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Effect of extraction solvents and encapsulation on the efficacy of certain medicinal plant extracts to inhibit enteric methane emission

By

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Submitted in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
ANIMAL SCIENCE

In the Faculty of Natural and Agricultural Sciences
Department of Animal Science
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July 2023

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Declaration

I, **Taofik Adam IBRAHIM**, declare that this thesis, which I hereby submit for the degree of Ph.D. in Animal Science at the University of Pretoria, is my research results, besides where references are cited. The thesis has not previously been submitted by me for any degree at this university or any other university or tertiary institution.

Signature 

Taofik Adam IBRAHIM

Dedication

This achievement is dedicated to my late father, Mr. Ibrahim Adeyemo Iyaniwura, and my lovely mother who sacrificed luxury to train me and my siblings, and to my entire family for their moral and spiritual support.

Acknowledgment

My deepest appreciation goes to my academic supervisor, Prof Abubeker Hassen, for his attention to detail, objective criticism, and tireless support throughout my study period. I will always consider it invaluable; the contribution, knowledge, and support I received from you during my study period. I will further like to thank Prof. Zeno Apostelides for his mentorship.

I would also like to express my appreciation to the Tertiary Education Trust Fund (TETFund) via Ahmadu Bello University Zaria, Nigeria, for taking care of my welfare during the period of my research study and National Research Foundation (NRF), South Africa, for funding various components of this research. My gratitude also goes to the University of Pretoria, South Africa, for the additional postgraduate bursary award.

I would like to thank the head of the department (Prof. Este van Marle-Köster) and the entire staff of the Department of Animal Science, University of Pretoria for their support, and all the laboratory technologists, especially Mr. Vutlhari and Ms. Loner who helped me with various laboratory procedures, as well as Mr. Roelf Coertze, Ms. Corlia Swanepoel and the staff at Small and Large Stock Division, Innovation Africa Ruminant Unit Experimental Farm, who assisted me during the *in vitro* and animal research period. Mrs. Kate Nkau has played a motherly role since I arrived at the department and she will always be remembered and appreciated for her kind and moral support.

I would like to thank Dr. Festus Adejoro, Dr. Abiodun Mayowa Akanmu and Dr. Abdullahi Yusuf for their moral and strong academic support; I will never forget your kind support throughout my study period which is imprinted in my heart. To all my academic friends and colleagues, especially Mr. Shehu L. Ibrahim and Mr. Addisu Zeru for their support and sharing of academic knowledge during the period of my research study.

I would like to express my profound gratitude to my lovely wife and my adorable children for their prayers and sacrifices during my away from home in pursuit of my Ph.D. degree, as well as my adorable mum and siblings for their spiritual support and being there for me at the time I most needed their support. You have all been the pillars of my strength and no amount of words I can use to show my appreciation, God bless you all.

To all the Professors, academic mentors, and colleagues, starting from my Head, Prof. Danjuma Zaharaddeen, Prof. S. B. Abdu, Prof. M. R. Hassan, Prof. M. Kabir, and Prof T. S. Olugbemi to mention a few at the Department of Animal Science, Ahmadu Bello University Zaria Nigeria, I

thank you all for your support. I will like to specially and sincerely appreciate and recognize the support and contributions of Prof Akpan and Prof S. Duru (of blessed memory), Prof Olufunmilola Alabi (former Dean, Faculty of Agriculture, ABU, Zaria), Prof I. U. Abubakar and Prof Ishiyaku (respectively former and current Director of the Institute of Agricultural Research, ABU Zaria) towards my nomination and securing of TETFund for my study, supports that are too memorable and invaluable to forget. May Almighty God reward you abundantly.

Finally, I thank Almighty Allah and of course will always thank Him for his mercy on me and my family before, during, and after this noble journey.

Preface

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

Peer-reviewed journals

Ibrahim TA, Hassen A, Apostolides Z. 2022. The Antimethanogenic Potentials of Plants Extracts; Their Yields and Phytochemical Compositions as Affected by Extractive Solvents. *Plant Special Issue "Phytochemical analysis"*. 11(23): 3296. https://Doi.org/10.3390/plants11233296.

Ibrahim TA, Hassen A. 2023. Characterization and *in vitro* methane inhibition of *Aloe vera* and *Moringa oleifera* plant extracts encapsulated with alginate or alginate-chitosan wall materials (TO be submitted to Animal Production Journal).

Ibrahim TA, Hassen A. 2023. Effect of alginate encapsulated *Moringa oleifera* plant extract additives on growth performance, nutrient digestibility, methane, and rumen fermentation of South African Mutton Merino lamb (To be submitted to Livestock Science Journal).

Conference presentation

Ibrahim TA, **Hassen A**, Apostolides Z. 2022. Phytochemical and in vitro methane inhibition of plant extracts as influenced by extractive solvents. An oral presentation at 2nd one-day symposium Animal Task Force and the EAAP Commission on Livestock Farming Systems 5 September, Brussels, Belgium.

This thesis has been prepared according to the style of the **Animal Feed Science and Technology** journal.

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List of Acronyms

ADF	Acid detergent fibre
ADL	Acid detergent lignin
AOAC	Association of Official Analytical Chemists
CH_3OH	Methanol
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
EE	Ether extract
EME	Encapsulated moringa extract
FCR	Feed conversion ratio
FID	Flame ionization detector
g	Gram
GC	Gas chromatography
GP	Gas production
H_2	Hydrogen molecule
H_2O	Water
HPLC	High performance liquid chromatography
IVOMD	*In vitro* organic matter digestibility
kg	Kilogram
mL	Millilitres
MO	*Moringa oleifera*
NDF	Neutral detergent fibre
NH3-N	Ammonia nitrogen
NME	Non-encapsulated moringa extract
OM	Organic matter
PAM	Protozoan associated methanogens
PCA	Principal component analysis
PD	Particle density
RCBD	Randomly complete block design
RT	Retention time
SA	South Africa
SAMM	South Africa Mutton Merino lamb
SEM	Scanning electron microscope
TGP	Total gas production
TMR	Total mixed ration
UME	Unencapsulated moringa extract
UPLC–MS	Ultra-performance liquid chromatography–mass spectrometry
VFA	Volatile fatty acids

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University of Pretoria

Degree: PhD in Animal Science

ABSTRACT

A series of *in vitro* and *in vivo* experiments were conducted to evaluate the antimethanogenic properties of four medicinal plants (*Aloe vera, Jatropha curcas, Moringa oleifera,* and *Piper betle*) extracts as alternative rumen modifiers to antibiotics to modulate rumen fermentation and inhibit methane production. In the first study, two solvents (methanol and water) were used in three different combinations (70, 85, and 100%) to extract bioactive compounds from the four studied medicinal plants as some of their metabolites have been reported to possess rumen modulation properties and improve nutrient utilization in ruminants, thereby reducing enteric methane emission per unit of animal product. The extracts were evaluated at 50 mg kg^{-1} DM feed as an additive to *Eragrostis curvula* hay substrate *in vitro*. Results showed an increase in extract yields with increasing water content in the extraction solvents. The effect of extraction solvents was also observed in the concentration of the bioactive compounds in each plant extract when analysed with UPLC-MS, these plant bioactive compounds showed different solubility values. Subsequently, promising plant extracts were selected based on yield and methane inhibition potentials for subsequent encapsulation with alginate as wall material. The encapsulated products were scanned using electron microscope for morphological characterisation and later their methane inhibition attributes were investigated using both *in vitro* and *in vivo* studies. The particle characterization of the encapsulated extracts was carried out using a scanning electron microscope (SEM) and images were generated for a morphological study. Gas measurements were taken at regular intervals of 3, 6, 12, 24, and 48 h during the incubation period. Methane emission was determined for each gas sample with the use of gas chromatography. During the first phase of the *in vitro* study, the plant extract treatments were incubated with feed samples to test their methane inhibition

potentials, their effect on total gas production (TGP), and their organic matter digestibility (IVOMD). In the second phase of the *in vitro* study, *Aloe vera* and *Moringa oleifera* extracts were encapsulated with alginate and/or alginate-chitosan wall materials and the final product is incubated with feed samples to evaluate their methane inhibition potentials, their effect on TGP, and IVOMD. The results from the first *in vitro* study indicated that the four plant extracts from three aqueous-methanol extractions (70%, 85%, and 100%) generally reduced methane emission in different proportions at 50 mg kg^{-1} DM without adversely affecting TGP and IVOMD of *E. curvula* hay. However, the methane inhibition potentials of the tested plant extracts were not influenced by the extraction solvents. For the second *in vitro* study, alginate encapsulated and alginate-chitosan encapsulated plant extracts were also tested together with non-encapsulated plant extracts on *E. curvula* hay and also reduced methane production. Higher methane inhibitions were noted for the alginate encapsulated and alginate-chitosan encapsulated extracts as against the non-encapsulated extracts, without negatively affecting the TGP and IVOMD.

Finally, encapsulated *M. oleifera* plant extract was selected for *in vivo* evaluation to determine its effect on enteric methane production, rumen fermentation parameters, growth performance, feed intake, apparent digestibility, and nutrient utilization of South African Mutton Merino (SAMM) lamb. A total of thirty (30) SAMM lambs were first ranked according to their body weight and divided into three groups with approximately equal body weight measurements. The three treatment groups were randomly allotted to one of the three treatments (two plant extract treatments and control). The plant extract treatments included non-encapsulated *M. oleifera* extract (NME) and encapsulated *M. oleifera* extract (EME) additives. These additives were provided to the lambs at 50 mg kg^{-1} feed DM intake by incorporating them into a ball-like molasses mix and presented at 7:30 am daily to the experimental lambs. All treatments were placed on the same total mixed ration formulated with a 42% roughage component. The growth performance study lasted for 14 weeks, including adaptation, and the feed digestibility study lasted for 14 days. The lambs were moved into the open circuit respiratory chambers for methane emission measurements in six batches. The methane emission measurements and digestibility study were done concurrently with the growth performance study. For the *in vivo* study, both the NME and EME reduced ($p < 0.05$) enteric methane emission by approximately 22.61% and 20.06%, respectively; reduced rumen ammonia nitrogen (NH_3-N), urinary nitrogen excretion and feed conversion

ratio but increased nitrogen retained as a percentage of intake without adverse effect on nutrient intake, apparent digestibility, and performance of SAMM lambs. Supplementation of SAMM lambs with NME and EME as dietary additives did not affect the rumen fermentation parameters. The alginate encapsulation of MO plant extract is safe and did not reduce the efficacy of MO plant extracts compared to non-encapsulated plant extracts, and is therefore recommended for practical use as antimethanogenic dietary additives in the feeding system of ruminant animals. Further study needs to be conducted to determine the effect of alginate encapsulated MO plant extracts on the rumen microbial populations and meat quality of the lambs. Also, other wall materials that can act as good carriers of active compounds in rumen medium should be evaluated for methane inhibition in livestock production.

GENERAL INTRODUCTION

Background

Increasing animal protein consumption is in tandem with the increase in the world population (Morgavi et al., 2010). The global consumption of meat is expected to rise from 229 to 465 million tonnes and milk from 580 to 1043 million tonnes by 2050 (FAO, 2006). This will cause the demand for meat and milk among other protein sources to be increasingly high and possibly surpass supply. Therefore, there is a need to increase the productive potentials of livestock species through dietary manipulation, especially those competing less with humans for cereal grains and are capable of utilizing low-quality forages like ruminant animals (Morgavi et al., 2010). Ruminant animals can efficiently utilize feeds with a high level of fibre which is less nutritious and not easily digested by other species of animals to produce meat, milk, and various fibre products. Therefore, ruminants occupy a significant ecological niche for meeting the global demand for high-quality and nutritious food (Bayat & Shingfield, 2012). However, an increase in ruminant animal production to meet the projected increase in protein demand of the world population will lead to environmental pressure that can be manifested in the form of pollution, an increase in greenhouse gas production of which enteric methane emission is the major one (Knapp et al., 2014). Greenhouse gases such as methane and carbon dioxide can raise the Earth's temperature through the absorption of long-wave radiation (Lovett et al., 2005; Jordan et al., 2006). Thus, increasing livestock production like ruminants to meet the rise in the population without adverse effects on the environment becomes practically useful (Akanmu & Hassen, 2018). Previous research showed that rumen and dietary manipulation are among the key strategies utilized to improve production efficiency and reduce environmental footprints (Beauchemin et al., 2003; Ibrahim & Hassen, 2022; Patra, 2014). Therefore, any scientific strategy that will increase protein production in the livestock industries should be environmentally friendly and be able to reduce carbon footprints (Knapp et al., 2014).

Justification

A study on enteric methane emission has attracted significant attention in the last decade because methane emission from ruminants contributes to global greenhouse gas emissions and represents a loss of feed energy (Patra et al., 2017). Rumen enteric methane emission accounts for about 17% of global methane emission (Knapp et al., 2014) while 30-40% of

methane emission from total agricultural sources is from ruminants (Moss et al., 2000). About 2–12% of the ingested feed energy is also lost as methane (Patra et al., 2017). Many researchers have attempted to develop and evaluate interventions to mitigate enteric methane production using antimethanogenic compounds and plant secondary metabolites (Adejoro & Hassen, 2018; Akanmu & Hassen, 2018; Patra & Saxena, 2010; Patra, 2014; Patra & Yu, 2013; Ibrahim & Hassen, 2021).

Some antimethanogenic plant-based phytochemicals such as tannins, saponins, and nitrates are likely to be toxic to animals, and/or could limit rumen microbial activities if provided in large quantities. These phytochemicals may cause reduced feed intake, nutrient digestion, and rumen fermentation even if included at levels that will effectively reduce enteric methane emissions (Patra & Yu, 2013). These adverse effects and toxicity may be averted by using medicinal plants with multiple antimethanogenic compounds. These plant active compounds have been safe to use if applied at appropriate doses and combinations to directly inhibit the activities of methanogens (Patra et al., 2017) or ciliate protozoa. Medicinal plants and plant extracts such as *Aloe vera*, *Jatropha curcas*, *Moringa oleifera*, and *Piper betle*, have been reported to potentially modulate the rumen environment, reduce methane production, and have antimicrobial properties (Ratshilivha et al., 2014; Akanmu & Hassen, 2018). However, the potentials of these medicinal plants as dietary feed additives are not yet fully explored in commercial livestock production due to a number of limitations which includes lack of standardized product, actually recommended dosages, stability during feed mixing and processing, and effective and safe method of administration. For example, the oral drenching of ruminants with crude plant extracts like it has been in the past (Akanmu, 2018) was effective but not realistic commercially as it may impose stress on animals. It is also labour intensive and may not even be achievable in some production systems. To minimize the potential loss in the efficacy of plant extracts, encapsulation with an inert material that will enhance the delivery of the bioactive molecules (core materials) in the rumen is therefore required. In addition, encapsulation of plant extracts could assist to mask and reduce the adverse/bitter effects of plants' phytochemicals like tannins, and alkaloids (Adejoro & Hassen, 2018; Ibrahim & Hassen, 2022).

Besides encapsulation of plant-based extracts, identifying suitable solvents for extraction is a scientific strategy that helps to increase the quantity of plant extracts and/or improve the

efficacy of antimethanogenic plant extracts. In an attempt to increase methane inhibition, solvent extraction is vital as different solvents have been used in the extraction of medicinal plants, and positive results on methane inhibition obtained (Liwiński et al., 2002; Sirohi et al., 2009, 2012; Yejun et al., 2019). The solvent extraction technique is paramount in the determination of yield and concentration of bioactive compounds in plant extracts. Increasing extract yields from plant materials requires a good knowledge of the plant material, the metabolites of interest, and appropriate solvents which could be organic, ionic, or combined (Sultana et al., 2009; Ballesteros et al., 2014; Nn, 2015). This is because phytochemicals have different solubility values in different solvents, which determines the relative availability of the bioactive compounds in the plant extract. No universal extraction method is ideal for all types of plants, and each extraction procedure is unique to the targeted plant compounds (Nn, 2015) and utilization purpose. The use of a combination of different solvents will affect the extract yields and concentration of bioactive compounds due to differing levels of solubility, interaction, and ionic activity of the biologically active compounds (Sultana et al., 2009; Ballesteros et al., 2014; Nn, 2015).

Therefore, it is important to consider a more suitable method to increase the quantity of antimethanogenic plant-based extracts using the right technics that are simple and practicable to effectively reduce methane production and ultimately the carbon footprints in ruminant production. The main challenge, however, is for research to develop methane inhibition technologies that are adaptable, show long-term effects, and sustainably improve the efficiency of energy and nitrogen utilization in ruminant production systems. There is a limited literature information on the encapsulation of plant-based extracts that can be used as dietary additives to mitigate enteric methane in ruminants. In this regard, a review of the literature was conducted to understand the mechanism of methanogenesis, identify knowledge gaps, formulate working hypotheses, and test innovative approaches that modify or replace existing technologies currently used in ruminant production systems.

CHAPTER ONE

Review of Literature

1.1 Background on enteric methane production

Methanogenic archaea responsible for methane synthesis in ruminant animals are located mainly in the rumen while some reside in the lower parts of the large intestines of ruminant animals. According to Patra et al. (2017), methane-producing bacteria use hydrogen molecules produced during rumen fermentation to form enteric methane when they react with carbon dioxide, methylamines, or formic acid. Research on methane emissions from livestock has been topical in the last few years. Ruminants are known to significantly contribute to global greenhouse gas emissions, a process that gives rise to gross energy loss from the feed consumed ranging from 2-12% (Patra et al., 2017). Rumen enteric methane emission accounts for about 17% of global methane emission (Knapp et al., 2014) while 30-40% of methane emission from total agricultural sources is from ruminants (Moss et al., 2000). In recent times, researchers have concentrated on explaining the factors that determine and affect the archaeal structure, compositions, and diversity of methane-producing bacteria in the rumen. Simultaneously, a number of researchers have attempted to develop suitable technologies to reduce methane production using antimethanogenic compounds and plant secondary metabolites to mitigate methane emission (Adejoro & Hassen, 2018; Akanmu & Hassen, 2018; Patra & Saxena, 2010; Patra, 2014; Patra & Yu, 2013; Ibrahim & Hassen, 2021).

This review will give briefly an overview of the rumen methanogens and methanogenesis, and briefly discuss some methane reducing bioactive compounds that had been tested both *in vitro* and *in vivo*.

1.2 An overview of rumen methanogens

The rumen methanogens have small diversity when compared to the rumen bacteria, with the total small subunit (SSU) ribosomal (r)RNA archaea making up about 6.8% of the overall SSU rRNA bacteria present in the rumen (Ziemer et al., 2000). According to Janssen & Kirs, (2008), the rumen archaea account for less than 4% of the overall rRNA while about eight species of rumen methanogens were isolated and cultured. These species of the isolated methane-producing bacteria include *Methanosarcina barkeri*, *Methanobrevibacter*

ruminantium, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanoculleus olentangyi*, and *Methanomicrobium mobile*. In recent time, five new species of archaea were additionally isolated and cultured from the ruminants, which include *Methanobrevibacter boviskoreani* that was isolated and cultured from cattle rumen (Lee et al., 2013), *Methanobacterium beijingense* that was cultured from the rumen fluid of a goat, *Methanosarcina mazei*, *Methanoculleus marisnigri* and *Methanoculleus bourgensis* which were all isolated and cultured from the rumen fluid of cattle (Patra et al., 2017). Hence, the knowledge of the archaeal interactions in the rumen is key to understanding enteric methane formation and identification of target steps and pathways for the reduction of enteric methane emission.

1.3 Methanogenesis and archaeal interactions in the rumen

Rumen micro-organisms known as the rumen methanogen or archaea synthesize methane. They perform this methane production by converting carbon dioxide and hydrogen molecules to enteric methane and water. This will cause a reduction in the number of hydrogen molecules in the rumen (Gerber et al., 2013). Methane production represents the major pathway for hydrogen molecules utilization in the rumen, hence, redirecting the available hydrogen molecules in the rumen for a useful process like bio-hydrogenation are available strategies in recent time. (Gerber et al., 2013). The mechanisms involving the conversion of hydrogen molecules to animal lipids via bio-hydrogenation in the rumen as well as the implications on methane inhibition have been documented (Goiri et al., 2010; Jafari et al., 2016). There are two major types of methanogens in the rumen; the hydrogenotrophic methanogens which are more in population compared to the acetoclastic methanogens. These two groups of methanogens usually source hydrogen and carbon dioxide molecules produced as a by-product of rumen microbial fermentation and convert them to methane (Rother et al., 2010). These groups of rumen methanogenic bacteria can also make use of methylamine and formic acid available in the rumen to synthesize enteric methane (Rother et al., 2010). In a review study by Patra et al. (2017), methanogenic archaea usually interact with other rumen bacteria, protozoa, and fungi via interspecies hydrogen molecules transfer. Generally, such production and removal interaction enhances rumen fermentation as it can lower the accumulation of hydrogen molecules through redirecting into other useful activities like bio-hydrogenation (Patra et al., 2017). A few number of methanogenic archaea are resided in the rumen either as endosymbionts or ectosymbionts while a large amount of the methanogens

reside freely in the rumen or adhere to feed particles (Lambie et al., 2015). *In vivo* experiments similarly revealed that suppression of methanogenic bacteria in the rumen reduces the ratio of acetate to propionate indicating more propionic acid production and fewer acetate production (Patra et al., 2017; Patra & Yu, 2013). A good understanding of how methanogens and other rumen microbes interact in the rumen provides better knowledge for the identification of suitable strategies that can help mitigate methane emissions from ruminants.

1.4 Methane inhibition strategies

Several methane mitigation strategies have been investigated with major intervention in animal management, rumen modulation, dietary manipulation, and the use of antimethanogens (Gerber et al., 2013; Knapp et al., 2014; Patra et al., 2017). Among a number of mitigation options, reducing the growth/population of methanogenic archaea or interfering with their metabolic pathway is the most suitable and effective approach (Patra et al., 2017). This interference in the metabolic pathway can be achieved using a strategy that either decreases the production of hydrogen molecules and/or increases propionate production (Patra et al., 2017). Also, the H_2 gas produced in the rumen can be diverted into other useful functional activities such as biohydrogenation, etc. Although several reports have been presented in the literature, there are significant differences between studies vis-à-vis the magnitude of the efficacies of the methods and the adverse effect on fermentation, apparent digestibility, and nutrient utilization (Islam & Lee, 2019; Patra et al., 2017). The methane reduction strategies from livestock origin are a time-demanding issue worldwide. There are different methane inhibition strategies adopted so far but still lack sustainability (Islam and Lee, 2019). Figure 1.1 summarised the vital methane inhibition strategies in ruminant animal production. The rectangular white boxes are probably the target for enteric methane inhibition while the shaded boxes depict the different options which have been studied either *in vitro* or *in vivo* to mitigate enteric methane emission. The use of bioactive compounds obtained from plant-based extracts in mitigating enteric methane emissions is reviewed in sections 1.5 and 1.6.

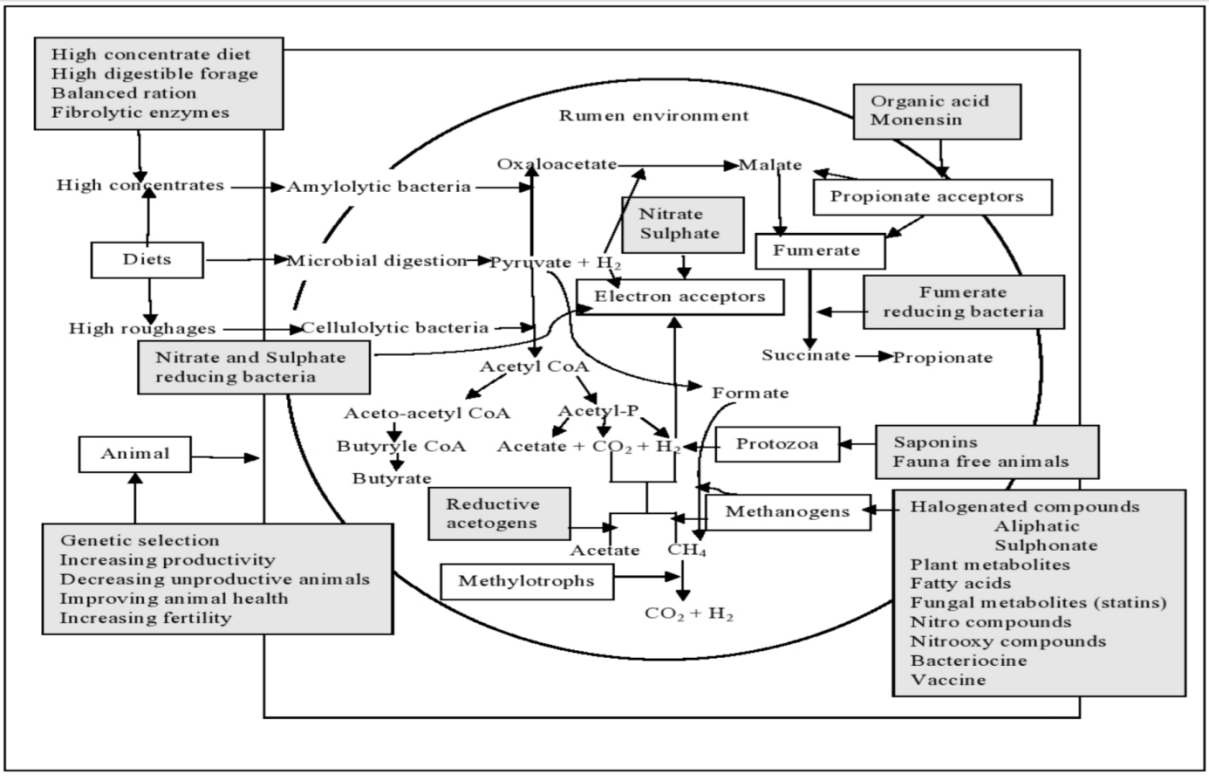


Figure 1.1. A schematic illustration of possible targets to mitigate enteric methane emissions in ruminants (Islam & Lee, 2019).

1.5 The use of plant secondary metabolites as antimethanogens

The secondary metabolites in plant material such as alkaloids, flavonoids, phenolic acids, and other biologically active substances have antimicrobial activities against many types of micro-organisms (Patra, 2012). Several plant extracts have been identified as potential inhibitors of rumen methanogens and methane emission (Akanmu & Hassen, 2018; Cieślak et al., 2013; Patra & Saxena, 2009; Patra & Saxena, 2010). However, the effectiveness of plant extracts to suppress methane emission differs largely depending on the types of diet, the amount of inclusion of the extracts or the natural products, and the physicochemical properties of the products among other factors. (Patra et al., 2017). Phenolic compounds and flavonoids are the two major groups of plant secondary metabolites (Patra & Saxena, 2010), with antimicrobial, antioxidant, and antimethanogenic properties. The medicinal plants with secondary compounds considered in this study are *Aloe vera*, *Jatropha curcas*, *Moringa oleifera*, and *Piper betle*.

1.6 The antimethanogenic impact of flavonoids and phenols

Flavonoids as antimethanogenic compounds have not been extensively evaluated on the methanogenic archaea in the rumen (Patra & Saxena, 2010). The study by Oskoueian et al. (2013) showed the methane inhibition potential of flavonoids. The inclusions of flavone, kaempferol, myricetin, quercetin, naringin, and rutin at 4.5% of dry matter feed significantly decreased *in vitro* methane emission by 8.1 to 43.0% with myricetin having the highest methane inhibition potential. The author also ranked their potency as myricetin > kaempferol > flavone > quercetin > naringin > rutin > catechin. Catechin reduced methane emissions both *in vitro* (Becker et al., 2014) and *in vivo* (Aemiro et al., 2016). Flavonoids, when fed at a dosage of 0.2 g/kg DM reduced methanogenic archaea of hydrogenotrophic origins while the inclusion of *Citrus aurantium* extract rich in flavonoids such as naringin and neohesperidin better suppress methanogens (Seradj et al., 2014). The flavonoids have a suppressing effect on methanogenic microbes (Oskoueian et al., 2013; Seradj et al., 2014); and also act as hydrogen molecule sinks through the splitting of the ring structure (eg catechin and epicatechin) and reductive dihydroxylation (Becker et al., 2014). Several plants have tannins as major phenolic compounds and their biological significance is associated with protection against attacks by animals (Broucek, 2018). Tannin is a polyphenolic compound produced in many plants that are either in hydrolyzable or condensed form (Broucek, 2018). The non-tannin phenols efficiently reduced methane production *in vitro* (Jayanegara, 2009). The use of caffeic acid, cinnamic acid ferulic acid, and p-coumaric acid as 5 mM DM feed effectively reduced *in vitro* methane emission in the following order: caffeic acid > p-coumaric acid > ferulic acid > cinnamic acid without any adverse effect on organic matter digestibility (Jayanegara, 2009). Understanding the effects of medicinal plant extracts as antimethanogenic substances on animal performance will help to determine their suitability as methane-reducing agents.

1.7 Impact of medicinal plants on methane inhibition and animal performance

Secondary plant compounds decreased in vitro methane. These compounds can be used as antimethanogenic agents without adverse effects on feed digestibility (Akanmu et al., 2018). According to Chaturvedi et al. (2015), crude plant extracts of *Azadiracta indica, Emblica officinalis, Ocimum sanctum, Clerodendrum phlomidis,* and *Curcuma longa* had no adverse effect on *in vitro* gas production and feed digestibility; however, these crude plant extracts effectively decreased *in vitro* methane and ammonia production. Some of these compounds

are practically useful, acting as anti-methanogens and serving as alternatives to the use of antibiotics in ruminant production, and may likely improve animal performance (Akanmu et al., 2018). Jerónimo et al. (2016) reported that polyphenolic compounds like tannins have the potential to prevent rumen acidosis and bloat and enhance better utilization of proteins in ruminants. Tannins also help to regulate internal parasites and enhance tissue attrition in beef, and milk production in dairy animals. Tannins usually act by iron deprivation and interfere with useful proteinase enzymes in the rumen. An alkaloid has been described as a deoxyribonucleic acid intercalator and an inhibitor of topoisomerase. Saponins, on the other hand, chelate sterols residing in the plasma membrane of microbes, thereby initiating some damage to the cell membrane and consequent breakdown of cells (Cheng et al., 2014). The risks associated with the use of crude plant extracts in methane mitigation include a decrease in TVFA production in the rumen, a reduction in dry matter, and the apparent digestibility of NDF (Cobellis et al., 2015). Crude plant extracts have been reported to contain a blend of active compounds. These crude plant extracts vary in their compositions which are largely affected by genotype and other biological factors, production techniques, and the conditions of storage (Baert et al., 2011). According to Yang et al. (2009), the efficacy of crude plant extracts is largely influenced by the genetic difference in the plants, agronomic practices, age of harvest, extraction techniques, inclusion levels, and compatibility with the substrates/feed ingredients. All these conditions are likely to impose unstable effects on animal performance and welfare; and can be toxic when included in animals' feeds at an unreasonable dosage (Akanmu et al., 2018).

1.8 Mitigation mechanism of medicinal plants on enteric methane production

Medicinal plants and their extracts have been examined to be antimicrobial and anti-methanogenic as they possess compounds that inhibit some ruminal microbes (Amaglo et al., 2010; Nouman et al., 2016; Akanmu et al., 2018). Some studies have substantiated that phytochemicals suppress methane production (Adejoro et al., 2018; Akanmu et al., 2018), and the activity/population of the methanogenic archaea in the rumen most likely by binding the proteins and enzymes of microbes leading to a collapse of cells (Tavendale et al., 2005). Tannins also directly inhibit some protozoa in the rumen and indirectly affect the associated rumen methanogens, especially the protozoa-associated methanogens (Tavendale et al., 2005). Plant secondary metabolites most likely limit the activity of cellulolytic bacteria (Patra & Saxena, 2010; Waghorn, 2008) and, consequently, fermentation of structural

polysaccharides to volatile fatty acids, and acetate in particular, thereby decreasing carbon dioxides and hydrogen molecules productions that are required for methanogenesis in the rumen. In this study, four medicinal plants (*A. vera, J. curcas, M. oleifera,* and *P. betle*) with secondary compounds were selected to review document information related to their secondary plant compounds and their potential as methane mitigation agents.

1.9 Antimethanogenic, antimicrobial, and antioxidant potentials of *A. vera, J. curcas, M. oleifera,* and *P. betle*

1.9.1 Aloe vera

A. vera leaf is a rich source of anthrone/anthraquinone. Anthrones are phenolic compounds with laxative effects on the gastro intestinal tracts and result in easy movement of bowels with good antibiotic properties. It exhibits strong antimicrobial activity against bacteria, viruses, and yeasts (Kedarnath et al., 2012). Anthrone in *A. vera* extract has anti-inflammatory action with a wide range of antimicrobial activity (Aysan et al., 2010). Other phytochemicals that are present in the *A. vera* plants include tannins, saponins, flavonoids, and terpenoids (Kedarnath et al., 2012). The previous research studies showed that acetone and methanol extraction of *A. vera* leaf decreased *in vitro* methane production. (Sirohi et al., 2009; Akanmu et al., 2018). *A. vera* crude extract contains two main biologically active substances which include aloe soluble polysaccharide/amylose which is present in aloe filet and the anthraquinone derivatives residing in the leaves of *A. vera* plants (Pugh et al., 2001). Aloe-emodin, Aloin A, and B have been reported present in *A. vera* leaf extracts with 60% ethanolic extraction (Logaranjan et al., 2013). These bioactive compounds had been identified as active principles by their activity against pathogenic fungi such as *Colletotrichum gloeosporides* and *Cladosporium cucumerinum* (Wrede et al., 2012). *A. vera* and its extracts had been reported to exhibit anti-bacterial activity against *Helicobacter pylori, Streptomyces greseus,* and *Candida albicans* (Bawankar et al., 2013).

1.9.2 Jatropha curcas

In a study earlier carried out by Santra et al. (2012), a large number of plants were evaluated for their methane inhibition potentials, and ethanolic extracts of *J. gossipifolia* reduced methane production by about 31%. The extracts of *J. gossipifolia* reduced the population of the rumen protozoa *in vitro* and were attributable to the presence of tannin and saponin in the extracts of *J. gossipifolia*. The activity of antinutrients; their various compounds and other

various phytochemicals in different parts and species of Jatropha have been reviewed (Devappa et al., 2010; Sabandar et al., 2013). The leaf extract of *J. curcas* has been reported to exhibit methane-reducing properties and increase the organic matter digestibility at 25, 50, 75, and 100 mg kg^{-1} DM substrate (Akanmu & Hassen, 2018).

1.9.3 Moringa oleifera

The Moringa plants contain crypto-chlorogenic acid, isoquercetin, and astragalin in their leaves (Vongsak et al., 2014). The various portions of the *M. oleifera* tree are rich in glucosinolates, flavonoids, and phenolic acids (Amaglo et al., 2010; Coppin et al., 2013). According to a previous study, among flavonoids compounds, flavonol glycosides (which include the glucosides and their derivatives) of quercetin, and kaempferol are mainly present in the *M. oleifera* leaves (Coppin et al., 2013). The quantity of quercetin and kaempferol in *M. oleifera* ranges from 0.07–1.26% and 0.05–0.67%, respectively. Nouman et al. (2016) characterized seven varieties of *M. oleifera* plants with more emphasis on their phenolic compounds and antioxidant potential. The apigenin, quercetin, and kaempferol derivatives of hydromethanolic extracts of *M. oleifera* were reported to be 20.9, 47.0 and 30.0%, respectively of the total flavonoids.

1.9.4 Piper betle

P. betle is a perennial creeper plant that has been useful to man for a long period (Emon et al., 2020). It plays a vital role in the traditional institution as it is used in the treatment of abscesses, boils, cuts, injuries, affected gums, halitosis, and constipation (Fathilah, 2011; Emon et al., 2020) and to treat bad breath. Although the leaf extracts of *P. betle* have been evaluated *in vitro* for enteric methane emission in ruminants (Akanmu et al., 2018), the characteristics and properties of these leaf extracts make it a valid medicinal plant to evaluate further. Also, there is a paucity of information on the methane inhibition of *P. betle* leaf extracts. Freshly harvested *P. betle* leaves contain nutrients like carbohydrates, proteins, vitamins, minerals, and essential oils (Basak et al., 2015). The essential oils present in *P. betle* leaves contain anti-bacteria, anti-protozoan and anti-fungal properties. According to Dwivedi & Tripathi (2014), phytochemical screening on leaves revealed the presence of alkaloids, amino acids, carbohydrates, steroids components, and tannins, while the leaves contain majorly Betle oil and betle phenol (Chavibetol and Chavicol). A study was conducted by Nalina & Rahim (2007) on the aqueous extract of *P. betle* and its antibacterial activity

against *Streptococcus mutans*. The result of the micrographic transmission of electrons showed that the leaf extracts of *P. betle* damage the plasma cell membrane of *S. mutans* and initiate coagulation of the nucleoid. The crude plant extract was also noted to effectively reduce the capacity of the bacteria to produce acid. Lee et al. (2015) conducted a study that involves the extraction of crude extract from dried *P. betle* leaves using deionized water and 70% ethanol and evaluated major phytochemicals from the extracts using qualitative analysis. The study revealed the presence of alkaloids, alcoholic compounds, phenolic compounds, organic acids, and other solvent-soluble compounds with good antimicrobial and antioxidant activity.

1.10 Chitosan, its potential for methane inhibition and organic matter fermentation

Chitosan is described as a linear polysaccharide compound comprised of two repeated units which include D-glucosamine and N-acetyl-D-glucosamine, chemically joined together by β-(1→4)-linkages. Chitosans are characterized by their molecular mass/weight, viscosity, and level of deacetylation (Jiménez-Ocampo et al., 2019). Chitosan is a collective name for a group of partly or completely deacetylated chitin from large biopolymer, it is a natural biologically active compound with many advantages as it is non-toxic, it is biocompatible, biodegradable, and contains bioactive compounds with good antimethanogenic properties, and has been reported to be safe for use in food and animal studies (No et al., 2007; Mohammad El-Aidie, 2018; Jiménez-Ocampo et al., 2019). Chitosan is a high molecular weight polymer with cationic properties, and it is the second most abundant polysaccharide next to cellulose. Chitosan can be found in the exoskeleton of lower animals like crustaceans, insects, mollusks, fungi, and some algae, but largely obtained from marine crustaceans (Li et al., 2018). It has moderate to strong antimicrobial properties against many microbes, viruses, and filamentous fungi, including yeast. Chitosan also exhibits some health-promoting benefits like anti-tumor and anti-inflammatory activities (Divya et al., 2017; Duffy et al., 2018).

In recent animal nutrition studies, the focus has been shifted to studying the methane inhibition potential of chitosan and as rumen modifiers in beef or dairy cattle as well as nutrient digestibility in cattle (Araújo et al., 2015; Henry et al., 2015; Belanche et al., 2016; Gandra et al., 2016). Chitosan has been reported to exhibit effects on the voluntary intake of animal feed/diet, apparent digestibility, nutrient utilization, fermentation parameters, and

enteric methane emission. Conversely, the results obtained from different studies differ between the *in vitro* and *in vivo* (Jiménez-Ocampo et al., 2019). In the *in vitro* tests, Belanche et al. (2016) found that chitosan altered the rumen fermentation patterns and increased propionate production. The authors also noted an increasing trend in the amylolytic bacteria but a decrease in cellulolytic bacteria like Butyrivibrio, Fibrobacter, and Ruminococcus as well as hemicellulolytic bacteria like Eubacterium. This has led to the prediction by Jiménez-Ocampo et al. (2019) that the electrostatic interaction of the bioactive compounds in chitosan and the interference with cell membrane suppressed methanogens and/or methanogenic pathways and lowered methane emission by 10 to 42%. In an *in vitro* study, Goiri et al. (2010) revealed that chitosan has effective inhibitory action against bio-hydrogenation by increasing the amount of unsaturated fatty acids such as C18:1 t11 and the conjugated linoleic acid proportions with no preference to the type of dietary lipids. These results can be related to the interaction with negatively charged free fatty acids and support the contention that chitosan changes the population of the rumen protozoa. (Jiménez-Ocampo et al., 2019). This also conforms with the results of Wencelová et al. (2013) who reported that chitosan did not affect fatty acid profile but lowered the apparent digestibility of feed dry matter and TGP and showed little influence on enteric methane emission.

1.11 Pre-extraction procedures and extraction of plant extracts from medicinal plants

Medicinal plants and their liquid extracts have recently gained much interest and awareness due to their application in traditional medicine. They are used to treat common diseases like colds, fever, and other similar diseases (Nn, 2015). The study of medicinal plants usually begins with the extraction of crude plant extracts. Different extraction procedures have different impacts and critical roles in the extraction outcomes such as the yields of crude plant extracts and the composition of the biologically active compounds in the crude plant extracts (Nn, 2015). A wide range of technologies for solvent extractions is available nowadays (Nandave et al., 2009; Das et al., 2010). However, this study review will only focus on aqueous-organic solvents extractions methods.

1.12 Pre-extraction processing procedures and choice of extraction solvents

The initial stage in the study of medicinal plants involves the preparation of plant materials. The handling procedure needs to ensure the preservation of the bioactive compounds in the medicinal plants (such as the leaf, stem, root, etc.) before the extraction of the actual crude

plant extracts and determination of biologically active compounds (Nn, 2015). Plant extractions can be conducted on different parts of the plant samples like leaves, roots, tree bark, and fruits of fresh or dried plant material. Other pre-extraction processing and preservation procedures of plant materials such as freezing, refrigeration, grinding, and drying influence the compositions of phytochemicals in the final extracts (Nn, 2015). The presence of useful bioactive metabolites in plant extracts is mainly determined by the type of solvent used in the extraction. A good solvent for plant extractions should exhibit qualities such as low toxicity, ease of evaporation at low heat, and enhancement of rapid physiologic absorption of the extracts. In addition, a suitable solvent should have a good preservative property and must not cause the medicinal plant extract to dissociate/complex (Sultana et al., 2009; Ballesteros et al., 2014; Nn, 2015). Some key factors influencing the choice of extraction solvent are the quantity of bioactive compounds that need to be extracted, the diversity of bioactive compounds, the rate of extraction, ease of subsequent handling of the extracts, and the potential health hazard of the extractive solvents. The choice of solvent could also be influenced by the purpose, functional activity of the extracts, and targeted compounds to be extracted (Nn, 2015). Since the crude plant extracts may contain traces of residual solvent used in the extraction, the solvent should be non-toxic and should not impact negatively the analysis of constituent metabolites, both quantitatively and qualitatively (Ncube et al., 2008; Das et al., 2010). The solvents used for the extraction of bioactive compounds in medicinal plants are selected based on their ionic interactions and the polarity of the solute of interest. Solvents with the same polarity as their solutes will better dissolve the solute and more bioactive compounds will most likely be made available in the final extraction (Remadi et al., 2017). Many solvents had been used sequentially and/or in combined form to increase the number of different bioactive compounds of interest in crude plant extracts. The solvent polarity has been ranked, from most to least polar, of a few common solvents, which include Water > Methanol > Acetone > Ethylacetate > Chloroform > Hexane (Remadi et al., 2017).

1.13 The potential of water and alcohol as solvents for extraction in plant materials

Water is a universal solvent, and can be used to obtain crude extracts from plant material. Though traditional healers use mainly water for the extraction of biologically active compounds from medicinal plants, plant extracts from organic solvents are more consistent in their antimicrobial activity as compared to the extractions with distilled water (Das et al.,

2010). To increase the type and amount/quantity of plant secondary metabolites from plant materials, a number of solvents of different polarities are preferable (Wong et al., 2006). Moreover, studies have revealed that solvents like methanol increased the antioxidants and antimicrobial activities of crude plant extracts. In a report by Bidie et al. (2011), methanol as an extraction solvent increased the amount of biologically active compounds from whole walnut fruit compared to the solvent ethanol. The higher antioxidant activity of the alcoholic extracts as compared to the aqueous extracts of medicinal plants is attributable to the presence of higher amounts of polyphenol compounds in alcoholic extracts compared to aqueous extracts. Additionally, alcohols easily penetrate the cellular membrane of plant tissues to extract content from the plant material (Wang et al., 2010). This signifies that alcohol can better degrade plant cells to cause biologically active compounds released from the cells. (Lapornik et al., 2005). However, water can also support the extraction of active compounds and increase the occurrence of microorganisms (Lapornik et al., 2005). Higher amount of bio-flavonoids was recorded with 70% solvent ethanol due to its higher polarity/ionic interaction than 100% ethanol. With the addition of 30% water to the pure ethanol to prepare 70% ethanol solvent, the polarity/ionic interaction of the aqueous-ethanol solvent was increased (Bimakr et al., 2011). The yield from aqueous extraction (1.57 g/10 g) was slightly higher than the 70% ethanol extraction (1.23 g/10 g) in *P. betle* leaf. Also, the percentage antimicrobial function for the aqueous extraction from *P. betle* leaf was 96.0% which is also higher than 83.7% for the 70% ethanol extraction (Lee et al., 2015). The higher extract yield from the aqueous extraction of plants compared to the water-ethanol extraction may be because of higher polarity of deionized water and a shorter chain than ethanol (Pin et al., 2010).

1.14 Methods of preparing crude extracts from medicinal plants

Plant extraction can be described as the separation of medicinally or biologically active portions of plants (leaf, stem, root, etc.) using selective solvents through standard procedures (Nn, 2015). The main objective of extractions is to separate the soluble bioactive compounds/metabolites in plant materials from the insoluble cellular residue. The crude plant extracts obtained from solvent extractions contain a complex mixture or blend of phytochemicals/metabolites like alkaloids, flavonoids, glycosides, phenolics, and terpenoids (Nn, 2015). Some of the initially obtained crude plant extracts may be suitable for use as medicinal agents in the form of solution/fluid extracts while some plant extracts most likely

require further processing (Ballesteros et al., 2014; Nn, 2015). Methods to adopt in the extraction of medicinal plants depend on the type of solvents, the particle size of the plant tissues, the solvent-to-sample ratio, the length of the extraction period, and the temperature and pH of the solvent (Ballesteros et al., 2014; Nn, 2015). The different extraction methods include decoction, infusion, maceration, and percolation, among others. The more advanced extraction procedures such as sonication, microwave, and ultrasound-assisted methods of extraction are all extraction procedures that require good skill and technical know-how (Handa et al., 2008; Nn, 2015). However, this study only adopted a maceration method of extraction and therefore, will limit review to maceration, infusion, and decoction which are solid-liquid extractions, as well as their strengths and limitations.

1.15 Maceration, infusion, and decoction: merits and their demerits

Maceration can be described as a solid-liquid extraction (or shake/soft extraction) method. It could be regarded as a technique that has wide application in winemaking and the study involving medicinal plants (Handa et al., 2008; Nn, 2015). Maceration as an extraction technique involves the soaking of plant materials/samples, whether grainy or milled in a clean container with a solvent and allows the mixture to stand at room temperature for a minimum duration of three days with continuous agitation (Stéphane et al., 2021; Nn, 2015). The main purpose of the process is to soften and degrade the plant's cell wall to release soluble bioactive compounds. After a minimum of three days, the mixture is passed through a sieve, collecting the filtrate below the sieve, and leaving behind the cellular/insoluble residues. This method usually involves heat transfer via conduction and convection. The choice of solvent combination will most likely determine the type of bioactive compounds extracted from the samples (Handa et al., 2008; Nn, 2015). In the maceration, milled or un-milled plant materials are dissolved in a solvent for a defined period with continuous and fixed revolution speed/agitation until the soluble constituents are dissolved and removed from the cellular matrix. This method is relatively more suitable for thermo-labile drugs and phytochemicals (Ncube et al., 2008). The use of infusion and decoction on the other hand apply a similar principle as maceration. Both methods involve soaking in deionized water, however, the maceration period is longer while the infusion is shorter but for decoction; samples are heated in a specified amount of water ranging from a ratio of 1:4 to 1:16 for a particular period of time (Nn, 2015). A decoction method is more suitable for extracting

heat-stable phytochemicals/bioactive compounds such as those present in hard plant materials like tree bark and roots (Nn, 2015).

The maceration, infusion, and decoction techniques are the easiest and simplest methods of extraction from medicinal plants. However, proper recycling of organic waste may be a major challenge since a large volume of solvents is most often used and adequate handling of the waste is required (Handa et al., 2008; Nn, 2015). Alterations in temperature and choice of solvents enhanced the extraction process on medicinal plants and reduce the volume of the solvents needed for extraction (Stéphane et al., 2021; Handa et al, 2008). Heating *Centella asiatica* at 90°C increased the amount of polyphenols and improve their antioxidant activities but increase/decrease extract pH with prolonged extraction time (Nn, 2015). After the extraction of crude plant extracts from medicinal plants using a suitable method, it is important to further identify and quantify the available metabolites. This will help to get a better understanding of the type and amount of phytochemicals present in the crude plant extracts, and their antimicrobial, antioxidant, and antimethanogenic properties.

1.16 Metabolomics and plant metabolites

Metabolomics involves the identification and/or quantification of biologically active compounds in biological materials such as medicinal plants (Kumar et al., 2017). Natural products from plants/plant-derived materials are considered a useful source of valuable biologically active compounds in scientific studies (Salem et al., 2020). Plant extracts are usually comprised of several phytochemicals, whereby the biological activity of the extracts can be represented by synergism among various metabolites. Conversely, isolating every single metabolite from a medicinal plant extract is not usually feasible due to the complex biochemical nature and presence of most metabolites/secondary compounds at very low levels (Salem et al., 2020). Metabolomics in recent years has been considered an invaluable tool for the bioassay of several metabolites/phytochemicals from crude plant extracts giving rise to useful scientific research (Kumar et al., 2017; Salem et al., 2020). Metabolite profiling in large-scale studies has allowed many researchers to access the global data sets of metabolites of both plant and animal origins and their various pathways in a biological system (Kumar et al., 2017). Metabolites are an essential part of plant metabolisms as they affect plant biomass (Turner et al., 2016). In recent years, metabolomics study has been regarded as one of the main innovations in science and has given rise to accurate metabolite

identification and quantification in microbes, plants, and animals (Dam & Bouwmeester, 2016; Kumar et al., 2017). Metabolomics can identify and quantify a vast collection of metabolites from a single crude plant extract, hence allowing quick and accurate analysis of metabolites in a sample (Kumar et al., 2017).

1.17 Devices for metabolites Identification and quantification

Metabolite separation has helped to increase precision in metabolomics studies. Metabolites from plant extracts or natural products are identified and quantified using Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), or Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS), while the MS device helps to provide additional information on the structures of the separated metabolites or bioactive compounds (Salem et al., 2020). There are a number of MS-based databases and software tools that are used for metabolites/bioactive compounds identification. These databases include the Golm Metabolome Database (GMD) which makes use of the GC retention indices, a decision tree search that provides information regarding the substructure of unknown metabolites/bioactive compounds, and METLIN, which is an MS/MS database that can predict the structures of metabolites with much higher accuracy (Murray et al., 2013; Salem et al., 2020).

1.18 Encapsulation of plant extracts

Plant extracts and essential oils are natural products that have several scientific and economic applications. The extractions of plant extracts and essential oils from plants are carried out by classical and innovative methods (Asbahani et al., 2015). Encapsulation is a technology that has increasing applications in animal nutrition. Encapsulation enables proper protection of sensitive biologically active compounds, especially during feed processing, and improves the condition of storage (Tolve et al., 2021). Encapsulation also helps to mask the bitter and unpalatable taste of bioactive compounds like tannin and alkaloids (Tolve et al., 2021). Several encapsulation methods/processes have been performed in recent times with the purpose of improving the efficacy of bioactive molecules, nanocrystals, vitamins, minerals, amino acids, oils, and also essential oils for different applications like *in vitro* diagnosis, therapy, cosmetics, textile, food, and in animal nutrition study (Belščak-Cvitanović et al., 2011; Isailović et al., 2012; Rijo et al., 2014; Tolve et al., 2021). Essential oils and plant-based tannins encapsulation has led to several new formulations with new applications

(Asbahani et al., 2015; Ibrahim & Hassen, 2022; Tolve et al., 2021). This ensures the protection of fragile oil or tannin and controlled release. The most commonly prepared carriers are alginate beads, polymer particles, liposomes, and solid lipid nanoparticles (Belščak-Cvitanović et al., 2011; Isailović et al., 2012; Asbahani et al., 2015). Sections 1.19 to 1.22 review the production, biosynthesis, features, advantages, and application of alginate beads.

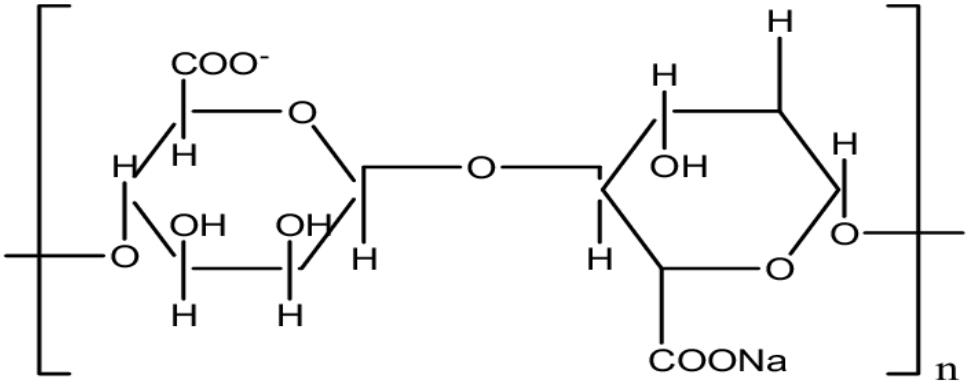
1.19 Alginate and its Production

Alginate can be described as a polysaccharide that has many applications in the food/feed and drug manufacturing industries (Urtuvia et al., 2017). This polysaccharide has a chemical structure made up of subunits of (1-4)-β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). The properties of an alginate molecule are mainly affected by the composition of the monomers and their molecular weight (Urtuvia et al., 2017). Alginate molecules are common in seaweed and can be commercially extracted from the seaweed in large quantities. In addition, alginate can also be produced from the bacteria *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (Hay et al., 2013). Alginate with various molecular weights and reproducible physicochemical properties can be produced via the manipulation of culture conditions during fermentation (Hay et al., 2013; Urtuvia et al., 2017). Alginate can be produced from seaweeds through washing, dissolution, and filtration with additional treatments. The fresh or dry seaweeds will be properly washed in dilute acids to remove cross-linking ions; these ions will cause the alginate to become insoluble. After washing the seaweeds in dilute acids, they are further dissolved in dilute sodium hydroxide. A gelatinous solution of alginate with some cellular debris will then be produced which is further filtered to get rid of the cellular debris. Depending on the purity requirement, the solution may be treated further to remove colour leaving behind a clean alginate solution. The clean alginate solution is finally subjected to a controlled drying process and packaged in a clean and well-labeled stoppered container (Borowitzka et al., 2007; MacArtain et al., 2007; Corona et al., 2017).

1.20 Structure and description of alginate

Sodium alginate has been described as a polysaccharide or natural amylose carbohydrate. It is usually distilled from algae or seaweed. It has a wide application in food, cloths, medicine, dyeing, printing, paper manufacturing, and chemicals such as thickeners, emulsifiers,

stabilizers, and binders. It has a molar mass of 398.316 g mol^{-1} and melting and boiling points are >300ºC and 495.2ºC respectively (Homayouni et al., 2007). The molecular formula and structure of sodium alginate are presented in Figure 1.2.



$(C_6H_7O_6Na)n$

Figure 1.2. Molecular formula and structure of sodium alginate

1.21 Biosynthesis of alginate from bacteria

There are two major genera of bacteria that are known to produce or secrete alginate. These include *Azotobacter* and *Pseudomonas*. Most of the studies to reveal the mechanisms involving the biosynthesis of alginate from bacteria have been carried out on *Azotobacter vinelandii* and pathogenic bacteria *Pseudomonas aeruginosa* (Hay et al., 2014). These two major groups of bacteria have been found to utilize highly similar molecular mechanisms to synthesize alginate; however, the *Azotobacter* and *Pseudomonas* produce alginate for different purposes with differing material properties (Hay et al., 2013, 2014). Some *P. aeruginosa* strains have the capacity to secrete a large quantity of alginate to assist in the formation of thick highly structured biofilms (Nivens et al., 2001; Hay et al., 2009), whereas the *Azotobacter* produce stiffer alginate with high α-L-guluronic acid residue (Hay et al., 2009).

1.22 Features and practical applications of alginate encapsulating aqueous plant extracts

The features of alginate make it a good wall material for the encapsulation of aqueous extracts. Alginate has been used as a wall material for encapsulating plant extracts and other

natural products. Alginate molecules are large polymers with mechanical and hosting properties. These properties are mainly influenced by alginate compositions, the ratio, and the order of distribution of α-L-guluronic acid and 1,4-linked β-D-mannuronic acid residues (Hay et al., 2009). The alginate rich in guluronic residues belongs to the group of high-quality immobilization matrixes (Belšćak-Cvitanović et al., 2011). The department of United State Food and Drug Administration has established and approved alginate as an encapsulating or coating material with an optimum level of safety for consumption. (Hay et al., 2013). It is a commonly used material for the encapsulation of different bioactive compounds (e.g. tannins, probiotics, prebiotics, enzymes, yeast, etc.) in foods/feeds because it has several advantageous features. These features include non-toxicity, ease of production, good biocompatibility with natural products, thermally and chemically stable (Zohar-Perez et al., 2004; Belšćak-Cvitanović et al., 2011; Hay et al., 2013). Also, dried alginate forms can be used to improve the shelf life of products (Zohar-Perez et al., 2004). The encapsulation efficiency of alginate beads varies in the range of 50-99.9% depending on the method adopted (Chan et al., 2010; Belšćak-Cvitanović et al., 2011; Lević et al., 2015). The use of alginate as a coating material preserves the total antioxidant content of the core material/bioactive compounds. This phenomenon has been validated with Fourier transform infrared analysis, which revealed that there were no chemical interactions between extracted compounds and alginate (Belšćak-Cvitanović et al., 2011). The addition of a filler substance, such as inulin (Isailović et al., 2012) and chitosan (Belšćak-Cvitanović et al., 2011) in the dried product reduced alginate roundness distortion and improved the retention of the physicochemical properties of the products during the process of drying. Thyme polyphenolic compounds had been reported to be chemically stable and the antioxidant activity was preserved when the product was immobilized with calcium alginate. This has helped to prevent the excessive leakage of bioactive compounds like polyphenols and then achieve desirable release profiles (Belšćak-Cvitanović et al., 2011).

Conclusions

In conclusion, commitment to climate-smart livestock production is important in agriculture for the provision of animal protein, enhancing adaptability and sustainability of animal production and the environment. The crude plant extracts are good agents for methane inhibition with no adverse effect on animal performance when fed at the right dosage. However, the phrase “right dosage” is still subjective in the application of plant extracts as

alternative rumen modifiers since previous researchers reported some variations in animal performance resulting from variations in the concentration of plant bioactive compounds and methane inhibition of plant extracts. Different extraction solvents were used for various plant materials in previous research with differing results reported on the yield and chemical composition of plants' bioactive compounds. Water as an extraction solvent could influence positively the yield and phytochemicals constituents of medicinal plant extracts, especially when combined with organic solvent in the right proportion. This right proportion for the combination of extraction solvents has not been fully established. Furthermore, proper identification and quantification of plant secondary metabolites, especially those associated with methane inhibitors are important for the standardization of plant extracts consistent in reducing methane production. Precise identification and quantification of plant secondary metabolites is a major research gap and this led to the working hypotheses of this study. Also, the antimicrobial, antioxidant, and antimethanogenic properties and chemical activities of crude plant extracts or natural products had been reported to be positively affected by encapsulation with good bio-compatible materials such as alginate, etc. From the document works of literature, the potential of alginate as an encapsulating material for plant extract with the purpose of providing a co-benefit in reducing methane in ruminants has not been explored, hence motivating the current research objectives and working hypotheses.

General objective

The general objective of this study is to optimize plant extract yields and develop alginate encapsulated plant extracts as dietary additives to modulate rumen fermentation and reduce enteric methane emission without affecting negatively the growth performance of South Africa Mutton Merino lambs.

Specific objectives

- To evaluate the effect of three different proportions of aqueous-methanol extractions (70, 85, and 100% CH_3OH) on yields and phytochemical constituents of *A. vera, J. curcas, M. oleifera,* and *P. betle* leaf extracts.
- To evaluate the effect of three different aqueous-methanol extracts of *A. vera, J. curcas, M. oleifera,* and *P. betle* on rumen *in vitro* gas production and organic matter digestibility of *Eragrostis curvula* hay.

- To evaluate alginate and chitosan as wall material for encapsulation of medicinal plant extracts and characterize alginate-chitosan encapsulated plant extract in terms of morphology and particle density.
- To comparatively evaluate the efficacy of the crude plant extract versus alginate-chitosan encapsulated plant extracts on *in vitro* gas production and organic matter digestibility of *E. curvula* hay.
- To evaluate the effect of alginate encapsulated *M. oleifera* plant extracts additives on enteric methane production, feed intake, and apparent digestibility; nitrogen utilization, weight gain, and rumen fermentation parameters in South African Mutton Merino lamb.

Working hypotheses

H_o: The aqueous-methanol extractions (70, 85, and 100% CH_3OH) have no effect on yields and phytochemical constituents of *A. vera, J. curcas, M. oleifera*, and *P. betle* leaf extracts.

H_o: The aqueous-methanol extracts of *A. vera, J. curcas, M. oleifera*, and *P. betle* have no effect on rumen *in vitro* gas production and organic matter digestibility of *E. curvula* hay.

H_o: The encapsulation of *A. vera,* and *M. oleifera* extract with alginate or alginate-chitosan has no effect on micro-particle morphology and particle density on the products.

H_o: The alginate and alginate-chitosan encapsulation of *A. vera,* and *M. oleifera* extracts have no effects on *in vitro* gas production and organic matter digestibility of *E. curvula* hay.

H_o: The alginate encapsulated *M. oleifera* plant extracts additive has no effect on enteric methane production, feed intake, and apparent digestibility; nitrogen utilization, weight gain, and rumen fermentation parameters in South Africa Mutton Merino lamb.

List of experiments undertaken

❖ The Antimethanogenic Potentials of Plant Extracts; Their Yields and Phytochemical Compositions as Affected by Extractive Solvents

❖ Characterization and *in vitro* methane inhibition of *A. vera* and *M. oleifera* plant extracts encapsulated with alginate or alginate-chitosan wall materials

❖ Effect of alginate encapsulated *M. oleifera* plant extract additives on growth performance, nutrient digestibility, methane and rumen fermentation of South African Mutton Merino lamb

CHAPTER TWO

The antimethanogenic potentials of plants extracts; their yields and phytochemical compositions as affected by extractive solvents

Abstract

Plant phytochemicals are an important area of study in ruminant nutrition, primarily due to their antimethanogenic potential. Plant extract yields, their bioactive compounds, and their antimethanogenic properties are largely dependent on the nature of the extractive solvents. This study evaluated the yields and phytochemical constituents of four plant extracts, as affected by the aqueous-methanolic (H_2O-CH_3OH) extraction and their antimethanogenic properties on the in vitro methane production. The plant extracts included *Aloe vera, Jatropha curcas, Moringa oleifera,* and *Piper betle* leaves with three levels of extractions (70, 85, and 100% CH_3OH). The crude plant extract yields increased with the increasing amount of water. *M. oleifera* crude extracts yields (g/10 g) increased from 3.24 to 3.92, *A. vera,* (2.35 to 3.11) *J. curcas* (1.77 to 2.26)*, and P. betle* (2.42 to 3.53). However, the identified and quantified metabolites showed differing degrees of solubility unique to the plant leaves in which they exist, while some of the metabolites were unaffected by the extraction solvents. The methane mitigating potentials of these extracts were evaluated as additives on *Eragrostis curvula* hay at a recommended rate of 50 mg kg^{-1} DM. The plant extracts exhibited antimethanogenic properties to various degrees, reducing ($p < 0.05$) in vitro methane production in the tested hay, *A. vera, J. curcas, M. oleifera*, and *P. betle* reduced methane emission by 6.37–7.55%, 8.02–11.56%, 12.26–12.97, and 5.66–7.78 respectively compared to the control treatment. However, the antimethanogenic efficacy, gas production, and organic matter digestibility of the plant extracts were unaffected by the extraction solvents. Metabolites, such as aloin A, aloin B, and kaempferol (in *A. vera*), apigenin, catechin, epicatechin, kaempferol, tryptophan, procyanidins, vitexin-7-olate and isovitexin-7-olate (in *J. curcas*), alkaloid, kaempferol, quercetin, rutin and neochlorogenic acid (in *M. oleifera)* and apigenin-7,4′-diglucoside, 3-*p*-coumaroylquinic acid, rutin, 2-methoxy-4-vinylphenol, dihydrocaffeic acid, and dihydrocoumaric acid (in *P. betle*) exhibited a methane reducing potential and hence, could be regarded as promising plant secondary metabolites for methane inhibition.

Keywords: plant extracts, methanolic extractions, metabolomics, methane, *in vitro*

2.1 Introduction

One of the significant atmospheric methane contributors is ruminant animals. Methane emission contributes to the global warming effect and thus remains a topic of great interest, globally (Broucek, 2018). The contribution of enteric methane emission to total methane production from agricultural sources is quite significant (Jiménez-Ocampo et al., 2019). The need to find solutions to reduce enteric methane emissions without compromising the performance and welfare of ruminants becomes inevitable. Many efforts, such as the supplementation of diets with concentrates (Broucek, 2018) and lipids (Ungerfeld, 2015), the use of probiotics and prebiotics (Mwenya et al., 2004), as well as the addition of medicinal plant extracts (Akanmu & Hassen, 2018; Goel et al., 2008; Patra & Yu, 2013), have been used to mitigate enteric methane production. However, antimethanogenic plant-based compounds, such as tannins, saponins, nitrates, and halogenated aliphatic hydrocarbons have been reported to limit some useful rumen microbial activities and reduce the animal performance, especially when added at higher doses, to achieve effective methane mitigation (Patra & Yu, 2013). These adverse effects and toxicities may be averted through the use of multiple antimethanogenic compounds that may exert synergistic actions when used at appropriate doses, to inhibit the methanogenic archaea and other rumen protozoa that promote the methane production in an additive or complementary manner (Patra et al., 2017). Some medicinal plants and their extracts, such as *Aloe vera*, *Jatropha curcas*, *Moringa oleifera*, and *Piper betle* have been reported to potentially modulate the rumen environment, reduce methane emissions, and have antimicrobial activities (Ratshilivha et al., 2014; Akanmu & Hassen, 2018). This may be due to the combined activity of multiple bioactive compounds in the plant extracts. The abundance of medicinal plants/herbs in Africa has deepened the efforts of researchers to exploit plant-based compounds as potential natural alternatives to antibiotic growth promoters to enhance livestock productivity (Makkar et al., 2007), including the decreased methane emission (Soliva et al., 2004; Akanmu & Hassen, 2018).

The characteristics, antioxidant, antimicrobial, and chemical constituents of *A. vera, J. curcas, M. oleifera,* and *P. betle* have been discussed in the previous study (Akanmu & Hassen, 2018). *A. vera* leaf is a rich source of anthraquinone, a phenolic compound that has stimulating effects on the bowels and antibiotic properties (Kedarnath et al., 2012). It contains saponins, which are soapy substances found in the gel and capable of cleansing. It

exhibits antimicrobial activity against bacteria (Kedarnath et al., 2012) and shows anti-inflammatory action, with a wide range of antimicrobial activity (Aysan et al., 2010). *J. curcas* is an ideal biodiesel crop in most arid areas of Asia, South America, and Africa, because of its high oil (43–61%) seed kernel (Huang et al., 2014). Traditionally, *Jatropha* plants have been used to produce oil, soap, and medicinal compounds (Huang et al., 2014). The various parts of the *M. oleifera* tree have been established as being good sources of glucosinolates, flavonoids, and phenolic acids (Amaglo et al., 2010; Coppin et al., 2013). Among the flavonoid compounds, flavonol glycosides of quercetin > kaempferol > isorhamnetin had been reported to be predominantly present in various parts of the tree, except in the roots and seeds (Coppin et al., 2013). *P. betle* is an evergreen perennial creeper (Emon et al., 2020). Traditional healers used the betle leaf to treat halitosis, boils and abscesses, constipation, swelling of the gums, cuts, and injuries. In the South East Asia region, *P. betle* was among the plants that have been used to control caries and periodontal diseases (Fathilah, 2011) and to treat bad breath. Fathilah (2011) reported that the crude aqueous extract of the *P. betle* leaves exhibits antibacterial activities towards *Streptococcus mitis, Streptococcus sanguis,* and *Actinomyces viscosus,* some of the early colonizers of dental plaque. Although the betle leaf has been tested *in vitro* for enteric methane emission in ruminants (Akanmu & Hassen, 2018), the characteristics and properties of this plant make it a unique medicinal plant to further investigate.

The solvent extraction technique is significant in the determination of crude plant extract yield and the concentration of bioactive compounds in plant extracts. Increasing the efficiency of solvents in the extraction of plant-based compounds in medicinal plants requires the choice of the right combination of extraction medium (Sultana et al., 2009; Ballesteros et al., 2014; Nn, 2015). This may be because some bioactive compounds in plant materials are relatively hydrophobic and others hydrophilic. It has also been stated that no universal extraction method is ideal and each extraction procedure is unique to the targeted plant compounds (Nn, 2015). Water is a universal solvent, used to extract plant products with antimicrobial activity. Though plant extracts from organic solvents give more consistent antimicrobial activity, compared to water extract, water-soluble phenolics are mostly important as antioxidant compounds (Das et al., 2010). In order to extract the different phenolic compounds from plants with a high degree of accuracy, various solvents of different polarities have to be used (Wong et al., 2006). Studies had shown that highly polar solvents,

such as methanol, have high effectiveness as antioxidants (Bidie et al., 2011) and antimicrobial resistance (Akanmu & Hassen, 2018). However, the yields/concentrations of biologically active compounds in plant extracts could be increased further by the addition of a more polar solvent, such as water. The higher concentrations of bioactive flavonoid compounds were recorded with aqueous alcohol (30:70), due to its higher polarity than pure alcohol (Bimakr et al., 2011).

Solvents with different polarities have been used to obtain extracts in plant-based materials and different results concerning the yields and antimicrobial efficacy were reported. Hence, this study investigated the effect of an extraction efficiency of various aqueous-methanol concentrations on yields, phytochemical constituents, and the antimethanogenic potential of *A. vera, J. curcas, M. oleifera,* and *P. betle* leaf extracts. It is hypothesized that the use of different proportions (70%, 85, and 100% methanol) of aqueous-methanol solvent extractions may have a useful effect in improving the crude plant yields, phytochemical concentrations, and subsequently the methane mitigation potential of *A. vera, J. curcas, M. oleifera,* and *P. betle* leaf extracts.

2.2 Materials and Methods

2.2.1 Study area and collection of the plant materials

The study was conducted at the departmental laboratory of the Department of Animal and Wildlife Sciences, University of Pretoria, South Africa, following its ethical approval (NAS336/2019). The *Moringa oleifera* leaves (A11NA) used in this study were harvested fresh from two year old growing and blooming trees at Lefakong farm in Pretoria North at 399 Thabaya Batho Boplaas, South Africa, while *Piper betle* leaves (cultivar Marakodi) of eight month old plants were collected from a farm in Durban at Tongaat Kwazulunata, South Africa. In the month of March, Pretoria has a maximum day temperature of 27.7^{o}C and as low as 14.4^{o}C at night. The rainfall is moderate with an average of 72 mm, 62% humidity, and 255 h sun during the whole month. In the month of May, Durban has maximum day temperature of 26^{o}C and 14^{o}C at night. *Jatropha curcas* (IARJAT-S1) from three year old trees and *Aloe vera* (Taxon A) leaves of six month old were way billed via air in to South Africa. They were harvested in Kaduna state, a northwestern region in Nigeria, with the permission of the Department of Agriculture (P0095290), South Africa. Kaduna state is located 586 meters above sea level with average yearly temperature of 28.46^{o}C and about 93

mm rain fall. All leave samples were collected between the month of March and June from five to ten plants of the sample species. The plants were transported to the laboratory in ice boxes.


Aloe vera


Jatropha curcas


Moringa oleifera


Piper betle

Plant leaves from which the crude plant extracts collected

2.2.2 Methanolic extraction

Moringa oleifera, *Jatropha curcas*, *Aloe vera*, and *Piper betle* aqueous-methanolic extracts were prepared using methanol (CH_3OH) at 70, 85, and 100% concentrations as a modification to the previous method (Akanmu & Hassen, 2018). The plant materials were first freeze-dried for 96 h and stored in plastic bags pending further use. Dried samples of each plant leaf were milled through a 1 mm sieve and extracted by dissolving 10 g of milled dried leaf material into a 300 mL extraction bottle containing 200 mL (1:20 *w*/*v*) aqueous methanol [70% (3 mL H_2O: 7 mL CH_3OH), 85% (1.5 mL H_2O: 8.5 mL CH_3OH), and 100% (CH_3OH only)]. The extraction bottles were arranged into an Incoshake incubator and agitated at 130 rpm and 20 °C for 96 h. Extracts from each bottle were filtered by squeezing through a sieve with a 150 µm aperture. The filtrate was placed in a fume cubicle for 24 h until partially dried. The semi-dried extracts were freeze-dried for 72 h. All freeze-dried extracts were stored in plastic bottles at 4 °C until further use.

2.2.3 Ultra-performance liquid chromatography-mass spectrometry (UPLC–MS) analysis of the bioactive molecules in plant extracts

The phytochemical identification of the crude extracts was carried out using ultra-performance liquid chromatography-mass spectrometry (UPLC–MS). A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for the high-resolution UPLC–MS analysis. Electrospray ionization was used in negative mode with a cone voltage of 15 V, a desolvation temperature of 275 °C, a desolvation gas at 650 L/h, and the rest of the MS settings optimized for the best resolution and sensitivity. Data

were acquired by scanning from m/z 150 to 1500 m/z in resolution mode, as well as in the MSE mode. In the MSE mode, two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40−100 V), to obtain the fragmentation data. Leucine enkaphalin was used as the lock mass (reference mass) for accurate mass determination, and the instrument was calibrated with sodium formate. The separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 µm column. An injection volume of 2 µL was used, and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B. The gradient started at 100% solvent A for 1 min and changed to 28% B over 22 min in a linear way. It then went to 40% B over 50 s and a wash step of 1.5 min at 100% B, followed by re-equilibration to the initial conditions for 4 min. The flow rate was 0.3 mL/min, and the column temperature was maintained at 55 °C. The identification was performed, based on its measured mass, compared to the theoretical mass (<5 ppm), the molecular formula, and the characteristic fragments for each compound, finding the differences and similarities between the samples analysed. The library database was used to identify compounds present in the crude extracts. Data was reprocessed using MSDIAL and MSFINDER (RIKEN Center for Sustainable Resource Science: Metabolome Informatics Research Team, Kanagawa, Japan) to utilise the fragmentation data contained in the Waters MSe acquisition (Tsugawa et al., 2015). The peak height data from MSDial was used to determine the mean abundance of the identified metabolites (Yu et al., 2020).

2.2.4 Buffer mineral solution, collection of the rumen fluid from donor steer, and the in vitro gas production

The in vitro gas production studies were carried out following the procedure of Menke et al. (1979), with the modifications detailed in Adejoro & Hassen (2018). The prepared buffer solution was preserved in a water bath at 39 °C and constantly purged with CO_2 until the solution turned colourless. The rumen fluid was collected from three rumen-cannulated Holstein breed of cattle fed Lucerne hay *(Medicago sativa) ad libitum*. A 40 mL prepared solution of rumen fluid was used to incubate 400 mg of the substrate in a 120 mL in vitro bottle in triplicates and four successful runs known as biological replicates were conducted. Gas pressure was taken at 2, 4, 8, 12, 24, and 48 h after the commencement of the incubation, while gas samples were taken inside Hamilton syringes for the analysis of methane concentration. Three blanks were included to correct the methane produced from the

inoculum in each run. Methane concentration was analysed with gas chromatography (8610C BTU Gas Analyser GC System; SRI Instruments GmbH, Bad Honnef, Germany). The GC was pre-equipped with a solenoid column, packed with silica gel and a flame ionization detector (FID). Methane concentration values were related to the total gas production, in order to estimate its concentration. Methane concentration was subsequently converted to mass values (Adejoro & Hassen, 2018). Eragrostis hay of known chemical composition was used as a substrate and incubated with the crude extracts of four medicinal plants prepared using different percentages of methanol/aqueous solvent extraction (70, 85, and 100%). Plant extracts were reconstituted in distilled water and added to the test the feed at 50 mg kg^{-1} DM. For the control treatment, there was an equal amount of distilled water without plant extracts.

2.2.5 Determination of the in vitro organic matter digestibility (IVOMD)

Extracts of *Aloe vera, Jatropha curcas, Moringa oleifera,* and *Piper betle* were evaluated as additives to 1 mm particle size Eragrostis hay substrate using the IVOMD procedure (Akanmu & Hassen, 2018). Briefly, the first stage involved a 48h rumen degradation phase, followed immediately by another 48h acid-pepsin digestion phase. During the first phase, 200 mg of the feed samples were incubated in triplicate under anaerobic conditions with 20 mL of rumen liquor for 48h at 39 °C with the inclusion of blanks and standards in every batch of incubation. This was followed by a 48h acid-pepsin digestion phase at 39 °C under anaerobic conditions. Following 96 h of incubation, the residual plant materials were collected and oven-dried at 105 °C for 18h. The ash contents were determined by combustion in a muffle furnace at 250 °C for 2h and later at 600 °C for 4h, and the in vitro organic matter digestion was estimated.

2.2.6 Chemical analyses

The feed sample, *Eragrostis curvula* hay, was analysed for the dry matter (DM) and total ash (Thiex et al., 2012). The ether extract was determined using the ether extraction in the Tecator Soxtec (HT6) system (McCormick, 2014). 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), as described (Van Soest et al., 1991), nitrogen (Sáez-Plaza et al., 2013) (FP2000 Nitrogen/Protein Analyser, Leco Instrumente GmbH, Kirchheim, Germany), and the crude protein was obtained by multiplying nitrogen by 6.25.

2.2.7 Statistical analysis

The crude extract yield of the plant samples was evaluated in triplicate, and the data collected were analysed using the GLM procedure of SPSS (version 20) with the model Yij = μ + Tj + eij. where Yij is the mean of the individual observations (crude extract yield), μ is the overall mean, Tj is the treatment effect (extraction solvent), and eij is the residual error. The means were separated using the Tukey test, and the significance was declared at $p < 0.05$. For the in vitro gas production study, individual bottles within each run served as analytical replicates, while each run represented a statistical replicate. Data were analysed using the GLM procedure in SPSS software (version 20) with the model Yij = μ+ Bi + Tj + eij. Where Yij = mean of the individual observation (gas production), μ = overall mean, Bi = block effect (replicate), Tj = treatment effect, and eij = residual error. Mean separations were performed using the Tukey test, and the significance was declared at $p < 0.05$. The principal component analysis was carried out using the PAST 4 software, version 4.11.

2.3 Results and Discussion

2.3.1 The yield of plant crude extracts

The crude extract yields (Table 2.1) from the medicinal plants were influenced by the treatment solvents with visible trends and increased with a decrease in the amount of methanol (CH_3OH) solvents, replaced by an equivalent amount of distilled water (H_2O) in the mixture for all of the plant samples. The yields of the *M. oleifera* crude extracts increased from 3.24 to 3.92 g/10 g, *A. vera* (2.35 to 3.11 g/10 g), *J. curcas* (1.77 to 2.26 g/10 g), *and P. betle* (2.42 to 3.53 g/10 g). For *A. vera* and *M. oleifera*, the 70% CH_3OH are statistically higher ($p < 0.05$) than the 85% and 100% CH_3OH while *J. curcas* and *P. betle* are significantly higher at 70% CH_3OH than 100% CH_3OH. These results are within the average value of the 30% extract yields of green tea earlier reported (Perva-uzunalic et al., 2006). The extraction procedures and solvent type play a critical role in the determination of crude extract yields and the concentration of bioactive compounds in medicinal plants (Nn, 2015). The results of this study show that the four plants used in the study are probably water-soluble, with *M. oleifera* having more relatively hydrophilic phytochemicals than *A. vera*, *J. curcas*, and *P. betle*. The increase in yield of plant crude extracts with increased distilled water in the solvent mixtures could mean that most bioactive compounds in the plant leaves are hydrophilic and could easily leach out, preferentially, in the presence of polar solvents. To increase the efficiency of the extraction in medicinal plants, the use of solvents with

different polarities is critical (Wong & Kitts, 2006). The combination of CH_3OH and H_2O as extraction solvents in this study could have softened the plant samples in the mixture better than pure CH_3OH and promoted the rapid physiological absorption of the extracts due to the higher polarity of H_2O (VegaArroy et al., 2017; Nn, 2015; Quispe et al., 2018).

Table 2.1 Effects of the solvent extraction on the yield of crude extracts (g/10 g) dry sample

Leave extract	70% CH_3OH	85% CH_3OH	100% CH_3OH	SEM	*p*-value
A. vera	3.11 ^{A}	2.42 ^{B}	2.35 ^{B}	0.10	0.009
J. curcas	2.26 ^{A}	2.06 ^{AB}	1.77 ^{B}	0.05	0.006
M. oleifera	3.92 ^{A}	3.38 ^{B}	3.24 ^{B}	0.12	0.013
P. betle	3.53 ^{A}	3.07 ^{A}	2.42 ^{B}	0.14	0.002

SEM = standard error of means, *p*-value = probability value, uppercase letters compare the means of the extract yield among all solvent extraction techniques of each plant material across the row. Means with different letters across the row for each parameter are significantly ($p < 0.05$) different.

2.3.2 Phytochemical identification and the concentration of plant metabolites

2.3.2.1 Aloe vera

The study reveals the presence of bioactive compounds in the aqueous-methanolic extraction of *A. vera* with a detailed characterisation of the identified compounds (Table 2.2). The concentrations (mg L^{-1}) of aloesin, nataloin A, nataloin B, 10-hydroxyaloin A, 10-hydroxyaloin B, and caffeoyl ester of aloesin were more abundant at 100% methanol extraction than at 70% methanol extraction, indicating they were relatively less water-soluble. While kaempferol-7-*O*-glucoside and 3-*p*-coumaroylquinic acid were relatively hydrophilic, the extraction solvents had no clear effect on the abundance of aloin A, aloin B, and aloe emodins as the concentrations of these metabolites were likely affected by the relative solubility behaviour of other metabolites in the crude plant extracts. Aloin A, aloin B, and aloe-emodin have been reported in a few aloe species crude extracts (Logaranjan et al., 2013; Wu et al., 2013; Kanama et al., 2015; El Sayed et al., 2016). Kaempferol, 10-hydroxyaloin A and B, and caffeoyl ester of aloesin and aloesin have also been identified in the solvent extracts of the aloe species (Wu et al., 2013; El Sayed et al., 2016). Quispe et al. (Quispe et al., 2018) reported the presence of aloin A, aloin B, aloe emodin diglucoside, 10-hydroxyaloin A, 6-malonylnataloin A (nataloin A), and caffeoyl ester of aloesin in *A. vera* aqueous extracts. Additionally, 6-malonylnataloin A and B, aloinoside A/B, and aloeresin have been identified in three to six species of aloe (El Sayed et al., 2016). From the few

identified metabolites in *A. vera*, kaempferol-7-*O*-glucoside and 3-*p*-coumaroylquinic acid were relatively water-soluble and had a direct relationship with the crude plant extracts (in Table 2.1) while other metabolites were either hydrophobic or unclearly affected by the extraction solvents. The possible reason is that there are other unidentified metabolites for which a relative solubility is yet to be understood. However, this study focused more on the metabolites in crude plant extracts capable of reducing the enteric methane production and how they are influenced by the extraction of solvents. The principal constituent of the compounds in *Aloe vera* is anthraquinone/anthrone with a broad spectrum of biological activities, such as anticancer, anti-inflammatory, antimicrobial, diuretic, vasorelaxing, and phytoestrogen indicating their possible clinical use in several diseases (Chien et al., 2014).

Table 2.2 Identification and mean abundance of the phytochemicals (mg L^{-1}) in *Aloe vera* extracts using 70%, 85%, and 100% aqueous-methanol

RT (min)	Molecular Formula	Measured Mass (*m/z*)	Error *m/z* (ppm)	MS Fragment	UVmax (nm)	Compound	Classification of the Compounds	70%	85%	100%
10.94	$C_{16}H_{18}O_8$	337.0928	−0.3	273,245,202	309	3-*p*-coumroyl quinic acid	Phenolic acid	7.65	8.42	2.40
11.64	$C_{19}H_{22}O_9$	393.1185	−0.3	273245202	296	Aloesin	Chromone (*C*-glycosylated chromone)	59.51	281.46	293.30
14.02	$C_{27}H_{30}O_{15}$	593.1526	−0.7	473,383,353	332	Aloe emodin-diglucosid	Anthrone (Anthracene compound)	5.04	30.51	25.99
15.15	$C_{27}H_{30}O_{15}$	593.1455	−6.1	431,311,297, 283,282,269	269,335	Aloe emodin-diglucoside isome	Anthrone (Anthracene compound)	28.45	92.67	91.18
16.25	$C_{21}H_{20}O_{11}$	448.1304				Kaempferol-7-*O*-glucoside	Flavonoid (*C*-glycosylated flavoinoid)	8.32	6.50	5.22
16.66	$C_{21}H_{22}O_{10}$	433.1138	1.6	313,270	304	10-hydroxyaloin B	Anthrone	1.45	60.38	74.93
16.89	$C_{21}H_{22}O_{10}$	433.1133	0.9	313,270	305	10-hydroxyaloin A	Anthrone	0.91	58.23	70.58
18.61	$C_{24}H_{26}O_{12}$	505.1343	−1.6	343,297,257	264,301	6-Malonylnataloin B (nataloin B)	Anthrone	3.04	77.87	83.30
19.24	$C_{24}H_{26}O_{12}$	505.1355	−4.4	343,325,297, 257	264,301	6-Malonylnataloin A (nataloin A)	Anthrone	1.97	55.97	59.22
20.16	$C_{29}H_{30}O_{12}$	569.1669	0.2	407,243,161	300	Caffeoyl ester of aloesin	Chromone	3.14	43.32	44.84
21.01	$C_{21}H_{22}O_9$	417.1194	0.0	297	297,354	Aloin B	Anthrone (Anthracene compound)	123.34	275.75	265.68
21.84	$C_{21}H_{22}O_9$	417.1176	−2.4	297	297,354	Aloin A	Anthrone (Anthracene compound)	163.05	310.38	303.57

2.3.2.2 Jatropha curcas

The principal components of the phenolic compounds identified in the J. curcas leaf extract (Table 2.3) in this study are flavonoids with a few procyanidin compounds. The concentration (mg L^{-1}) of vitexin-7-olate, isovitexin-7-olate, kaempferol-7-*O*-glucoside, apigenin-7-*O*-rutinoside, and apigenin-6-*C*-arabinosyl-8-*C*-arabinoside were not affected by the extraction solvents. The concentration of catechin, epicatechin, and procyanidins are affected by the extraction solvents, with the greater concentration at 100% methanol extraction, and the lowest at 70% methanol extraction. However, the difference in the concentration of catechin, tryptophan, and procyanidin dimer B1 is relatively small. Catechin and epicatechin are phytochemicals mostly reported in *Jatropha*, in recent times; they were identified in methanolic extracts of *J. curcas* and *J. cinerea* kernel meal (Vega-Arroy et al., 2017), catechin in the methanol extracts of the *J. curcas* leaf (Rejila et al., 2012), and catechin and its derivatives were reported in the *J. macrantha* stems (Benavides et al., 2006). Catechin and epicatechin have been reported to exhibit methane reducing properties (Vega-Arroy et al., 2017). Vitexin-7-olate and isovitexin-7-olate have been detected in the ethanolic extracts of the *J. curcas* leaves (Huang et al., 2014) and the crude leaf extracts of *J. gossipifolia* (Pilon et al., 2013). Other similar compounds that have been reported in *Jatropha*, include apigenin and its glycosides in *J. platyphylla* (Ambriz-Pérez et al., 2016) and kaempferol in *J. curcas* (Diwani et al., 2009), however, procyanidin dimers B1, B2, and procyanidin trimer C1 and C2 have not been reported in any species of the *Jatropha* plants. Most of the plant compounds identified in *J. curcas* have therapeutic activities. For example, apigenin has anticancer, anti-depressing, antidiabetic, and health-promoting properties with learning and memory-enhancing activities (Salehi et al., 2019), while procyanidins reportedly exhibited antioxidant, anticancer, anti-inflammatory, immunosuppressive, and antiallergy activities with protection against chronic diseases and metabolic disorders (Chen et al., 2022; Dasiman, 2022). Vitexin and isovitexin both possess antioxidant, anti-inflammatory, antidiabetic, anticancer, and neuroprotective properties (Azubuike-osu et al., 2021).

Table 2.3. Identification and the mean abundance of phytochemicals (mg L^{-1}) in *Jatropha curcas* extracts using 70%, 85%, and 100% aqueous-methanol.

RT (min)	Molecular Formula	Measured Mass (*m/z*)	Error *m/z* (ppm)	MS Fragment	UVmax (nm)	Compound	Classification of the Compounds	70%	85%	100%
7.73	$C_{45}H_{38}O_{18}$	865.2037	3.7	577,407,289,125	279	Procyanidin trimer C1	Flavonoid	10.54	30.67	34.64
8.98	$C_{11}H_{12}N_2O_2$	203.0821	−0.3	149	279	Tryptophan	Amino acid	43.36	46.64	49.83
10.4	$C_{30}H_{26}O_{12}$	577.1321	−4.9	407,289,125	279	Procyanidin dimer B	Flavonoid	96.38	104.34	109.56
10.78	$C_{30}H_{26}O_{12}$	577.1322	−4.9	407,289,125	279	Procyanidin dimer B2	Flavonoid	49.05	95.19	99.05
11.06	$C_{15}H_{14}O_6$	289.0712	−1.0	245,203,151,103	279	Catechin	Flavonoid	292.48	298.98	312.01
11.44	$C_{45}H_{38}O_{18}$	865.1969	−1.3	577,407,289,125	279	Procyanidin trimer C2	Flavonoid	56.84	72.59	74.58
13.16	$C_{15}H_{14}O_6$	289.0716	−1.4	245,203,151,103	279	Epicatechin	Flavonoid	101.19	125.93	143.53
15.47	$C_{26}H_{28}O_{14}$	563.1395	−2.1	443,383,353	271,335	Apigenin-6-*C*-arabinosyl-8-*C*-arabinoside	Flavonoid (*C*-glycosylated flavoinoid)	141.07	137.38	137.52
15.69	$C_{21}H_{20}O_{11}$	448.093	0.7	357,327,300	269,349	Kaempferol-7-*O*-glucoside	Flavonoid	7.89	6.31	6.37
16.91	$C_{21}H_{20}O_{10}$	431.0966	−1.9	341,311,283	268,335	Vitexin-7-olate	Flavonoid (*C*-glycosylated flavoinoid)	142.42	143.18	144.79
17.44	$C_{21}H_{20}O_{10}$	431.097	−2.1	341,311,283	271,335	Isovitexin-7-olate	Flavonoid (*C*-glycosylated flavoinoid)	188.89	187.05	190.77
19.81	$C_{27}H_{30}O_{14}$	577.1558	0.7	269	267,335	Apigenin-7-*O*-rutinoside	Flavonoid (*O*-glycosylated flavoinoid)	78.32	77.60	80.49

RT = retention time

2.3.2.3 Moringa oleifera

The phenolic profile of the *M. oleifera* leaf extracts shown in Table 2.4 consists of phenolic, flavonoid, and polyamine alkaloid compounds. These include chlorogenic acid, neochlorogenic acid, 3-*p*-coumaroylquinic acid, feruloylquinic acid, rutin, quercetins, kaempferols, and alkaloids. The concentrations of these metabolites in moringa leaf extracts were not affected by the extraction solvents, except the alkaloids. The principal compound present in the *M. oleifera* leaf extract observed in this study was the flavonoids, which validated a previous report (Amaglo et al., 2010). A number of similar quercetin and kaempferol derivatives and isomers, such as kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-acetyl-glucoside, and rutin, have been previously reported in *M. oleifera* leaves (Bennett et al., 2003; Amaglo et al., 2010), while some flavonoids, such as kaempferol and its derivatives (Rocchetti et al., 2019), have been detected. Chlorogenic acid (5-caffeoylquinic acid) and neochlorogenic acid (3-caffeoylquinic acid) have been reported to be present in *M. oleifera* leaf extracts (Amaglo et al., 2010; Nouman et al., 2016). Feruloylquinic acid, quercetin-3-*O*-acetyl-glucoside (quercetin-3-acetyl-glucoside), cinnamoylquinic acid (3-*p*-coumaroylquinic acid), kaempferol-3-*O*-glucoside, and quercetin-3-*O*-hexoside and their isomers have also been reported (Nouman et al., 2016). A phytochemical screening study by Onyekaba et al. (2013) revealed that flavonoids, terpenoids, phenolics, and alkaloids characterizing the *M. oleifera* leaf extracts possessed a marked antibacterial potential against *E. coli* and *Pseudomonas aeruginosa*, and this strong antibacterial activity probably needs to be explored in the methane studies. This study also confirms the presence of polyamine alkaloids in *M. oleifera*, which is also in tandem with the earlier findings of Leone et al. (2016). Alkaloids have muscle relaxant, antioxidant, anticancer, antimicrobial, and amoebicidal properties (Kaur et al., 2015). Kaempferol has been widely used in treating cancer, cardiovascular diseases, metabolic complications, and neurological disorders (Hussain et al., 2022). Quercetin is another flavonoid with a wide variety of biological activities, such as antioxidant, broad-spectrum antibacterial, and antiparasitic properties, cardiovascular protection, anti-immunosuppression treatment, and reduce the toxicity of mycotoxins (Yang et al., 2020), while rutin has been reported as a strong antioxidant with cancer preventive properties (Satari et al., 2021). Chlorogenic acid has liver and kidney protective properties, antioxidant, anti-tumor, antibacterial, and anti-inflammatory, as well as regulation of glucose and lipid metabolism (Wang et al., 2022).

Table 2.4. Identification and mean abundance of phytochemicals (mg L^{-1}) in *Moringa oleifera* extracts using 70%, 85%, and 100% aqueous-methanol

RT (min)	Molecular Formula	Measured Mass (*m/z*)	Error *m/z* (ppm)	MS Fragment	UVmax (nm)	Compound	Classification of the Compounds	70%	85%	100%
9.29	$C_{16}H_{18}O_9$	353.0864	−2.5	191,179,135	325	neochlorogenic acid	Phenolic acid	203.93	205.09	198.07
10.85	$C_{16}H_{18}O_8$	337.0922	−0.3	191,173,163	305	3-*p*-coumaroylquinic acid	Phenolic acid	108.21	110.85	107.67
11.72	$C_{16}H_{18}O_9$	353.088	−1.4	191	325	chlorogenic acid	Phenolic acid	30.44	47.73	19.94
12.11	$C_{17}H_{20}O_9$	367.1014	−1.9	193,134	323	Feruloylquinic aci	Phenolic acid	39.14	0	0
12.57	$C_{39}H_{19}NO_7$	612.1063	−1	97	344	Alkaloid	Alkaloid	142.73	265.40	272.63
13.99	$C_{27}H_{30}O_{15}$	593.1519	2.5	473,383,353,297	270,334	Kaempferol-3-*O*-rutinoside (isomer)	Flavonoid (*O*-glycosylated flavoinoid)	77.03	78.23	64.81
17.08	$C_{27}H_{30}O_{16}$	609.1464	−0.3	300,271,255	256,354	Rutin	Flavonoid	102.18	105.07	101.61
17.56	$C_{21}H_{20}O_{12}$	463.0873	−0.2	300,271,255	351	Quercetin-3-*O*-hexoside	Flavonoid (*O*-glycosylated flavoinoid)	94.24	94.18	89.58
18.36	$C_{23}H_{22}O_{13}$	505.0983	0.2	300,271,255	354	Quercetin-3-*O*-(6”- acetyl-glucoside	Flavonoid (*O*-glycosylated flavoinoid)	84.60	82.54	86.07
18.71	$C_{27}H_{30}O_{15}$	593.151	−1.9	285,271,255	265,348	Kaempferol-3-*O*-rutinoside	Flavonoid (*O*-glycosylated flavoinoid)	79.98	82.57	85.09
19.18	$C_{21}H_{20}O_{11}$	448.0924	−0.7	285,255,227	265,348	Kaempferol-3-*O*-glucoside	Flavonoid (*O*-glycosylated flavoinoid)	146.74	146.99	157.28
20.31	$C_{23}H_{22}O_{12}$	489.1047	3.5	285,255	265,348	Kaempferol-3-*O*-acetyl-glucoside	Flavonoid (*O*-glycosylated flavoinoid)	108.11	115.14	108.24

RT = retention time

2.3.2.4 Piper betle

The phenolic profile of the *P. betle* leaf extract is presented in Table 2.5. In contrast to *Jatropha curcas*, the principal components of the phenolic compounds in the *P. betle* leaf extract identified in this study are phenolic acids with a few flavonoids. Phenolic acids consist of coumaric acid and its derivatives and compounds of caffeic acid, while the flavonoids are rutin and apigenin-7,4′-diglucoside. The relative metabolite concentrations (mg L^{-1}) of 100% methanol extraction were low for coumaric acid, 3-*p*-coumaroylquinic acid, dihydrocaffeic acid, and dihydrocoumaric acid while 70% and 85% methanol extractions recorded higher concentrations, indicating that these metabolites were relatively more water-soluble. On a contrary, rutin, apigenin, and methoxy-4-vinylphenol are relatively less water-soluble and had a higher concentration at 100% methanol extraction. Similar compounds were noted by Lee et al. (2015), who investigated the antimicrobial, antifungal, and antioxidant activities of the *P. betle* leaf. Rutin and coumaric acid (chavibetol) have been reported to be present in chloroform extracts of the *P. betle* leaf (Yazdani et al., 2013), while Purba and Paengkoum (2019) identified compounds, such as rutin, coumaric acid, caffeic acid, and apigenin in different solvent extracts of *P. betle*. Caffeic acid is abundant in coffee and tea with good antioxidant, anti-inflammatory, anticancer, and neuroprotective properties (Alam et al., 2022), while coumaric acid possesses bioactivities, such as antioxidant, anti-inflammatory, analgesic, and antimicrobial, prevent liver damage and exhibit an amoebostatic activity against *Entamoeba histolytica* (Rubí et al., 2021).

Table 2.5. Identification and mean abundance of phytochemicals (mg L^{-1}) in the *Piper betle* extracts using 70%, 85%, and 100% aqueous methanol

RT (min)	Molecular Formula	Measured Mass (m/z)	Error m/z (ppm)	MS Fragment	UVmax (nm)	Compound	Classification of Compounds	70%	85%	100%
7.45	$C_{15}H_{18}O_{10}$	357.0825	0.8	345,195	326	Dihydrocaffeic acid 3′-*O*-β*D*-glucuronide	Phenolic acid	34.36	54.73	44.62
8.35	$C_{15}H_{18}O_{10}$	357.0829	2.0	195,129,75	325	Dihydrocaffeic acid 4′-*O*-β*D*-glucuronide	Phenolic acid	25.14	27.52	21.13
9.91	$C_{15}H_{18}O_9$	341.0859	0.6	195,163,119	312	Dihydro-*m*-coumaric acid 3′-*O*-β-*D*-glucuronide	Phenolic acid	98.09	101.57	88.52
10.39	$C_{15}H_{18}O_9$	341.0862	−3.2	195.163	308	Dihydro-*p*-coumaric acid 4′-*O*-β-*D*-glucuronide	Phenolic acid	52.65	50.67	30.85
13.72	$C_{16}H_{18}O_8$	337.0925	−0.3	191,173,163	305	3-*p*-coumaroylquinic acid	Phenolic acid	96.74	106.49	91.72
14.05	$C_9H_8O_3$	163.04	−0.5	163	339	Coumaric acid	Phenolic acid	155.02	190.66	12.17
15.03	$C_{16}H_{18}O_8$	337.0929	1.8	191,173,163	305	3-*p*-coumaroylquinic	Phenolic acid	128.56	129.21	105.34
15.51	$C_{27}H_{30}O_{16}$	609.1448	1.8	489,429,357, 327,309	348	Rutin	Flavonoid	73.76	98.27	103.70
16.42	$C_{27}H_{30}O_{15}$	593.1487	−1.7	413,293	268,334	Apigenin-7,4′-diglucoside	Flavonoid (*O*-glycosylated flavoinoid)	83.36	118.99	128.32
22.12	$C_9H_{10}O_2$	149.0603				2-Methoxy-4-vinylphenol	Phenolic acid	675.93	775.91	814.11

RT = retention time.

2.3.3 In vitro organic matter fermentation

This section evaluated the antimethanogenic potentials of three different combinations (70, 85, and 100%) of two extractive solvents (CH_3OH and H_2O) of *A. vera, J. curcas*, *M. oleifera*, and *P. betle* on *Eragrostis curvula* hay. The substrate, *E. curvula* hay, used in this study had 92% DM while crude protein, ash, and ether extract, respectively, contained 5.12, 9.1, and 1.3% of DM. The NDF, ADF, and ADL contents were 75.5, 44.5, and 8.1% of DM, respectively. The high content of NDF, ADF, and ADL in the feed causes an increase in the amount of methane (CH_4) formed in the rumen fermentation. The CH_4 emission, total gas production, and organic matter digestibility of *E. curvula* hay are presented in Table 2.6, while the principal component analysis (PCA) of the CH_4 emission, total gas production, and organic matter digestibility of *E. curvula* hay fermented with crude plant extracts of *A. vera, J. curcas*, *M. oleifera*, and *P. betle* are presented in Figure 2.1 while the correlation results are illustrated in Table 2.7. The plant extracts reduced ($p < 0.05$) *in vitro* the methane production in the tested hay. *Aloe vera, Jatropha curcas, Moringa oleifera*, and *Piper betle* reduced the methane emission by 6.37–7.55%, 8.02–11.56%, 12.26–12.97, and 5.66–7.78, respectively, compared to the control treatment. However, the extraction solvents did not affect the antimethanogenic efficacy, gas production, and organic matter digestibility of the crude plant extracts. The aqueous-methanolic extractive solvents were observed to increase ($p < 0.05$) the crude extract yields of the test leaves, compared to the pure methanolic extraction (Table 2.1); however, these yields did not affect the methane-reducing potentials of the leaf extracts. This may be due to the little variation in the physical and chemical activities of the two combined solvents (CH_3OH and H_2O). The use of different solvents to obtain extracts in plants increased the variation in bioactive compounds in the crude extracts, compared to the proportionate mixing of the two solvents (Math et al., 2011). According to Sabandar et al. (2013), the variation in the antimicrobial activity of the *J. unicostata* extract was higher between the solvents chloroform and methanol, compared to their proportionate combinations. The little intra-variation in the solvent properties, the little/no difference in the concentration of the methane-reducing metabolites, and the patterns in which the metabolites in the crude plant extracts affect fermentation could probably be responsible for the non-significant variation in the methane production between extraction solvents. For example, in the PCAs of *A. vera* (Figure 2.1A), aloin A, aloin B, and kaempferol were metabolites associated with the CH_4 reduction. The 70% methanol extraction of *Aloe vera* had a higher concentration of kaempferol but a lower concentration of aloin A and aloin B, while the 85%

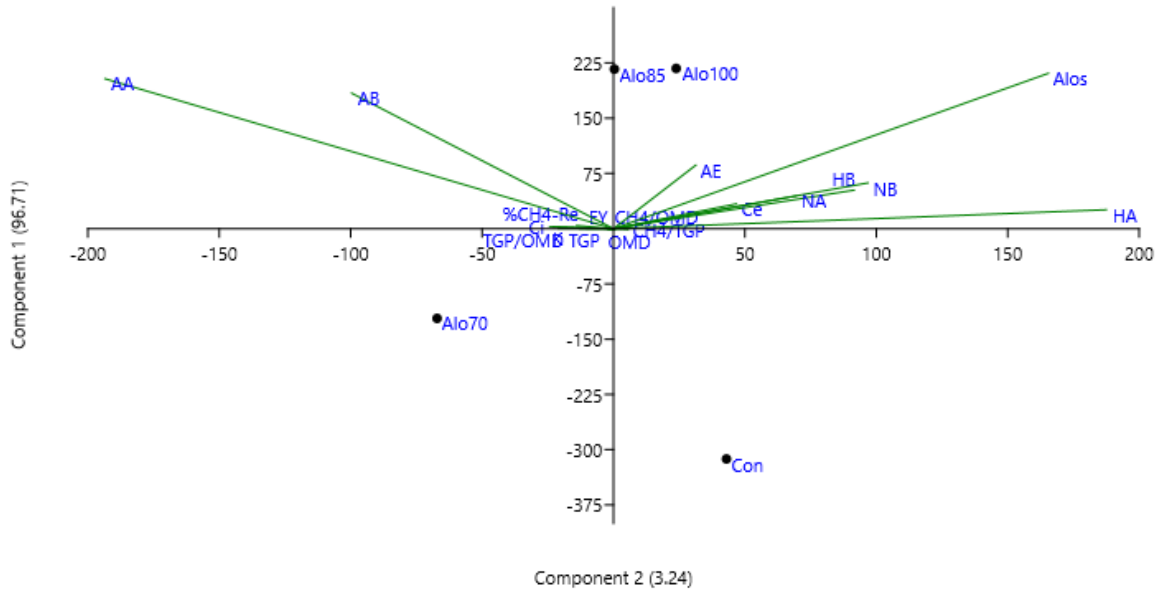
and 100% methanol extractions had a higher concentration of aloin A and aloin B, but lower kaempferol (Table 2.2). In *J. curcas* (Figure 2.1B), catechin, apigenin, kaempferol, vitexin-7-olate, and isovitexin-7-olate were more associated with methane reduction in the 70% methanol extraction, while epicatechin, tryptophan, procyanidin dimer B1, and procyanidin trimer C2 were more associated with methane reduction in 85% and 100% methanol extraction. Furthermore, the differences in the concentrations (Table 2.3) of catechin, vitexin-7-olate, isovitexin-7olate, apigenin, kaempferol, and procyanidin dimer B1 in the crude plant extracts of *J. curcas*, were very small and not affected by the extraction solvents. In *M. oleifera* (Figure 2.1C), kaempferol, quercetin, rutin, neochlorogenic acid, and 3-*p*-coumaroylquinic acid had more associative effects for the CH_4 reduction in the 85% and 100% methanol extractions while alkaloid was more associated with the CH_4 reduction in the 70% methanol extraction. In addition, the percentage difference in the concentrations of these antimethagenic metabolites (Table 2.4) in the crude plant extracts was little and not influenced by the extraction solvents. In *P. betle* (Figure 2.1D), apigenin, rutin, and 2-metoxy-4-vinylphenol were more associated with the CH_4 reduction in 100% methanol extraction while *3-p*-coumaroylquinic acid, dihydroxycaffeic acid, and dihydroxycoumaric acid were more associated with CH_4 mitigation in the 70% and 85% methanol extractions. While the concentrations of rutin, apigenin-7,4′-diglucoside, and 2-metoxy-4-vinylphenol were low in the crude plant extract with the 70% methanol extraction, this could probably be balanced with other methane-reducing metabolites which were relatively higher in concentration. These associations of the metabolites with methane inhibition are further corroborated by the correlation results (Table 2.7). The primary objective of identifying the metabolites in the crude plants' extracts is to establish or validate their antimethanogenic potentials. Some of the metabolites of *A. vera*, *J. curcas*, *M. oleifera*, and *P. betle* extracts identified in this study have been reported to possess antimicrobial activities with good antimethanogenic properties. The presence of alkaloids, flavonoids, and phenols in plant extracts had been attributable to reducing the enteric methane in ruminants (Agidigbi et al., 2014; Akanmu et al., 2018). Furthermore, alkaloids, due to their bitter taste (Gautam et al., 2007; Ojiako, 2014) in moringa, could create an undesirable condition for some ruminal microbes. Flavonoids have been evaluated for rumen methanogenesis (Patra & Saxena, 2010). Oskoueian et al. (2013) reported that the inclusion of flavone, myricetin, naringin, rutin, quercetin, or kaempferol decreased the *in vitro* methane emission by 5 to 9 mL g^{-1} DM

while catechin reduced methane production both *in vitro* (Becker et al., 2014) and *in vivo* (Aemiro et al., 2016). All the flavonoids, when fed at 0.2 g kg^{-1} DM, noticeably decreased the relative abundances of the hydrogenotrophic methanogens. Flavonoids have been reported to directly suppress methanogens (Oskoueian et al., 2013; Seradj et al., 2014) and also likely act as H_2 sinks via the cleavage of the ring structures (e.g., catechin) and the reductive dihydroxylation (Becker et al., 2014). The anthraquinones and flavonoids (Kedarnath et al., 2012) in *A. vera* had been reported to exhibit strong antimicrobial activities against bacteria and fungi, while an early study revealed that acetone and methanolic extraction of aloe (Sirohi et al., 2009) and the pure methanolic extraction of aloe (Akanmu & Hassen, 2018), reduced *in vitro* the methane production. The aqueous extraction of *P. betle* showed the presence of alkaloids, phenolic compounds, and alcoholic compounds with a good antimicrobial function (Pin et al., 2010) and effective inhibitory action against microorganisms (Dwivedi et al., 2014). According to Santra et al. (2012), the ethanolic extracts of *J. gossipifolia* reduced *in vitro* methane production by 31% and also inhibited the growth of the rumen protozoal population, due to the presence of phenolic compounds in the extracts. The antimethanogenic property of 3-*p*-coumaroylquinic acid, dihydroxycaffeic acid, and dihydroxycoumaric acid had been previously reported in compounds with similar chemical activities, such as cinnamic acid, caffeic acid, and coumaric acid, respectively (Jin et al., 2020). The use of 2mM of cinnamic acid, caffeic acid, and coumaric acid decreased the *in vitro* methane production without reducing the organic matter digestion (Jin et al., 2020), while the use of caffeic acid reduced the *in vitro* methane production (Jayanegara, 2009). The reduction in enteric methane ($p < 0.05$) observed in this study for all of the treatment plant extracts is consistent with previous findings (Akanmu & Hassen, 2018), except for *P. betle*, which increased methane in a previous study (Akanmu & Hassen, 2018) against the reduction ($p < 0.05$), observed in this study. Plant extracts have a complex blend of bioactive components with many variations in their composition, due to biological factors, production techniques, and storage conditions (Ojiako, 2014), while parameters that affect the efficacy of the plant extracts are genetic variations of the plant, the age of the plant, dosage, extraction technique, harvest time, and compatibility with other ingredients (Gautam et al., 2007). All these conditions will probably impose variations on the parameters of interest.

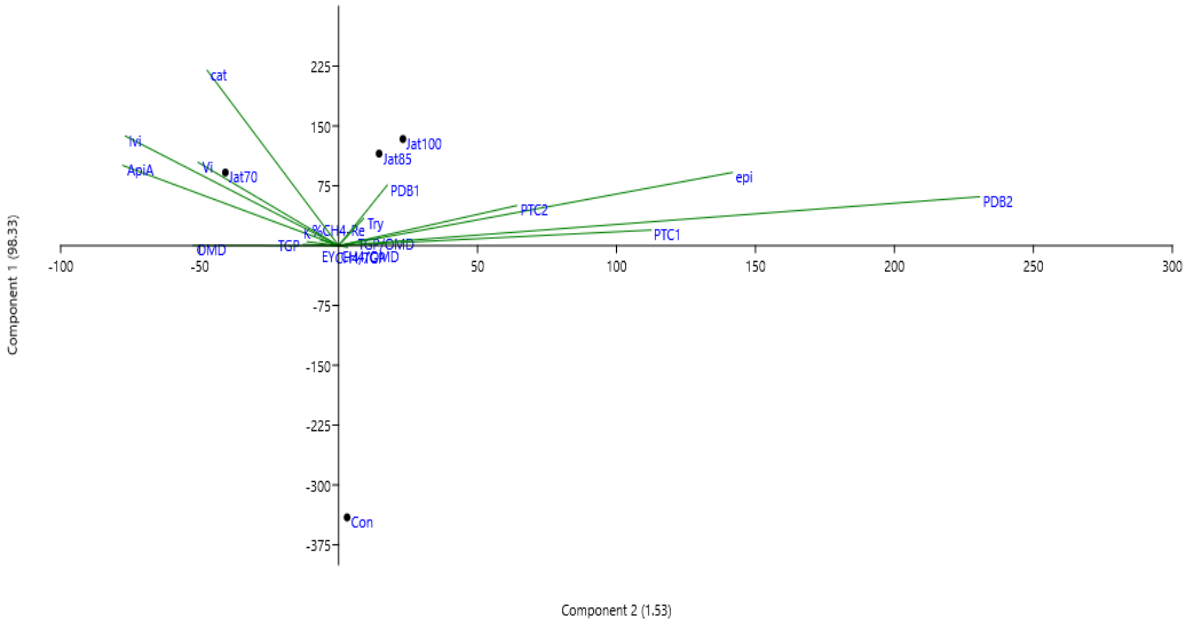
Table 2.6. Methane, TGP, IVOMD and their relative estimation on Eragrostis hay, as affected by the plant extract yields of three different % solvent CH_3OH:H_2O (70, 85, and 100%) combinations

Extract (50 mg kg^{-1} DM)	CH4 (mL g^{-1} DM)	TGP mL g^{-1} DM	CH4/TGP ($\times 10^{-3}$)	IVOMD (g kg^{-1} DM)	TGP/IVOMD (mL kg^{-1} DM)	CH4/IVOMD (mL kg^{-1} DM)
Control	4.24^{A}	166.50^{B}	25.64^{A}	608.41	273.97	6.96^{A}
Aloe vera 70%	3.97^{B}	170.49^{A}	23.39^{B}	607.40	280.91	6.54^{B}
Aloe vera 85%	3.92^{B}	167.73^{AB}	23.49^{B}	608.43	275.44	6.44^{B}
Aloe vera 100%	3.94^{B}	167.72^{AB}	23.68^{B}	604.08	277.10	6.53^{B}
SEM	0.05	1.10	0.41	10.39	3.82	0.12
p-value	0.01	0.04	0.01	0.97	0.35	0.04
Control	4.24^{A}	166.50	25.64^{A}	608.41	273.97	6.96^{A}
Jatropha curcas 70%	3.79^{B}	169.93	22.52^{B}	616.17	275.20	6.15^{B}
Jatropha curcas 85%	3.90^{B}	172.30	22.78^{B}	604.03	284.89	6.45^{B}
Jatropha curcas 100%	3.75^{B}	164.91	22.95^{B}	608.37	271.01	6.16^{B}
SEM	0.14	2.78	0.45	9.79	8.32	0.13
p-value	0.04	0.13	0.003	0.63	0.41	0.04
Control	4.24^{A}	166.50	25.64^{A}	608.41	273.97	6.96^{A}
Moringa oleifera 70%	3.70^{B}	165.46	22.69^{B}	608.67	271.95	6.09^{B}
Moringa oleifera 85%	3.71^{B}	165.23	22.73^{B}	613.33	269.55	6.07^{B}
*Moringa oleifera*100%	3.69^{B}	165.65	22.55^{B}	613.00	270.02	6.02^{B}
SEM	0.08	2.42	0.38	6.40	2.41	0.12
p-value	0.002	0.95	0.001	0.87	0.35	0.001
Control	4.24^{A}	166.50	25.64^{A}	608.41	273.97	6.96^{A}
Piper betle 70%	3.97^{B}	168.42	23.65^{B}	614.72	274.19	6.47^{B}
Piper betle 85%	4.00^{B}	171.89	23.41^{B}	609.29	282.45	6.57^{B}
Piper betle 100%	3.91^{B}	166.36	23.66^{B}	613.97	270.92	6.37^{B}
SEM	0.06	2.18	0.43	6.08	3.87	0.09
p-value	0.03	0.15	0.01	0.74	0.09	0.02

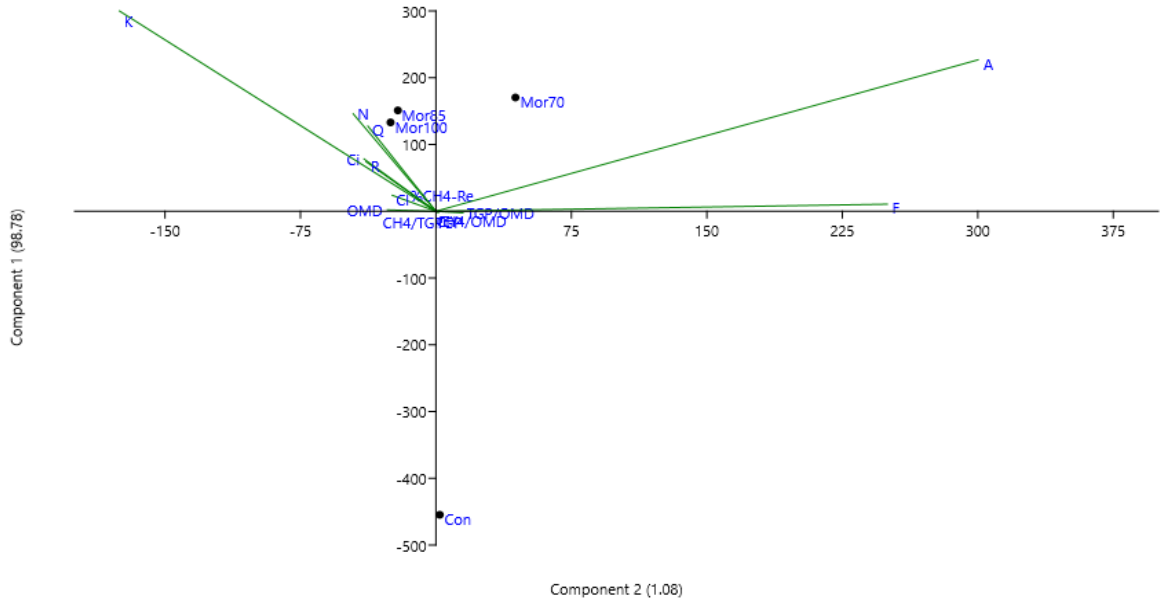
SEM: standard error of mean, CH_4: methane TGP: total gas production, IVOMD: in vitro organic matter digestibility. A-C: The letters compare the means of extraction solvents for each plant species. The means with different letters within the column are significantly different ($p < 0.05$).


AA
AB
Alo85
Alo100
Alos
AE
HB
NB
NA
Ce
HA
%CH4-Re
EY
CH4/OMD
TGP/OMD
TGP
OMD
Alo70
Con
Component 1 (96.71)
Component 2 (3.24)

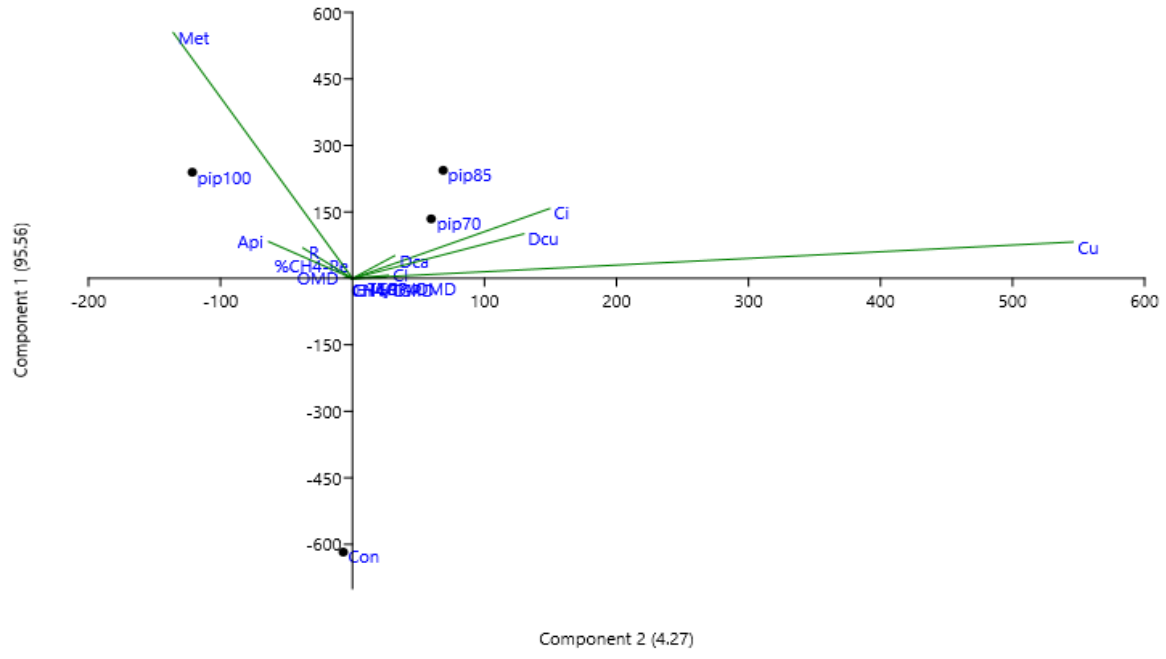
(A)


cat
Ivi
ApiA
Yi
Jat70
Jat100
Jat85
PDB1
PTC2
epi
PDB2
PTC1
Try
TGP
OMD
Con
Component 1 (98.33)
Component 2 (1.53)

(B)



(**C**)



(**D**)

Figure 2.1. Principal component analysis plot 1 vs. plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with three different aqueous-methanol (70, 85, and 100%) extractions of *Aloe vera* (A), *Jatropha curcas* (B), *Moringa oleifera* (C), and *Piper betle* (D) leaf extracts (letters in the figures interpreted in Table 2.7)

Table 2.7. Correlation showing the effects of extraction solvents, extract yields and phytochemicals of plant extracts on CH_4 emission, TGP, and IVOMD of *Eragrostis curvula* hay

Variable/metabolites	%CH_4-Re	TGP	CH_4/TGP	OMD	TGP/OMD	CH_4/OMD	EY
%CH_4-Re (percentage methane reduction)	1						
TGP (total gas production)	.544	1					
CH_4/TGP	-.980**	-.698	1				
OMD (organic matter digestibility)	-.385	-.071	.344	1			
TGP/OMD	.537	.973*	-.686	-.283	1		
CH_4/OMD	-.997**	-.538	.977*	.316	-.514	1	
Extract Yield (EY)	-.750	.997*	-.815	.368	.927	.506	1
***Aloe vera* (Alo)**							
Aloin A (AA)	.930*	.205	-.841	-.487	.226	-.924*	-.992*
Aloin B (AB)	.900*	.128	-.798	-.477	.149	-.895	-.990*
Aloe emodin (AE)	.815	-.041	-.685	-.461	-.018	-.811	-.990*
Aloesin (Alos)	.771	-.104	-.633	-.516	-.063	-.762	-.999*
Caffeoyl ester of aloesin (Ce)	.698	-.213	-.546	-.494	-.170	-.691	-.999*
3-*p*-coumaroylquinic acid (Ci)	.769	.681	-.816	.262	.538	-.807	.470
Hydroxyaloin A (HA)	.693	-.987	.861	-.444	-.893	-.433	-.997*
Hydroxyaloin B (HB)	.654	-.246	-.499	-.591	-.174	-.637	-.995*
Kaempferol hexoside	.901*	.845	-.967*	-.193	.805	-.904*	.943
Nataloin A (NA)	.677	-.239	-.521	-.502	-.193	-.668	-.999*
Nataloin B (NB)	.678	-.237	-.522	-.509	-.189	-.669	1.00**
***Jatropha curcas* (Jat)**							
Apigenin (Api)	.960*	.386	-.995**	.133	.249	-.935*	.788
Catechin (Ca)	.964*	.342	-.985**	.085	.230	-.932*	-.996*
Epicatechin (Epi)	.935*	.220	-.923*	-.084	.193	-.883	-.980
Kaempferol hexoside	.955*	.406	-.994**	.304	.198	-.952*	.788
Procyanidin dimer B1 (PDB1)	.957*	.325	-.975*	.035	.235	-.920*	-.975
Procyanidin dimer B2 (PDB2)	.809	.211	-.810	-.345	.295	-.729	-.847
Procyanidin trimer C1 (PTC1)	.716	.086	-.687	-.470	.239	-.623	-.889
Procyanidin trimer C2 (PTC2)	.923*	.309	-.940*	-.095	.275	-.870	-.864
Tryptophan (Try)	.960*	.311	-.973*	.038	.222	-.923*	-.994*
Vitexin-7-olate (V)	.960*	.372	-.991**	.104	.248	-.931*	-.995*
Isovitexi-7-olate (Ivi)	.963*	.367	-.992**	.116	.240	-.935*	-.594
***Moringa oleifera* (Mor)**							
Alkaloids (A)	.979*	-.943*	-.977*	.458	-.756	-.971*	.997*
chlorogenic acid (Cl)	.793	-.956*	-.791	.669	-.797	-.795	.140
3-*p*-coumaroylquinic acid (Ci)	.998**	-.958*	-.997**	.612	-.864	-.997**	-.083
Feruloylquinic acid (F)	.325	-.301	-.318	-.530	.191	-.288	.961
Kaempferol (K)	.998**	-.955*	-.998**	.616	-.867	-.998**	-.534
neochlorogenic acid (N)	.997**	-.959*	-.997**	.597	-.854	-.996**	.605
Quercetin (Q)	.998**	-.952*	-.998**	.590	-.851	-.997**	.999*
Rutin (R)	.997**	-.959*	-.997**	.613	-.865	-.997**	-.088

Table 2.7 Cont'd. Correlation showing the effects of extraction solvents, extract yields and phytochemicals of plant extracts on CH_4 emission, TGP, and IVOMD of *Eragrostis curvula* hay.

***Piper betle* (Pip)**							
Apigenin	.944*	.495	-.948*	.516	.195	-.916*	-.911
chlorogenic acid (Cl)	.629	.831	-.842	.314	.617	-.592	.854
3-*p*-coumaroylquinic acid (Ci)	.918*	.626	-.995**	.588	.315	-.893	.772
Coumaric acid (Cu)	.389	.894	-.668	.078	.767	-.344	.817
Dihydrocaffeic acid (Dca)	.893	.692	-.986**	.440	.408	-.855	-.170
Dihydrocoumaric acid (Dcu)	.892	.646	-.985**	.581	.340	-.869	.894
Methoxy-4-vinylphenol (Met)	.972*	.506	-.986**	.612	.184	-.951*	-.939
Rutin (R)	.954*	.510	-.966*	.543	.203	-.927*	-.900

*correlated at $p<0.05$, **correlated at $p<0.01$

2.4 Conclusions

The crude extract yields of the four plant leaves used in this study increased with an increase in the amount of distilled water in the extraction solvents, as a replacement for methanol. *M. oleifera* crude extracts yields (g/10 g) increased from 3.24 to 3.92, *A. vera,* (2.35 to 3.11) *J. curcas* (1.77 to 2.26), *and P. betle* (2.42 to 3.53). However, the identified metabolites showed differing degrees of solubility unique to their plant leaves while most of the metabolite yields were unaffected by the extraction solvents. Although the *A. vera, J. curcas, M. oleifera,* and *P. betle* leaf extracts reduced the *in vitro* methane gas emission at the dosage of 50 mg kg^{-1} DM of *E. curvula* hay, decreasing the methane emission by 6.37–7.55%, 8.02–11.56%, 12.26–12.97, and 5.66–7.78, respectively, compared to the control treatment, the extraction solvents did not affect their methane reducing potential, total gas production, and organic matter digestibility. Furthermore, aloin A, aloin B, and kaempferol (in *A. vera*), apigenin, catechin, epicatechin, kaempferol, tryptophan, procyanidins, vitexin-7-olate, and isovitexin-7olate (in *J. curcas*), alkaloid, kaempferol, quercetin, rutin and neochlorogenic acid (in *M. oleifera)* and apigenin-7,4′-diglucoside, 3-*p*-coumaroylquinic acid, rutin, 2-methoxy-4-vinylphenol, dihydrocaffeic acid, and dihydrocoumaric acid (in *P. betle*) exhibited the methane reducing potential and hence, are promising plant secondary metabolites for methane inhibition.

CHAPTER THREE

Characterization and *in vitro* methane inhibition of *Aloe vera* and *Moringa oleifera* plant extracts encapsulated with alginate or alginate-chitosan wall materials

Abstract

The methane-reducing potential of *Aloe vera* and *Moringa oleifera* extracts still necessitates further investigations due to the bitter taste of the extracts and the loss of efficacy over time. Encapsulation of plant bioactive compounds with an inert material will assist to reduce the bitter effects of plants' phytochemicals like alkaloids, and flavonoids, to improve particle density and particle distribution and to facilitate better delivery of bioactive compounds in the rumen medium. Alginate is a good encapsulant (or a wall material) commonly used mainly for encapsulation due to its biocompatibility and low density, among other factors. This study was conducted in two phases. In the first phase of this study, *A. vera* and *M. oleifera* plant extracts were encapsulated with alginate or alginate and chitosan and evaluated in terms of microscopic morphological characteristics and particle density. Whereas in the second phase, using *in vitro* gas production studies the potential of *A. vera* and *M. oleifera* plant extracts encapsulated or non-encapsulated products were evaluated as dietary additives to inhibit *in vitro* methane production from *Eragrostis curvula* hay substrate. In the first study, electron microscope scanning of the freeze-dried alginate or alginate encapsulated plant extracts of *A. vera and M. oleifera* revealed morphologically spongy and skin-like appearances while the electron microscope of chitosan revealed scatter particle distribution of different sizes with irregular shape. A change in particle density was recorded for the encapsulated plant extract products compared to the non-encapsulated product due to the inclusion of alginate or alginate-chitosan wall material. The particle density for the encapsulated products range from 4.94 g/10 mL (recorded in alginate) to 8.71 g/10 mL (recorded in chitosan). In the *in vitro* gas production study, the inclusion of both the encapsulated and non-encapsulated plant extract of *A. vera* or *M. oleifera* additives significantly ($p < 0.05$) reduced methane emissions compared to the control group. No adverse effect was observed due to the inclusion of the wall materials in terms of *in vitro* organic matter digestibility and total gas production. However, the encapsulated *A vera* plant extracts resulted in a higher methane inhibition of 30.72% (for alginate-extract encapsulated) and 23.09% (for alginate-extract-chitosan encapsulated) against 17.55% reduction recorded for the raw extracts. Similarly, the

encapsulated *M. oleifera* plant extracts inhibited methane by 30.02% (alginate-extract encapsulated) and 30.48% (alginate-extract-chitosan encapsulated) against 19.4% recorded for in *M. oleifera* raw extracts. It is observed that alginate and chitosan also had methane-reducing effects when tested as additives without the plant extracts. However, the differences in particle densities of the micro-particles from the encapsulated and non-encapsulated plant extract additives did not affect their methane inhibition. Thus the wall materials (alginate or alginate-chitosan) generally improved the *in vitro* methane inhibition of encapsulated plant extracts mainly by intensifying the inhibition of methane directly and indirectly by improving the distribution of bioactive compounds in the rumen for effective delivery to the target rumen methanogens. Bioactive compounds like alkaloid, coumaric acid, kaempferol, neochlorogenic acid, quercetin, and rutin (in *M. oleifera*), and aloin A, aloin B, aloesin and aloe-emodin (in *A. vera*) were associated with the reduction in methane emission. *In vivo* animal evaluation studies are recommended to validate the methane inhibition efficacy of the encapsulated plant extract products and their effect on animal performance and the quality of the target animal product.

Keywords: *A. vera, M. oleifera*, encapsulation, morphology, density, phytochemicals, *in vitro*

3.1 Introduction

Reducing enteric methane emission in ruminants presents two major merits: firstly, it provides a climate-smart atmosphere (Lassey, 2008; Benchaar et al., 2011), and secondly, increasing the efficiency of production in livestock systems, especially those that include ruminants. The conditions within the rumen are favorable for hydrogenotrophic methanogens (Methanobrevibacter, Methanomicrobium, Methanosphaera, Methanosarcina, Methanobacterium, and rumen cluster C) that reduce CO_2 with metabolic H_2 produced (87–90% in the rumen) during anaerobic fermentation of glucose, which results in the synthesis of CH_4 (Hill et al., 2016; Mirzaei-Aghsaghali et al., 2016). Over the last decade, strategies have been put in place to intensively reduce methane emissions by several research groups. These strategies include the inoculation of exogenous bacterial strains (Lee et al., 2013), vaccine development, biological control, prebiotics, probiotics (Takahashi, 2014), defaunation (protozoa or methanogens), and the identification of natural compounds in plants used as feed

additives (Patra, 2013; Akanmu & Hassen, 2018). Dietary manipulation in ruminants is important because the ruminant's diet has a strong influence on methane emission (Wallace et al., 2014; Broucek, 2018); therefore, in the last decade, more intensive research on natural compounds for livestock has increased intending to reduce rumen CH_4 without negatively affecting rumen fermentation and energy utilization mainly on *in vitro* trials (Bodas et al., 2008; Patra et al., 2011). The conditions for using natural compounds are that they should be safe for use in animals and humans, be effective in the long-term with different feedstuffs, have a low cost to mitigate emissions in ruminants, and increase productivity in the livestock system (Jiménez-Ocampo et al., 2019).

The efficacy of plants' extracts could be enhanced by the use of wall materials which probably provide multiple benefits such as protective roles to bioactive compounds in the extracts when used as wall material, overcoming bitter test of some of the bioactive compounds, slow release of the bioactive compounds, ease of administration as part of the feed mixing and in certain cases an additional benefit of methane inhibition due to the inherent characteristics of the wall material. This protection has been used to enhance action duration and reportedly provided a controlled release for tannin-based additives (Adejoro & Hassen, 2018). Recent studies have suggested that encapsulation techniques/use of wall materials may help mask or reduce the negative bitter effects associated with plants' phytochemicals like tannins in the feed or food industry (Adejoro & Hassen, 2018). This may be the reason why Akanmu & Hassen (2018) recommended that plant extracts such as *Aloe vera* and *Moringa oleifera* be encapsulated for ease of administration by farmers to ruminant animals. This may help to avoid the stress associated with drenching ruminants while using liquid extracts to reduce methane emissions.

Alginate (or hydrogel) is a wall material widely used in the food and drug industries. It has numerous benefits as it possesses attributes such as bio-compatibility, low density, ease of production, non-toxicity, and chemical and thermal stability (Belščak-Cvitanović et al., 2011; Rijo et al., 2014; Asbahani et al., 2015; Hecht et al., 2016). Heavier particle-density compounds are likely to settle down in the rumen fluid and possibly have limited mobility, leading to uneven distribution of metabolite (Golchin-Gelehdooni, 2011; Moyo & Nsahlai, 2018). The low density of alginate if used as wall material could probably help to increase particle mobility and better delivery of metabolites to the target sites in the rumen. Chitosan is a non-toxic material with antimicrobial activity and also possesses antimethanogenic

properties (Jiménez-Ocampo et al., 2019). It is reported to have a moderate to strong enteric methane mitigating potential (Goiri et al., 2010) and decreased rumen protozoa population (Jiménez-Ocampo et al., 2019). While crude extracts from a medicinal plant may likely target rumen methanogens (Islam et al., 2019) and/or possibly ciliate protozoa, chitosan inhibition effects are more related to reducing protozoa associated with methanogens (Jiménez-Ocampo et al., 2019), and/or by acting as an alternative H_2 sink (Goiri et al., 2010), as they redirect the utilization of H_2 to biohydrogenation process thereby limiting its supply for methanogenesis. Also, chitosan has been described as a good delivery material for food and drug (Li et al., 2018) while the polyphenolic antioxidant of medicinal plants has been enhanced with alginate-chitosan encapsulation (Belščak-Cvitanović et al., 2011). Hence, the combination of medicinal plant extracts and chitosan are most likely will have an additive effect or a synergy in the inhibition of enteric methane production.

In some previous studies (Akanmu & Hassen, 2018; Ibrahim et al., 2022), the methanolic extraction of *A. vera* and *M. oleifera* leaves exhibited good antimethanogenic properties but the use of wall material to help increase particle mobility and longer residence time in the rumen medium for better delivery of metabolites to the target rumen microbes becomes an area of research interest that received little attention. Therefore, the current study was conducted to evaluate the morphological attributes, particle density, and methane inhibition potential of chitosan, and alginate-chitosan wall material used to encapsulate *A. vera* and *M. oleifera* extracts. It is hypothesized that the particle density and methane inhibition of alginate or alginate-chitosan encapsulated *A. vera* and *M. oleifera* extracts are influenced by using alginate or alginate-chitosan as wall material.

3.2 Materials and Methods

3.2.1 Study site and research ethics

The study was carried out in the Nutrilab of the Department of Animal Sciences, University of Pretoria, Pretoria South Africa, following its ethical approval (NAS336/2019).

3.2.2 Plant collection, extraction procedure, phytochemical identification, and quantification

Fully matured *A. vera* (Taxon A) leaves were harvested and imported from Nigeria with the permit number (P0095290) while the *M. oleifera* (A11NA) leaves were collected fresh from multiple plants at a farm in Pretoria North, South Africa. The leaves were freeze-dried for 96 h and stored in plastic bags. The *A. vera* and *M. oleifera* leaf extracts were obtained with

aqueous methanol using the maceration method (Ibrahim et al., 2022). Dried samples of each plant leaf were milled through a 1 mm sieve and extracted by dissolving 10 g of milled dried leaf material into a 300 mL extraction bottle containing 200 mL (1:20 w/v) aqueous methanol (30%H_2O:70%CH_3OH). The bottles were placed into an Incoshake and agitated at 130 rpm and 20°C for 96 h. The extracts were filtered by squeezing through a sieve with a 150 µm aperture. The filtrates were placed in a fume cubicle for 24 h until partially dried. The semi-dried extracts were freeze-dried for 72 h and stored in plastic bottles at 4°C until further use. The phytochemical identification and quantification of metabolites used in the computation of principal component analysis (Figure 5) were obtained following approved procedures (Tsugawa et al., 2015; Yu et al., 2020). The metabolites data used to compute the principal component analysis (PCA) in this study was obtained from a parallel study that used the same extraction and was reported in a previously published study (Ibrahim et al., 2022).

3.2.3 *Chemicals, preparation of alginate and freeze-dried encapsulated plant extracts*

Low-viscosity sodium alginate and Chitosan (molecular weight: 100,000–300,000) were obtained from Acros organic (New Jersey, USA). Alginate-extract beads were produced following the methods described by Rijo et al. (2014) with some modifications. Briefly, 1 g of sodium alginate powder was dissolved in 100 mL of distilled water contained in a 300 mL beaker and properly homogenised. 0.4 g of dried chitosan was added to the mixture and further homogenised. 1 g of dried crude extracts was added to the mixture and thoroughly mixed to obtain an alginate-chitosan-extract (at a ratio of 2.5:1:2.5) solution. The formed encapsulated mixtures were thoroughly mixed. Another encapsulated mixture was also produced with sodium alginate and plant extracts at a ratio of 1:1. The two forms of encapsulated mixtures produced (alginate-extracts and alginate-chitosan-extract mixtures) were frozen at − 80°C for 12 h before freeze-drying (− 50 °C at a pressure of 1.1 Pa) for 4 days (GAMMA Martin Christ, GmbH, Osterode am Harz, Germany). The freeze-dried encapsulates were properly stored in sterile sample bottles at 4°C pending further analysis.

3.2.4 Morphological characterisation of alginate-chitosan encapsulated A. vera and M. oleifera micro-particles using scanning electron microscopy

The morphology of micro-particles was evaluated by scanning with an electron microscope (Aylor et al., 2009). Freeze-dried beads each of empty alginate, alginate-extract, and alginate-chitosan-extract of *A. vera* and *M. oleifera* plants were mounted on a slide with double-sided

tape and coated with carbon before sputtering with gold under an argon atmosphere using an Emitech K950X (Ashford, UK) vacuum carbon evaporator. The structure of gold-sputtered encapsulates was then viewed for their micro-particles under a Field Emission Scanning Electron Microscope (FE-SEM), ZEISS ULTRA PLUS (JEOL, Tokyo, Japan).

3.2.5 Particle density determination for alginate, chitosan, raw, and encapsulated plant extracts

The weight of an empty 2 ml vial was measured and recorded. Later, distilled water was filled into the 2 ml vial and the weight was also measured and recorded as a standard reference to evaluate the density of each variable. The milled powder of each material was carefully filled into the 2 ml space vial and the weight was measured. The formula d=(m-a)/(v-a) was used to estimate the particle density of each variable. Where 'd' is the particle density, 'a' is the weight of the empty vial, 'm' is the weight of each variable plus the empty vial and 'v' is the weight of distilled water representing the space volume of the vial plus the weight of the empty vial. Measurements were carried out five different times to get an average value and recorded in milligrams. The obtained values were then converted to g 10 mL^{-1}. The results are presented in Figure 3.2.

3.2.6 Buffer mineral solution, rumen fluid collection, and in vitro gas study

In vitro gas production studies were carried out in line with the procedure of Menke et al. (1979), with the modifications detailed (Adejoro & Hassen, 2018). The buffer mineral solution was prepared and preserved in a water bath at 39°C and continuously purged with CO_2 until the solution turned colourless. Rumen fluid was taken from three rumen-cannulated Holstein breeds of cattle fed Lucerne hay *(Medicago sativa) ad libitum*. 40 mL of prepared solution of rumen fluid was used to incubate 400 mg of *Eragrostis curvula hay* in a 150 mL serum bottle in triplicates and four different runs (biological replicates) were conducted. Gas pressure was taken at 2, 4, 8, 12, 24, and 48 h after the commencement of incubation, while gas samples were taken inside Hamilton syringes for the analysis of methane concentration. Three blanks were included to correct the methane produced from the inoculum in each run. Methane concentration was analysed with gas chromatography (8610C BTU Gas Analyser GC System; SRI Instruments GmbH, Bad Honnef, Germany). The GC was pre-equipped with a solenoid column, packed with silica gel and a flame ionization detector (FID). Methane concentration values were related to the total gas production in order to estimate its

concentration. Methane concentration was subsequently converted to mass values (Adejoro and Hassen, 2018). *E. curvula* hay of known chemical composition was used as substrate and incubated with the *A. vera* and *M. oleifera* crude extracts, alginate, chitosan, and encapsulated extracts of *A. vera* and *M. oleifera* each at 50 mg kg^{-1} DM. For the control treatment, an equal amount of distilled water was added without plant extracts.

3.2.7 Evaluation of in vitro organic matter digestibility

The *in vitro* organic matter digestibility of *A. vera* and *M. oleifera* crude extracts, alginate, chitosan, and encapsulated extracts of *A. vera* and *M. oleifera* each at 50 mg kg^{-1} DM were evaluated as additives to *E. curvula* hay substrate as previously described (Akanmu and Hassen, 2018). Briefly, the first phase consisted of 48 h rumen degradation and was followed immediately by an additional 48 h acid-pepsin digestion phase. During the first phase, 200 mg of feed samples were incubated in triplicate under anaerobic conditions with 20 ml of rumen liquor for 48 h at 39ºC with the inclusion of blanks and standards in every batch of incubation. This was followed by a 48 h acid-pepsin digestion phase at 39ºC under anaerobic conditions. Following the 96 h incubation, the residual plant materials were collected and oven-dried at 105ºC for 18h. The ash contents were evaluated by combustion in a muffle furnace at 250ºC for 2 h and later at 600ºC for 4 h and *in vitro* organic matter digested was estimated.

3.2.8 Chemical analyses

The test substrate feed sample, *E. curvula* hay, was analysed for dry matter (DM) and total ash following standard procedures (Thiex et al., 2012). The ether extract was determined using ether extraction in the Tecator Soxtec (HT6) system (McCormick, 2014). 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) as described (Van Soest et al., 1991), nitrogen (Sáez-Plaza et al., 2013) (FP2000 Nitrogen/Protein Analyser, Leco Instrumente GmbH, Kirchheim, Germany), and the crude protein was obtained by multiplying nitrogen by 6.25.

3.2.9 Statistical analyses

The *in vitro* organic matter digestibility and gas production study were statistically analysed, individual bottles within each run served as analytical replicates while each run represented a statistical replicate. Data were analysed using the GLM procedure in SPSS software (version

20); with the model: Yij = μ+ Bi + Tj + eij. Where, Yij= mean of individual observation (gas production), μ= overall mean, Bi = block effect (replicate), Tj = treatment effect, and eij= residual error. The means comparisons were done using the Tukey test and significance was declared at $p < 0.05$. The principal component analysis was carried out using the PAST 4 software, version 4.11.

3.3 Results and Discussion

3.3.1 Characterisation of alginate or alginate-chitosan encapsulated plant extracts

The results of scanning electron microscope (SEM) examination of micro-particles in chitosan, alginate, alginate-extract, and alginate-chitosan-extract beads are presented in Figure 3.1, while the particle densities of alginate, chitosan, non-encapsulated (raw) and encapsulated plant extracts are shown in Figure 3.2. The density of the particles of antimethanogenic plant extracts and biologically active compounds constituting it may have an effect on residence time in the rumen (Golchin-Gelehdooni, 2011; Moyo and Nsahlai, 2018) and most likely their methane-modulating properties. The physical properties of the primary structure of freeze-dried alginate beads range from clusters of masses with some visible pores in the empty alginate beads; to spongy and skin-like layers in the bead-containing extracts of *A. vera* and *M. oleifera*. The micro-particle morphology of the bead-containing extracts of *A. vera* and *M. oleifera* revealed the presence of micro-particles held together by and within a skin-layer mass. Na-alginate is the sodium of a natural amylose carbohydrate distilled from alga (Draget et al., 2005) with the empirical formula $(C_6H_7O_6Na)n$ (Rofifah, 2020). The spongy-like agglomeration of particles observed in this study had almost similar looks to the findings of Isailović et al. (2012) and Lević et al. (2015) when *Pterospartum tridentatum* and flavour (D-limonene) were respectively encapsulated in Ca-alginate produced by electrostatic extrusion and also Istenič et al. (2015) who encapsulated resveratrol (3, 4′, 5 trihydroxystilbene) into Ca-alginate. This study intended to explore the antimethanogenic properties of chitosan as well as the low density of alginate to increase methane inhibition in ruminants. Particle density analysis revealed that alginate had the lowest particle density, followed by the non-encapsulated plant extracts while the highest particle density was noted in chitosan. The lower density of the alginate encapsulated plant extracts compared to non-encapsulated (raw) plant extracts is likely attributable to the alginate wall materials, hence, making the particles of encapsulated plant extracts lighter. This could probably help to increase the mobility of the micro-particles of the encapsulated

plant extracts in the lower liquid phase of the ruminal medium and improve the flow of the plant extracts to the next compartment within a short period for brief delivery of the core materials to the target microbes in the rumen (Moyo & Nsahlai, 2018). In contrast, the alginate-chitosan encapsulated plant extracts had higher particle density as compared to non-encapsulated plant extracts due to the inclusion of chitosan. This could probably modify the mobility of the encapsulated plant extracts to move into the solid phase of the rumen thereby improving the residence time within the rumen (Golchin-Gelehdooni, 2011).

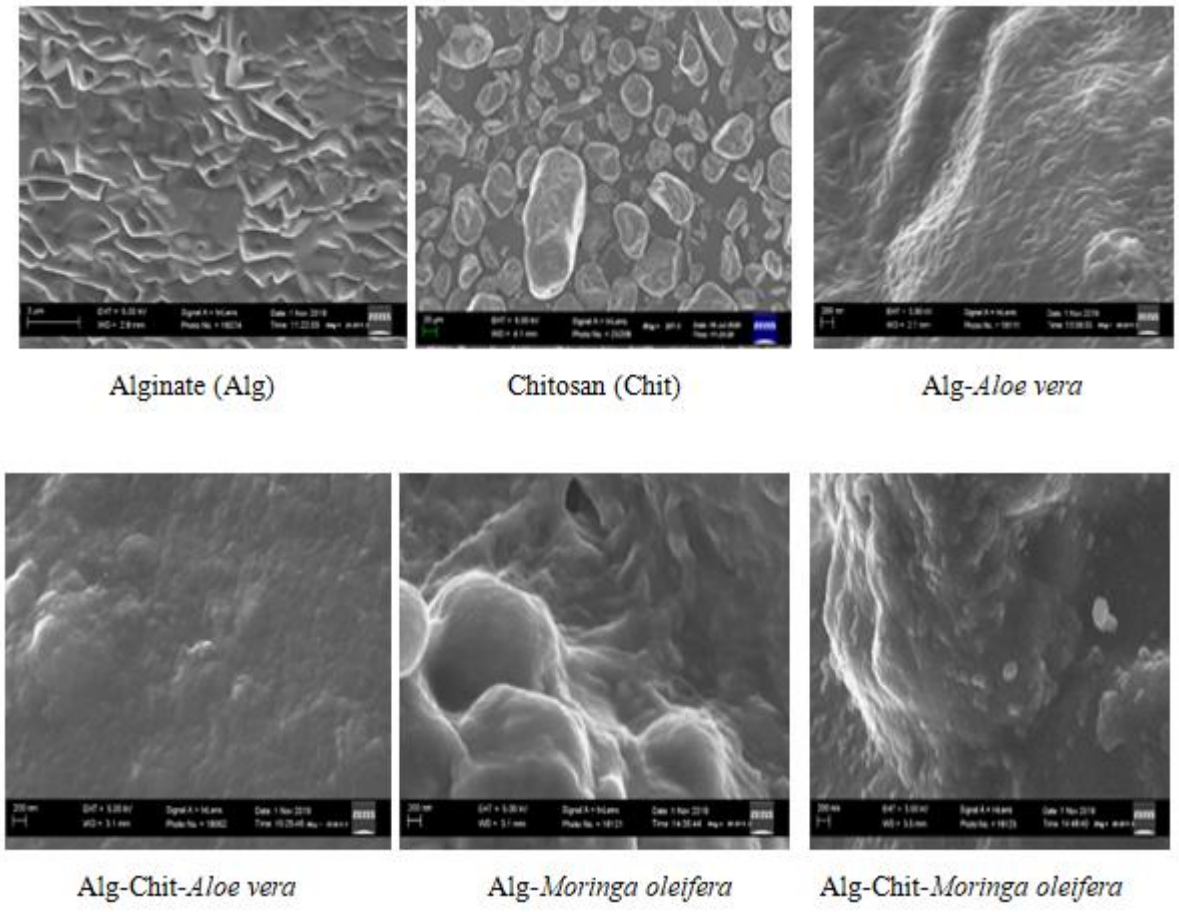


Figure 3.1 The scanning electron microscope shows the morphological features of micro-particles of alginate, chitosan, and encapsulated plant extracts.

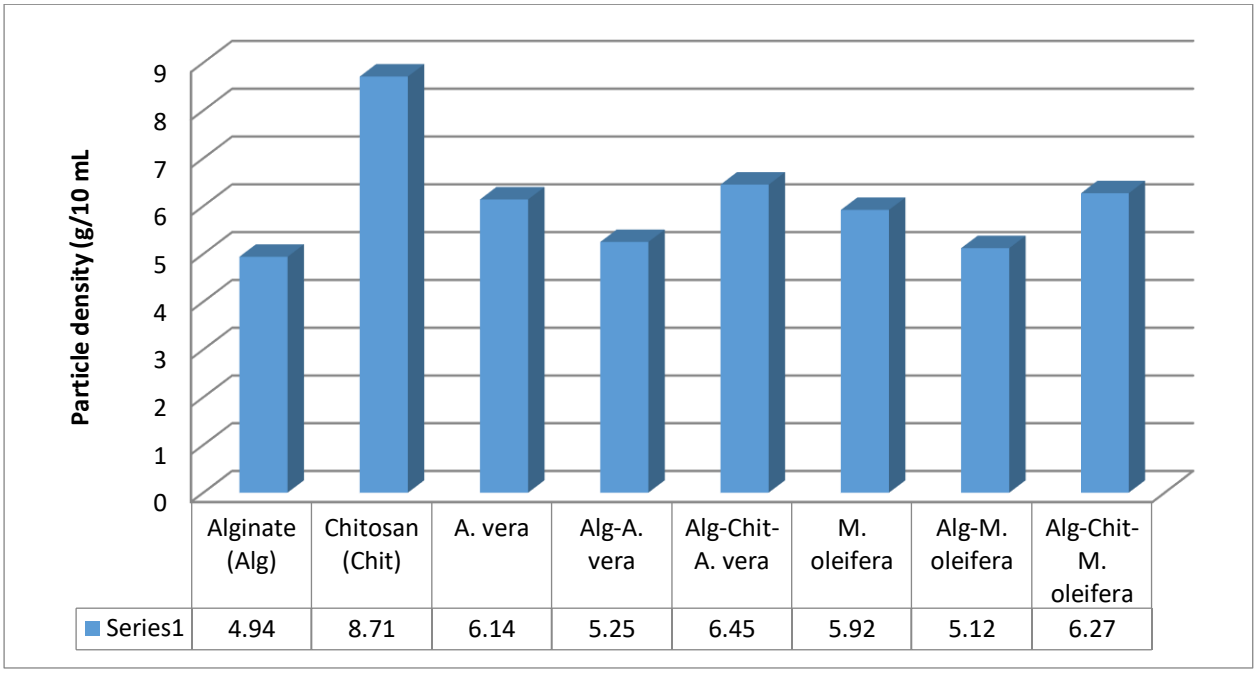


Figure 3.2 The particle density of chitosan, raw, and encapsulated plant extracts influenced by alginate as wall material

3.3.2 Effect of encapsulated plant extracts on in vitro total gas and CH_4 production, and organic matter digestibility (IVOMD) of E. curvula hay.

The *in vitro* methane production, total gas production, and IVOMD of *E. curvula* hay treated without or with the encapsulated *A. vera* and *M. oleifera* extracts are presented in Table 3.1, while the overall relationship between percentage CH_4 reduction and IVOMD as influenced by the inclusion of alginate or aliginate-chitosan wall material, raw and encapsulated plant extracts are graphically illustrated in Figure 3.3. Compared to the control group, the inclusion of alginate, chitosan, *A. vera*, and *M. oleifera* separately as additives reduced methane production per gram DM of *E. curvula* hay ($p < 0.05$) by 15.24% (3.67 mL), 14.78% (3.69 mL), 17.55% (3.57 mL) and 19.40% (3.49 mL) respectively. The encapsulated extracts of *A. vera* and *M. oleifera* also effectively reduced ($p < 0.05$) methane by 30.72% (3.00 mL), 27.71% (3.13 mL), 30.02% (3.03 mL) and 30.48% (3.01 mL) for *A. vera* encapsulated with alginate, *A. vera* encapsulated with alginate-chitosan, *M. oleifera* encapsulated with alginate, and *M. oleifera* encapsulated with alginate-chitosan, respectively (Table 3.1). The inclusion of the wall materials, non-encapsulated (raw), and encapsulated plant extracts as additives have no adverse effect on TGP and IVOMD compared to the control treatments. The principal component loadings plot and principal component analysis of all the fermentation parameters and phytochemicals composition of the plant extracts are shown in Table 3.2 and

Figure 3.3 respectively. The principal component (PC) loadings show the variations in the principal component analysis. PC1 and PC2 have 90.81% and 8.86% variations, respectively where most of the metabolites were positively correlated with CH_4 inhibition. The OMD is inversely correlated with most metabolites on PC2. In the principal component loadings (Table 3.2) and analysis (Figure 3.3), the alkaloids, coumaric acid, kaempferol, neochlorogenic acid, quercetin, and rutin were associated with *in vitro* methane inhibition in *M. oleifera* extracts and its encapsulated products while aloin A, aloin B, aloesin and aloe-emodin are responsible for methane inhibition in *A. vera* and its encapsulated products. The observed relationship between these metabolites and methane inhibition in this study is consistent with the previous results (Akanmu & Hassen, 2018). Alkaloids in plant extracts lowered enteric methane emission (Agidigbi et al., 2014; Akanmu & Hassen, 2018) while alkaloids in moringa adversely affect ruminal microbes especially methanogens (Gautam et al., 2007; Ojiako, 2014). Huang et al. (2014) reported the reducing potential of kaempferol, quercetin, and rutin in *in-vitro* methane emission by 5 to 9 mL g^{-1} DM. The anthraquinones (Kedarnath et al., 2012) in *A. vera* extracts reportedly elicited antimicrobial activity against bacteria and fungi, while acetone and methanolic extraction of aloe decreased *in vitro* methane emission (Sirohi et al., 2012). Although, from the principal component loadings (Table 3.2) and analysis (Figure 3.3), the particle density (PD) of the micro-particles did not show an effective association with methane inhibition of the encapsulated products, the lighter density of the encapsulated plant extracts (Encapsulated *M. oleifera* extract, 5.12 g 10 mL^{-1}, and Encapsulated *A. vera* extract, 5.25 g 10 mL^{-1}) due to the influence of alginate wall material (Figure 3.2) may likely mean faster mobility of the particle with better and more uniform distribution of metabolites within the lower liquid-solid phase of the rumen compared to the heavier unencapsulated extracts (*M. oleifera* crude extract, 5.92 g 10 mL^{-1}, and *A. vera* crude extract, 6.14 g 10 mL^{-1}). The alginate-chitosan-extract encapsulated products (of *A. vera* and *M. oleifera*) have a higher density than the raw plant extracts and also exhibited higher methane inhibition, this higher methane inhibition could be attributable to the additional effect provided by the chitosan in combination to the effect of the alginate and the plant extract in the products. In the current study, the particle density does not affect methane inhibition most likely because the *in vitro* gas production study is an enclosed system with constant agitation/revolution speed thereby putting the particles in a continuous motion. Another possible reason is that alginate also exhibited methane inhibition. Although

alginate (wall material) has not been reported to be antimethanogenic, its low density, non-toxicity, high encapsulation efficiency (up to 100%), and biocompatibility potential (Belščak-Cvitanović et al., 2011; Lević et al., 2015) were considered in selecting it as a wall material. The observed methane inhibition by alginate (15.24%) as noted in this study means that the use of alginate as a wall material has a potential co-benefit of reducing methane, which may be attributable to its chemical structure, antimicrobial protective property as well as biosynthesis from seaweeds; pathogenic *Pseudomonas aeruginosa* and the soil-dwelling *Azotobacter vinelandii* (Urtuvia et al., 2017). Also, alginate is a soluble polysaccharide (Rijo et al., 2014), which probably makes it capable of acting as a hydrogen sink in the medium like soluble amylose carbohydrates. In contrast, chitosan has been known not only for its antimicrobial properties but also as an antimethanogenic compound (Jiménez-Ocampo et al., 2019). The methane inhibition property of chitosan observed in this study was in accordance with the previous reports. Goiri et al. (2010) reported a reduction in enteric methane emission with chitosan, and in another study decreased the rumen protozoa population (Jiménez-Ocampo et al., 2019). In a different study, chitosan inhibited bio-hydrogenation *in vitro* by increasing C18:1 t11 and conjugated linoleic acid proportion (Goiri et al., 2010), thereby channeling H_2 from methanogenesis to the biohydrogenation process.

Table 3.1 Effect of alginate, chitosan, alginate, and/or chitosan encapsulated plant extracts on *in vitro* CH_4 production, TGP, and IVOMD of *E. curvula* hay

Treatments (50 mg kg^{-1} DM)	CH_4 (mL g^{-1} DM)	TGP (mL g^{-1} DM)	CH_4/TGP ($\times 10^{-3}$)	IVOMD (g kg^{-1} DM)	TGP/IVOMD (mL kg^{-1} M)	CH_4/IVOMD (mL kg^{-1} DM)
Control	4.33^{A}	159.0	28.0^{A}	607.3	262.0	7.33^{A}
Alginate (Alg)	3.67^{B}	160.0	22.7^{B}	607.4	263.6	6.00^{AB}
Chitosan (Chit)	3.69^{B}	156.9	22.6^{B}	604.7	259.7	5.99^{AB}
A. vera	3.57^{BC}	159.0	22.4^{B}	602.0	264.0	5.95^{AB}
Alg encap. *A. vera*	3.00^{C}	145.7	23.0^{B}	601.0	242.3	5.33^{B}
Alg-Chit encap. *A. vera*	3.13^{C}	146.6	22.8^{B}	598.3	245.7	5.58^{B}
M. oleifera	3.49^{BC}	159.3	21.9^{B}	609.3	266.3	5.73^{AB}
Alg encap. *M. oleifera*	3.03^{C}	145.7	22.0^{B}	609.0	239.0	5.33^{B}
Alg-Chit encap. *M. oleifera*	3.01^{C}	143.0	23.1^{B}	594.0	241.3	5.66^{B}
SEM	0.31	6.24	0.23	1.54	0.33	0.51
p-value	0.01	0.21	0.01	0.97	0.56	0.024

$^{A-C}$Means with different letters along the same column for each parameter are significantly ($P < 0.05$) different, SEM: standard error of the mean, TGP: total gas production, IVOMD: in vitro organic matter digestibility, *p*-value=probability value. For encapsulated extracts, the core and wall materials contained 50 mg each while for encapsulated extracts and chitosan, 42 mg of wall materials, 42 mg of extracts, and 16 mg of chitosan

Table 3.2 Principal component loadings of all phytochemicals and fermentation parameters of *E. curvula* hay fermented with encapsulated plant extracts.

Variables/metabolites	PC 1×100	PC 2×100
%CH_4-Re	1.29	4.23
OMD (organic matter digestibility)	0.69	-1.85
Alkaloid (A)	55.94	6.65
Aloe-emodin (AE)	-2.32	15.33
Aloesin (Alos)	-4.12	27.23
Aloin A (AA)	-11.30	74.61
Aloin B (AB)	-8.55	56.44
Caffeoyl ester of aloesin	-0.22	1.44
Chitin (CH)	-0.63	-3.38
Chlorogenic acid (Cl)	4.83	0.57
Coumaric acid (Cu)	16.64	5.54
Feruloyl quinic acid (F)	6.21	0.74
Hydroxyaloisin (HA)	-0.22	1.05
Kaempferol (K)	64.76	11.57
Nataloin (NA)	-0.38	2.29
Neochlorogenic acid (N)	32.25	3.84
Particle density (PD)	0.10	0.75
Quercetin (Q)	28.37	3.37
Rutin (R)	16.21	1.93
Sodium alginate (SA)	-0.53	-2.82
% variance	90.81	8.86

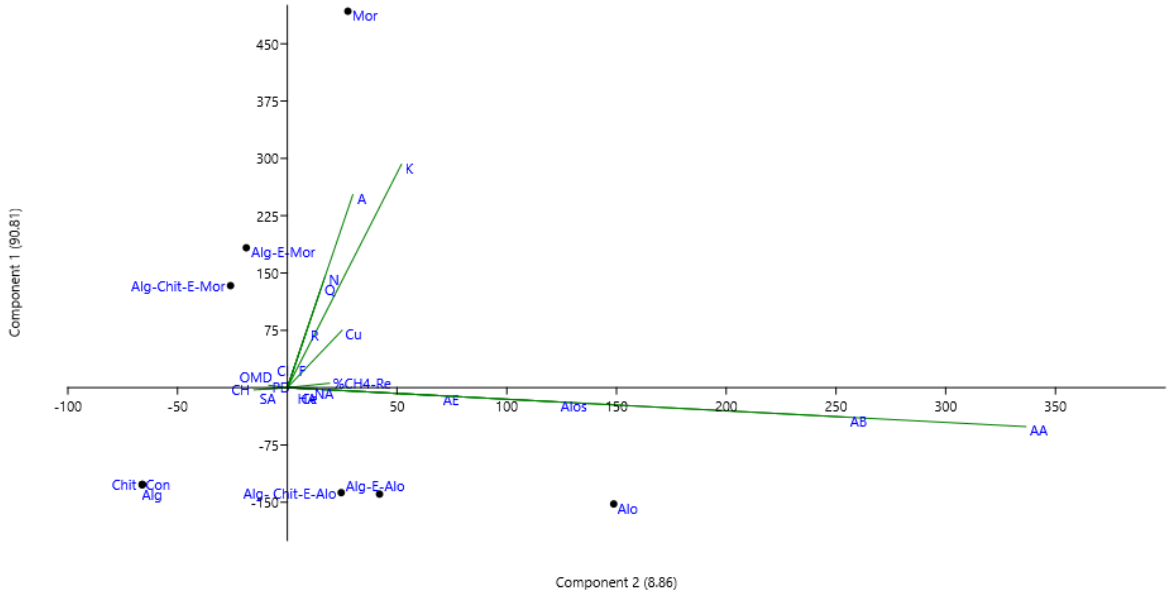


Figure 3.3 Principal component analysis Plot 1 vs Plot 2 of all phytochemicals and fermentation parameters of *E. curvula* hay fermented with encapsulated plant extracts (letters interpreted in Table 3.2).

Key: Figure 3.3

Con: Control
Alg: Alginate (wall material)
Chit: Chitosan
Alo: *Aloe vera*
Mor: *Moringa oleifera*
Alg-E-Alo: Alginate encapsulated *Aloe vera*
Alg-chit-E-Alo: Alginate-chitosan encapsualted *Aloe vera*
Alg-E-Mor: Alginate encapsulated *Moringa oleifera*
Alg-chit-E-Mor: Alginate-chitosan encapsualted *Moringa oleifera* with chitosan

3.4 Conclusions

The alginate and chitosan as observed via electron microscope have different morphological features with a skin-like appearance in alginate and scattered particles of different shapes in chitosan. The alginate (4.94 g 10 mL^{-1}) and chitosan (8.71 g 10 mL^{-1}) also differ in particle density and these influenced the density of the micro-particles of the encapsulated plant extracts. For the *in vitro* study, though, no adverse effect was noted in terms of *in vitro* organic matter digestibility and total gas production, the encapsulated plant extracts of *A. vera* and *M. Oleifera* effectively reduced ($p < 0.05$) methane emission on average between

27.71% to 30.48% as against methane inhibition by their raw extracts which ranged between 17.55% to 19.40%. The alkaloid, coumaric acid, kaempferol, neochlorogenic acid, quercetin, and rutin (in *M. oleifera*), and aloin A, aloin B, aloesin and aloe-emodin (in *A. vera*) were responsible, among other phytochemicals for the reduction in methane emission. However, the differences in particle densities of the raw vs encapsulated plant extract micro-particles did not affect methane inhibition. Alginate and chitosan also had a reducing effect ($p < 0.05$) on methane emissions when tested individually as additives. The use of alginate and alginate-chitosan as wall materials provided a co-benefit to the plant extracts to increase methane inhibition by an additional 13.17% (for alginate encapsulated *A. vera*), 10.62% (for alginate encapsulated *M. oleifera*), 10.19% (for alginate-chitosan encapsulated *A. vera*), and 12.93% (for alginate-chitosan encapsulated *M. oleifera*).

CHAPTER FOUR

Effect of alginate encapsulated *Moringa oleifera* plant extract additives on growth performance, nutrient digestibility, methane and rumen fermentation of South African Mutton Merino lamb

Abstract

Moringa plant extracts are known to have rumen modulating potentials, reducing methane productions in ruminants, however, they have limitations in terms of attaining consistent efficacy due to variations in palatability and thermal stability associated with some phytochemicals and modes of feeding. Encapsulation with encapsulant like alginate that has a good bio-compatibility, anti-microbial protective properties, ability to reduce bitter taste and improve the shelf-life of bioactive phytochemicals in the plant-based extracts has the potential to protect the efficacy of phytochemicals and improve animal performance. This study evaluated the efficacy of alginate-encapsulated and non-encapsulated *Moringa oleifera* (MO) leaf extracts on enteric methane inhibition, growth performance, nutrient digestibility, and rumen fermentation parameters of South African Mutton Merino (SAMM) lambs. A total of thirty lambs were randomly allotted to three different groups and balanced for weight. The three groups comprised the control diet which consisted of total mixed ration (TMR) only, TMR + non-encapsulated MO extract (NME), and TMR + alginate-encapsulated MO extract (EME). Lambs on NME and EME were administered iso-concentration of MO extracts, which was fixed at a dose of 50 mg kg^{-1} DM of TMR feed. Supplementation of NME and EME additives did not affect feed intake, nutrient digestibility, rumen fermentation parameters, and growth performance but reduced ($p < 0.05$) feed conversion ratios (4.90 and 5.08, respectively) of the lambs. Inclusions of NME and EME reduced ($p < 0.05$) urinary nitrogen excreted and increased nitrogen retained as a percentage of intake (g/kg N-intake) of the lambs. Lambs that received EME had lower ($p < 0.05$) rumen ammonia nitrogen (18.2 mg/dL) than those on the control treatment (26.9 mg/dL). Supplementation of NME and EME reduced ($p < 0.05$) methane emission (g/head/day) by 22.61% and 20.06%, respectively; and also reduced methane emission in g per unit of DM and NDF intake, per unit of digested DM and NDF. In conclusion, alginate encapsulation of MO extract is safe and did not reduce the efficacy of MO extracts compared to non-encapsulated extracts. Further study needs to be conducted to determine the effect of alginate-encapsulated MO

extracts on the rumen microbial populations and meat quality of the same lambs from this study in order to further evaluate the safety use of alginate as a wall material and whether or not it has additional benefit compared to the non-encapsulated *M. oleifera* extract.

Keywords: methane emission, nutrient utilization, rumen fermentation, weight gain, SA Merino lamb

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4.1 Introduction

The attempt to reduce methane emissions from livestock species has attracted global interest and attention. This is because methane gas has a detrimental effect on the climate and it has a much higher global warming potential than CO_2 (Patra et al., 2017). Since CO_2 is a precursor for methane production in the rumen of ruminants in the presence of hydrogen molecules and rumen methanogens, any effort to reduce enteric methane emission will include either inhibiting the activities of rumen methanogens or removing hydrogen molecules and/or CO_2 from the system (Patra et al., 2017; Islam et al., 2019). One of the means to achieve this is the use of bioactive plant extracts from medicinal plants. The methane-mitigating potential of medicinal plants and efficiency in feed utilization by livestock has been attributed to the presence of a number of plant secondary metabolites such as alkaloid, kaempferol, quercetin, rutin, chlorogenic acid, and neochlorogenic acid (Ibrahim et al., 2022) and while other types of identified plant secondary metabolites are MIF 4.44_609.1462, MIF 4.53_433.1112, MIF 9.06_443.2317 and MIF 15.00_487.2319 (Zeru et al., 2022) in the Moringa plant extracts. Extracts from *Moringa oleifera* are effective against diseases and pathogenic microbes (Ojiako, 2014; Prabakaran et al., 2018) and methanogens (Akanmu and Hassen, 2018; Ibrahim et al., 2022; Zeru et al., 2022), which inhibit growth and animal performance. *M. oleifera* extracts have been shown to be beneficial to animal agriculture due to the presence of different bioactive compounds that are capable of mitigating methane emission with no adverse effect on organic matter digestibility (Ibrahim et al., 2022). However, the methane-reducing efficacy of some of these antimethanogenic compounds might fade with time due to changes in bioactive compounds during extended storage time and exposure to heat and light during feed processing (Zeru et al., 2023). The use of plant extracts as rumen modifiers has limitations in terms of attaining consistent results due to variability in the method of feeding, palatability, and thermal stability during feed processing. Encapsulation of plant-based

extracts has gained a useful application in animal nutrition as it has the potential to protect the efficacy of bioactive compounds in plant extracts and improve animal performance, expecially when the encapsulant (or wall material) has a good bio-compatibility with the plant's bioactive compounds (Tolve et al., 2021). Previous study has shown that bioactive compunds like tannins has been enhanced by the use of wall materials or encapsulation which likely increased the mobility of the micro-particles and may enhance the controlled delivery of bioactive compounds in the rumen (Adejoro et al., 2019; Ibrahim and Hassen, 2022).

Alginate is an abundant marine biopolymer, ranking second to cellulose, and is mostly found in the intracellular spaces of brown seaweed (Rijo et al., 2014). Alginate is a wall material commonly used in the food and drug industries. It has multiple benefits as it possesses features like bio-compatibility, low density, ease of production, non-toxicity, and chemical and thermal stability (Belšćak-Cvitanović et al., 2011; Rijo et al., 2014; Asbahani et al., 2015; Hecht et al., 2016). Heavier particle-density compounds are likely to settle down in the rumen fluid and possibly have limited mobility which may lead to uneven distribution of bioactive compounds (Ibrahim & Hassen, 2021). The low density of alginate if used as wall material could probably help to increase particle mobility and better delivery of metabolites/bioactive compounds within the rumen (Moyo & Nsahlai, 2018). Previous research has shown that alginate has been used successfully to encapsulate *M. oleifera* plant extracts and it is found that alginate encapsulated Moringa plant extract increase *in vitro* methane inhibition from *Eragrostis curvula* hay substrate without any negative effect on organic matter digestibility of the feed (Chapter Three). This was because the alginate as a wall material provided a co-benefit to the *M. oleifera* extract to increase *in vitro* methane inhibition. However, there is no documented information if the alginate encapsulated Moringa plant extract has a similar effect when used as an additive in the diet of ruminant animals and whether there is any positive or negative effect in terms of its effect on feed intake, nutrient digestibility, growth performance and *in vivo* methane inhibition from ruminant animals. Thus there is a need for *in vivo* evaluation of alginate-encapsulated *M. oleifera* plant extract to validate its potential and repeatability in mitigating enteric methane without negative effects on feed digestibility and animal performance. This study was conducted with the objective to evaluate the effect of alginate-encapsulated *M. oleifera* extracts on enteric methane emission, feed intake and digestibility, growth performance, and rumen fermentation parameters of SA Mutton Merino lambs.

4.2 Materials and Methods

4.2.1 Study site and ethical consent

The trial was carried out at the University of Pretoria Innovation Africa Ruminant Unit on the Hillcrest campus, South Africa, between June and September 2021. The annual rainfall in Pretoria is about 573 mm and the city is located at 1700 m above sea level. The study was approved by the Animal Ethics Committee of the University of Pretoria (NAS-336-19).

4.2.2 Plant collection and purchase of sodium alginate

The leaves of *Moringa oleifera* (A11NA) plants were harvested from commercial crop farm in April 2019 at Lefakong farm in Pretoria north. These plant materials were placed in cool black boxes immediately after harvest and transported to the Nutrilab, Department of Animal Science where they were stored at -20 °C on arrival. Low-viscosity sodium alginate was obtained from Acros organic (New Jersey, USA).

4.2.3 Extraction of crude plant extracts from M. oleifera leaves

The preparation of crude plant extracts from *M. oleifera* was carried out following a procedure reported by Ibrahim et al. (2022). A 30:70 ratio of aqueous-methanol (H_2O: CH_3OH) solvent was initially prepared and this preparation was only done during extraction of the crude plant extract. The collected *M. oleifera* leaves were first freeze-dried for 96 h and later milled through a 1 mm sieve. The extraction was carried out by dissolving 1 kg of milled dried *M. oleifera* leaves material into a 25 L gallon containing 10 L of aqueous-methanol solvents. The gallon was placed into an Incoshake incubator and agitated at 130 rpm and 20°C for 96 h. The mixtures of the extracts were then filtered by gradually squeezing through a sieve with a 150 µm aperture. The filtrates were placed in a fume cubicle for 24 h until partially dried. The semi-dried extracts were freeze-dried for 72 h and then stored in clean plastic bottles at 4°C until further use.

4.2.4 Preparation of alginate-encapsulated MO plant extracts

The production of alginate-extract bead followed the method described earlier (Rijo et al., 2014) with some modifications reported by Ibrahim et al. (2022). Briefly, 100 g of sodium alginate powder was dissolved in 1 L of distilled water contained in a 2 L beaker and properly homogenized. 100 g of dried crude extracts were added to the mixture and thoroughly mixed to obtain an alginate-extract (at a ratio of 1:1) solution. The formed encapsulated mixtures were thoroughly mixed and the mixture was later frozen at − 80°C for

12 h before freeze-drying (− 50°C at a pressure of 1.1 Pa) for 6 days (GAMMA Martin Christ, GmbH, Osterode am Harz, Germany). The freeze-dried encapsulate was milled and properly stored in sterile sample bottles at 4°C pending further analysis.

4.2.5 Preparation and administration of MO plant extracts as a dietary additive

The plant extracts were administered as dietary additives by making a ball consisting of the *Moringa oleifera* crude plant extract (or the encapsulated MO plant extract), molasses, water, and concentrate feed. Briefly, 300 g of molasses was poured into a 1 kg container, and 100 g of water was added and mixed thoroughly after which 10 g of the MO plant extracts treatment was added and homogenized. Then 600 g of concentrate diet was gradually added to the mixture and stirred until a proper mixture was obtained. A similar preparation was made for the control treatment without the inclusion of non-encapsulated or encapsulated MO plant extracts. The treatment mixtures were molded into balls, weighed, refrigerated, and administered to the lambs daily at 8:00 am at a dosage of 50 mg kg^{-1} DM diet, as recommended earlier (Akanmu and Hassen, 2018). Each lamb is presented with a treatment ball (with or without plant extracts) on the palm daily to ensure minimal discomfort to the lambs as against the drenching method previously utilized to administer a mixture of plant extract with water. The actual treatment dosages given to lambs were adjusted fortnightly according to their average feed DM consumption recorded in the previous week.

4.2.6 Experimental design, feed, and growth performance

Thirty 4-month-old South African Merino lambs with a mean live weight of 25.6 ± 0.9 kg were used for this trial. The experimental lambs were ranked according to their body weight and randomly allotted to three dietary treatment groups using a randomized complete block design. The experimental treatments consisted of total mixed ration (TMR) + non-encapsulated *M. oleifera* plant extract (NME), TMR + alginate-encapsulated *M. oleifera* plant extract (EME), and TMR without MO plant extracts or control treatment. All experimental lambs were fed a formulated TMR containing approximately 42% roughage. The ingredients and chemical composition of the TMR are shown in Table 1. Lambs were initially adapted to the experimental feed for 7 days and gradually given the assigned plant extract balls starting with low dosages to the recommended level within 7 days period while the lambs were monitored closely for any health challenges. Two animals were kept in a pen, and animals in each pen received a different ball size of the plant extract, which was estimated based on the

previous week's average dry matter intake of the pen. The growth study lasted for 12 weeks. Each lamb was weighed fourthnightly and weight gain was recorded throughout the trial. The average daily weight gain (muscle attrition) was obtained by dividing weekly body weight gain against time. The feed offered to experimental lambs and orts were recorded daily for each pen and divided by 2 to obtain the average voluntary dry matter intake per lamb. The feed offered was adjusted to 5% of the new body weight. Before the start of the experiment, animals were vaccinated (with Multivax-P Plus) and dewormed. The experimental lambs were fed at 7:30 am and 2:00 pm daily. The plant extracts balls were given before morning feeding and clean drinking water was provided *ad libitum* throughout the study.

4.2.7 Determination of dry matter intake and digestibility

A total of 15 lambs (five per treatment) of approximately equal weights were selected from each block for the digestibility and balance trial. Lambs were allowed 5 days' adaptation to the metabolic cages and the faecal bags. Lambs were given 6 g of treatment balls containing MO plant extracts equivalent to 50 mg kg^{-1} feed intake at 07:30 am prior to morning feeding. Digestibility and balance data were collected for five 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 and stored in the freezer pending further analysis. The daily dry matter of the faeces was determined by transferring 100 g of sub-sampled faeces to a 105°C oven for 48 hrs. The urine produced by the lambs was collected through urine pans into containers in which 20 ml of 72% sulphuric acid was added and thereafter, samples taken were frozen at -20°C for later analysis of the sample for nitrogen.

4.2.8 Methane emission measurement

A total of 18 lambs were transferred in a batch of three animals to the open circuit respiration chamber for measurement of methane emission. Each batch consisted of three lambs selected from the same block and represented the three treatments. Details of the set-up of open-circuit respiratory chambers were described previously (Gemeda et al., 2015).

In each cycle, the three lambs that represented each of the three treatment groups were rotated every day to pass through a different chamber. Lambs were kept in the chamber for four days, which consisted of one day for adapting to the chamber and three 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 three blocks per treatment. The lambs were fed daily and the plant extracts treatments were offered at 50 mg kg^{-1} DM intake. Collection of orts, cleaning of the pen, and rotation of animals were done daily before morning feeding. Prior to the start of introducing the lambs, the chambers were calibrated by determining the recovery percentage of each chamber before the lambs were introduced into the methane collection chambers. The recovery percentages obtained for the three chambers ranged from 89% to 98%. Methane was analyzed in the laboratory from the gas collected from the sheep in the chambers with gas chromatography fitted with a flame ionization detector, which had a solenoid column packed with silica gel (8610C Gas Chromatograph (GC) BTU Gas Analyser GC System, SRI Instruments, Germany).

4.2.9 Rumen fluid collection

After the completion of the trial, all 30 lambs, representing ten from each treatment group, were slaughtered and rumen fluid was collected about five minutes after slaughtering from the rumen of each slaughtered lamb. The rumen fluid for every slaughtered sheep was filtered through a four-layer of cheesecloth to remove particles for each treatment group and mixed thoroughly before a representative sample was taken for each lamb. About 30 ml of rumen fluid sample from each lamb was filled into a container with 7.5 ml 50% H_2SO_4 for NH_3-N analysis (Broderick et al., 1980), and another 30 ml rumen fluid was preserved with 25% ortho-phosphoric acid for volatile fatty acid analysis (Ottenstein et al., 1971).

4.2.10 Chemical analysis of experimental feed, faeces, and urine

The dry matter (DM), ash, and crude protein of the total mixed ration (TMR) diet used in this study and faeces were evaluated following the standard procedure described by the Leco/Dumas method all according to AOAC (2000) as indicated in procedure 934.01, 942.05 and 968.06, respectively. The neutral detergent fibre (NDF), and acid detergent fibre (ADF) were determined using Ankom technology 200/220 (Ankom Technology, Fairport, NY, USA) as described by Van Soest et al. (1991). The ether extract was determined using ether extraction in the Tecator Soxtec (HT6) system (McCormick, 2014).

4.2.11 Statistical analysis

The raw data generated complied with the assumption of analysis of variance and the error terms were normally distributed. The data were subjected to the analysis of variance (ANOVA) using the general linear model (GLM) procedures of SPSS (version 20). Means

with significance were compared using the Tukey test and significantly different means were declared at $p < 0.05$. The following model was used:

$Yij = \alpha i + \beta j + \mu + e_{ij}$ where

Yij = treatment response of different parameters measured;

αi = overall effect of treatment

βj = effect of initial body weight of lamb (block)

μ = overall mean

e_{ij} = residual error effect

4.3 Results

4.3.1 Ingredient and chemical compositions of experimental diet

The ingredient and chemical compositions of the experimental diet are presented in Table 1. The percentage of dietary dry matter (DM) of the TMR is 89.38%, while the crude protein, ether extract, and ash were 20.21%, 2.17%, and 5.73%, respectively. The neutral detergent fiber (NDF) and the acid detergent fiber (ADF) respectively represented 35.35% and 17.95%.

Table 4.1. Ingredients and chemical composition of total mixed ration used in the study.

Items	Levels
Ingredients composition (%)	
Soybean meal	17.0
Yellow maize	28.0
Alfalfa hay	20.0
Eragrostis curvula hay	22.7
Molasses	6.00
Wheat offal	5.00
Urea	0.80
Vitamin premix	0.50
Total	100
Chemical composition (%)	
Dry matter	89.4
Crude protein	20.2
Neutral detergent fiber	35.4
Acid detergent fiber	17.9
Ether extract	2.17
Ash	5.73
Metabolizable energy (MJ kg^{-1} DM)	9.03

4.3.2 Intake, apparent digestibility, and growth performance of SA Mutton Merino lamb

The growth performance, nutrient intake/apparent digestibility, and nitrogen balance are presented in Tables 2, 3, and 4, respectively. The supplementation of non-encapsulated and encapsulated *M. oleifera* extracts did not show significant differences ($p > 0.05$) in nutrient intake, apparent digestibility, nitrogen intake, and weight gain when compared to the control group. However, the feed conversion ratios and urinary nitrogen excretion were lower while the nitrogen retained as a percentage of intakes was higher ($p < 0.05$) for the lambs administered non-encapsulated and encapsulated *M. oleifera* extracts compared to the control. There was no difference ($p > 0.05$) in the nitrogen balance and feed conversion ratio between the lambs administered with non-encapsulated and encapsulated *M. oleifera* extracts.

Table 4.2. Growth performance of alginate-encapsulated *Moringa oleifera* leaf extract (50 mg kg^{-1} DM) of South African Mutton Merino lambs.

Parameters	Control	NME	EME	SEM	*p*-value
Initial weight (kg)	25.4	25.4	26.1	1.95	0.40
Final weight (kg)	48.2	47.8	48.4	2.34	0.61
Total weight gain (kg)	22.7	22.4	22.3	0.88	0.91
Average daily gain (g $head^{-1}$ day^{-1})	271	267	266	10.5	0.81
Daily feed intake (kg $head^{-1}$ day^{-1})	1.47	1.31	1.38	0.07	0.13
Feed conversion ratio	5.43[a]	4.90[b]	5.08[b]	0.22	0.04

[ab] =means with different superscripts differ significantly at $p < 0.05$, NME=Non-encapsulated moringa extract, EME=Encapsulated moringa extract, SEM=Standard error of mean.

Table 4.3. Effect of alginate-encapsulated *Moringa oleifera* leaf extract (50 mg kg^{-1} DM) on nutrient intake and apparent digestibility of South African Mutton Merino lambs.

Parameters	Control	NME	EME	SEM	*p*-value
Nutrient intake (g $head^{-1}$ day^{-1})					
Dry matter	1094	1050	1061	72.4	0.14
Organic matter	1031	1001	1007	83.5	0.17
Crude protein	187	179	181	9.13	0.21
Neutral detergent fiber	403	352	378	26.9	0.23
Apparent digestibility (g kg^{-1})					
Dry matter	692	711	716	20.2	0.20
Organic matter	707	751	746	21.9	0.19
Crude protein	753	766	767	12.9	0.79
Neutral detergent fiber	501	553	541	35.1	0.50

NME=Non-encapsulated moringa extract, EME=Encapsulated moringa extract, SEM=Standard error of mean.

Table 4.4. Effect of alginate-encapsulated *Moringa oleifera* leaf extract (50 mg kg^{-1} DM) on nitrogen balance of South African Mutton Merino lambs.

Parameters	Control	NME	EME	SEM	*p*-value
Nitrogen intake (g $head^{-1}$ day^{-1})	30.2	28.1	28.6	2.70	0.81
Faecal nitrogen (g $head^{-1}$ day^{-1})	11.0	10.4	10.2	1.04	0.83
Urinary nitrogen (g $head^{-1}$ day^{-1})	6.53[a]	4.12[b]	3.94[b]	1.40	0.03
Nitrogen excreted (g $head^{-1}$ day^{-1})	17.6	14.5	14.2	2.75	0.48
Nitrogen retained (g $head^{-1}$ day^{-1})	12.8	13.6	14.2	2.21	0.93
Faecal nitrogen (g kg^{-1} N-intake)	362.3	368.6	359.3	19.8	0.94
Urinary nitrogen (g kg^{-1} N-intake)	202.4[a]	150.2[b]	136.1[b]	20.3	0.04
Retained nitrogen (g kg^{-1} N-intake)	435[b]	481[a]	504[a]	23.4	0.04

[ab] =means with different superscripts differ significantly at $p < 0.05$, NME=Non-encapsulated moringa extract, EME=Encapsulated moringa extract, SEM=Standard error of mean.

4.3.3 Methane emission and rumen fermentation parameters of SA Mutton Merino lamb

Methane emission and rumen fermentation parameters of lambs on non-encapsulated and alginate-encapsulated *M. oleifera* plant extract additives for SA Mutton Merino lambs fed TMR are shown in Tables 5 and 6, respectively. The supplementation of non-encapsulated *M. oleifera* plant extracts (NME) and alginate-encapsulated *M. oleifera* plant extracts (EME) at 50 mg kg^{-1} DM reduced ($p < 0.05$) methane emissions per day by 22.61% and 20.06%, respectively. Compared to the control, the NME and EME additives both reduced methane emission in g per unit of DM intake, per unit of NDF intake, per unit of digested DM, and per unit of digested NDF. Lambs that received EME additives had lower ($p < 0.05$) rumen NH_3-N concentrations than those on the control treatment. There was no significant ($p > 0.05$) difference in total volatile fatty acids (TVFA) and the individual fatty acids between lambs that received both NME and EME additives vs the lambs on the control diet though, there was an increasing trend in TVFA ($p=0.14$) for the lambs supplemented with NME and EME compared to the lambs on the control diet.

Table 4.5. Effect of alginate-encapsulated *Moringa oleifera* leaf extract (50 mg kg^{-1} DM) on enteric methane inhibition of South African Mutton Merino lambs.

Methane indices	Control	NME	EME	SEM	*p*-value
CH_4 (g $head^{-1}$ day^{-1})	31.05[a]	24.03[b]	24.82[b]	1.08	0.004
CH_4 (g kg^{-1} dry matter intake)	20.51[a]	16.35[b]	16.42[b]	0.33	0.002
CH_4 (g kg^{-1} neutral detergent fiber intake)	61.03[a]	50.52[b]	51.53[b]	1.06	0.041
CH_4 (g kg^{-1} dry matter digested)	31.29[a]	23.01[b]	23.11[b]	0.93	0.036
CH_4 (g kg^{-1} neutral detergent fiber digested)	129.67[a]	92.37[b]	94.12[b]	4.52	0.006

[ab] =means with different superscripts differ significantly at $p < 0.05$, NME=Non-encapsulated moringa extract, EME=Encapsulated moringa extract, SEM=Standard error of mean.

Table 4.6. Effect of alginate-encapsulated *Moringa oleifera* leaf extract (50 mg kg^{-1} DM) on rumen fermentation parameters of South African Mutton Merino lambs.

Rumen parameters	Control	NME	EME	SEM	*p*-value
pH	6.01	5.78	5.84	0.16	0.41
Rumen NH_3-N (mg dL^{-1})	26.9^{a}	21.6^{ab}	18.2^{b}	2.29	0.02
TVFA (mmol L^{-1})	125	146	155	13.3	0.14
Molar proportions of VFAs (mol/100mol)					
Acetic acid	54.1	54.8	54.3	3.45	0.98
Propionic acid	23.3	24.9	24.4	2.36	0.79
Butyric acid	15.6	13.7	15.1	1.85	0.61
Isobutyric acid	2.20	1.72	1.60	0.45	0.41
Valeric acid	1.89	1.74	1.67	0.33	0.79
Isovaleric acid	2.92	3.13	2.95	0.35	0.82
Acetate: propionate ratio	2.36	2.29	2.46	0.41	0.92

[ab] =means with different superscripts differ significantly at $p < 0.05$, NME=Non-encapsulated moringa extract, EME=Encapsulated moringa extract, SEM=Standard error of mean

4.4 Discussion

The non-encapsulated and encapsulated *M. oleifera* extracts effectively reduced methane emission in SA Mutton Merino lamb at 50 mg kg^{-1} DM compared to the control group. Encapsulation of *M. oleifera* extracts with alginate provided a co-benefit in increasing *in vitro* methane inhibition compared to the non-encapsulated *M. oleifera* extract (Ibrahim and Hassen, 2023, Unpublished data). Although this co-benefit could not be replicated in the current *in vivo* study, no adverse effects were observed for lambs in terms of weight gain and nutrient intake with the use of alginate as a wall material to encapsulate *M. oleifera* extract and this finding confirms earlier results that reported the non-toxicity features of alginate (Asbahani et al., 2015; Hecht & Srebnik, 2016; Rijo et al., 2014) which makes it safe to use as wall material to encapsulate the plant extracts as a feed additive for practical livestock production. The most likely reason could be attributed to the differences in particle density between the encapsulated (5.12 g 10 mL^{-1}) and the non-encapsulated (5.92 g 10 mL^{-1}) *M. oleifera* extracts (Ibrahim and Hassen, 2023, Unpublished data). The lower particle density for the EME could probably reduce its residence time in the rumen (Golchin-Gelehdooni et al., 2011; Moyo and Nsahlai, 2018) where the methanogenesis predominantly occur. In Moringa plant extracts phytochemicals like alkaloids, chlorogenic acid, neochlorogenic acid, feruloylquinic acid, kaempferol, 3-*p*-coumaroylquinic acid, quercetin, and rutin have been reported to be associated with methane inhibition in *M. oleifera* (Ibrahim et al., 2022). Alkaloids from Moringa extract reduced rumen methanogens (Ojiako, 2014) while the use of plant secondary metabolites such as kaempferol, quercetin, and rutin reduced *in vitro*

methane production by up to 9 mL g^{-1} DM (Huang et al., 2014). The reported mode of action for the methane inhibition properties of plant metabolites could probably be related to the ability of the metabolites to reduce the rumen methanogens population (bactericidal), or make the methanogens inactive (bacteriostatic) and/or interfere with the process of methanogenesis (Patra et al., 2017; Islam et al., 2019).

The higher nitrogen balance observed in lambs that received NME and EME additive suggested better nitrogen utilization due to higher retained nitrogen in the lambs supplemented with NME and EME. Urinary nitrogen is mainly comprised of urea and is more quickly converted to nitrous oxide and released to the atmosphere and/or nitrate which may contaminate water bodies (Eckard et al., 2010). The lower concentration of rumen NH_3-N for the lambs fed NME and EME in contrast to the control group is consistent with the work of Akanmu and Hassen (2018) who evaluated the methane inhibition of *M. oleifera* extract *in vitro* and found that *M. oleifera* extract reduced methane production and lowered rumen NH_3-N. Also, the lower rumen NH_3-N for the lambs that received NME and EME additives compared to the control diet is in agreement with the previous report (Abdel-raheem et al., 2021). Rumen protozoa are considered an important source of rumen NH_3-N through ingestion and proteolysis of bacterial protein (Abdel-raheem et al., 2021). The supplementation of the lambs with EME may probably have a better regulatory role in the metabolism of NH_3-N in the digestive tract of ruminants most likely by reducing the degradation and deamination of ruminal protein by rumen protozoa which eventually decrease rumen NH_3-N (Sampath, 2012; Abdel-raheem et al., 2021). In this study, the reduced proteolytic activity of undesirable rumen protozoa on bacterial protein is most likely responsible for the lower rumen NH_3-N and lower methane emission for the lambs on the NME and EME diet. Protozoa can engulf and degrade bacteria, and digest their protein, excreting NH_3-N as an end-product into the rumen (Bannink, 2014) thereby increasing the rumen NH_3-N. They are also closely associated with methanogens. Protozoa usually sequestrate and engage in a predator–prey interactions with rumen bacteria, which negatively affect feed conversion efficiency via the recycling of microbial cells in the rumen (Bannink, 2014). The higher rumen NH_3-N for lambs on the control diet could be associated with inefficient nitrogen metabolism and thus may have resulted in the higher urinary nitrogen excretion for the lambs on the control diet compared to the greater nitrogen utilization in the lambs supplemented with NME and EME additives. This could further be supported by the

results of Abdel-raheem & Hassan (2021) who reported a significant decrease in rumen total protozoa and NH_3-N in buffalo calves fed *M. oleifera* leaf meal. The supplementation of EME additive to the diet of lambs could most likely reduce the excessive proteolytic activities of undesirable rumen protozoa on bacteria protein through direct or indirect effects. Most extracts from medicinal plants have direct or indirect effects on rumen methanogens and methanogenesis (Islam et al., 2019). Some proteolytic rumen protozoa are associated with methanogenesis (Bannink, 2014); hence, the supplementation with NME and EME as additives could be said to interfere with rumen protozoa and reduce methanogenesis in South African Mutton Merino (SAMM) lambs. The increase in the concentration of TVFA and lower rumen NH_3-N as observed in this study is also in accordance with the previous reports (Abdel-raheem & Hassan, 2021; Kholif et al., 2015). The TVFA concentration was increased while the rumen NH_3-N was lowered in Anglo-Nubian goats fed with different levels of *M. oleifera* replacing sesame meal as a protein source (Kholif et al., 2015), and also as *M. oleifera* leaf meal dietary inclusion in buffalo calves (Abdel-raheem et al., 2021).

4.5 Conclusions

The encapsulated *M. oleifera* extracts given as an additive to SAMM lambs at 50 mg kg^{-1} DM of TMR feed reduced enteric methane emissions, rumen NH_3-N, nitrogen excretion, and feed conversion ratio without any adverse effect on nutrient intake and weight gain. However, the co-benefit of alginate-encapsulated *M. oleifera* extract in increasing methane inhibition compared to the non-encapsulated extracts as observed previously in the *in vitro* study is not replicated *in vivo*. Additional studies need to be conducted to determine the effect of alginate encapsulated *M. oleifera* plant extracts on the rumen microbial populations (with a focus on rumen protozoa and methanogens) and meat quality of the same lambs from this study in order to further determine the safe use of alginate as a wall material and whether or not it has additional benefit compared to non-encapsulated *M. oleifera* extract.

CHAPTER FIVE

General conclusions, recommendations, and critical review

5.1 General conclusions and recommendations

This study aimed at improving the methane inhibition potential of some medicinal plant extracts without adversely affecting feed digestibility, nutrient utilization, or animal performance by increasing the quantity of crude extracts and their methane-reducing bioactive compounds. It is anticipated that this can be achieved through i) the use of the right combination of extraction solvents and also to improve the antimethanogenic efficacy of plant extracts and ii) encapsulation of plant extracts to enhance the efficacies of the plant extracts (plant bioactive compounds) in the rumen when used as dietary additives to mitigate enteric methane production from ruminants. The first study, which dealt with the extraction of plant leaves extracts of *A. vera, J. curcas, M. oleifera,* and *P. betle* using aqueous-methanol to determine the yields of crude extracts, identify and quantify the phytochemicals in the crude plant extracts, was crucial for the subsequent trials that were conducted. The followings are the conclusions from the study:

❖ The aqueous-methanolic extraction (at a ratio of 3:7) of the studied medicinal plant leaves increased the amount of plant crude extracts compared to the pure methanol extraction; however, many of the identified and quantified metabolites showed various degrees of solubility unique to the plant leaves in which they exist, while some of the metabolites were unaffected by the extraction solvents.

❖ The four medicinal plant extracts derived from *A. vera, J. curcas, M. oleifera,* and *P. betle* leaves reduced *in vitro* methane emission when applied at a dose of 50 mg kg^{-1} DM *Eragrostis curvula* hay. However, the methane reducing potentials of the plant extracts, total gas production, and organic matter digestibility were not influenced by the extraction solvents and extracts yields. Metabolites, such as aloin A, aloin B, and kaempferol (in *A. vera*), apigenin, catechin, epicatechin, kaempferol, tryptophan, procyanidins, vitexin-7-olate and isovitexin-7-olate (in *J. curcas*), alkaloid, kaempferol, quercetin, rutin and neochlorogenic acid (in *M. oleifera)* and apigenin-7,4′-diglucoside, 3-*p*-coumaroylquinic acid, rutin, 2-methoxy-4-vinylphenol, dihydrocaffeic acid, and dihydrocoumaric acid (in *P.*

betle) were associated with *in vitro* methane inhibition and hence exhibited a methane reducing potential.

❖ Plant extracts from *A. vera* and *M. oleifera* leaves were successfully encapsulated by using alginate as a wall material. The micro-particle for the encapsulated product was characterized as a spongy cluster of masses containing plant extracts with visible particles held together around and within a skin-layer mass. However, the observed changes in the density of the micro-particles of alginate (wall material) and chitosan encapsulated *A. vera* and *M. oleifera* extracts did not influence *in vitro* methane inhibition; though, encapsulated *A. vera* and *M. oleifera* extracts further reduced *in vitro* methane production at 50 mg kg^{-1} DM compared to the non-encapsulated extracts, with no adverse effect on total gas production and organic matter digestibility of *E. curvula* hay.

❖ Evidence presented from the *in vivo* study showed that the encapsulated *M. oleifera* extracts reduced enteric methane emission, rumen ammonia nitrogen, urinary nitrogen excretion, and feed conversion ratio of SA Mutton Merino lamb when supplemented to the lambs at 50 mg kg^{-1} DM, and this has been achieved without any adverse effect on animal performance.

The following recommendations are coined from the conclusions of this study;

❖ The use of 70% aqueous extraction for *M. oleifera* leaf is recommended due to the reduced cost of extractive solvents, cheaper and availability of Moringa plants in South Africa, especially in the Gauteng province. Furthermore, 70% aqueous-methanolic extractions of *A. vera, J. curcas,* and *P. betle* are recommended for practical use in regions where they exist in abundance and are cost-effective.

❖ Plant extracts could be more promising and hence, further study is necessary to explore other extraction methods to increase extract yields from methane-reducing medicinal plants such as *A. vera, J. curcas, M. oleifera,* and *P. betle*.

❖ Additional studies should be carried out to quantify and confirm the methane-reducing potential of metabolites associated with *in vitro* methane inhibition such as aloin A, aloin B, kaempferol, apigenin, catechin, epicatechin, tryptophan, procyanidins, vitexin-7-olate, isovitexin-7-olate, alkaloid, quercetin, rutin, neochlorogenic acid, 3-*p*-coumaroylquinic acid,

2-methoxy-4-vinylphenol, dihydrocaffeic acid, and dihydrocoumaric acid. Subsequently, follow-up research needs to be conducted to use some of these metabolites with confirmed methane-reducing potential to produce a standardized plant extract-based product that guarantees consistent methane inhibition efficacy.

❖ The use of encapsulated *M. oleifera* extracts as a dietary additive to the South African Mutton Merino lamb is safe and practicable like the non-encapsulated plant extract. Further study needs to be conducted to determine the effect of alginate encapsulated *M. oleifera* plant extracts on the rumen microbial populations and meat quality of the lambs.

5.2 Critical review

One of the limitations of this study is that the plant leaves used were obtained from the same location, and are the same strain and species, according to their identification. There has been a report that suggests one can expect some variations in terms of the efficacy of these medicinal plant leaves obtained from different locations owing to differences in the types and quantity of bioactive chemical constituents (metabolites) responsible for the observed effects in this trial. Different trials have earlier reported that metabolites composition and concentration can be influenced by genetic factors while ecological factors and agronomic practices will also probably affect the production and/or composition of desired active ingredients in the leaves. Thus, it is important to describe the crude plant extract in terms of the type and concentration of key metabolites in order to develop a standardized product. This has been overlooked in the current study and can be regarded as a weakness of the current study.

The pre-extraction procedure, which includes rinsing, sun-drying, oven-drying, shade-drying, or freeze-drying, can influence the efficacy of leaf extracts. For instance, plant materials with latex like *A. vera* are better kept frozen immediately after harvesting so that useful ingredients are largely retained. Stacking of fresh plant materials together can lead to heat generation which can cause loss of heat-labile metabolites in the plant material; this can be aggravated as cells within plant materials continue to respire, even after harvesting.

Other factors include the milling of dried plant material, which has to be homogenous to improve the kinetics of analytical extraction and provide a good surface area to volume ratio to improve the contact surface for solvent extraction. During the present trial, adequate care

was taken to ensure that fine plant materials already soaked in extraction solvents did not escape inside the extracts to avoid an erroneous increase in the yield of the dried leaf extract as well as a reduction in the efficacy of the active ingredients due to dilution effect or loss of active ingredients during the procedure. This study optimized the solvent-to-substrate ratio but the ratio of the H_2O to CH_3OH needs to be further optimized, as it was already reported that solvents strongly affect the amount of plant extracts obtained. Also, optimizing the ratio of H_2O to CH_3OH solvent could probably affect the resulting outcome of both the yields, phytochemical compositions, and the methane-reducing property of the plant extracts. To avoid loss of useful bioactive compounds from the plant leaves, while sieving, the sieve was completely immersed in the solvent with frequent agitation to avoid locking away important bioactive compounds in a stack of non-soluble residue.

Reconstitution of dried plant extract to the desired concentration can be a tricky process. It was already reported that high or low gas volume could be caused by differences in the particle sizes of the reconstituted extracts when not fully dissolved and a large particle size in a single vial caused a large error in gas production. Therefore, the plant extract solution was prepared by dissolving a weighed amount of plant extract into a beaker with a known amount of water and allowed for continuous agitation by placing it in a magnetic stirrer for several hours without using heat until the solution was fully homogenized.

Encapsulation is another process that must be carefully managed; homogenization is a major factor to consider when encapsulating so as to allow even distribution of the core materials in and around the wall material. Also, the ratio of the core to wall material as well as their compatibility is very important. Where the core material and the wall material are not compatible, the wall material will most likely interfere with or limit the effectiveness of the active ingredient (core material). During the *in vitro* and *in vivo* trials, as much as possible, errors were kept to a minimum. Where errors were observed, the procedure was repeated.

During the *in vivo* study, plant extracts were made into a small ball, and a calculated known amount was presented to the sheep daily in the morning, based on their average feed consumption (50 mg kg^{-1} DM) during the previous week. This could probably be an improvement over the previous study where a drenching gun was used to administer liquid extracts which imposed some levels of discomfort on the animals and was not practicable in commercial farming. While the method of administering plant extracts in this study may

eliminate discomfort to the animals and be relatively more practicable than the previous trial, the encapsulation of plant extracts, and the accurate amount of the extracts to be incorporated into the molasses and concentrate mix require technical know-how.

Methane emission measurement was a complicated process during this study. However, this study followed a laid down procedure from the previous study which helped to eliminate some possible errors in the measurements of enteric methane emission. Initially, the methane reduction outcome for the administered treatment extracts was not impressive, careful observation revealed that the selection of feed particles was a problem because the feeding troughs were mounted at a relatively lower height from the floor. This error was immediately corrected by adjusting the positions of the feeders. Therefore, the subsequent study should consider the use of pelleted feed to easily eliminate such errors.

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