running the opening of collection tubes in front of each chamber. In each cycle, the four animals that represented each of the four treatment groups were rotated every day to pass through a different chamber. Animals were kept in the chamber for five days, which consisted of one day for adapting to the chamber and four days of data collection. Thus, the design was a randomized complete block design, where each cycle of methane measurement stood as a block, which translates to a total of five blocks per treatment. All animals were all given fixed amounts of TMR and plant extracts were administered as described in Section 5.2.4.

Collection of refusals, cleaning of the pen and rotation of animals was done once a day before morning feeding. Before introducing animals into the chambers, several preliminary studies and optimization procedure were carried out to determine the recovery percentage of each chamber. Methane recovery percentage was tested by releasing a known volume of 99.5% CH_4 into each chamber for one to four hours with a mass flow meter connected to a pressurized methane cylinder. Gas samples that left the chamber through the extraction pipes during the recovery tests and during animal measurement were sub-sampled with a peristaltic pump into a collection bag. The methane gas concentration for each collection bag was determined by injecting five separate samples from each bag using gas chromatography fitted with flame ionisation detection in the laboratory. The amount of methane recovered was calculated from the total volume of air that has been extracted from the chamber in a specified time and multiplied by the net concentration of methane estimated for each chamber to determine recovery percentages for each of the chamber. The recovery percentages obtained for the four chambers ranged from 85% to 97%. Methane was analysed in the laboratory with gas chromatography fitted with a flame ionisation detector, which had a solenoid column packed

with silica gel (8610C Gas Chromatograph (GC) BTU Gas Analyser GC System, SRI Instruments, Germany).

Figure 5.1a. Open-circuit respiratory chamber for small ruminants, University of Pretoria.

Figure 5.1b. Open-circuit respiratory chamber for small ruminants, University of Pretoria.

Figure 5.2 Experimental animals two hours before slaughter.

Figure 5.3 Experimental animals in digestibility crates during digestibility trial.

Figure 5.4 Experimental animals during growth study.

Figure 5.5 Moisture-free (freeze-dried) crude plant extract of *Jatropha curcas* leaves used in the study.

Figure 5.6 Moisture-free (freeze-dried) crude extracts of *Moringa oleifera* leaves used in the study.

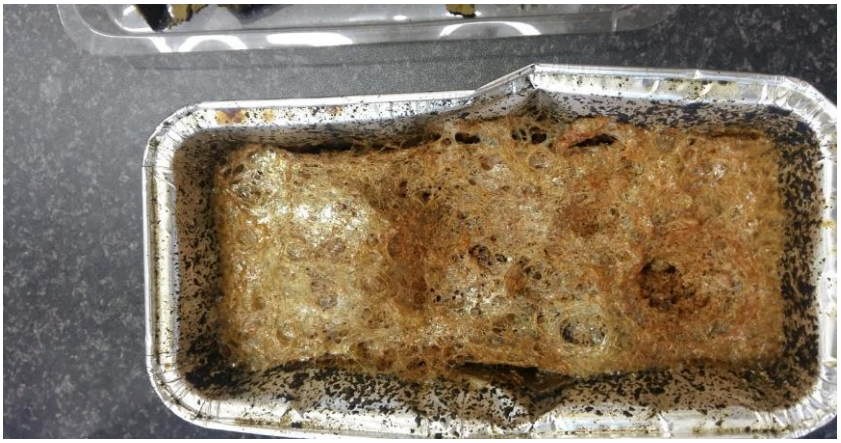


Figure 5.7 Stiffened jelly-like extracts obtained after freeze-drying methanolic plant extract of *Aloe vera*.

5.2.7 Data collection and analysis

Growth performance, nutrient and dry matter intake

Body weights of animals were recorded for three consecutive days prior to the start of the experiment and thereafter at seven day intervals before the morning feeding until the end of the growth experiment. Final weights of animals were also recorded for three consecutive days before the morning feeding. Average daily gain (ADG) was calculated by regressing the weekly body weight against time in weeks. Feed offered to experimental animals and orts were recorded daily for each pen. This was divided by 2 to estimate average voluntary DM intake and refusals per animal. Samples of fresh feed and orts were analysed by oven drying at 55 ^{o}C for 48 hours or until constant weight was achieved. Dried samples were milled to pass through a 1-mm sieve and analysed later for nitrogen, NDF, ADF, and ash by the procedure described earlier in this section.

5.2.7 Statistical analysis

All data collected were analysed using the general liner model (GLM) procedures of SAS (version 9.4; SAS, Cary, USA). Means when significant were separated using the Tukey test. The following model was used:

$Yij = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$ where

Yij is the response of different treatments in terms of all parameters measured;

α_i is the overall effect of treatment (β_j) is the effect of different body weight of sheep (block);

ε_{ijk} is the effect of random error.

Table 5.1. Composition and chemical analysis of total mixed ration fed to SA Mutton Merino sheep receiving various plant extract dosages

Ingredient	Composition (%)
Soybean meal	17.0
Yellow maize	28.0
Alfalfa hay	20.0
Eragrostis curvula hay	22.2
Molasses	6.0
Wheat offal	5.0
Urea	0.8
Vitamin premix	0.5
Total volume	100.0
Chemical composition	
CP (%)	18.3
Starch (g/kg)	181
NDF (g/kg)	345
ADF (g/kg)	206
Lignin (g/kg)	24.5
Ash (%)	6.4
ME (MJ/kg)	9.1

Key to abbreviations: CP:crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, ME: metabolizable energy.

5.3 Results and discussion

5.3.1 Chemical composition of experimental feed

The average chemical composition of TMR used in this study is reported in Table 5.1. The crude protein (CP) of the TMR is 17.2%. The g/kg DM content of starch, NDF, ADF and lignin were 64.8, 315.8, 241.5 and 24.5, respectively. The TMR was formulated to meet the nutritional needs of growing lambs to achieve an average daily weight gain of 200 g per day. To obtain a better response for methane-reducing potential of plant extracts of MO, JA, and AV, the fibre content of the TMR was kept high during formulation as plant extracts tended to perform better when low-quality roughage is used (Chapter 4).

5.3.2 Total tract digestibility and nitrogen balance

Significantly higher (P<0.05) DM and OM matter intakes were recorded on sheep that received plant extracts of MO, JA and AV as an additive compared with the control. However, the CP, NDF, ADF and starch intake per/head/day did not differ (P>0.05) across all treatments. Generally, the apparent digestibility of SAMM sheep recorded for treatments MO, JA and AV plant extracts was higher than that of the control (Table 5.2). Dry matter digestibility of diet by sheep that received MO, JA and AV plant extracts as additives was significantly higher (P<0.05) compared with sheep on the control treatment. Organic matter digestibility recorded for sheep on MO, JA and AV extracts was slightly higher than that obtained for sheep on the control diet.

Nitrogen balance results are shown in Table 5.3. No significant (P>0.05) difference in nitrogen intake was observed among the experimental treatments. Nitrogen balance was lower for sheep dosed with MO extract compared with JA and AV extracts, but the difference observed between MO extract and the control was not significant (P>0.05). Urinary N was significantly higher (P<0.05) in the MO treatment compared with the control and other treatments. Extracts of AV and JA recorded the highest N balance of 27.89 g/head/d and 27.29 g/head/d, respectively, compared with MO.

Higher DM and OM intakes by sheep that received medicinal plant extracts could to some extent be an indication of a faster rate of passage and shorter resident time of digesta in the rumen, which could have resulted in a higher feed intake and total tract digestibility of the TMR. Feeding ruminants a high roughage diet has been linked to the increased activity of cellulolytic bacteria in the rumen (Patra 2016), which are responsible primarily for fibre degradation, and produce acetate, CO_2 and hydrogen as products of fermentation. In contrast, feeding high concentrate diets would increase the activities of amylolytic bacteria, while protozoa in the rumen aid in the conversion of H_2 to CH_4. Increase in the population of amylolytic microbes increases the production of more oxaloacetate, a precursor to malate and fumarate, which helps in the production of more propionate. Monensin and organic acid have been reported by Patra (2016) to work as propionate enhancers. Plant extracts MO, JA and AV could have increased feed degradation in this study through actions similar to monensin (Akanmu and Hassen 2017), by attacking the complex polysaccharide chain, thereby loosening up substrates for easier microbial degradation. The breakage of the polysaccharide chain through the cleavage of β-phenyl ether bond of lignin by phytochemicals in plant extracts,

especially anthraquinone (see Chapter 2), could have influenced the rate of feed degradation and passage in the rumen and consequently lowered the rate of multiplication of cellulolytic bacteria in the rumen, as judged by higher DM, OM, NDF, ADF and CP intake values recorded for sheep on MO, JA and AV treatments. These were complemented by higher total tract digestibility compared with those on the control. This agrees with *in vitro* findings reported in previous chapters, where the addition of plant extracts of MO, JA and AV was shown to increase *in vitro* feed organic matter digestibility.

A significant increase in CP intake by MO group was reported by Fadiyimu *et al*. (2010), when MO leaves was supplemented to West African Dwarf sheep, which could be partly due to the higher CP content and rich amino acid profile in MO. A positive relationship between higher CP intake and higher N excretion was reported by Dabiri and Thonney (2004). These authors attributed the higher N excretion in the urine to a possible endogenous loss because of increased dietary protein intake. In the present study, all experimental animals were fed the same diet. The significantly higher N in the urine of sheep supplied with MO additive could be due to the action of the experimental treatment on the digestibility of the nitrogen fraction. Sultana *et al.* (2015) reported higher urinary N excretion when diets containing MO were fed to goats. High urinary excretion observed in sheep that received MO could have resulted from the increased contribution through the action of phytochemicals and the rich amino acid profile of MO plant extracts on TMR, which could produce more N than is normally required by microbes in the rumen. Brooker *et al*. (1994) explained that this surplus ammonia would be converted to urea and consequently excreted by the animal, as witnessed in this study. Anthraquinone glycoside, a recognized phenolic compound in plant extracts of MO, JA and AV, could have affected digestibility (Lewis et al. 2003; Al-Kassie 2009; Sharifi et al. 2013; Naeemasa *et al*. 2015; Omar *et al.* 2016).

Anthraquinone is a laxative agent. It increases peristalsis movement in the colon and reabsorbs water from the colon, thereby reducing the retention time of digesta. It also makes the stool more liquid and enhances easier passage. As witnessed in this study, higher feed intake and total tract digestibility for animals on experimental treatments, especially AV and MO, could be due to the effect of anthraquinone, which affected the rumen environment by influencing the digestion of fibre components in the feed (Akanmu & Hassen 2017). Only a few literature studies are available on the use of methanolic plant extracts as rumen modulators. However, several studies on poultry nutrition, which involved the supplementation of various medicinal plant extracts to broiler feed (Lewis *et al.* 2003; Al-Kassie 2009; Sharifi *et al.* 2013; Naeemasa

et al. 2015; Omar *et al.* 2016), achieved improved ADG and feed conversion efficiency. Authors credited this improvement to the presence of phytochemicals in the extracts that have antimicrobial and digestion-stimulating properties. In all cases, plant extracts have not reduced broiler growth rate. The higher feed digestibility response could be partly associated with the higher roughage content of TMR formulated for this study because plant extracts have been found to be more effective when used on lower-quality feed as reported in previous studies (Chapter 4).

Table 5.2. Nutrient intake and total tract digestibility of total mixed ration when SA Mutton Merino sheep are dosed with various medicinal plant extracts

Parameters	Control	MO	JA	AV	SEM	P-Value
Feed intake						
DM g/head/d	1425c	1552a	1502b	1530ab	30.0	0.0001
OM g/head/d	1370c	1443a	1410b	1432ab	26.2	0.0002
NDF g/head/d	490.0	532.5	512.0	522.5	32.2	0.4429
ADF g/head/d	294.0	318.7	310.0	314.5	22.2	0.3264
CP g/head/d	261.0	287.0	274.5	280.0	9.41	0.0540
Starch g/head/d	257.0	265.0	270.0	275.0	6.02	0.5548
Dry matter g/kg $W^{0.75}$/d	78.7	84.4	81.1	86.2	4.51	0.1403
Organic matter g/kg $W^{0.75}$/d	75.67	78.37	76.19	80.75	4.16	0.3382
Total tract digestibility (%)						
Dry matter	70.09c	72.98b	75.59a	73.45ab	1.43	0.0014
Organic matter	79.78b	80.24b	81.41a	80.74ab	0.67	0.0291
NDF	47.85	51.04	52.81	48.32	4.40	0.3769
ADF	40.97	42.71	47.14	45.91	5.68	0.2376
CP	78.26b	81.85a	82.21a	80.12ab	1.47	0.0096
Starch	98.34	99.27	99.13	99.40	0.85	0.1669

*$W^{0.75}$: metabolic body weight, DM: dry matter, OM: organic matter, NDF: neutral detergent fibre, ADF: acid detergent fibre, CP: crude protein
*Means across the row with different letters are significantly different

Table 5.3. Nitrogen balance of SA Mutton Merino sheep dosed with various plant extracts

Parameters	Control	MO	JA	AV	SEM	P-Value
N intake (g/head/d)	42.96	46.03	44.91	44.78	2.53	0.3222
Faecal N (g/head/d)	9.11	8.32	7.87	8.63	0.72	0.0839
Urinary N (g/head/d)	9.03b	16.98a	9.74b	8.27b	3.18	0.0017
N balance (g/head/d)	24.82	20.73	27.29	27.89	4.91	0.0129
N balance/N intake	0.57a	0.45b	0.61a	0.62a	0.08	0.0245
N balance (g/kg $W^{0.75}$)	76.24b	78.59ab	80.04a	78.34ab	1.64	0.0452

*Means across the row with different letters are significantly different

5.3.3 Volatile fatty acids, ammonia nitrogen and rumen pH

Volatile fatty acid concentration, rumen pH and ammonia nitrogen values are presented in table 5.4. The rumen pH in sheep on MO, JA and AV extracts was 5.9, 6.1 and 5.95, respectively, compared with the 6.02 for the control. A significantly ($P<0.05$) higher rumen NH_3-N concentration was recorded for the control (23.6 mg/100 ml) compared with sheep on MO (15.1 mg/100 ml), JA (18.1mg/100 ml) and AV (16.4 mg/100 ml) extracts. Increase in rumen NH_3-N production corresponds to the findings of *in vitro* study reported in Chapter 3. Total VFA production for all treatments was not significantly different. The molar proportions of individual VFA were significantly ($P<0.05$) different in acetic and propionic acid concentrations. The use of MO increased the propionic acid concentration in the rumen compared with sheep on JA and AV and the control. A lower acetate/propionate ratio recorded for animals on MO is favourable for protein synthesis (meat production) because an increased volume of propionate production enhances production of lactose, which is a precursor to glucose synthesis via the gluconeogenesis pathway (Schmidt & Zsedely 2011). Previous studies (Holter & Young 1992; Duan *et al*. 2006) reported that increasing the quantity of concentrate at the expense of forage in TMR favours the production of more propionic acid. MO could have enhanced propionic acid production by favouring the amylolytic bacteria that are responsible for fermenting starch or could have caused a rapid fermentation of the substrate in the rumen, thus reducing the amount of acetic acid produced, which would normally amass from slow fermentation (Kung 2014; Schmidt & Zsedely 2011).

Table 5.4. Effect of plant extracts supplementation on the pH, ammonia nitrogen and volatile fatty acids of SA Mutton Merino sheep

Ruminal fluid characteristics	Control	MO	JA	AV	SEM	P-value
pH	6.02	5.90	6.10	5.95	0.17	0.44
NH_3-N (mg/100ml)	23.60a	15.16c	18.11b	16.42bc	1.55	<0.01
TVFA (mg/100ml)	306.39	312.55	310.58	304.29	10.1	0.48
Molar proportions of VFAs						
Acetic acid	61.78	52.07	64.96	66.78	7.72	0.08
Propionic acid	16.06b	25.42a	17.63b	14.34b	2.76	0.04
Butyric acid	12.95	13.74	10.33	11.18	4.26	0.66
Iso-butyric acid	1.78	2.09	2.06	1.66	0.81	0.85
Valeric acid	4.12	3.39	1.92	2.42	1.66	0.29
Iso-valeric acid	3.30	3.27	3.09	3.61	1.09	0.93
Acetic/propionic acid	3.91	2.08	3.69	4.65	1.79	0.20

*Means across the row with different letters are significantly different.

5.3.4 Growth performance and carcass yield

Table 5.5 shows the growth performance characteristics of SA Mutton Merino sheep that received plant extracts. The initial and final body weights were not statistically different (P>0.05). However, ADG of animals on MO and JA extracts were slightly higher than the control and AV extracts. MO tended to improve ADG compared with the control and AV treatments. The lowest average daily DM intake was recorded for sheep on JA treatment, while the highest was on MO, although the values were not statistically significant (P>0.05). Similarly, the total weight gain was significantly (P<0.05) higher for sheep on MO and JA treatments compared with the control and AV treatments. Carcass dressing percentage and cold carcass weight were not affected by the plant extracts compared with the control. Likewise, feed conversion efficiency did not differ significantly, although MO and JA tended to have lowered feed to gain ratio when compared with the control and AV treatments. Judging from the improved total tract digestibility and VFA data and improved propionate production for MO plant extract, one could expect higher ADG in MO than in the other two plant extracts. Generally, propionate production favours protein synthesis, which leads to more meat production.

Table 5.5. Growth performance and carcass yield of male SA Mutton Merino sheep dosed with various medicinal plant extracts

Parameters	Control	MO	JA	AV	SE	P-Value
DMI (kg/head/day)	1.19	1.23	1.17	1.19	0.11	0.4371
Initial BW (kg)	28.80	28.50	28.90	28.80	3.06	0.8714
Final BW (kg)	46.51	48.89	49.14	46.56	3.37	0.6988
Total weight gain (kg)	17.71b	20.42a	20.32a	17.81b	1.44	0.0262
ADG (g/sheep/day)	259.7	299.2	272.6	255.2	22.7	0.0713
FCE (kg feed DMI/kg gain)	5.03	4.54	4.33	5.03	0.38	0.158
Cold carcass weight (kg)	21.67	22.93	21.89	21.74	0.37	0.7458
Dressing percentage (%)	47.60	47.90	45.50	47.70	3.22	0.141
Carcass fat score	2	2	2	2	--	--

*BW: body weight, ADG: average daily gain, DMI: dry matter intake, FCE: feed conversion efficiency
*Means across the row with different letters are significantly different

5.3.5 Methane emission measurement

Methane (CH_4) emissions were significantly ($P<0.05$) higher CH_4 (g/head/day) for sheep on the control group (58.46), compared with sheep on MO (46.01), JA (37.98) and AV (41.97) plant extracts. Dry matter and OM intake per head per day were not significantly different ($P> 0.05$). This was expected as the sheep were offered the same amount of feed every day. Methane emission by lambs per kg of DM intake was significantly higher ($P<0.05$) for sheep in the control treatment compared with sheep on the other plant extract treatments. Plant extracts of MO, JA and AV significantly ($P<0.05$) reduced CH_4/OMI (methane/organic matter intake); CH_4/DOMI (methane/digestible organic matter intake); CH_4/DDMI (methane/digestibe dry matter intake) by 22, 34.8, 28.2%; 24.5, 39.8, 31.6%; 21.5, 36.2, 28.9%, respectively. Methane emission per kg of DM intake recorded for lambs on MO, JA and AV plant extracts was lower compared with the control. The observed significant CH_4 reduction from all the plant extracts could have resulted from the beneficial effect of phytochemicals in MO, JA and AV. The lower CH_4 emission observed for MO plant extract was expected as the sheep that received MO plant extract additive recorded a lower acetate/propionate ratio, lower rumen pH, higher propionic acid concentration and better growth performance. Lower pH in the rumen positively affects

the amylolytic bacteria and negatively affects the population of protozoans, which aided in the formation of CH_4in the rumen. Methane could also have been reduced owing to a faster rate of feed passage in the rumen, slower fermentation of poor-quality feed that might have led to the production of more acetic acid, which favours the production of CH_4 because of the extra H_2 by-product associated with acetic acid formation.

Various *in vitro* studies have documented the effectiveness of plant secondary compounds against methanogens. Compounds that are mainly responsible for the observed effect included condensed tannins, saponins and essential oils (Rochfort *et al.* 2008; Alexander *et al.* 2008; Tan *et al.* 2011; Akanmu & Hassen 2017). Tannins, saponins and essential oils were expected to be present in varied proportions in MO, JA and AV plant extracts used in this study. Dey *et al*. (2014) reported *in vitro* CH_4 reduction of wheat straw supplemented with MO leaves. As indicated in Figure 1.3, protozoans and the methanogen populations are closely linked with methane production. Part of the strategy of reducing methane emission is the eradication or suppression of activities of methanogens or protozoans through the use of saponins, halogenated compounds and plant metabolites. In this study, MO, JA and AV could have reduced CH_4 (g/head/day) because of their antimicrobial, antifungal and antiprotozoal activities. Numerous compounds in these plants (as reviewed in Chapter 2) have negative effects on protozoans and methanogens. Curcin, which is present in JA, is toxic, and JA could have reduced methane emission from sheep by direct inhibition of methanogens and protozoans in the rumen. Srivastava et al. (2012) reported the inhibitory ability of JA plant extract against Penicillium and Aspergillus spp. On the other hand, AV is rich in alkaloids and could have reduced methane production through its direct effect on methanogens because it has antibacterial, antifungal, and antiseptic properties (Patra 2014).

Popp *et al*. (2016) reported a decrease in methane production by up to 53% when alkaloid gramine was added in an anaerobic co-digestion of grass silage and cow manure. These authors indicated that the decrease in CH_4 gas production could have resulted from the shift from a well-balanced mixture of five phylotypes towards a strong dominance of Methanosarcina. The methane-reducing effect witnessed in this study could have also resulted from a shift in archaea population in the rumen, which is caused by the secondary metabolites dosed to sheep. Unfortunately, in this study, crude methanolic plant extracts were used, and thus the plant compounds associated with the responses observed in this study were unknown. As presented in Table 5.6, the calculation of CH_4 g/kg mutton resulted in higher CH_4 production from animals on the control treatment. Significantly ($P<0.05$) higher CH_4 was emitted (90.35g) per

kg of mutton produced by animals on the control treatment compared with 64.1, 48.5 and 65.1 g methane emitted per kg mutton produced from animals dosed with MO, JA and AV plant extract treatments, respectively.

Table 5.6. Effect of various medicinal plant extracts on methane production of SA Mutton Merino sheep

Parameters	Control	MO	JA	AV	SEM	P-Value
DM intake (g/head/d)	1550	1560	1540	1550	40.1	0.8860
OM intake (g/head/d)	1410	1430	1410	1420	33.0	0.8912
CH_4 (L/head/d)	89.12a	70.14b	57.90b	63.98b	9.07	0.0023
CH_4 (g/head/d)	58.46a	46.01b	37.98b	41.97b	5.95	0.0022
CH_4 g/kg DMI	41.31a	32.22b	26.93b	29.65b	4.19	0.0022
CH_4 g/kg OM intake	37.79a	29.48b	24.64b	27.13b	3.83	0.0023
CH_4 g/kg DDM intake	83.51a	63.01b	50.22b	57.11b	7.98	0.0005
CH_4 g/kg DOM intake	65.11a	50.97b	41.54b	46.26b	6.48	0.0015
CH_4 g/kg Mutton	90.35a	64.10b	48.54c	65.09b	3.01	0.0011

*Means across the row with different letters are significantly different

*DM: dry matter, OM: organic matter, DDM: digestible dry matter, DOM: digestible organic matter

5.4 Conclusion

Moringa oleifera (MO), *Jatropha curcas* (JA), and *Aloe vera* (AV) methanolic plant extracts are important anti-methanogenic agents that are capable of reducing methane emission from sheep when used at 50 mg/kg of feed. MO and JA can also increase feed digestibility, thereby increasing weight gain and capable of reducing methane production per kg of mutton produced.

CHAPTER SIX

Effect of plant extracts of *Moringa oleifera*, *Jatropha curcas* and *Aloe vera* supplementation on haematology, serum biochemistry and overall performance of SA Mutton Merino sheep

Abstract

Many medicinal plants have been reported to be toxic when used inappropriately at a higher dose, and could cause mild to acute disease or clinical conditions in animals. This study was conducted to test the long-term effect of supplementing selected methanogenic plant extracts of *Moringa oleifera* (MO), *Jatropha curcas* (JA) and *Aloe vera* (AV) on animal welfare and toxicity by monitoring the blood haematology and serum biochemical properties of SA Mutton Merino (SAMM) sheep. The plant extracts were prepared from fresh leaves of MO, JA and AV by freeze-drying them, milling them and dissolving them in pure methanol. All plant extracts were fully dried and reconstituted by dissolving 96 g of each plant extract in 120 ml distilled water and kept in a cold room for later use. A total of 40 SAMM lambs were used for the experiment after randomly dividing animals with similar body weight into four groups, which received one of the four experimental treatments. The treatments included a control (without any additive) treatment (T1) and three other plant extracts from MO, JA and AV, which represented T2, T3 and T4, respectively. Plant extract solutions were drenched to animals at the rate of 50 mg/kg dry matter (DM) and were given to these animals twice daily. Blood samples were taken twice to represent the conditions before and after the experiment, analysed for haematology and serum biochemistry using standard procedures. The results of haematological analysis generally indicated no significant ($P>0.05$) differences among treatments, especially in terms of haemoglobin, red blood cell (RBC), haematocrit, mean corpuscular haemoglobin (MCV), mean corpuscular haemoglobin concentration (MCH), red cell distribution, monocyte and platelet count. However, significantly ($P<0.05$) higher white blood cell (WBC) and lymphocytes counts were obtained for the control and animals treated with AV plant extract when compared with animals treated with MO and JA. All serum biochemical properties (except alkaline phosphatase) were not significantly ($P>0.05$) different between control animals and animals treated with plant extracts. The alkaline phosphatase was relatively low in animals treated with AV plant extract when compared with the control, but alkaline phosphatase among animals treated with plant extracts was not different ($P>0.05$). The

result of the study showed that plant extracts of MO, JA and AV were not toxic to sheep at the dose of 50 mg/kg DM feed that is recommended for reducing enteric methane emission. A co-benefit was noted for some plant extracts in modulating the health condition of animals. Therefore, these plant extracts could be used safely as alternative dietary additives to reduce enteric methane emission and boost the performance of animals.

Keywords: haematology, blood biochemistry, Merino sheep, medicinal plant extracts

6.1 Introduction

Although natural alternatives are important and are preferred to the use of antibiotics in animal feed, a great deal of consideration should be given to the impact of these 'natural alternatives' on the health of animals. Recently, the use of medicinal plants as additives has been an area of focus as researchers tried to exploit the beneficial effect of medicinal plants by supplementing them to animals feed to achieve improved performance. However, many of these medicinal plants have developed various metabolites against grazing animals, coupled with anti-nutritional factors that might be toxic to animals, depending on the type of plant or parts consumed (Liwiński *et al*. 2002; Kumar *et al*. 2016). This forms the main reason that grazing animals in particular do not find some medicinal plants acceptable.

Plant extracts of *Moringa oleifera* (MO), *Jatropha curcas* (JA) and *Aloe vera* (AV) have shown a potential beneficial effect in reducing methane emission from ruminants without adversely affecting feed digestibility *in vitro* (Akanmu & Hassen 2017). The use of these plant extracts also promotes feed efficiency owing to the presence of various phytochemicals, which rapidly break down the complex polysaccharide chains in roughages, thereby releasing the nutrients available for animals. But *Jatropha curcas* (JA) was reported to be toxic when seeds were fed to animals, causing severe disease and high mortality (Adams & Magzoub 1975). *Jatropha curcas* contains phorbol esters, which are mainly responsible for its toxicity. Its antimicrobial and antiprotozoal activities in the rumen have been linked to the presence of phorbol esters (Akanmu & Hassen 2017), though this has not been certain. *Aloe vera* has a bitter taste that is orchestrated by anthraquinones and phenolic compounds. AV ingestion is associated with diarrhoea, kidney failure, phytotoxicity and hypersensitive reactions (Guo & Mei 2016). *Moringa oleifera* toxicity has not been reported in animal experiments. Akanmu and Hassen

(2017) reported no adverse effects on fermentation *in vitro* when leaves of MO, JA and AV were extracted using pure methanol.

To exploit and fully recommend the beneficial effects of extracts of MO, JA and AV on methane reduction, improvement in feed digestibility and fermentation parameters, there is a need to test whether supplementing 50 mg extracts of MO, JA and AV per kg of feed is toxic to animals. This study, therefore, tests the effect of supplementation of 50 mg/kg feed of *Moringa oleifera*, *Jatropha curcas* and *Aloe vera* on the haematology and blood chemistry of SAMM sheep fed total mixed ration (TMR).

6.2 Methodology

6.2.1 Study area

The study was conducted at the University of Pretoria Experimental Farm, Hatfield, South Africa. The annual rainfall in Pretoria is about 573 mm and the city is located at 1700 m above sea level. Maximum and minimum temperatures around the time of the study (December 2016 to March 2017) were 39 and 18 °C.

6.2.2 Collection and preparation of plant extracts

Plant materials were obtained and extracted as discussed in Section 5.2.2

6.2.3 Animals, blood collection and analysis

This study was approved by the Animal Ethics Committee of the University of Pretoria (ECO-030-14). Forty (40) four-month-old male SAMM sheep with an average live weight of 28.8 ± 0.4 kg were used for the experiment. The animals were first ranked according to their body weight and then blocked into four groups using stratified sampling achieved by distributing weights across the groups to achieve similar weights for all the groups. All four groups had an average weight of 29 ± 0.7 kg and were randomly allocated to the four treatments, that is, control, MO, JA and AV. There were ten animals per treatment, and two animals of the same treatment were housed in a pen.

Blood samples were taken from all 40 animals, and collection was done twice from the jugular vein, before and on the last day of the experiment during the growth study which lasted a total of 103 days as reported in Chapter 5. Five ml blood samples were collected into BD vacutainer tubes (BD-Plymouth UK) which contained ethylene diamine tetra acetic acid (EDTA) for

haematological analysis and another 5 ml was collected into BD vacutainer tubes for serum samples and blood urea analyses. The samples were immediately transferred to the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, for processing.

Complete blood analysis was done using a multi-parameter automated haematology analyser (ADVIA 2120i, Siemens, South Africa) and blood chemistry was analysed using the Cobas Integra 400 Plus (Roche, South Africa). Total mixed ration (TMR) and crude protein (CP) were determined by a nitrogen/protein analyser (Leco Instruments, Germany). Fibre analyses were obtained using Ankom 200/220 fibre analyser and ether extract was determined by standard procedure (AOAC 2000).

6.2.4 Statistical analysis

Data obtained from this study were analysed using the general linear model (GLM) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The statistical model included treatment effect, block and random error. Where F-test revealed significant difference, means were separated using the Tukey test of the same package. In addition, multivariate analysis was performed to investigate the overall effects of plant extracts on animal welfare and performance by integrating performance data presented in Chapter 5 with welfare data in Chapter 6.

6.3 Results and discussion

6.3.1 Experimental diet

The experimental diet used for the study was presented in Chapter 5 (Table 5.1). Roughages (alfalfa and *E. curvula* hay) make up 42% of the ration and the TMR has crude protein value of 17.2%. The diet was formulated to meet the needs of growing lambs to achieve a growth rate of 200 g/head/day.

6.3.2 Blood haematology

The blood haematology of SAMM sheep drenched with extracts of MO, JA and AV is presented in Table 6.2. Haemoglobin, red blood cells (RBC), haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), monocytes, eosinophil and platelet counts did not differ ($P>0.05$) significantly among the treatments. The observed values for haemoglobin, RBC, MCV, white blood cells (WBC) were within the range reported by Lepherd *et al*. (2009) for SAMM sheep.

Relatively higher ($P<0.05$) WBC counts were recorded for animals on the control diet (8.42 $x10^9$/L) compared with animals that received MO (6.78 $x10^9$/L) and JA (5.99 $x10^9$/L) plant extracts, which had significantly lower values. In contrast, the WBC count (8.21 $x10^9$/L) for animals on AV did not differ significantly from values recorded for JA, MO and the control treatments. One of the functions of WBC is to defend the body against infections caused by pathogens such as viral, bacterial and fungal organisms.

A high WBC value recorded for the control group would probably suggest that immunity level of the animals on the control treatment were lower than those drenched with plant extracts. Plants extract of MO, JA, and AV have antifungal and antibacterial properties (Ganatra Tejas *et al.* 2012; Ke *et al.* 2012; Kumar *et al.* 2016), which might have played a positive role in modulating the immune system and subsequently health condition of animals. This might have contributed to the observed lower levels of WBC for animals on MO and JA plant extract additives. It also indicates that animals were not under any disease condition or threat when blood samples were taken. Similarly, a significantly ($P<0.05$) higher lymphocyte count was recorded for animals in the control (4.73 $x10^9$/L) and those that received AV (4.75 $x10^9$/L) plant extract as compared with MO (3.48 $x10^9$/L) and JA (3.36 $x10^9$/L). Lymphocyte is a component of WBC, which is fundamental to the immune function of the animal, and the results indicated good health conditions of all animals on the experimental treatments.

Table 6.1. Blood haematology of SA Mutton Merino sheep drenched with extracts of *Moringa oleifera*, *Jatropha curcas* and *Aloe vera*

Parameters		**Control**	***M. oleifera***	***J. curcas***	***A. vera***	**SEM**	**P value**
Haemoglobin	(g/L)	120.4	120.1	119.8	115.5	8.31	0.1254
Red blood cells	(H x 10^12/L)	11.67	11.18	11.27	11.07	1.02	0.0911
White blood cells	(x 10^9/L)	8.42a	6.78bc	5.99c	8.21ab	1.59	0.1452
Haematocrit	(L/L)	0.35	0.34	0.34	0.34	0.02	0.0751
MCV	(fL)	30.15	30.94	30.49	30.91	2.11	0.1112
MCH	(pg)	10.49	10.87	10.75	10.62	0.81	0.4785
MCHC	(g/dL)	34.77	35.15	35.28	34.42	1.39	0.9652
Red cell distribution	(%)	17.71	17.78	17.92	17.41	1.34	0.7541
Segmented neutrophil	(x10^9/L)	3.39	3.03	2.67	3.04	1.16	0.2532
Lymphocytes	(x10^9/L)	4.73a	3.48b	3.36b	4.75a	1.02	0.0235
Monocyte	(x10^9/L)	2.22	4.36	2.11	3.10	1.71	0.0652
Eosinophil	(x10^9/L)	0.05	0.06	0.05	0.07	0.02	0.9251
Basophil	(x10^9/L)	0.02	0.00	0.00	0.00	0.02	0.2411
Platelet count	(x10^9/L)	508.2	628.2	636.0	575.6	211	0.5521

Means with different letters across the rows differed significantly (P<0.05)

MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration.

Table 6.2. Blood biochemical indices of SA Mutton Merino sheep drenched with extracts of *Moringa oleifera*, *Jatropha curcas* and *Aloe vera*

Parameters		**Control**	***M. oleifera***	***J. curcas***	***A. vera***	**SEM**	**P value**
Urea nitrogen	(H mmol/L)	8.78	9.41	9.04	10.02	1.57	0.343
Glucose	(H mmol/L)	3.33	3.42	3.39	3.25	0.39	0.785
Cholesterol	(mmol/L)	1.42	1.47	1.53	1.49	0.32	0.896
Total serum protein	(g/L)	66.58	64.07	64.31	65.60	4.31	0.544
Albumin	(g/L)	36.58	37.58	35.06	36.68	2.81	0.291
Globulin	(g/L)	30.00	26.48	29.24	28.92	3.66	0.311
AST	(H U/L)	106.7	113.1	152.7	114.1	61.3	0.371
ALT	(U/L)	14.58	14.55	22.74	15.63	4.17	0.549
ALP	(H U/L)	335.6a	259.6b	267.3b	221.1b	10.2	0.047

Means with different letters across the rows differed significantly ($P<0.05$)

AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase.

6.2.3 Serum biochemistry

According to Hammond (1997), digestible protein in the diet of ruminants can either be degraded while in the rumen or escapes to the abomasum and small intestine for further degradation into amino acids and peptides, which are then absorbed in the blood system. Feeding increasing levels of dietary protein while holding energy intake at a constant level increases blood urea nitrogen (BUN). In addition, BUN concentration can be increased significantly with greater dietary solubility or degradability. Thus, the BUN test is used to determine kidney functionality by measuring how much urea nitrogen is in the blood of animals. Urea in the blood is generally considered a waste product, which should be filtered off from the blood by the kidneys unless reutilized in the rumen via the urea cycle. Hyper or hypo values checked against control animals or known references could indicate serious physiological problems with the animal, which can be linked with liver damage, kidney and urinary tract issues. In ruminants, various authors have reported a linear relationship between BUN and rumen degradable protein (RDP), the concentration of BUN could be significantly increased based on dietary RDP (Sarwar *et al.* 2007). BUN recorded for this study showed no difference when plant extracts of MO, JA and AV were used as additives compared with the control, although it appears that higher BUN values were recorded for animals that received the three plant extracts. The potential increment could be attributed to the activities of phytochemicals present in the plant extracts MO, JA and AV, which could have increased the proteolytic activity, resulting in the release of more N in the rumen, since all treatments were fed the same total mixed ration (TMR). The values for BUN recorded for this study showed that plant extracts MO, JA and AV had not affected the animal negatively.

Blood glucose, cholesterol, and total serum protein showed no difference ($P>0.05$) among the treatments. The source of glucose for ruminants is gluconeogenesis from volatile fatty acids (VFA), and the fraction of VFA responsible for this is the propionate. The concentrations of total serum protein and cholesterol in the blood are regulated to balance physiological functions that cater for immunity, coagulation, small molecule transport and inflammation, and any huge variation in the concentration of serum proteins might be a result of disease (Tothova *et al.* 2016). The glucose level for sheep that received MO, JA and AV plant extract additives was comparable with that of the control, which indicates that providing animals with MO, JA and AV plant extract did not affect glucose production as a result of negative interference digestibility. The slightly higher numeric values obtained for glucose from MO treatment might be related to the higher molar propionic acid concentration observed for this treatment (Chapter

5). This study also confirms the authors' previous *in vitro* finding (Akanmu & Hassen 2017), which indicated that the use of MO, JA and AV plant extracts at a dosage of 50 mg/kg of feed did not affect feed digestibility negatively.

Results obtained for aspartate transaminase (AST) and alanine transaminase (ALT) were not significantly ($P>0.05$) different among treatments. However, significant ($P<0.05$) reduction in alkaline phosphatase (ALP) was recorded in sheep that received AV (221.1 H U/L) plant extract when compared with the control (335.6 H U/L), but the observed differences between AV and MO (259.6 H U/L) and JA (267.3 H U/L) were not significant ($P>0.05$). In the diagnosis of animal health, AST and ALT are important enzyme tests that are used to check the conditions of important organs in the body – such as heart and liver – of the experimental animals. Higher elevations of AST, ALT and ALP could signal serious liver conditions. When the liver is damaged, additional AST is released into the bloodstream. The amount released usually corresponds with the extent of tissue or organ damage. ALP resides in the wall of intra and extra biliary ducts that connect the cells in the liver and also functions in the cells that connect the liver to the gall bladder. An elevation of this enzyme in the blood can also be related to malfunction or blockage of the ducts inside the liver. Various factors, such as feeding animals with toxic supplements, can also influence the concentration of AST, ALT and ALP in the blood serum.

A higher ALP value recorded for the control animals, compared with animals that received the plant extract treatments, showed that the SAMM sheep can tolerate the dosage level of all three plant extracts. According to some researchers, JA contains toxic phorbol esters and curcin (Adams *et al.* 1975). The JA and AV plant extracts tested at 50 mg/ kg of feed did not put pressure on the organs of animals. This suggests that there is a possibility of testing higher levels of these plant extracts to optimise further the rumen fermentation, methane reduction and modulation of the health condition of the animal, including performance. In contrast to the findings of this study, Adams et al. (1975) reported goat toxicity leading to mortality when JA seed (0.25 to 10 g/kg/day) was fed to goats. This mortality was caused by haemorrhage in the rumen, kidney and heart. The present study indicated that drenching JA (50mg/kg DM feed) extracted with methanol did not lead to any disease condition or mortality. However, other studies have shown that AV contains glycosides, which can be metabolised by the intestinal bacteria, causing mucus production and water in the colon, resulting in diarrhoea. As reported by Kumar *et al*. (2010), the use of organic solvent for extraction of *J. curcas* might have reduced phorbol esters to a safer level. Overall, the use of MO, JA and AV plant extracts as

additives to Merino sheep diet did not lead to disease or mortality, perhaps due to a safer dosage level tested in this study.

6.3.4 Analysis of overall response of animals to the plant extracts using multivariate approach

To highlight the overall effect of using the plant extract on rumen fermentation, nutrient digestibility, animal performance and health utilization, principal component analysis (PCA) was carried out using all parameters with the intention of identifying important variables that were influenced by these plant extract treatments. Key parameters that had shown at least 5% contribution to the total variation in at least one of the components are reported in Table 6.4. The data matrix comprised 82 variables and was used for the PC analysis. The first three components of the PCA expressed 99.9% of the total variation. PC1 accounts for 73.5% while PC2 and PC3 are responsible for 18.2% and 8.2% of total variations, respectively.

Positive correlations with all three principal components were witnessed for methane (CH_4 L/head/d) and all the parameters that involved methane (CH_4 g/head/d; CH_4 g/kg DMI; CH_4 g/kg OMI; CH_4 g/kg DDMI; CH_4 g/kg DOMI). The plotted PCs revealed that animals on the control treatment were associated positively to methane emission and negatively to ADG, as reported in Chapter 5. Moreover, ADF digestibility, urinary N, ADG, propionic acid and TVFA were negatively correlated with PC1. This is in line with a higher ADG and urinary N obtained for MO plant extract treatments in the growth study (Chapter 5). The most important factor associated with PC2 was TVFA, which is positively correlated with MO and JA plant extracts and subsequently associated with improved ADG of sheep that received these two plant extracts as an additive.

Figure 6.1 presents a plot of PC1 against PC2. It can be observed that animals on the control treatment had higher values of CH_4 L/d, CH_4 g/d, CH_4 g/kg DMI, CH_4 g/kg OMI, CH_4 g/kg DDMI, CH_4 g/kg DOMI, and NH_3-N/100 ml compared with other treatments. On the other hand, AV treatment was associated with increased molar proportion of acetic acid in the rumen, while JA additive were associated with reduced ADF digestibility when compared with AV. In contrast to AV, animals on MO treatment increased ADG due to the observed higher TVFA, DMI and molar concentration of propionic acid in the rumen. The PCA also revealed that the overall effects of the plant extract on blood haematology and biochemical properties were not high as only the haemoglobin component contributed more than 5% to the three dominant PCs.

The dendrogram also revealed three main groupings of treatments tested in this study (Figure 6.2). Generally, the control group differed from the plant extracts by 47% while within the plant extract additives MO treatment differed from JA and AV groups by about 16%. JA and AV had more or less similar characteristics as judged by their overall response in terms of rumen fermentation, nutrient digestion, animal performance and blood profile parameters.

Table 6.3. Principal component loadings of all parameters tested *in vivo*

		Principal components x100		
Parameters tested	Key	1	2	3
ADF digestibility %	17	-2.794	-2.879	-6.383
Urinary N g/d	23	-0.900	11.54	16.91
ADG g/head/d	29	-13.53	37.77	38.31
TDM intake	33	-0.641	1.161	7.460
CH_4 L/head/d	38	16.77	7.901	16.48
CH_4 g/head/d	39	11.00	5.183	10.81
CH_4 g/kg DMI	41	7.153	3.210	6.437
CH_4 g/kg OMI	43	7.818	3.508	7.035
CH_4 g/kg DDMI	44	17.90	7.718	17.29
CH_4 g/kg DOMI	45	12.59	5.926	12.78
NH_3-N mg/100ml	46	4.587	1.441	-6.035
Acetic acid molar conc.	47	1.016	-11.91	-19.05
Propionic acid molar conc.	48	-2.694	9.230	11.39
Butyric acid molar conc.	49	0.765	1.836	5.158
Total VFA nM	54	-9.001	25.54	40.61
Haemoglobin g/L	57	0.744	6.016	-0.7811
Eigenvalue		5581.5	1383.6	625.8
% Variance		73.53	18.23	8.24

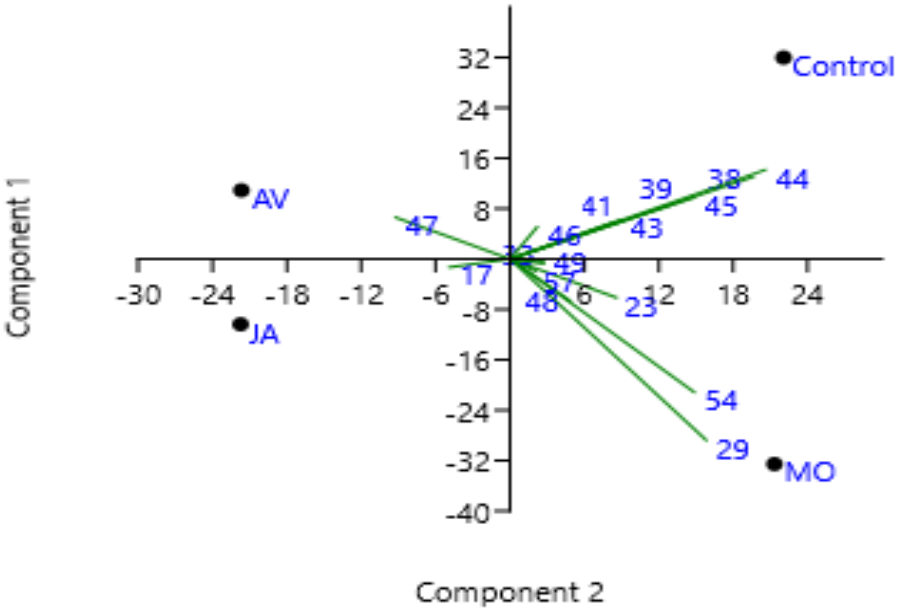


Figure 6.1. Principal component plot PC1 vs PC2 of growth performance, carcass characteristics, total tract digestibility, methane emission measurement, ammonia nitrogen and volatile fatty acids and blood profile of SA Mutton Merino sheep treated with plant extracts *Moringa oleifera*, *Jatropha curcas* and *Aloe vera*.

N.B. Parameters selected for PC plots were based on those that had variation of at least 5% in either direction from the principal component loadings, as shown in Table 6.4

KEY:

17	ADF digestibility%	44	CH_4 g/kg DDMI
23	Urinary N g/d	45	CH_4 g/kg DOMI
29	ADG g/head/d	46	NH_3-N mg/100ml
33	TDM intake	47	Acetic acid molar conc.
38	CH_4 L/head/d	48	Propionic acid molar conc.
39	CH_4 g/head/d	49	Butyric acid molar conc.
41	CH_4 g/kg DMI	54	Total VFA nM
43	CH_4 g/kg OMI	57	Haemoglobin g/L

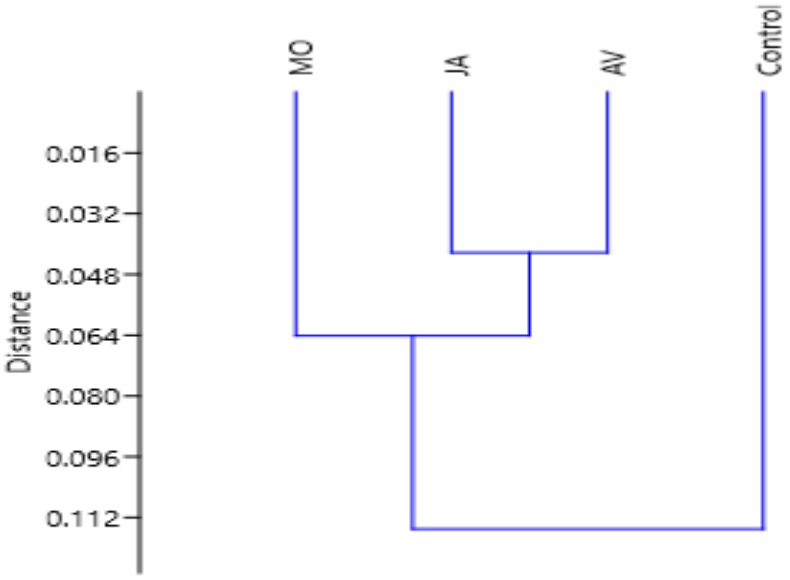


Figure 6.2. Dendrogram showing the overall relationship among experimental treatments in terms of growth performance, methane emission, feed digestibility, rumen fermentation parameters and blood profile of SA Mutton Merino sheep dosed with various plant extracts.

6.4 Conclusion

There were no incidences of diarrhoea or disease in all animals during the experiment. Methanolic extracts of MO and JA as additives to sheep diets boosted their immunity as evidenced by reduced WBC, lymphocytes and ALP values. The overall effect of addition of plant extracts of MO, JA and AV to sheep showed a better response than for those on the control. In conclusion, methanolic extracts of *M. oleifera*, *J. curcas* and *A. vera* supplementation to sheep were not toxic. Thus, despite the use of these plant extracts as additives, the welfare of sheep was not compromised. Plant extracts of MO, JA and AV can thus be used as alternative additives at a dosage of 50 mg/kg DM feed to achieve improved performance and boost the immune system of lambs in feedlot or in an organic mutton production system.

CHAPTER SEVEN

Criticial review, conclusions and recommendation

7.1 General conclusion and recommendation

This study aimed at assessing the potential of medicinal plant extracts as additives to reduce methane emission from ruminants without adversely affecting nutrient digestibility, animal performance or the health of the animal. The first study, which dealt with the *in vitro* screening of plant extracts and determination of the optimum dosage of application, was a pre-requisite to the subsequent studies that were designed. These are the conclusions from the study:

- Extraction of plant secondary compounds using pure methanol proved effective for plant species used in this study.
- Plant extracts of AV, AZ, JA, TD, CP and MO were effective rumen modulators and were able to reduce methane emission *in vitro.* These plant extracts increased feed digestibility without significantly increasing methane production.
- Plant extracts of PB were able to modulate rumen fermentation by increasing digestibility while increasing methane production.
- Cocktails of effective plant extracts AV, AZ, JA, TD, CP and MO increased propionic acid production *in vitro.* However, the extent of methane reduction recorded for these cocktails is not as low as the level recorded for individual plant extracts.
- Plant extracts of AV, AZ, JA, TD, CP and MO improved digestibility while reducing methane emission, regardless of the substrate type. However, they were more effective when used on low-quality forage than high-quality feeds such as lucerne and total mixed ration.
- Storing plant extracts of AV, AZ, JA, TD, CP and MO under refrigeration for over 12 months did not affect the activity of these plant extracts at reducing methane production *in vitro.*
- Plant extracts of MO, JA and AV reduced methane emission from SA Mutton Merino (SAMM) sheep while increasing feed digestibility in Merino sheep confirming the repeatability of in vitro results during the in vivo evaluation stage.
- In addition, plant extracts of MO and JA increased growth performance of SAMM sheep and had no adverse effect on the health of these animals.

These recommendations are based on the results of this study:

- More research could be conducted to test the effectiveness of medicinal plants used in this study from various regions of the world on their potential to reduce methane and increasing the digestibility of feeds.
- Research should be conducted to test the effect of supplementing more than 50 mg plant extracts per kg of feed *in vivo*, since animals responded well to the dosage administered. Improved performance and lower methane emission may be obtained for some of the plant extracts if the dosage is increased.
- Plant extracts of MO, JA and AV could be combined with fillers or encapsulation for easy administration by end users or farmers.

7.2 Critical review

One of the limitations of this study is that plant materials used throughout were obtained from the same location, and are possibly of the same strain and species, according to their identification. There might be a little variation in terms of the performance of the plant materials obtained from a different location owing to differences in the types and quantity of chemical constituents responsible for the observed effect in this study. Several studies have already confirmed that metabolites and ecological factors of various growing locations could affect the production or composition of desired active ingredients in the plants.

The extraction procedure, which includes the pre-washing, drying of plants materials under the sun, air drying under shade, oven drying or freeze-drying, can greatly influence the effectiveness of plant extracts. For plant materials with latex, for example *Aloe vera*, it is important to keep them frozen immediately after harvesting so that important ingredients are not lost due to pressing during handling. Dumping and clogging fresh plant materials together can lead to over-heating and loss of important phytochemicals in the plant materials, as cells within a plant continue to respire, even after harvesting.

Other factors include milling of dried plant material, which has to be uniform and homogenous in order to improve the kinetics of analytical extraction and provide good surface area to volume ratio to improve the contact surface for solvent extraction. During the current study, adequate care was taken to ensure that fine plant material that was already soaked in methanol did not escape inside the extracts. Any escape would increase the yield of the dried plant extract unnecessarily and reduce the effectiveness of the active ingredients owing to dilution effect or

loss of active ingredients during the procedure. The solvent to substrate ratio should also be optimized, as it was observed during the preliminary study that using too little solvent can affect plant extract production. To avoid loss of important secondary compounds from the plants, during the sieving process, the sieve or cheesecloth was completely immersed in the solvent, while continuous agitation was performed to avoid locking away secondary compounds in a stack of residue. Afterwards, the filtrate containing the extracted compounds was passed several times through the sieve until it was clear there were no solid residue left.

Reconstitution of dried plant extract to the desired concentration can be a tricky process. During the pre-trial experiment, it was found that certain vials under plant extract treatment produced high volumes of gas while other vials produced really low gas volumes. After several repetitions and elimination of possible errors, the author found that not all plant extract particles were fully dissolved in water. A big or large particle in a single vial can effect a large error in gas production. Therefore, plant extract solution should be prepared by first dissolving a weighed amount into a beaker with a small level of water and placing in a magnetic stirrer for several hours without using heat. After all the plant extracts are fully dissolved, the solution should be placed under a homogeniser for about two minutes to ensure all is properly mixed and no particles left. After homogenization, a coloured but clear solution should be obtained, depending on the type of plant material. Lack of a clear solution might be an indication of impurities in the extract. Some plant materials might have passed through the sieve and be regarded as extract, or the extract could have been contaminated during hand-working of dissolved plant material through the sieve or cheesecloth.

During the *in vitro* and *in vivo* study, as much as possible, errors were kept to the minimum. Where errors were found, the procedure was repeated.

During the *in vivo* study, plant extracts were drenched to animals twice a day, based on their feed consumption, which was determined in the previous week with the aid of drench gun. However, this is not practicable in commercial farming and was done only for experimental purposes to ensure all animals were given the required dosage.

Methane measurement was a complicated process during this study. The fully automated system, which involves automatic airflow reading measurement, gas sampling and consequent gas analysis using the MGA 3000 gas analyser, was substituted because of the performance of sensors used to measure airflow from the duct. To alleviate some of these errors, a modified workable set-up was obtained after a lot of optimization procedures. The first was to sample

gas samples from all the chambers at the same time using an 8-channel peristaltic pump. This pump was able to continuously sample and dump in the collection bags at low revolutions per minute (70) throughout 24 hours before the collection bag was changed the next day. This would allow the collection of representative sample of air throughout the 23 hour period of the day used to estimate daily methane production. Gas samples collected were then analysed in the laboratory with gas chromatography (GC) fitted with flame ionization detection (FID), which had a lower error margin compared with the gas analyser. The second was the purchase of two handheld anemometers, a hot wire and vane anemometers. Manual air flow samples were taken from several points and depths in the pipe during recovery tests. This method of testing was done to correct the continuous airflow measurement, which was done using a fixed probe hot-wire anemometer inserted in the duct at a fixed depth, which was half way from the wall of the duct. With the manual measurement, the values obtained were compared with those on the automatic recorder, and thereafter a correction factor was used to compensate for loss of performance of the sensor due to continuous exposure to air contaminated by dust particles.

Higher methane values recorded in this study for the ambient measurement could have been due to the location of the open circuit respiratory chamber. The structure was set up close to the dairy unit of Proefplaas Experimental Farm where dairy cows were camped within a few meters of the open-circuit structure. Dairy cows during milking (three times daily) pass in front of the open circuit respiratory chamber, and this could have contributed to the high ambient methane concentration recorded in the study.

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