The effect of *Helinus integrifolius* (Lam.) Kuntze crude leaf extract on ruminant gas production and fodder digestibility

by

Blessing Mdaka

DISSERTATION

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Botany has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

_____ _____

Surname, Initials (title) **Date**

DEDICATION

A special dedication to my late grandmother, Margaret Sitiba who always believed in me, even in moments when I did not believe in myself. My late uncle Eugene H Nghonyama who always said, “set goals that seem impossible, so when you look back you stutter in disbelief”. May they rest in peace.

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ABSTRACT

There have been numerous attempts to alleviate enteric methane emissions by ruminants in both commercial and subsistence farming. This is because methane is the most potent greenhouse gas responsible for atmospheric contamination leading to the greenhouse effect, global warming and climate change. Although, carbon dioxide emissions via the use of fossil fuels and in industrial production have had considerable attention, livestock production continues to contribute substantial amounts (80 Mt per year) of methane to the atmosphere. Ruminants are a source of meat and dairy products that mankind cannot do without. Eliminating them is definitely not a viable option hence strategies such as feed, feeding management and nutrition, rumen modifiers and animal genetic production and other management approaches have been proposed. Recently studies have focused on the use of plants with high content of secondary metabolites. Plant secondary metabolites possess numerous desirable biological activities which lead to their exploitation as nutraceuticals, pharmaceuticals/recreational drugs and supplements. Properties such as antifungal, antimicrobial and anti-inflammatory effects have prompted their use in attempts to reduce enteric fermentation's major by-product i.e. methane. This study's main objective was to investigate the effect of a methanolic crude saponin extract and leaf powder of *Helinus integrifolius* on *in vitro* ruminant total gas production and fodder digestibility. A methanolic crude extract was obtained by sequential extraction in Soxhlet apparatus starting with n-hexane and then methanol. Total gas production and feed digestibility were quantified using a ruminant gas production/digestion bioassay. The gas released was analysed with the aid of gas chromatography. The methanolic crude extract was found to contain 286.0±0.60 mg saponins/g of dry material by colorimetric spectrophotometry. In numerous studies the anti-methanogenic effect in ruminants is attributed to condensed tannin activity. However, no tannins were detected in the methanolic crude saponin extract when a tannin test was performed. The methanolic crude saponin extract showed substantial reduction of total gas release (99%) and increased fodder digestion (55%) compared to the control treatment. Leaf powder inclusion did not reduce total gas release. However, digestion was slightly improved but less than 50% of dry material was digested. It was evident that leaf powder inclusion had minimum effect on ruminant enteric microbial activity. The methanolic crude saponin extract imposed much

greater effects in both total gas production and fodder digestibility compared to the leaf powder. These observations indicate that the *Helinus integrifolius* plant may be one of the ideal candidates with potential to reduce ruminant methane production. Furthermore, its methanolic crude saponin extract may contain the ideal phytochemicals to be investigated further as a ruminant feed additives/supplement. They may aid in the alleviation of enteric fermentation and increased feed utilisation efficiency while improving dairy and meat yield in livestock production.

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ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
°C	Degrees Celsius
C	Carbon
CFC	Chlorofluorocarbon
CH_4	Methane
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
COX-1/2	Cyclooxygenase assay(s)
CT	Condensed tannins
DM	Dry material
DMSO	Dimethyl sulfoxide
EO	Essential oil
F	Front
FAO	Food and Agriculture Organisation
FID	Flame ionization detector
GC	Gas chromatography
GHG	Greenhouse gas
GP/E	Gas production/emission
HOAc	Acetic acid (glacial)
HPLC	High performance liquid chromatography

HT	Hydrolyzable tannins
IVDMD	*In vitro* dry material degradability
IVOMD	*In vitro* organic matter degradability
IPCC	Intergovernmental Panel for Climate Change
MeOH	Methanol
N_2O	Nitrous oxide
NADH	Nicotinamide adenine dinucleotide hydride
NP/PEG	Natural product - polyethylene glycol
O_3	Ozone
PPM	Parts per million
PSM	Plant secondary metabolite
R_f	Relativity to front
S	Start
SIL G/UV	Silica gel/ultra-violet
UV	Ultra-violet
VIS.	Visible light
TLC	Thin layer chromatography

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The change in global average temperatures overtime has elevated concern for humanity and environmental safety. This is evident as the perception of climate change is gradually moving from being a scientific phenomenon to virtual reality. Recently, drought, heatwaves and extreme temperatures in various parts of the world have been reported. These effects have severe health implications to our livelihood. Climate change may be generally described as change in climate imposed by global warming. The concept of global warming is a natural phenomenon that maintains the earth's temperatures to human habitable standards, through the 'greenhouse effect'. This is achieved by the presence of the atmosphere on earth which sustains and regulates the planet's thermal energy from the sun. This also enables regulation of thermal energy globally and in turn induces weather patterns (Miller and Spoolman, 2009).

There are numerous natural and anthropogenic activities that contribute to global warming. When such activities disturb the equilibrium of the thermal and climatic systems, any input may shift the system to a new state over a period of time. Thus, any interference in the climate system will in turn have severe consequences. Hence, since the industrialisation of human civilisation mankind has been contributing to a global scale climate change, without realising it (Dahlman et al., 2011). Activities that have contributed immensely to change in climate are agriculture (livestock production), creation of landfills, the use of fossil fuels and deforestation among others. These contribute greenhouse gas and disturb the natural carbon sequestration processes, exposing more of the gas into the atmosphere. Carbon dioxide has received ample attention as a contributor to climate change. However, the most considerable contributor to climate change is methane (CH_4) gas which is approximately 21 times more potent than carbon dioxide (Bodas et al., 2012).

Besides production by anthropogenic activities, methane gas may also be produced naturally by volcanic eruptions, wetlands, decomposition of organic matter and by animals during digestion. The agriculture and forestry sector contributes an enormous quantity of methane emissions, currently at 30% of overall global greenhouse gas estimates (Crank and Jacoby, 2015). These emissions are attributed to livestock production e.g. enteric fermentation, manure management and

land use. This is because ruminants can convert in-digestible cellulose-rich plant material into meat and milk. Hence, they are a reliable source of food. However, their complex digestive system contains methanogens in the rumen, which induce formation of enteric methane that forms part of greenhouse gases. This makes ruminants the largest source of anthropogenic global greenhouse methane emissions (25%) (Buddle et al, 2011). The reduction in ruminant farming is not a viable option, since the demand for meat and dairy products increases directly in proportion with human population (FAO, 2008). Therefore, effective policies on livestock management, good farming practices (climate smart agriculture) and enteric methane mitigation strategies must be developed and implemented.

The need to develop good farming practices and substantially inhibit or reduce enteric methane production has recently gained considerable attention. Numerous mitigation strategies have been proposed and tested by various researchers (Buddle at al., 2011; Hart et al., 2008). The major strategies as suggested by Knapp et al., (2014) may be categorised as: (i) feeds, feeding management and nutrition, (ii) rumen modifiers and (iii) genetic animal improvement. Of the latter strategies, rumen modifiers with the use of plant secondary metabolites show great potential. Hence, there is a world-wide search for animal feed additives that can reduce enteric methane emissions from livestock production. The ideal candidates are plant extracts with high concentrations of plant secondary metabolites. Although there are a few contradictory reports of the effects of plant secondary metabolites on the reduction of enteric methane production, it is still one of the most viable, inexpensive and sufficient strategies for enteric methane production mitigation (Knapp et al., 2014).

The livestock production in agriculture and domestic farming imposes a threat to food security and the environment. This is because besides contributing to anthropogenic methane emissions which influence climate change, methane production also accounts for energy loss (2–15%) by the ruminant. Methane produced via enteric fermentation by ruminants must be reduced to decrease the burden on the environment (Cobellis et al., 2016). The task to produce adequate quantities of food without any major environmental impact is becoming a challenge. Mitigation strategies should be economical, sustainable, inexpensive and easy to

implement. Thus, feed additives and supplements seem to be the most relatively favourable strategies. Plant secondary metabolites such as condensed tannins have been established to reduce methane production by up to 63% when incorporated into ruminants' diet (Tan et al., 2011). Saponins have also demonstrated great potential as they contain anti-protozoal activity which may aid in manipulation of rumen microflora (Åby et al., 2013). In the rumen microflora, methanogens and homoacetogens form part of microbes responsible for methane and volatile fatty acid production respectively. The manipulation of rumen activities such as fermentation and microbial composition may be the solution to influencing methanogenic and homoacetogenic activities.

Buddle et al. (2011) state that methane is formed in the rumen by methanogens which out-compete homoacetogens for carbon dioxide (CO_2) and hydrogen (H_2). This is because methanogens utilise H_2 to reduce CO_2 to CH_4. If excess H_2 is channelled towards homoacetogens then production of volatile fatty acids (VFAs) like propionic acid will be favoured. According to Bodas et al. (2012) the volatile fatty acids are in turn utilised by the ruminant as source of energy and carbon. This makes VFA production a significant sink for H_2. So, it is necessary to manipulate rumen activities towards non-greenhouse outputs such as VFA production. Plant secondary metabolites with enteric methane mitigation capabilities should be vigorously investigated. These plant secondary metabolites should substantially reduce methanogenic activity and enhance homoacetogenic activity. Furthermore, the ruminant's dairy and meat yield or production must be improved.

The use of plant secondary metabolites has always been an essential part of mankind. Examples are the use of plant materials for oils, perfumes and food flavouring agents. Hence the healing properties of plants lead to elucidation of phytochemicals for biological effects. A myriad array of phytochemicals exhibits pharmacological properties such as antibacterial activity, antifungal activity, antiinflammatory effects, anticarcinogenic activity, antimalarial effect and inhibition of cholesterol synthesis (Wadood et al., 2013). Their role has become more significant as human dietary constituents since a prolonged intake has proven to be beneficial for the treatment of chronic diseases (Crozier et al., 2006). These pharmacological

properties make plants the ideal chemical library for the search for nutraceuticals and for biological agents to aid with enteric fermentation reduction.

Plant extracts have been investigated for their potential to reduce methanogenesis. Bunglavan (2014) states that triterpenoid and steroid saponins of *Acacia concinna* exhibit an inhibitory effect on ruminal protozoa. A decrease in ruminal CH_4 production was also observed from plant extracts of *Terminalia chebula* (seed pulp) and *Allium sativum* (garlic oil). The prospect of enteric methane reduction by plant secondary metabolites has great potential and seems to be a viable solution.

1.2 PROBLEM STATEMENT

Enteric methane is produced by ruminants through enteric fermentation processes. These are natural processes that aid in digestion and feed conversion. According to Wang et al. (2009), the degradation of dry matter is not adversely affected by saponin-containing plants or extracts, but methane production may be substantially decreased. However, a decrease in digestion may be attributed to the inhibition of fungi and cellulolytic bacteria's growth. Whereas methane inhibition is predominantly attributed to decreases in protozoal populations (Patra and Sexena, 2010). In addition to the detrimental effects methane has to our environment, it also accounts for the loss of feed energy in ruminants. The production of methane in the rumen is aided by methanogenic microflora in the gut in a process called methanogenesis (Bodas et al., 2012). Ruminant methane emission accounts for 25% of the anthropogenic greenhouse methane (Buddle et al., 2011). The relationship between emitted methane and dry material intake or gross energy intake is not constant. However, methane emission may decrease with increases in dry material intake (Knapp et al., 2013). The reduction of livestock numbers as a means of reducing methane production is not a feasible option since the demand for meat and milk increases directly in proportion to human populations (FAO, 2008). This raises a challenge of meeting demands for food without any further impact on the environment. Hence there is a need to search for animal feed additives with potential to reduce ruminal methanogenic activity whilst improving fodder digestibility. There is also a need for introducing good farming practices in line with already established climate change policies which are enforced by authorities.

1.3 SCOPE OF THE RESEARCH

This study explored the use of a methanolic crude saponin extract and leaf powder of *Helinus integrifolius,* 'soapbush' to reduce methane emission by *in vitro* enteric fermentation bioassay as well as their effects on fodder digestibility. The phytochemical constituents of the crude extract were also investigated. The major aspects covered included:

i. The extraction of a crude saponin extract by sequential Soxhlet extraction with n-hexane and methanol. Screening the crude extract for the presence of various phytochemicals, through thin layer chromatography analysis. The quantification of major phytochemicals in the extract (saponins, total phenolics, flavonoids and tannins) was done with the aid of colorimetric spectrophotometry analysis.
ii. The methanolic crude extract and leaf powder were examined for their effect on ruminant total gas production and fodder digestion in 24 hour intervals for a period of 96 hours using *in vitro* ruminant enteric fermentation assay.
iii. The emitted gas was further analysed by gas chromatography.

1.4 STRUCTURE OF DISSERTATION CHAPTERS

Chapter 1: General introduction*.* This chapter gives an overview of global warming and climate change with emphasis on how ruminant livestock production contributes to climate change. The literature covers the use of plant secondary metabolites as biological agents to alleviate biogenic methane emissions by ruminants. The scope of the research, problem statement and structure of dissertation chapters is also covered.

Chapter 2: Phytochemical analysis of *Helinus integrifolius* (Lam.) Kuntze leaf extract. This chapter focuses on the phytochemical content of *H. integrifolius*. The literature review outlines the significance of plant secondary metabolites and their applications. The analysis and quantification of various phytochemicals within the extract is also covered.

Chapter 3: In vitro effect of *Helinus integrifolius* (Lam.) Kuntze on ruminant total gas emission. This chapter focuses on the use of plant secondary metabolites

(triterpenoid saponins) to reduce ruminant gas emission. The literature review covers various enteric fermentation mitigation strategies proposed by researchers. It provides an overview of the manipulation of rumen processes and the need to reduce biogenic gas production. This chapter also focuses on one of the proposed enteric methane mitigation strategies, as a solution for livestock production, management and environmentally friendly farming.

Chapter 4: In vitro effect of *Helinus integrifolius* (Lam.) Kuntze on ruminant fodder digestibility. This chapter focuses on the use of plant secondary metabolites to enhance fodder digestion. The literature review covers aspects such as ruminal microbial communities and methanogenesis reduction imposed by plant secondary metabolites. The main objectives included the effect of the crude saponin extract on feed digestibility in relation to total gas production. The comparison between the effect due to crude saponin extract and leaf powder was established.

Chapter 5: Conclusion. This final chapter provides a summary of the research findings in relation to proposed aims and objectives. It outlines significant observations on the use of the crude saponin extract and leaf powder as biological agents for the reduction of ruminant methane gas production. It also highlights the ability of plant secondary metabolites to substantially enhance feed digestion. The knowledge gaps were identified and recommendation for further research suggested.

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CHAPTER 2

PHYTOCHEMICAL ANALYSIS OF *HELINUS INTEGRIFOLIUS* (LAM.) KUNTZE LEAF EXTRACT

2.1 INTRODUCTION

The ever increasing human population imposes pressure on the agricultural industry. This is because the demand for dairy products and meat also increases. Hence the quantity of livestock production must be drastically improved to satisfy demands. These must be achieved with no major impact on the environment. This is because livestock production and the forestry sectors are the highest anthropogenic contributors to climate change (Dick et al., 2015). Ruminants produce methane through their complex digestive system. This does not only pose a threat to environmental safety but also food security. Hence, climate-smart and environmentally friendly farming practices must be introduced to reduce greenhouse gas production by livestock farming. Numerous authors such as Hart et al. (2008); Patra and Sexena (2010) and Bodas et al. (2012), suggest that the most feasible enteric methane production mitigation strategy seems to be the use of plant secondary metabolites as rumen modifiers.

There have been several attempts to manipulate rumen fermentation to reduce methane gas emission by using plant secondary metabolites as supplements or additives in ruminant diets. This effect is attributed to the biological activity of plant secondary metabolites since most of them possess pharmacological properties. So plant secondary metabolites may be defined as phytochemicals that appear not to have any essential role in plant metabolism (Crozier et al., 2006). This is in contrast to plant primary metabolites that perform essential metabolic activities such as plant growth and development. Plant secondary metabolites may be grouped into three groups as; phenolics, terpenes or terpenoids and alkaloids. The presence of biologically active plant secondary metabolites has led to their exploitation for treatment of human ailments and other applications in various industries (Wadood et al., 2013).

The isolation of plant secondary metabolites and screening of medicinal plants for bioactive chemical properties was elevated in the early 19th century. This was prompted by the elucidation of the chemical structure of morphine and quinine among others in the 1920s (Sexena at al., 2013). Their discovery attracted considerable attention for biochemical and molecular research, which also directed the focus towards nutritional and pharmaceutical benefits of fruits and medicinal plants.

According to Sparg et al. (2004) biological properties such as anti-protozoal and anti-inflammatory effects led to their recent utilisation in an attempt to reduce rumen enteric fermentation. Of the three major groupings of plant secondary metabolites, considerable potential has been observed with the use of phenolics (condensed tannins) and terpenes (monoterpenes and triterpenes) as rumen modifiers. The latter's ability i.e., triterpenes (saponins) to reduce protozoal viability may be due to their haemolytic properties. This has also fuelled the utilization of triterpenes as potential enteric methane mitigation agents. Hence numerous authors (Hart et al., 2008; Patra and Sexena, 2010; Buddle et al., 2011; Bodas et al., 2012) conform to the use of phytochemicals as a feasible and efficient strategy for rumen fermentation manipulation and alleviation for methane emission.

2.1.1 Motivation of study

The *Helinus integrifolius* (Lam.) Kuntze (Rhamnaceae) plant commonly referred to as the soap plant or "seepbos" has various medicinal uses and bioactivities (van Wyk and Gericke, 2003; Pooley, 2005), but its chemical constituent profile is not established. It is said to have a number of bioactivities i.e., antibacterial and antimicrobial activity by Ray et al. (2004) and Shai et al. (2013), and it is used as a traditional medicinal plant (van Wyk and Gericke, 2003; Stafford et al., 2008; Shai et al., 2013).

According to Sparg et al. (2004), the Rhamnaceae plant family has triterpenoid saponins which generally have bioactive and pharmacological properties. Though the plant is used in traditional medicine for the treatment of hysteria, sandworms and gonorrhoea, black quarter in cattle and as prophylactic medicine (van Wyk and Gericke, 2003; Pooley, 2005), the only outlined phytochemicals are saponins. These phytochemicals give the plant the properties from which the common names are derived: soap plant or seepbos and 'ubhubhubhu' in English, Afrikaans and Zulu, respectively, which means "frothing". There is a need to characterise its chemical constituents to which the different uses and bioactivities can be ascribed to.

2.1.2 Aim of the study

The aim of the study was to screen and quantify the various phytochemicals from leaf extracts of *Helinus integrifolius*.

2.1.3 Objectives of the study were to:

i. To prepare crude nonpolar and polar extracts from *H. integrifolius* leaves by sequential extraction in n-hexane and methanol using Soxhlet apparatus.
ii. To screen the leaf extracts for the presence of various phytochemicals through thin layer chromatography.
iii. To quantify various phytochemicals (tannins, phenolics, saponins) in the leaf extracts through colorimetric spectrophotometry.

2.2 LITERATURE REVIEW

2.2.1 Plant secondary metabolites

Phytochemicals which appear not to have any essential role in plant metabolism (growth and development) are referred to as plant secondary metabolites (alkaloids, terpenoids and phenolics), in contrast to those such as common sugars, amino acids and chlorophylls which are primary metabolites because of their essential roles in plant metabolism (Walton and Brown, 1999). According to the authors the prior definition of 'secondary metabolites' suggests they are a less significant class of phytochemicals, but their biological activities suggest otherwise. Plant primary metabolites are universally distributed across all plants whereas certain plant secondary metabolites are found among specific plant families. The precise differentiation between 'primary' and 'secondary' metabolites is blurred in some instances (Crozier et al., 2006). However, plant secondary metabolites give aroma, colour and flavour to plants in addition to protection against pathogens and external damage. They also form part of the human diet with substantial health benefits when consumed in significant proportions (Taiz and Zeiger., 1991).

The major differentiation of phytochemicals as primary and secondary metabolites is based on their biological functions. Plant secondary metabolites may be further classified as phenolics, terpenes and alkaloids based on their functional groups, carbon-skeletal units, biosynthetic pathways, precursors and or bioactive properties. Phenolics are the most widely distributed class of plant secondary metabolites (Sexena et al., 2013). They have a distinctive aromatic ring with one or more hydroxyl groups attached. They range from simple, low molecular weight, single aromatic-ringed chemicals to more complex tannins derived from polyphenols. Their classification is based on the arrangement and number of carbon atoms, and is

usually adjoined to organic acids and sugars. They range from 7 to 15 carbon atoms and more i.e., C_6-C_1 (phenolic acids), C_6-C_2 (acetophenols), C_6-C_2 (phenylacetic acids), C_6-C_3 (hydroxycinnamic acids), C_6-C_3 (coumarins), C_6-C_4 (naphthoquinones), C_6-C_1-C_6 (xathones), C_6-C_2-C_6 (stilbenes), C_6-C_3-C_6 (flavonoids) and polyphenols such as tannins $(C_6\text{-}C_3\text{-}C_6)_n$ (Crozier, 2006; Taiz and Zeiger, 1991). These chemicals are synthesized from phenylalanine a product from the shikimic acid pathway and or malonate pathway. They have diverse functions including plant defence (pathogenic or herbivory), UV protection and as seed-dispersal attractants.

Another group of plant secondary metabolites are the alkaloids which are low molecular weight nitrogen-containing chemical compounds. They are biosynthetically derived from amino acids such as ornithine, lysine, tyrosine and tryptophan. Alkaloids may be linked to a five or six carbon cyclic system. They are a structurally diverse group of compounds found in about 20% of species in the plant kingdom. These phytochemicals may be classified into subclasses according to their carbon skeleton. The alkaloids' subclasses are pyrrolidine- (nicotine from *Nicotiana tabacum*), tropane- (cocaine from *Conium maculatum*), piperidine- (coniine from *Conium maculatum*), pyrrolizidine- (senecionine from *Senecio jacobaea*), quinolizidine- (lupanine from *Lipinus polyphyllus*), isoquinoline- (morphine from *Papaver somniferum*) and indole-alkaloids (vinblastine from *Catharanthus roseus*). These phytochemical groups form the majority of poisons, neurotoxins and social drugs such as jacobine, saxitoxin and caffeine (Kennedy and Wightman, 2011).

Terpenes are a class of diverse plant secondary metabolites with isopentenyl pyrophosphate and dimethylallyl pyrophosphate isomers as their building blocks. These interconvertible isomers are condensed in a sequential manner to produce geranyl, farnesyl, geranylgeranyl pyrophosphate, squalene and phytoene, which are precursors for the major class of terpene phytochemicals (Walton and Brown, 1999). Hence, they have a repetitive five-carbon unit based on isopentane. They are classified according to the number of isoprene units. A single isoprene unit is identified as a hemiterpene, two units (monoterpenes), three units (sesquiterpenes), four units (diterpenes), five units (sesterpenes), six units (triterpenes), eight units (tetraterpenes) and more than 8 isoprene units make up the polyterpenes (Kennedy and Wightman, 2011). The biological activities of terpenes are as varied as their structural diversity.

Crozier at al. (2006) state that they range from being phytoalexins in plant defence, plant growth regulators, insect attractants and have medicinal bioactivities too. These phytochemicals also form the structural part of major biologically active components in plants such as the phytol side-chain of chlorophyll, tetrahydrocannabinol in marijuana and Taxol an anti-carcinogenic drug. Saponins which are triterpenes also form a part of this class of bioactive phytochemicals. They are postulated to possess desirable effects on the reduction of ruminant enteric fermentation and methane emission alleviation (Sparg et al., 2004; Patra and Sexena., 2010; Buddle et al., 2011).

Sexena et al. (2013) describes saponins as a group of chemicals that include glycosylated steroids, triterpenes and steroid alkaloids. Monodesmoside saponins have one sugar molecule conjugated at C-3 position and bidesmoside saponins have a minimum of two sugar molecules conjugates at C-3 and C-22 positions. They form part of the plant's defence and protective systems and have biological effects such as antioxidant activity. They have substantial effects on animals' feed intake, reproduction and growth. According to Sparg et al. (2004) haemolytic and molluscicidal activities on protozoa and molluscs, respectively, have also been observed as properties of saponins. Furthermore, saponins are toxic to invertebrates but have no toxic effects on humans such that Asian fisherman would poison their hooks with saponin extracts to catch fish. Knapp et al. (2014) suggests that as the quest to elucidate plant natural products for advancements in ruminant enteric fermentation and biogenic methane mitigation, the bioactivities, pharmaceutical and medicinal properties of saponins remain the most optimistic solution.

2.2.2 Phytochemicals and their applications

The use of phytochemicals for various ailments dates back to many centuries ago. The isolation of alkaloids such as morphine and quinine, among others, in the early 19th century elevated the screening of medicinal plants for their bioactive chemical properties. French pharmacists (Peletier and Caventou) isolated quinine from the Cinchona tree in 1920 and aspirin was isolated from the willow bark (genus *Salix*) by German chemist Hoffmann in the mid-19th century (Sexena et al., 2013). Samuelsson and Bohlin (2009) discussed the significance of phytochemicals based on their bioactivities and isolated bioactive compounds in some plant species. This also

illustrates that phytochemicals are widely distributed in the plant kingdom and are commercially viable and beneficial to mankind.

Ray et al. (2004) illustrates properties of numerous medicinal plants (antifungal, antibacterial, antiviral activities) and isolated chemical compounds. The bio-effectiveness is furthermore attributed to synergistic relations and variations of phytochemical content within an extract. Phytochemicals such as pro-vitamin A, ascorbic acid, folate and fibre have been identified in the *Carica papaya* fruit which is ranked in the top five nutritionally beneficial fruits among others like guava, grapefruit, watermelon and kiwi fruit (Ikram et al., 2015). Fruits form part of neutraceutical products that have attracted considerable attention in recent years due to nutritional and pharmaceutical benefits when they form substantial amounts in human diets. Considerable amounts of neutraceuticals are derived from herbal tea e.g. *Camellia sinensis,* with suggestions that it may help prevent cancer (López-Gutiérrez et al., 2015).

Other phytochemicals like phenolics, phytosteroids and triterpenoid derivatives are exploited for pharmacological activities. Potential bioactivities including chemopreventive agents, antioxidant and cardioprotective effects have been demonstrated by studies on *Hibiscus* sp. plants (Maganha et al., 2010). Elekofehinti's (2015) review of saponins with antidiabetic properties covers a range of plant saponins from *Dioscorea rotundata, Garcinia kola, Ponax notoginseng* and *Solanum anguivi.* These have been reported to impose hypoglycemic activity making them ideal candidates for use as antidiabetic agents due to their antioxidant effects and ability to regulate plasma glucose levels. The saponin extract Quil A from the plant *Quillaja saponaria* (Rosaceae) is used as an adjuvant in a foot-and-mouth vaccine. Other applications include the usage as an expectorant (respiratory organ infections) and as a mild detergent in fine textiles (Samuelsson and Bohlin, 2009).

Bioactivities such as haemolytic, antiinflammatory, antifungal, antimicrobial, antiviral and antitumour have been attributed to saponins. Furthermore, their antiinflammatory properties have been demonstrated in numerous studies. Fawole et al. (2009) investigated twelve medicinal plants for the treatment of gastro-intestinal ailments in which a dichloromethane extract (62.5 µg/ml) of *Agapanthus campanulatus* exhibited 83.7% cyclooxygenase-2 inhibitory effect. This cyclooxygenases assay accentuated

the use of *A. campanulatus* in treatment of gastro-intestinal tract infections. The use of saponins in rumen fermentation may be attributed to their destructive effects on protozoa. Saponins bind with sterols in protozoa which lead to the destruction of the cell membranes. The effects of phytochemicals have been demonstrated in other studies to manipulate rumen fermentation and methane gas production (Tan et al., 2011; Bodas et al, 2012).

2.2.3 Occurrence of saponins in the plant kingdom

Saponins, like most phytochemicals are wide spread across numerous plant families as suggested by Sparg et al. (2004). The word 'saponin' is derived from the *Latin* words '*sapo, onis'* which means soap, which is a characteristic property but not limited to plants in the genus *Saponaria*. Plants in the genus *Saponaria (Saponaroa officinalis* L.*)* were historically used as a substitute for soap (Arora, 2010). Saponins are amphiphilic in nature, such that an aglycone unit (hydrophobic) is linked to one or more sugar molecules (hydrophilic). This gives them their distinctive soap-like property. They may be classified into two major sub-groups i.e. steroidal and triterpenoid saponins. Triterpenoid saponins have a triterpenoid aglycone and consist of a 30 carbon skeleton with a pentacyclic structure. Steroidal saponins have a steroidal aglycone and consist of a 27 carbon spirostane skeleton which lacks three methyl groups in contrast to triterpenoid saponins (Sparg et al., 2004; Vincken et al., 2007). However, Crozier et al. (2006) describes steroidal saponins as phytochemicals that contain a flat cyclopenta [*a*] phenanthrene skeleton and an aliphatic sidechain. Saponins' toxicity varies based on administration i.e. oral and intravenous. The latter is highly toxic, this prevents clinical applications of saponins as drugs. However, they may only be absorbed in small amounts in the intestines (Sparg et al., 2004).

Saponins' highly polar sugar residues make this class of phytochemicals difficult to isolate and purify. However, there are several crude triterpenoid saponin drugs with numerous applications. These include; ginsenosides (protopanaxadiol and protopanaxatriol) from *Aralia quinquefolia* (Araliaceae) formerly known as *Panax ginseng*, which are panacea and adoptogens; Aescin a mixture of saponins from *Aesculus hippocastanum* (Sapindaceae) used to treat chronic venous insufficiency; and Senegae radix from *Polygala senega* (Polygalaceae) which has presenegenin as its aglycone, is used as an expectorant (Samuelsson and Bohlin, 2009). According to

Sparg et al. (2004) inflammation disorders traditionally used to be treated with dry roots of *Bupleurum fruiticescens* (Apiaceae), which has a bioactive chemical called a saikosaponin. It is evident that saponins have various bioactivities and are distributed across numerous plant families in the plant kingdom. Their chemical elucidation may be a challenge, however crude extracts still seem to be biologically active with a wide array of pharmaceutical properties.

2.2.4 *Helinus integrifolius* and its properties

Helinus integrifolius (Lam.) Kuntze (Rhamnaceae) commonly known as soap bush or soap creeper (Figure 2.1), is a sprawling climber with unbranched tendrils. It is widely distributed in the savannah and open woodlands of the Northern provinces of South Africa, Eastern Cape, KwaZulu Natal, Namibia, Botswana and spreads through to tropical Africa and Kenya (Fabian and Germishuizen, 1997). It has small white to green flowers which are predominant between October and April, the fruits are edible and black when ripe (Pooley, 2003). The plant's common names are derived from the plant's properties since it was used as a soap substitute (van Wyk and Gericke, 2003).. Zukulu et al. (2012) indicates that the plant is used by young men seeking affection of women as a charm plant and diviners believe it drives competitors away. Ray et al. (2004) and Shai et al. (2013) illustrates that the dichloromethane *folium* and *cortex* extracts of *H. integrifolius* showed substantial activity against *Staphylococcus aureus, Candida albicans* and *Escherichia coli*. However, Gundidza (1987) highlights that aqueous extract of *H. integrifolius* leaves is substantially active against *S. aureus* and *C. albicans*.

Figure 2.1 The *Helinus integrifolius* (Lam.) Kuntze plant

2.2.5 Extraction and analysis of phytochemicals

The extraction of phytochemicals is the selective separation and or partitioning of potentially bioactive substances. This may be from plant parts known to contain phytochemicals i.e. leaves, stems, flowers or roots. The preliminary selection of plants and parts utilised is usually prompted by ethnobotanical researchers with prior knowledge from traditional healers and the general public. There are numerous factors that may influence the method of extraction such as extraction period, size of plant material, solvent(s) used and solvent to sample ratio. Soxhlet extraction, maceration, decoction, sonication, reflux, microwave-assisted extraction, percolation, infusion and serial exhaustive extraction procedures may be employed for adequate phytochemical extraction (Tiwari et al., 2011).

Once phytochemical extraction is completed an extract may be screened directly or dried further and diluted to desired concentrations. The extract is examined for the presence of myriad phytochemicals using preliminary screening techniques prior complete elucidation. Phytochemicals such as alkaloids, tannins, saponins, flavonoids may be detected by Wagner's test, gelatin test, froth/foam test, and alkaline reagent test respectively. Moreover, the identification may be done by thin layer chromatography, column chromatography and further elucidation may be via high pressure liquid chromatography-mass spectrophotometry (HPLC-MS) and nuclear magnetic resonance spectroscopy (NMR). These phytochemical analysis techniques are employed for specific organic chemical groups and structural identification (Wagner and Bladt, 2001).

2.3 MATERIALS AND METHODS

2.3.1 Plant collection and identification

The *Helinus integrifolius* twigs (stems with leaves, flowers and fruits) were collected during the summer season (February 2014) from their natural habitat in the Sekhukhune District of the Limpopo Province and identified (voucher specimen number, BMDK#2014/02) at the Larry Leach Herbarium (UNIN) at the University of Limpopo. The flowers and fruits assisted in the identification of the plant.

2.3.2 Extraction and extract preparation

The leaves were dried at ambient temperature and ground to a fine powder with a pestle and mortar. A powdered sample of 50 g was defatted by heating overnight in

300 ml n-hexane in Soxhlet apparatus. The hexane extract was collected by filtering through Whatman no.1 qualitative filter papers (12 cm). The leaf residues were extracted overnight for saponins and other secondary metabolites in 300 ml methanol and filtered. The hexane and methanol extracts were evaporated to dryness in a rotary evaporator (Büchi Rotavapor-R) and under a draft of air in a fume chamber in pre-weighed glass Petri dishes, to evaporate the n-hexane and methanol, respectively. The extracts were further dried overnight at 60 °C in an incubator (Gallenkamp Economy Incubator Size 1) and were stored at -21 °C until use. The dried extracts were used to make stock solutions of 50 mg/ml, which were stored at 4 °C for comparative phytochemical analysis.

2.3.3 Chemical constituent analysis

2.3.3.1 *Froth test*

A froth test for saponins was carried out according to the method of Raphael (2012). The n-hexane and methanolic crude leaf extracts (100 mg) were added to 10 ml distilled water in separate test tubes. The tubes were vigorously shaken (vortex) for 2 minutes on a Finsons Whirlmixer.

2.3.3.2 *Thin layer chromatography*

A graduated pasture pipette was used to spot (10 µl) of the methanolic crude saponin extract (stock solution of 1 mg/ml) on an aluminium TLC sheet (pre-coated TLC-sheets Alugram® SIL G/UV). The TLC sheets were placed in tanks containing solvent systems: (1) Chloroform-methanol-water (70:30:4) and (2) Chloroform-glacial acetic acid-methanol-water (64:32:12:8). As the front was reached the TLC-plate was air-dried, sprayed with spray reagents: 2, 2-diphenyl-1-picrylhydrazyl (DPPH, 0.1 mM), vanillin-phosphoric acid (VPA), natural products-polythylene glycol reagent (NP/PEG or NEU-reagent) and anisaldehyde-sulphuric acid (AS) as recommended by Wagner and Bladt, (2001). Digitonin, a steroidal saponin, and ginsenoside Rg_3, a triterpenoid saponin from *Digitalis purpurea* (Plantaginaceae) and *Aralia quinquefolia (*previously called *Panax ginseng)* (Araliaceae) respectively, were used as standards. According to Sparg et al, (2004) the plant family Rhamnaceae generally has bioactive triterpenoid saponins hence the above stated standards were used. The TLC-plates were then viewed under visible light (vis.) and at UV-365 nm, to visualise terpenoids or saponins and any antioxidant active bands by DPPH.

2.3.3.3 *Tannin test by radial diffusion*

The methanolic crude saponin extract was tested for the presence of tannins according to the method of Hagerman (1987). An acetate buffer (pH 5.0) of 0.05 M acetic acid and 60 µM ascorbic acid was prepared by dissolving a mass of 5.28 g ascorbic acid in water and 1.43 ml acetic acid was added. The volume was made up to 500 ml with water. A mass of 1.0 g agarose was added to 100 ml buffer and heated with shaking until it melted. It was cooled to 45 ºC in a water bath. Bovine serum albumin at 0.5% w/v was added and shaken to dissolve and the mixture kept at 35 °C. The mixture was dispensed into 90 mm Petri dishes, cooled and sealed with parafilm. For radial diffusion, three wells (9.0 mm diameter) were made and 100 µl of extract (methanolic crude saponin extract of 10.0 mg/ml) was added. The petri dishes were closed, sealed with parafilm and incubated at 35 °C for 96 hours. Tannin standards were prepared from a stock solution of 250 mg/ml tannic acid. A dilution series of 2.5; 5.0; 10.0 and 20.0 mg/ml was prepared. On separate wells 100 µl of each solution was added. After incubation, the diameter of tannin rings was measured. A standard curve was plotted and the tannin concentration of the extracts determined from the curve.

2.3.4 Colorimetric spectrophotometry analysis

2.3.4.1 *Total phenolics*

The Folin-Ciocalteau method was used to determine total phenolics with the aid of standard curve utilising gallic acid (Torres et al, 1987). A dilution series of 0.00, 0.25, 0.50, 1.0 and 2.00 mg/l gallic acid was made into 10 ml graduated test tubes. Absorbance of both standards and samples was read at 765 nm (Varian, Cary 1E UV-Visible Spectrophotometer). The concentration of phenolics in the methanolic crude saponin extract was determined as gallic acid equivalents.

2.3.4.2 *Flavonoids*

Total flavonoids were determined according to the ‘Aluminium chloride colorimetric assay’ according to Marinova et al. (2005). An aliquot of 200 µl of the extracts or catechin standard solution was transferred into a 10 ml graduated test tube. Distilled water of 2 ml was added followed by 1.5 ml of 5% sodium nitrate and mixed well. The tubes were incubated at room temperature for five minutes after which 0.15 ml of 10% aluminium chloride was added followed by 1 ml of 1M sodium chloride. Distilled water

was used to make up the volume to 10 ml. The tubes were mixed well and absorbance was read at 510 nm (Varian, Cary 1E UV-Visible Spectrophotometer). For the standard curve, 0.200 g catechin was dissolved in methanol to a final volume of 100 ml to make a stock solution of 2 000 mg/L. A dilution series of 0.00, 0.25, 0.50 and 1.00 mg/l was made in tubes in duplicates. The concentration of flavonoids in the methanolic crude saponin extract was calculated as catechin equivalents.

2.3.4.3 *Saponins*

The determination of saponin content was conducted by colorimetric spectrophotometry methods outlined by Li et al. (2010). The methanolic crude saponin extract was purified by dissolving 0.5 g in 4 ml distilled water. The solution was applied onto a Sep-Pak C18 cartridge, the column was washed with 10 ml water followed by 5 ml of 10 % methanol. The column was then eluded with 5 ml methanol. The methanol was evaporated and the residue was dissolved in acetonitrile. As standard solutions, a stock solution of 2.4 mg/ml diosgenin was prepared in acetonitrile, then 0, 5, 15, 20 and 25 µl of stock solution was transferred to 10 ml test tubes. Samples were prepared in triplicates, 25 µl of the methanolic crude saponin extract was added. In the blank 5 µl of water was added. The solvents were evaporated in a waterbath, thereafter freshly prepared 0.2 ml of 5 % vanillin-acetic acid and 1.2 ml of 70% perchloric acid were added. The test tubes were mixed well and incubated at 70 °C for 20 minutes, cooled under running water for 2 minutes and 5 ml ethyl acetate was added. They were cooled to room temperature, absorbance was read at 550 nm (Varian, Cary 1E UV-Visible Spectrophotometer) and a standard curve was plotted.

2.4 RESULTS

2.4.1 Chemical constituents

2.4.1.1 *Froth test*

The n-hexane extract did not dissolve in distilled water and no foam was observed after shaking vigorously. However, the methanolic crude extract formed stable, honey-comb shaped foam on a graduated test-tube for a period of over 24 hours confirming the presence of saponins. The n-hexane extract was then eliminated from any other experiments.

2.4.1.2 *Thin layer chromatography*

The chromatograms were sprayed with various spray reagents and viewed in visible light (vis.) and UV-365 nm (UV). In solvent system 1 (chloroform-methanol-water, 70:30:4) only three clear major bands were observed in visible light when sprayed with DPPH at R_f values; 0.1, 0.6 and 0.7 (Figure 2.2 A). The DPPH detected antioxidant active bands. In addition, a blue fluorescent band was visible when the plate was visualised in UV-365 nm at R_f 0.8. Red (R_f value 0.9) and blue (R_f values 0.8 to 0.9) bands were observed when the plate was sprayed with vanillin-phosphoric acid reagent and heated at 100 °C (Figure 2.2 B). In Figure 2.3 B, the standards used were (1) digitonin (R_f values 0.5 and 0.6), (2) ginsenoside Rg3 (R_f value 0.9), they were yellow and purple, respectively. In solvent system 2 (chloroform-glacial acetic acid-methanol-water; 60:32:12:8), plates were sprayed with 2,2 diphenyl-1-picrylhydrazyl (DPPH), natural product-polyethylene glycol (NP/PEG) and anisaldehyde-sulphuric acid (AS) reagents (Figure 2.3 A, B and C), respectively. Plate sprayed with natural product-polyethylene glycol reagent showed numerous fluorescent bands when viewed under UV-365 (Figure 2.3 B). With regard to the AS spray the ginsenoside Rg3 was observed as a purple band (Rf value 0.8) and digitonin bands were yellow (R_f values 0.5 and 0.7) and brown (R_f value 0.6). At Figure 2.3 C, the methanolic crude saponin extract showed grey bands between Rf values 0.1 and 0.5 and brown/yellow bands in between Rf values 0.4 and 0.7.

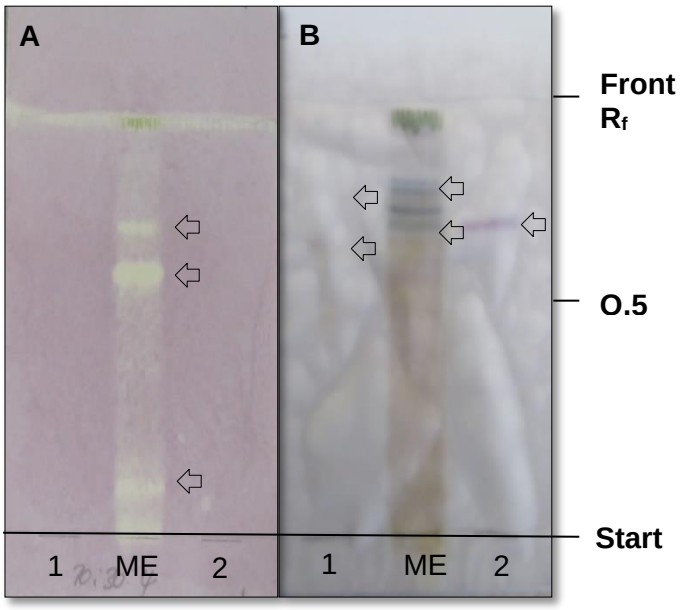

Figure 2.2 Methanolic extract on solvent system (chloroform-methanol-water; 70:30:4) chromatograms sprayed with A) DPPH and B) VPA in vis. (1: digitonin; ME: methanolic extract; 2: ginsenoside Rg_3). Arrows indicate significant bands discussed in section 2.5.1.2

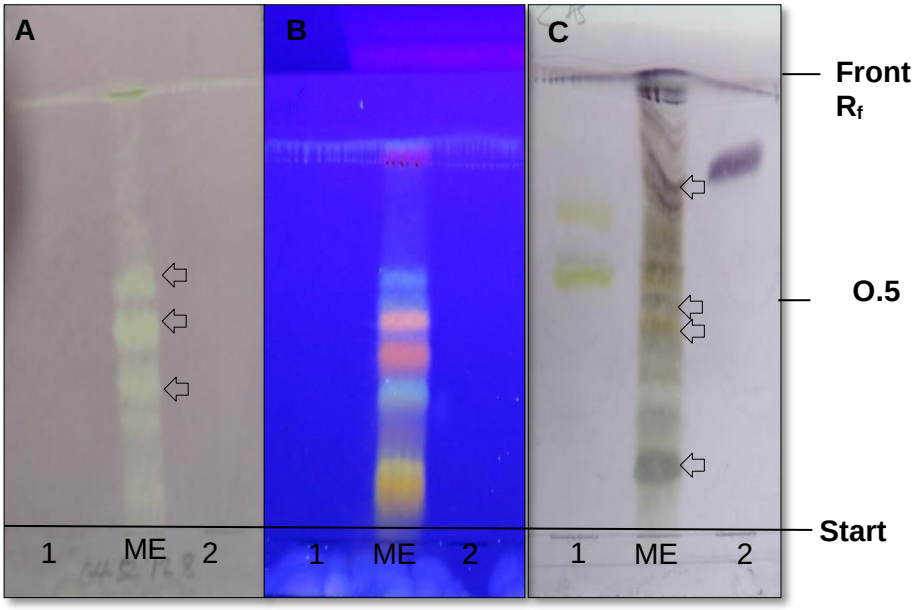

Figure 2.3 Methanolic extract on solvent system (chloroform-glacial acetic acid-methanol-water; 60:32:12:8) chromatograms sprayed with A) DPPH vis., B) NP/PEG in UV and (C) AS in vis. (1: digitonin; ME: methanolic extract; 2: ginsenoside Rg_3). Arrows indicate significant bands discussed in section 2.5.1.2

2.4.1.3 *Tannin test by radial diffusion*

The methanolic crude extract of *H. integrifolius* was investigated for the presence of tannins with tannic acid as a standard. The methanolic crude saponin extract did not exhibit any radial diffusion as indicated in Figure 2.4 (A and B). The wells in Figure 2.4 B had no radial variation to the initial wells before incubation.

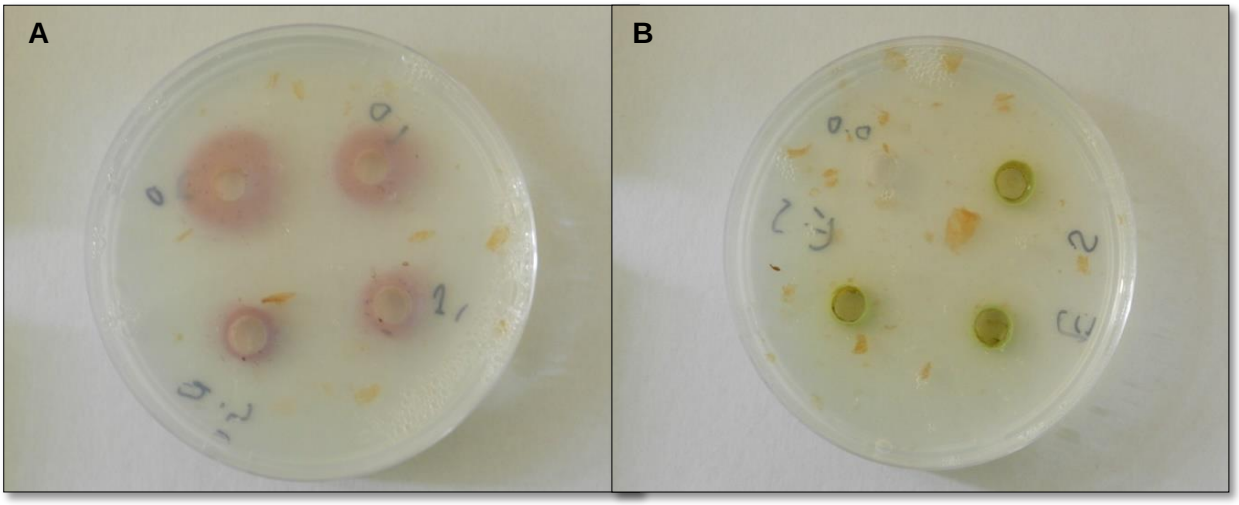

Figure 2.4 Determination of tannins from methanolic crude saponin extract. A) Tannin standards, tannic acid in varied concentrations 0.25; 0.50; 10.0; and 20.0 mg/ml and B) The leaf extract did not exhibit any radial diffusion

2.4.2 Colorimetric spectrophotometry

The phytochemical content of the methanolic crude saponin extract of *H. integrifolius* was analysed and compared to chemical content of other plant species. The saponin, flavonoid and phenolics content were recorded as 286.0±0.60, 19.0±0.40 and 23.3±0.50 mg/g, respectively, as shown in Table 2.1.

2.5 DISCUSSION

2.5.1 Chemical constituents

2.5.1.1 *Froth test*

A froth test was performed to establish the presence of saponins in the n-hexane and methanolic crude leaf extracts. Upon vigorous shaking, stable, honey-comb shaped foam was observed on the methanolic crude leaf extract. This was similar to observations in a study by Raphael (2012), in which a stable foam was formed on chloroform extracts from plants *Aloe vera* (Asphodelaceae) and *Azadirachta indica* (Meliaceae). This chloroform extracts were obtained via Soxhlet extraction. Tiwari et al. (2011) also suggests the froth test as a preliminary method to detect the presence of saponins in plant extracts. The current study is based on the use of a methanolic crude saponin extract, therefore the absence of foam in the n-hexane extract eliminated it from further experiments.

2.5.1.2 *Thin layer chromatography*

The methanolic crude saponin extract was analysed for phytochemical content by pre-coated TLC-sheets Alugram® SIL G/UV with various TLC solvent systems and spray reagents. Sparg et al. (2004) suggests that the plant family Rhamnaceae was observed to contain a wide variety of triterpenoid saponins. Thus, the plant *H. integrifolius* was also expected to contain “triterpenoid saponins” as it is classified into the same plant family. No similarities of the two yellow zones (Figure 2.3 C) or their R_f values (0.5, and 0.7) were observed on TLC fingerprints when digitonin (non-ionic detergent) was run as a standard with the methanolic crude saponin extract. Ditigonin is a steroidal saponin, found in *Digitalis purpurea* and *Digitalis lanata* (Samuelsson and Bohlin, 2009). However, a triterpenoid (ginsenoside Rg_3) standard was also utilised and the same R_f value (0.8) was recorded on both solvent system 1 and 2. Although the latter standard did not match any bands within the methanolic crude saponin extract, bands with similar colour where observed when vanillin-phosphoric

acid spray reagent was used (Figure 2.2 B). The natural reagent spray (NP/PEG) at Figure 2.3 B, visualised numerous orange, orange-yellow flavonol glycoside bands similar to those found in *Ginkgo folium*. Furthermore, the blue (R_f value 0.7) and orange-yellow (R_f value 0.6) bands observed also induced clear white zones when prayed with DPPH (Figure 2.3 A), another clear zone was at R_f value 0.3. This implies that the unidentified bands have antioxidant activity and may be flavonoids with various functional groups. It was also noted that similar blue and orange-yellow bands generated grey-blue (R_f value ~0.7) and brown-yellow (R_f value ~0.6) bands in anisaldehyde-sulphuric acid reagent (Figure 2.3 C). These bands were similar to those found in by *Sarsaparillae radix* and *Ginseng radix* extracts as observed by Wagner and Bladt, (2001). The observations raise questions to the specifications of these antioxidant active phytochemical bands. Are they triterpenoid saponins inducing different bioactivities due to their variation in functional groups? If so, this makes the crude extract ideal for various exploitations in attempts to identify bioactive phytochemicals for various applications. However, the TLC fingerprint did not confirm the presence of triterpenoid saponins.

2.5.1.3 *Tannin test by radial diffusion*

The methanolic crude saponin extract did not exhibit any radial diffusion (Figure 2.4 B), as observed in the tannic acid treatments in Figure 2.4 A). Thus, it was presumed that no tannins were present. According to Würger at al. (2014) an acetone extract of *Ziziphus mucronata* (Rhamnaceae) was established to contain tannins of 0.7 mg/ml gallic acid equivalent. However, the highest tannin content in the mentioned study was 6.2 mg/ml (plant family Anacardiaceae). The same bioassay was utilised to detect tannins in several varieties of *Lotus corniculatus* (Fabaceae), in which tannin content was between 0.3 to 1.0% on a dry material basis (Hedqvist et al., 2000). Maier et al. (2017) screened European medicinal herbs for their tannin content and potential as tannin agents in the leather industry. High tannin content of 32.1 and 38.4 w% in aqueous extracts of *Potentilla erecta* (Rosaceae) and *Rubi fruticosus* (Rosaceae) were recorded, respectively. In the current study tannin content was not quantified as the wells before and after incubation were of similar diameter.

2.5.2 Colorimetric spectrophotometry

The methanolic crude saponin extract was analysed for the various phytochemicals by colorimetric spectrophotometry assays. The saponin content was quantified and considered to be very high (286.0 mg/g) in comparison to a methanolic extract of *Yucca glosoria* (Asparagaceae) which is suggested to have high content (238.6 mg/g) of saponins by Skhirtladze et al. (2011). The methanolic crude saponin extract was further quantified for total phenolics and flavonoids (Table 2.1). This was established to emphasise that no individual chemical acts on its own to impose certain biological activities. Total phenolics and flavonoids were at lower quantities in relation to saponins hence the formation of stable honey-comb shaped foam upon vigorous shaking. Kaškonienė et al. (2015) observed that there are variations between fresh and dried material's total phenolic content and total flavonoid content. According to the authors' findings total phenolic content (expressed as rutin equivalents) was significantly reduced in dried plant material samples to a range of 70.6 to 126.8 mg/g compared to a range of 180.2 to 324.3 mg/g in fresh plant material samples. However, total flavonoid content of dried plant material samples increased by up to 2-fold and in some samples decreased 1.6 times in comparison to fresh plant material samples. This implies that the technique of drying plant material for phytochemical analysis may influence the quantification of phytochemical content. These may either present lower or higher phytochemical content than what is actually present in the fresh plant material.

TABLE 2.1 THE QUANTIFICATION OF PHYTOCHEMICALS USING COLORIMETRIC SPECTROPHOTOMETRY ASSAY COMPARED WITH OTHER FINDINGS IN LITERATURE

SPECIES	PHYTOCHEMICAL CONTENT (MG/G)			REFERENCES
	SAPONINS	FLAVONOIDS	PHENOLICS	
Helinus integrifolius	286,0±0.60	19,0±0.40	23,3±0.50	Experimental
Yucca glosoria L.	236,6±0.80	-	-	Skhirtladze et al. (2011)
Aspalathus linearis	-	6,7±0.02	-	Bramati et al. (2002)
Peltophorum africanum	-	4,5±0.15	12,2±0.03	Mulaudzi et al. (2011)
Chamerion angustifolium	-	14.3 - 41.0	180.2 - 324.3	Kaškonienė et al. (2015)

2.6 CONCLUSION

A froth test confirmed the presence of saponins in the methanolic crude extract of *Helinus integrifolius*. Furthermore, the saponin content was established to be 286.0±0.60 mg/g of dry material by colorimetric spectrophotometry analysis. The saponins were confirmed not to be steroidal saponins, hence they were presumed to be triterpenoid saponins. No substantial radial diffusion was induced by the methanolic crude saponin extract when a tannin test by radial diffusion was performed. Numerous bioactive bands were observed on thin layer chromatograms sprayed with reagents natural product-polyethylene glycol, anisaldehyde-sulphuric acid, vanillin-phosphoric acid and 2,2-diphenyl-1-picrylhydrazyl, respectively. However, thes bands presumed to be bioactive were not identified.

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CHAPTER 3

IN VITRO EFFECT OF *HELINUS INTEGRIFOLIUS* (LAM.) KUNTZE ON RUMINANT TOTAL GAS EMISSION

3.1 INTRODUCTION

The impacts of global warming are very detrimental to the overall structure of our environment. They lead to severe drought or high rainfall, melting of ice caps, and deforestation caused by overgrazing ultimately leading to erosion. Global warming is due to the accumulation of greenhouse gases (carbon dioxide, methane, nitrous oxide and carbon monoxide) beneath the ozone layer. This prevents radiant energy from escaping to the upper atmosphere. As a result, it is trapped between the earth's surface and lower atmosphere. This in turn induces a warming effect on the earth surface over time (Bodas et al, 2008). There are numerous anthropogenic activities that lead to the emission of greenhouse gases e.g. livestock production, landfills and burning fossil fuels. Carbon dioxide (CO_2) accounts for about 35 – 60% of anthropogenic gas emissions. While the global emission of methane (CH_4) is 16% its global warming potential is 21 times more than that of CO_2 (Scheehle and Kruger, 2006). Global methane emissions should be lowered just as much as all other anthropogenic activities that contribute to global warming. Bodas et al. (2008) state that livestock single-handedly produces 80 Mt of methane annually at a global scale. This makes them the major contributors of biogenic methane emissions. The reduction of biogenic methane emission may ultimately lead to reduced levels of methane concentrations in the atmosphere.

Biogenic methane in ruminants is produced during ruminal anaerobic fermentation of hydrolysed dietary carbohydrates. It forms part of loss in energy (~8% of gross energy) as suggested by Bhatta et al. (2011). This biogenic methane is produced by the activity of methanogenic archaea. The primary substrates for ruminal methanogenesis are carbon dioxide and hydrogen. The microbial fermentation of ammonia and volatile fatty acids also generates substantial quantities of methane. There are various techniques employed to quantify methane production in ruminants both *in vivo* and *in vitro*. The latter techniques have different advantages and disadvantages including cost efficiency and various ways of expressing the gas production. The reduction of methane emissions using techniques such as defaunation, feed additives and supplementation with unsaturated fatty acids, organic acids, sulphate supplements and herbal extracts has been extensively investigated according to Bunglavan (2014).

Plant extracts have been investigated for their potential to reduce methanogenesis which has been attributed to their phytochemical content. Bunglavan (2014), demonstrated that triterpenoids and steroid saponins of *Acacia concinna* exhibit an inhibitory effect on ruminal protozoa and a decrease in ruminal methane production was observed from plant extracts of *Terminalia chebula* (seed pulp) and *Allium sativum* (garlic oil). Saponins have a mechanism to inhibit microbial activities such as the breakdown on proteins in the rumen and antimicrobial properties, antifungal, anti-inflammatory, antibacterial, antitumor and antiviral activities (Sparg et al., 2004). *Helinus integrifolius* commonly known as “soap bush or seepbos” potentially has high concentrations of saponins based on its traditional uses (van Wyk and Gericke, 2003).

3.1.1 Motivation of the study

According to the Intergovernmental Panel for Climate Change (IPPC) (2007), it is evident that one of the major drivers of global warming is methane from agricultural activities. Thus, anthropogenic activities such as livestock farming, rice farming and dumping sites in addition to burning fossil fuels contribute substantial amounts of methane that adds on the global accumulation of greenhouse gases (Hannah, 2014). As global human population increases the demand for meat, milk and other products from livestock exponentially increases. This reliance on ruminants by mankind makes it impossible to eliminate or reduce livestock production in relation to demand (Dick et al., 2015).

The latter observations illustrate the need to establish good farming practices and/or cost-efficient dietary additives. Ruminants’ methane is produced primarily in the fore-stomach (rumen) due to methanogen activity (Bueno et al., 2015). The use of plant secondary metabolites to manipulate ruminant enteric fermentation may be the only viable option as recently demonstrated in numerous ‘ruminant gas reduction’ studies (Puchala et al., 2005; Bhatta et al., 2007; Animut et al., 2008; Bodas et al., 2008; Hart et al., 2008; Buddle et al., 2011; Bunglavan, 2014; Patra and Yu, 2014). This study investigated the potential use of a methanolic crude saponin extract and leaf powder of *H. integrifolius* as potential feed additives for the alleviation of *in vitro* ruminant total gas production.

3.1.2 Aim of the study

The aim of the study was to investigate the effects of a methanolic crude saponin extract and leaf powder of *Helinus integrifolius* on *in vitro* ruminant total gas production.

3.1.3 The main objectives were:

i. To quantify ruminant total gas emission due to varied crude saponin extract concentrations.
ii. To quantify ruminant total gas emission due to varied percentages of leaf powder inclusion.
iii. To analyse and identify gases produced during ruminant digestion in 24 hour intervals for a period of 96 hours.

3.2 LITERATURE REVIEW

3.2.1 Ruminant gas production

Rumen anaerobic fermentation is dependent on oxidation-reduction reactions which result in formation of methane. The production of methane represents a loss in energy. Furthermore, the activity of protozoa in the rumen promotes an abundance of hydrogen which may be utilised by either homoacetogens or methanogens. Protozoa and other microbial fermentation bacteria have membrane-bound hydrogenases that yield hydrogen by directly oxidising reduced electron carriers such as NADH or ferridoxin (Fenchel and Finlay, 1995). This leads to an accumulation of substantial amounts of hydrogen which affect the NADH-linked membrane-bound hydrogenases. It is methanogenic archea's responsibility to maintain vigorous fermentation by keeping low hydrogen pressure. Then the methanogens may efficiently utilise hydrogen to reduce carbon dioxide to methane as the end-product. However, at low hydrogen concentrations homoacetogens may utilise hydrogen and carbon dioxide for volatile fatty acid production which is used by the animal as a form of energy (Buddle et al., 2011).

The decarboxylation of metabolites and bicarbonate buffering during fermentation leads to abundance of carbon dioxide. The hydrogenation of carbon dioxide and partial oxidation of biomass yields formic acid and other by-products. Carbon dioxide and hydrogen are substrates for methane production by methanogens. However, they

may also utilise formic acid directly. The major genera of rumen methanogens are Methanobrevibacter *spp., Methanomicrobium spp., Methanocrococcus spp., Methanoculleus spp., Methanosphaera spp., Methanobacterium spp. and Methanosarcina spp.* The accumulation of methane in the rumen is mainly due to their activity. As the gas accumulates it may be released by either eructation or flatulation, which then exposes it to the atmosphere (Bodas et al., 2012).

3.2.2 Reduction of biogenic gas production

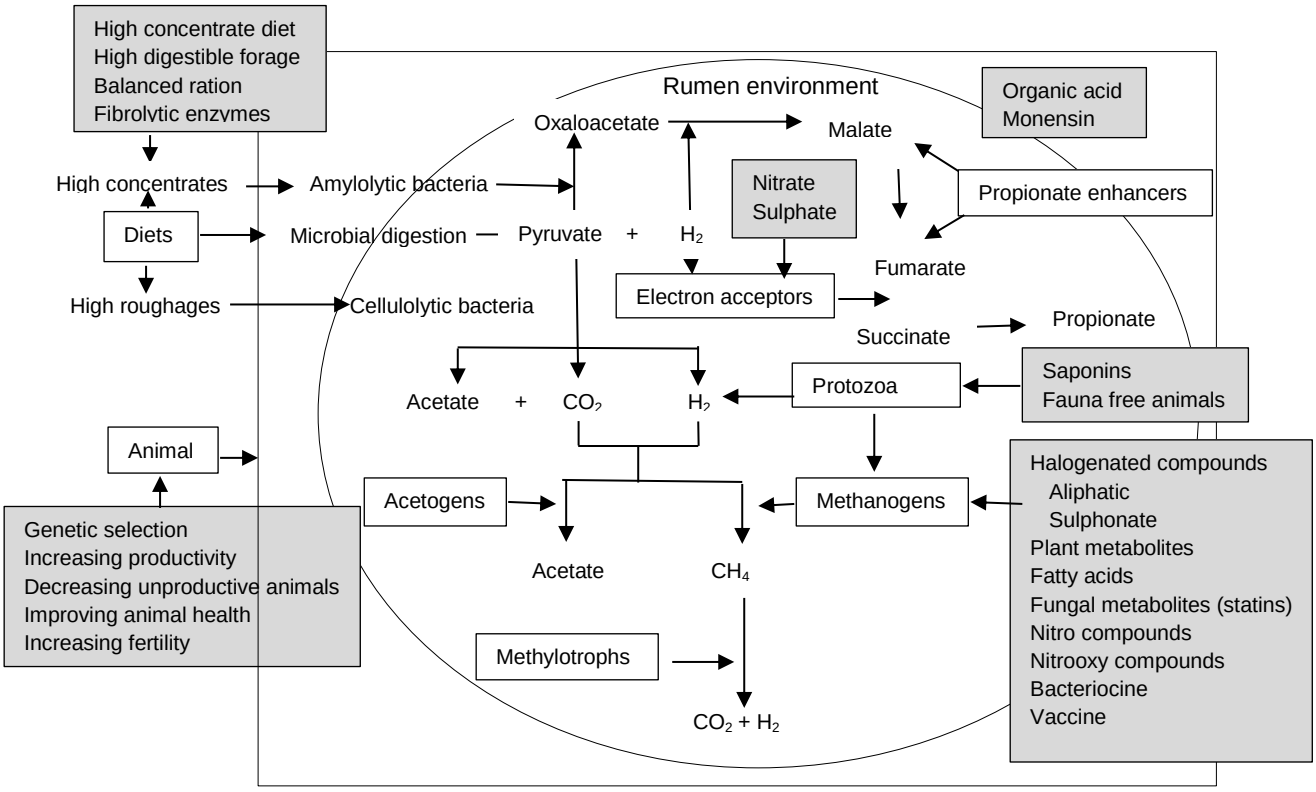
The substantial inhibition or reduction of gas production in ruminants has been demonstrated as a potent possibility in numerous studies both in vivo and in vitro (Animut et al., 2008; Buddle et al.,2011; Bunglavan, 2014; Bueno et al., 2015; Castro-Montoya et al., 2015). Although it remains a major challenge, plant additives such as forage rich in tannins, saponins, essential oils and other plant secondary metabolites show contrary effects on ruminant gas production alleviation (Bodas et al., 2012). According to Patra (2016), effective mitigation agents may be methanogen-specific inhibitors only if they do not affect other valuable microbes in the rumen (Figure 3.1. However, targeting protozoa may drastically reduce H_2 availability in turn decreasing CH_4 production. Plant secondary metabolites such as saponins exhibit antiprotozoal effect i.e., sterols in cell membranes of protozoa form a complex with saponins reducing access H_2 (Bodas et al., 2012). There are suggestions that saponins substantially reduce rumen fermentation in the foregut, resulting in increased fermentation in the hindgut, which promotes hydrogen for metabolic processes such as acetogenesis (Patra and Sexena, 2009).

3.2.3 Effects of secondary products on enteric fermentation

Plant secondary products such as saponins, essential oils, flavonoids and organosulphur compounds have yielded considerable effects in the modulation of rumen microbes which adversely impact on fermentation and methane production. These also influence nitrogen metabolism, bloating and acidosis which in turn may improve the health and productivity of the animal (Patra and Sexena, 2010). There has been established interest in the use of plant secondary products to mitigate enteric methane emissions (Patra and Sexena., 2009). Figure 3.1 illustrates various processes which may be targeted to reduce ruminant gas production (Patra, 2016).

Recent studies have investigated the potential of numerous plant secondary metabolites also highlighting challenges. Patra and Sexena (2010) and Castro-Montoya et al. (2015) indicate that only few studies show direct effects of essential oils on methanogenic archaea such as *Methanobrevibacter smithii*. The energy metabolism of *Streptococcus bovis* and *Selenomonas ruminantium* is inhibited by essential oils (Castro-Montoya et al., 2015). The addition of cinnamaldehyde to barley based diet (0.02 g/kg) only adversely inhibited *M. ruminantium.* However, phylogenetic distribution of methanogenic species (*M. smithii and Methanosphaera stadmanae*) was increased due to the supplementation. *In vitro* methane gas production was inhibited by methanol and ethanol extracts of fennel seeds (anethole, limonene, fenchone, terpinen-4-ol and estagole) and clove buds (cinnamon oils and eugenol) as indicated by Patra and Sexena (2010). Benchaar and Greathead (2011) indicate that in other studies methane, acetate and propionate production was not affected by cinnamaldehyde (132 mg/l) *in vitro*. According to Castro-Montoyo et al. (2015), *in vitro* observations do not reflect *in vivo* findings in some cases.

There has been considerable research about condensed tannins' effect on rumen methanogens and methanogenesis, with suggestions that low molecular weight tannins may be more effective inhibitors of methanogens as suggested by Patra and Sexena (2010). Authors such as Tan et al. (2011) clearly highlight the need to establish suitable supplementation levels with minimum effects of rumen fermentation. Reduction in *in vitro* methane production (33 to 63%) supplemented with *Leucaena leucocephala* hybrid-Bahru (CT/500 mg DM) was observed with increased condensed tannin levels. However, the author also indicated that a tannin extract of quebracho (20 g/kg DM) did not reduce methane emissions in cattle. A decline in acetate and propionate was observed upon condensed tannin inclusion of the latter extract. Bueno et al. (2015) suggest that the reduced condensed tannin effects on methane emissions in various experiments may be that inhibition between methanogenic archaea and protozoa micro fauna does not occur. Thus, it is evident that manipulation of methane emissions should focus on diverting hydrogen away from methane formation to alternative hydrogen disposal pathways in the rumen (Tan et al., 2011). Other plant secondary metabolites such as saponins are also exploited to enhance protein flow into duodenum and substantial inhibition of rumen methanogenesis (Patra and Sexena, 2010).



(Patra, 2016)

Figure 3.1 An overview of various targets that may reduce methane production in ruminants (highlighted in gray background are options and white background are target sites for reduced methane production, reproduced with permission from the correspondence author)

3.2.4 Effects of saponins on enteric fermentation

Saponins have a pronounced effect on ruminal total volatile fatty acid production and antiprotozoal activity (Goel and Makkar, 2012). They inhibit protozoa which are associated with symbiotic methanogens thus reducing a considerable amount of total methane production. High concentrations of saponins are required to directly inhibit methanogens. In most studies saponins have not had any effect of methanogens due to utilisation of low concentrations but a few studies have showe (Patra, 2016) hus, the effects of saponins are dependent on concentrations in the diet. Studies also postulate that these compounds inhibit fungal species and ruminal bacteria (Patra and Sexena, 2009).

Hart et al. (2008) outlines several effects of saponins by various authors, including effects on enteric fermentation, rumen protozoa, microbial degradation, bacteria and methanogens. Ruminal methanogens live in association with protozoa hence saponins' effects on protozoa is detrimental to CH_4 emissions. Other studies suggest saponins' may affect CH_4 production through diminished activity of CH_4 producing genes, thus methanogen activity is reduced with no change in methanogen populations in the rumen. Rira et al. (2015) reports on reduction of total archaeal populations by saponins from *Sesbania sesban* leaves (71%) and *Knautia* leaves (21%). These effects indicate impairment of protozoa structure and functionality, indirectly decreasing methanogenesis. Thus, the decrease is due to establishing an alternative route for H_2 disposal, with excess H_2 channelled to propionic acid production.

Hart et al. (2008) clearly states that there are numerous contradictory effects which may be due to the type of saponin extract and concentrations used, but emphasises on the potential of plant extracts to be exploited as rumen manipulation agents. Rira et al. (2015) also supports plant secondary metabolites as feed additives but highlights the need for detailed investigations on mechanisms of reducing CH_4 production, improving feed digestibility and animal performance.

3.2.5 Strategies to mitigate ruminant methane production

Knapp et al. (2014), outline and discuss three major strategies to reduce methane production in ruminants. These strategies are 1) feed, feeding management and nutrition, 2) rumen modifiers and 3) animal genetic production and other management approaches. Rumen modifiers exist in a wide variety e.g. organic acids and plant secondary metabolites. They may induce direct inhibition of methanogens or methanogenesis, provide alternative pathway for hydrogen disposal or suppress ciliate protozoa. Buddle at al. (2011), suggest a number of mitigation strategies that require consideration, such as vaccination against rumen methanogens. The approach would neutralise rumen methanogens by generating a high yield of antibodies in response to the vaccine. Gill et al. (2000) conform to vaccination of *Streptococcus bovis* (rumen bacterium) as it neutralises methanogenic activity. However, Williams et al. (2008) question stability of antibodies in rumen fluid and no significant methanogen

impairment was evident in addition to only minor changes in rumen microbial fauna (Williams et al., 2009).

The microbial interaction of rumen protozoa, fungi and bacteria (methanogens and homoacetogens) is vital in rumen fermentation, gas (H_2, CO_2, CH_4) production and volatile fatty acid production. Buddle et al. (2011) state that the defaunation (protozoa) approach has often been reported to lower gas emissions, primarily due to reduction in H_2 availability. Acetogens and methanogens compete for H_2 but rumen H_2 concentrations influence which microorganisms ultimately utilise it. Lower H_2 concentrations favour growth rate of acetogens which promote feed-efficiency by using H_2 to reduce CO_2 to acetate (VFA), in contrast to high concentrations which is used by methanogens for CH_4 production (Patra and Sexena, 2010). Therefore, there is a need to find means of lowering rumen H_2 concentrations and promote homoacetogen activity. These may be achieved by various approaches as demonstrated by Patra and Sexena (2010); Bodas et al. (2012); Knapp et al. (2014); and Rira et al. (2015).

It is evident that plant secondary metabolites have an effect on ruminal methanogenesis, population of microbial fauna, fodder digestion and feed-efficiency utilisation as demonstrated in numerous studies (Bodas et al., 2008; Bodas et al., 2012). In addition, investigations on toxicity and side-effects of phytochemical additives/supplements on host animal and comparative findings on *in vitro* and *in vivo* experiments, plant secondary metabolites impose a great potential in mitigation of ruminant gas production.

3.3 MATERIALS AND METHODS

3.3.1 Plant extract preparation

A methanolic crude saponin extract of *Helinus integrifolius* obtained by Soxhlet extraction (Chapter 2 section 2.2) was dissolved in dimethyl sulfoxide (DMSO, a polar aprotic solvent) to a concentration of 50.0 mg/ml. The prepared stock solution was used in the gas production bioassay as indicated in Table 3.1. The untreated ground leaf powder of *H. integrifolius* was also tested for its effect on gas production and the bioassay was set up as indicated in Table 3.2.

3.3.2 Rumen fluid inoculum preparation

Fresh cow rumen fluid was collected at Vencor Abattoir Polokwane, South Africa, immediately after the cow was slaughtered. The rumen fluid was cultured with buffers and substrates (*Zea mays* stover and *Medicago sativa* powder) as suggested by Fon and Nsahlai, (2012) and incubated at 38 °C for 72 hours before use. After incubation, the inoculum was used for *in vitro* ruminant fermentation and gas production bioassay.

3.3.3 Gas production bioassays

3.3.3.1 *Gas volume analysis*

Gas production assay was done according to the method of Getachew et al. (2004). The reactants were added in the reaction mixture to a final volume of 25.0 ml in each 50.0 ml serum bottle as shown in Table 3.1 and Table 3.2. All treatments were done in triplicates. The substrate was composed of *Zea mays* stover and *Medicago sativa* (1:1) and the buffer used was an anaerobic McDougall's buffer. The serum bottles were flushed with carbon dioxide to maintain an anaerobic condition in the reaction chamber. The bottles were incubated at 39 °C in a water-bath with occasional shaking. The volume of the gas produced was measured with a syringe.

3.3.3.2 *Gas chromatography*

After 24, 48, 72 and 96 hours of incubation the reaction chambers (50 ml serum bottles) in a 39 °C waterbath the gas was analysed according to the method of Tan et al. (2011), where the gas was withdrawn with a gas-tight syringe (Terumo micro syringe) for gas analysis using Shimadzu GC-2010 Plus series equipped with flame ionization detector (FID). The gas volume of 500 µl was injected into the split injector port at 250°C, with H_2 as the carrier gas at a flow rate of 4.0 ml/min and 100.7 kPa pressure. The column used for analysis was Zebron ZB-Wax *plus* 30 m x 0.25 mm x 0.25 µm df. Separation was detected at detection temperature of thermal conductivity detector at 255 °C.

TABLE 3.1 REACTANTS IN SERUM BOTTLES FOR *IN VITRO* RUMINANT TOTAL GAS RELEASE DETERMINATION DUE TO METHANOLIC CRUDE SAPONIN EXTRACT INCLUSION

		REACTANTS ADDED						
TREATMENT NO.	**CONCENTRATION (MG/ML)**	**DMSO (ML)**	**ALIQUOT (MG/ML)**	**ALIQUOT (ML)**	**SUBSTRATE (MG)**	**INOCULUM (ML)**	**BUFFER (ML)**	**TOTAL VOLUME (ML)**
1	0,0	0,5	0,0	-	250,0	1,5	23,0	25,0
2	1,0	-	1,0	0.5	250,0	1,5	23,0	25,0
3	2,0	-	2,0	1.0	250,0	1,5	22,5	25,0
4	4,0	-	4,0	2.0	250,0	1,5	21,5	25,0
5	8,0	-	8,0	4.0	250,0	1,5	19,5	25,0
6	10,0	-	10,0	5.0	250,0	1,5	18,5	25,0
7	12,0	-	12,0	6.0	250,0	1,5	17,5	25,0
DMSO, dimethyl sulfoxide; Buffer, McDougall's buffer; Aliquot, methanolic crude saponin extract (Stock solution, 50 mg/ml); Substrate, *Medicago sativa: Zea mays* stover (1:1); Inoculum, rumen fluid								

TABLE 3.2 REACTANTS IN SERUM BOTTLES FOR *IN VITRO* RUMINANT TOTAL GAS RELEASE DETERMINATION DUE TO LEAF POWDER INCLUSION

		REACTANTS ADDED				
TREATMENT NO.	**LEAF POWDER (%)**	**SUBSTRATE (MG)**	**LEAF POWDER (MG)**	**INOCULUM (ML)**	**BUFFER (ML)**	**TOTAL VOLUME (ML)**
1	0,00	250,0	0,0	1,5	23,5	25,0
2	0,25	250,0	0,6	1,5	23,5	25,0
3	0,50	250,0	1,4	1,5	23,5	25,0
4	1,00	250,0	2,7	1,5	23,5	25,0
5	2,00	250,0	5,4	1,5	23,5	25,0
6	4,00	250,0	10,8	1,5	23,5	25,0
7	8,00	250,0	21,6	1,5	23,5	25,0
8	16,00	250,0	43,2	1,5	23,5	25,0
Buffer, McDougall's buffer; Substrate, *Medicago sativa: Zea mays* stover (1:1); Inoculum, rumen fluid						

3.4 RESULTS

3.4.1 Total gas production

3.4.1.1 *Effect of extract concentration on gas production*

In Figure 3.2, it is observed that there was an increase in the volume of total gas released with increases in the methanolic crude saponin extract concentrations at incubation periods 48, 72 and 96 hours when the treatments were compared with the control. In the 24 hour incubation period the addition of methanolic crude saponin extract induced slight increases of gas released from 10.67±1.15 to 12.00±1.00 ml at concentrations 1.00 and 2.00 mg/ml, respectively. Whereafter, a gradual decrease of up to 3-fold which stabilised at 8.00 to 12.00 mg/ml of methanolic crude saponin extract concentrations was observed. In the 48 hour incubation period, a maximum volume of 25.93±0.1.79 ml of gas was released. The volume of gas released was stable between the extract concentrations of 1.00 and 2.00 mg/ml, then a gradual decrease of up to 8-fold was evident and stability in gas released was maintained between extract concentrations 8.00 and 12.00 mg/ml. The methanolic crude saponin extract inclusion in the 72 hour incubation period contributed to a gradual increase to a maximum gas production of 38.00±0.00 ml between extract concentrations 0.00 to 2.00 mg/ml. This was followed by a gradual decrease of up to 12-fold which then stabilised between the concentrations 8.00 and 12.00 mg/ml. In the 96 hour incubation period a maximum gas production of 31.07±0.12 ml at 2.00 mg/ml was obtained. This was followed by a gradual decrease of up to 4-fold then 16-fold at concentrations 8.00 and between 10.00 and 12.00 mg/ml, respectively. In all incubation periods gas was released to a maximum volume then decreased at concentration 8.00 mg/ml in which only substantial amounts of gas were released till the highest extract concentration of 12.00 mg/ml. The ideal incubation period for adequate gas releases analysis was 72 hours, since the highest and lowest gas releases were recorded.

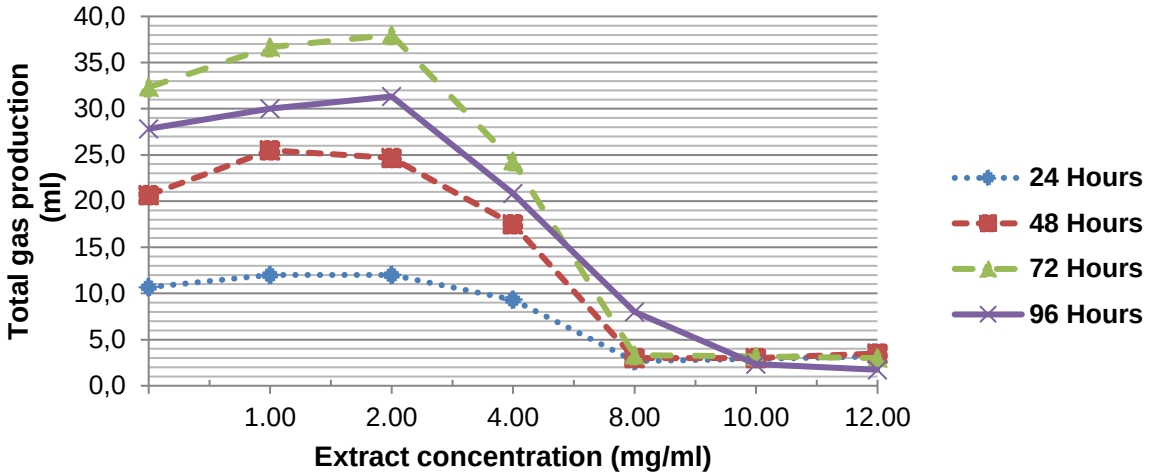


Figure 3.2 The effect of methanolic crude saponin extract on the volume of gas releases during the different incubation periods

One-way *ANOVA* of total gas release due to methanolic crude saponin extraction inclusion indicated that there were statistically significant differences *(F*(51.74105)=103.481, *p* = 0.000 at 24 hours, *F*(215.168) = 430.336, *p* = 0.000 at 48 hours, *F*(589.196) = 1178.392, *p* = 0.000 at 72 hours and *F*(1040.867) = 2081.734, *p* = 0.000 at 96 hours) between groups (Table 3.3). A Tukey post hoc was done with the main focus on the 72 hour incubation period (Appendix B and C). This test revealed that total gas release was substantially high at lower methanolic crude saponin extract inclusion (1.00 and 2.00 mg/ml) at which gas release was 36.67±1.15 and 37.33±1.56 ml, respectively with *p* = .948. It was also revealed that significantly low gas release was recorded at high methanolic crude extract inclusion (8.00, 10.00 and 12.00 mg/ml) at which gas volumes were 3.33±0.58, 3.16±0.29 and 3.00±0.00 ml, respectively with *p* = 0.999. There was no statistically significant difference within methanolic crude saponin extract inclusion of 1.00 and 2.00 mg/ml and no significant variation within high extract inclusion of 8.00, 10.00 and 12.00 mg/ml. However, low extract concentrations induced larger volumes of gas release and high extract concentrations substantially reduced gas release. The 8.00 mg/ml treatment was the lowest saponin extract concentration that induced substantially low total gas release.

TABLE 3.3 ONE-WAY ANOVA OF INCUBATION PERIODS (24, 48, 72 AND 96 HOURS) VERSUS CONCENTRATIONS OF METHANOLIC CRUDE SAPONIN EXTRACT

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
24H	Between Groups	351.540	6	58.590	103.481	.000
	Within Groups	7.927	14	.566		
	Total	359.467	20			
48H	Between Groups	1970.940	6	328.490	430.336	.000
	Within Groups	10.687	14	.763		
	Total	1981.627	20			
72H	Between Groups	4797.738	6	799.623	1178.392	.000
	Within Groups	9.500	14	.679		
	Total	4807.238	20			
96H	Between Groups	3080.967	6	513.494	2081.734	.000
	Within Groups	3.453	14	.247		
	Total	3084.420	20			

One-way *ANOVA* of total gas release due to methanolic crude saponin extraction inclusion indicated that there were statistically significant differences *(F*(149.562) = 299.124, *p* = 0.000 at 0.00 mg/ml, *F*(150.471) = 300.942, *p* = 0.000 at 1.00 mg/ml, *F*(385.276) = 770.551, *p* = 0.000 at 2.00 mg/ml, *F*(98.800) =1 97.600, *p* = 0.000 at 4.00 mg/ml, *F*(56.917) = 113.833, *p* = 0.000 at 8.00 mg/ml, *F*(1.137) = 2.274, *p* = 0.118 at 10.00 mg/ml and *F*(9.270) = 18.540, *p* = 0.001 at 12.00 mg/ml) between groups (Table 3.4). The ideal methanolic crude saponin treatment was 8.00 mg/ml, this was the lowest treatment at which minimum gas release was recorded. A Tukey post hoc test revealed total gas volumes at 24, 48 and 72 hour incubation period were 2.67±0.29, 3.00±0.00 and 3.33±0.57 ml respectively with *p* = 0.264. Those were significantly lower compared to the 96 hour incubation period with gas release of 8.00±0.50 ml, *p* = 1.000 (Appendix B and C). There was no statistically significant difference between gas release in incubation period 24, 48 and 72 hours (*p* = 0.264) at extract concentration 8.00 mg/ml.

TABLE 3.4 ONE-WAY ANOVA OF GAS RELEASES AT VARIOUS CONCENTRATIONS OF METHANOLIC CRUDE SAPONIN EXTRACT VERSUS INCUBATION TIME (24, 48, 72 AND 96 HOURS)

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
C0.00	Between Groups	803.896	3	267.965	299.124	.000
	Within Groups	7.167	8	.896		
	Total	811.063	11			
C1.00	Between Groups	978.063	3	326.021	300.942	.000
	Within Groups	8.667	8	1.083		
	Total	986.729	11			
C2.00	Between Groups	1107.667	3	369.222	770.551	.000
	Within Groups	3.833	8	.479		
	Total	1111.500	11			
C4.00	Between Groups	370.500	3	123.500	197.600	.000
	Within Groups	5.000	8	.625		
	Total	375.500	11			
C8.00	Between Groups	56.917	3	18.972	113.833	.000
	Within Groups	1.333	8	.167		
	Total	58.250	11			
C10.00	Between Groups	1.177	3	.392	2.674	.118
	Within Groups	1.173	8	.147		
	Total	2.350	11			
C12.00	Between Groups	5.377	3	1.792	18.540	.001
	Within Groups	.773	8	.097		
	Total	6.150	11			

3.4.1.2 *Effect of leaf powder on gas production*

In Figure 3.3, the inclusion of leaf powder did not lead to significant variation in the volume of gas released. There was a steady release of gas at an average of 8.20±0.30 ml from 0.00 to 8.00% leaf powder inclusion in the 24 hour incubation period. Thereafter, a slight increase in gas released was observed at 16.00% of leaf powder inclusion. This trend of stable gas release was also evident during the 96 hour incubation period. However, the gas volume was decreased from 15.20±1.05 ml to an average of 8.2±1.0 ml in between the leaf powder inclusion of 0.25 to 4.00%, after which an increase in gas production to a maximum of 15.80±0.80 ml was then observed. At the incubation periods of 48 and 72 hours total gas releases were of similar volumes at leaf powder inclusion of 0.25, 1.00, 8.00 and 16.00%. The

gas released without any leaf powder was of a similar volume as in the treatment of 16.00% leaf powder. However, the lowest gas released at 48 and 72 hours incubation periods were 3-fold higher than the lowest gas releases at 24 and 96 hours incubation periods.

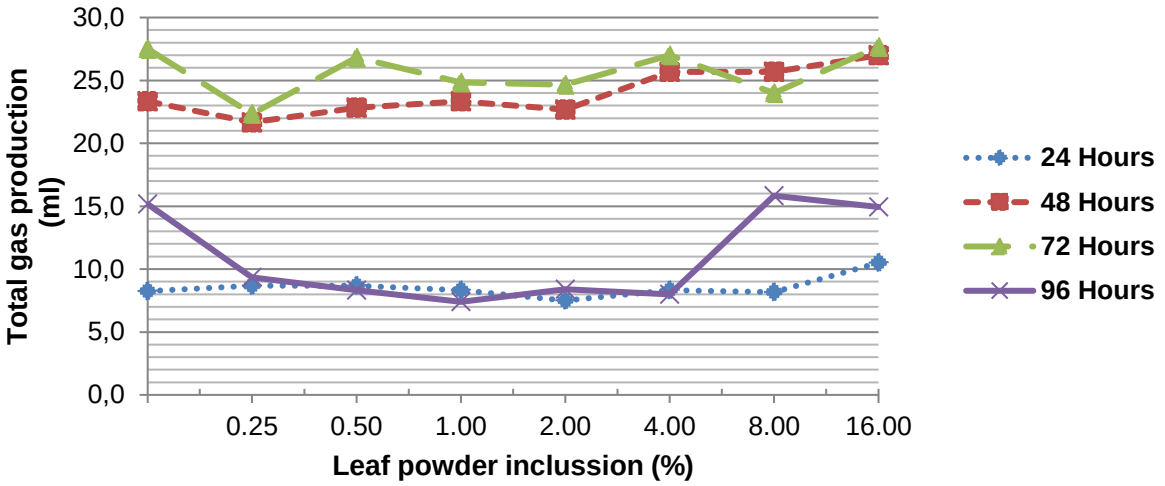


Figure 3.3 The effect of leaf powder on the volume of gas releases during the different incubation periods

One-way *ANOVA* of total gas release due to leaf powder inclusion indicated that there were statistically significant differences *(F*(4.176) = 8.352, *p* = 0.000 at 24 hours, *F*(12.356) = 24.711, *p* = 0.000 at 48 hours, *F*(1.931) = 3.862, *p* = 0.012 at 72 hours and *F*(26.601) = 53.201, *p* = 0.000 at 96 hours) between groups (Table 3.5). A Tukey post hoc test was done with the main focus on the 96 hour incubation period (Appendix D and E). This test revealed that total gas release was substantially high at 0.00, 8.00 and 16.00% (15.12±1.04, 15.83±0.77 and 14.93±0.12 ml, *p* = 0.900) leaf powder inclusion and low at 0.25, 0.50, 1.00, 2.00 and 4.00% (9.33±1.53, 8.33±0.58, 7.40±0.69, 8.40±0.53 and 8.00+1.00 ml, *p* = 0.189). This implies that there was no statistically significant difference in gas release due to leaf powder inclusion compared to the control treatment.

TABLE 3.5 ONE-WAY ANOVA OF INCUBATION PERIODS (24, 48, 72 AND 96 HOURS) VERSUS PERCENTAGES OF LEAF POWDER INCLUSION

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
24Hours	Between Groups	16.345	7	2.335	8.352	.000
	Within Groups	4.473	16	.280		
	Total	20.818	23			
48Hours	Between Groups	72.073	7	10.296	24.711	.000
	Within Groups	6.667	16	.417		
	Total	78.740	23			
72Hours	Between Groups	87.573	7	12.510	3.862	.012
	Within Groups	51.833	16	3.240		
	Total	139.406	23			
96Hours	Between Groups	284.272	7	40.610	53.201	.000
	Within Groups	12.213	16	.763		
	Total	296.485	23			

One-way *ANOVA* of total gas release due to leaf powder inclusion indicated that there were statistically significant differences *(F*(255.333) = 510.666, *p* = 0.000 at 0.00%, *F*(62.847) = 125.693, *p* = 0.000 at 0.25%, *F*(91.661) = 183.319, *p* = 0.000 at 0.50%, *F*(193.803) = 387.606, *p* = 0.000 at 1.00%, *F*(164.690) = 329.380, *p* = 0.000 at 2.00 %, *F*(273.908) = 547.816, *p* = 0.000 at 4.00 %, *F*(26.063) = 52.126, *p* = 0.000 at 8.00 % and *F*(7.905) = 15.810, *p* = 0.001 at 16.00 %) between groups (Table 3.6). A Tukey post hoc test revealed that total gas release at the highest percentage (16.00%) of leaf powder inclusion was 10.50±0.50 and 14.93±0.12 ml, *p* = 0.510 after 24 and 96 hours, respectively (Appendix D and E). After 48 and 72 hours it was 27.00±.0.00 and 27.67±7.50 ml, *p* = 0.996, respectively. There was no statistically significant difference in gas release within the two groups of incubation periods. However, low gas volumes were recorded at 24 and 96 hours and high gas volume at 48 and 96 hours. It is evident that the leaf powder inclusion had no effect in the reduction of gas release.

TABLE 3.6 ONE-WAY ANOVA OF GAS RELEASES AT VARIOUS PERCENTAGES OF LEAF POWDER INCLUSION VERSUS INCUBATION TIME (24, 48, 72 AND 96 HOURS)

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
P0.00	Between Groups	662.589	3	220.863	510.666	.000
	Within Groups	3.460	8	.433		
	Total	666.049	11			
P0.25	Between Groups	494.917	3	164.972	125.693	.000
	Within Groups	10.500	8	1.313		
	Total	505.417	11			
P0.50	Between Groups	814.396	3	271.465	183.319	.000
	Within Groups	11.847	8	1.481		
	Total	826.243	11			
P1.00	Between Groups	793.623	3	264.541	387.606	.000
	Within Groups	5.460	8	.683		
	Total	799.083	11			
P2.00	Between Groups	818.510	3	272.837	329.380	.000
	Within Groups	6.627	8	.828		
	Total	825.137	11			
P4.00	Between Groups	992.917	3	330.972	547.816	.000
	Within Groups	4.833	8	.604		
	Total	997.750	11			
P8.00	Between Groups	586.417	3	195.472	52.126	.000
	Within Groups	30.000	8	3.750		
	Total	616.417	11			
P16.00	Between Groups	671.089	3	223.696	15.810	.001
	Within Groups	113.193	8	14.149		
	Total	784.282	11			

3.4.2 Analysis of gas release by gas chromatography

3.4.2.1 *Effect of extract concentration on gas release*

The chromatograms of GC analysis of gas release were observed and indicated two main peaks with the control (Figure 3.4 A). The major peak, which is presumably for methane occurs at a retention time of about 2.5 minutes. The other peak, a minor one occurs at retention time of about 6.6 minutes. The effect of the methanolic crude saponin extract inclusion was evident as the decrease in methane peak area.

A slight increase was recorded at extract concentration 2.00 mg/ml across all incubation periods in comparison to 1.00 mg/ml extract concentration (Table 3.7). A gradual decrease is further observed till 8.00 mg/ml extract, at which from that point minimum peak areas were recorded. This trend was recorded across all incubation periods. The highest concentration of gas is observed at the control treatment and the lowest gas concentrations are at the high treatments of methanolic crude saponin extract inclusion (8.00, 10.00 and 12.00 mg/ml). The decreased methane peak area at the high extract concentration is accompanied by an increase in area of the peak at retention time 6.6 minutes (Figure 3.4 D). This suggests that the production of methane was not inhibited but that the methane produced was converted into other products.

TABLE 3.7 GAS CHROMATOGRAPHY METHANE PEAK AREAS IN VARIOUS CONCENTRATIONS OF METHANOLIC CRUDE SAPONIN EXTRACT INCLUSION IN RUMINANT GAS RELEASES IN 24 HOUR INTERVALS

		PEAK AREA			
		INCUBATION PERIOD (HOURS)			
TREATMENT NO.	EXTRACT CONC. (MG/ML)	24	48	72	96
1	0,00	69 261 848	23 087 283	30 783 044	41 044 058
2	1,00	16 043 567	5 347 857	7 130 475	9 507 299
3	2,00	18 322 815	6 107 607	8 143 474	10 857 965
4	4,00	7 557 677	2 519 228	3 358 970	4 478 625
5	8,00	164 568	54 860	73 146	97 525
6	10,00	226 002	75 339	100 450	133 931
7	12,00	159 395	53 138	70 848	94 460

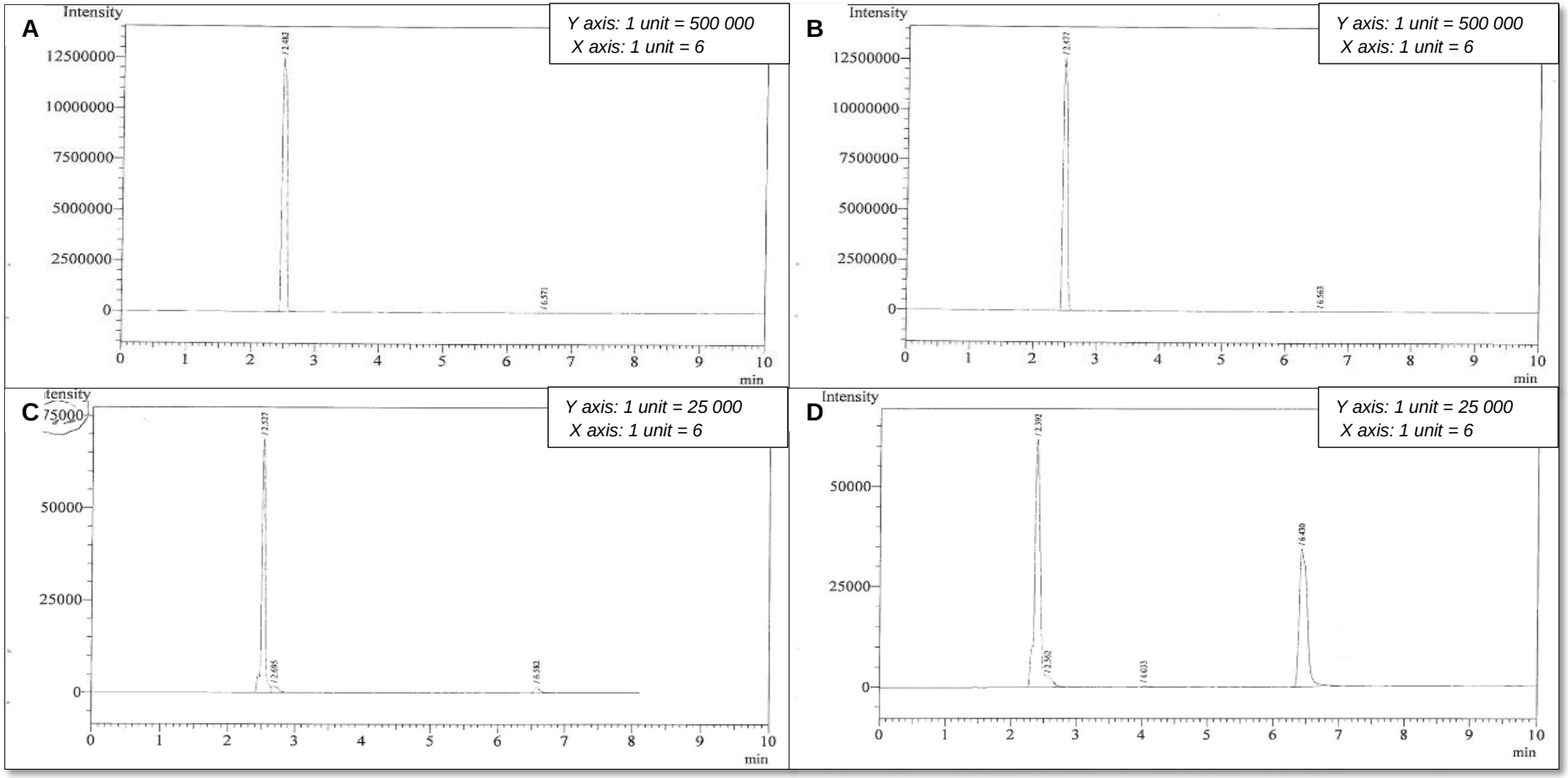


Figure 3.4 Gas chromatograms of gas releases due to methanolic crude saponin extract inclusion at concentrations A) 0.00, B) 1.00, C) 8.00 and D) 12.00 mg/ml after 72 hours

3.4.2.2 *Effect of leaf powder on gas release*

The chromatograms of GC analysis of gas release indicated a peak presumed to be methane and numerous unidentified ones (Figure 3.5). Chromatograms where leaf powder was added lead to the splitting of the peaks and the appearance of several small ones. The inclusion of leaf powder in various percentages had an effect on the volumes of gas released (peak area, Table 3.8). This was evident as an increase in gas release at 0.25% then a decrease at 0.50% was observed. The concentration of gas release was stabilised at leaf powder inclusion of 1.00 to 4.00%, then decreased to its lowest at 8.00%. However, an increase at 16.00% was recorded.

TABLE 3.8 GAS CHROMATOGRAPHY METHANE PEAKS IN VARIOUS PERCENTAGES OF LEAF POWDER INCLUSION

		PEAK AREA
		INCUBATION PERIOD (HOURS)
TREATMENT NO.	LEAF POWDER (%)	96
1	0,00	16 789 669
2	0,25	20 553 363
3	0,50	7 282 926
4	1,00	4 992 356
5	2,00	3 904 396
6	4,00	4 414 245
7	8,00	564 526
8	16,00	1 649 611

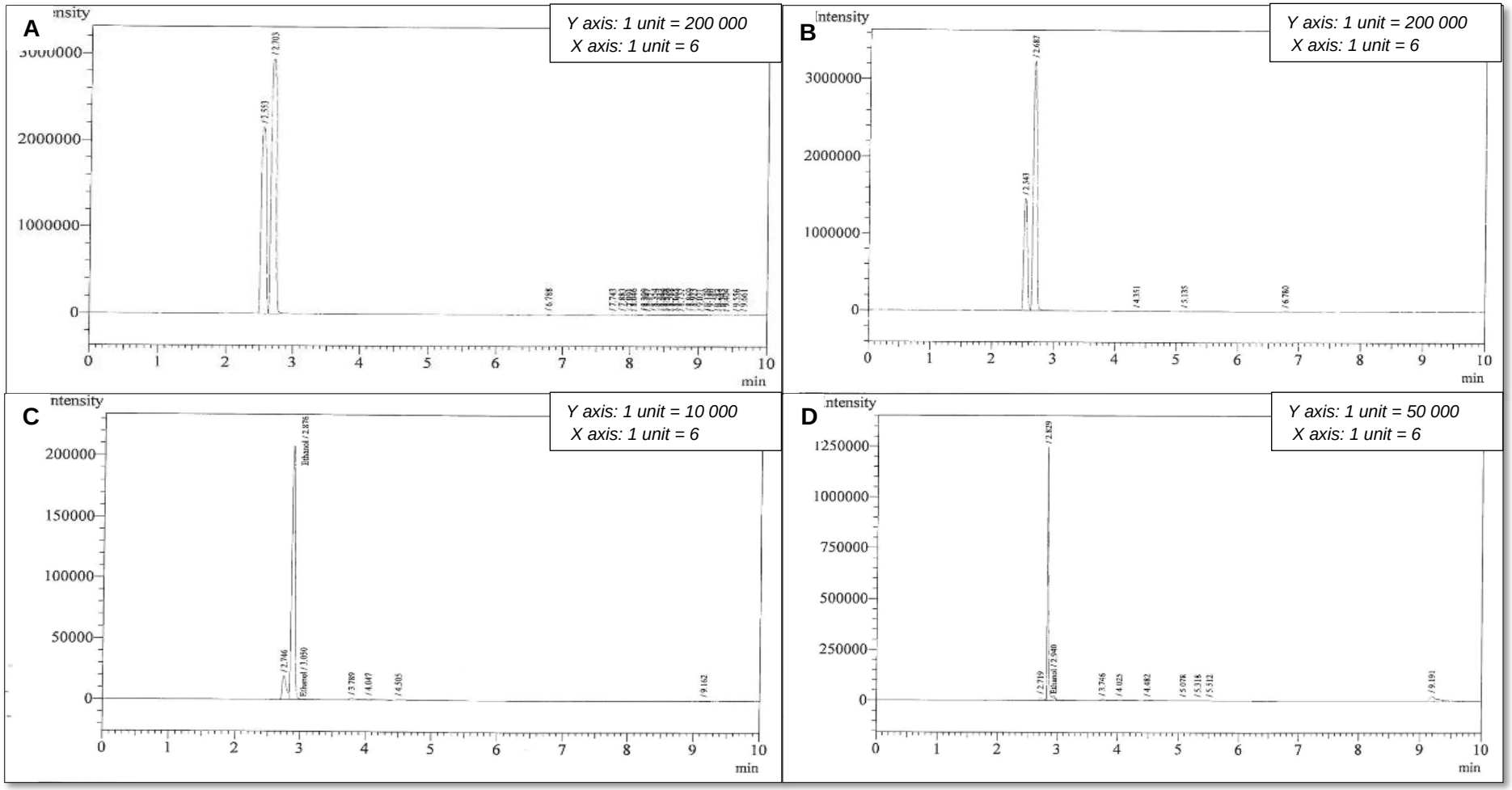


Figure 3.5 Gas chromatograms of gas releases due to leaf powder inclusion at percentages A) 0.00, B) 0.25, C) 8.00 and D) 16.00% after 96 hours

3.5 DISCUSSION

Various plant species have shown varied magnitudes of their effect on total gas/methane production in ruminant enteric fermentation. These variations may be due to the differences in phytochemical structures, molecular weights of the plant secondary metabolites and dosages administered (Crozier and Ashihara, 2006).

3.5.1 Total gas production

3.5.1.1 *Effect of extract concentration on gas production*

The addition of methanolic crude saponin extract induced a substantial increase in total gas production across incubation periods 48 to 96 hours. This increase reached a threshold at 2.00 mg/ml extract concentration during longer incubation periods (72 and 96 hours). Furthermore, after a gradual decrease in total gas release as extract concentrations increased a second threshold was observed at 8.00 mg/ml (Figure 3.2). At low or no extract inclusion (0.00 to 2.00 mg/ml) microorganisms establish themselves and actively metabolise exponentially. The point (2.00 mg/ml) of maximum gas released may represent the optimum activity of enteric fermentation. The crude saponin extract seem to have activated the microorganisms which resulted in rapid production of CO_2 and H_2. The availability of these gases allows the action of methanogens for methane production. This observation was also noted in gas chromatography analysis in which at low or no extract inclusion the methane gas concentrations are the highest across all incubation periods (Table 3.7). After the first threshold, the gradual decline in gas production may be a result of excess H_2 and CO_2 being utilised by the methanogens or homoacetogens. This implies that methanogens were active at low extract concentrations. According to Bodas et al. (2012), homoacetogens rarely out-compete methanogens for H_2 utilisation. Thus, methane is favoured instead of total volatile fatty acid production. Hence the higher concentrations of methane at treatment range of 0.00 to 2.00 mg/ml. The second threshold (8.00 mg/ml) is also recorded across all incubation periods at which total gas release stabilises to minimum volumes at the range 8.00 to 12.00 mg/ml treatments. High extract concentrations may have either substantially inhibited methanogenic activity and altered the rumen conditions or activated homoacetogen activity promoting the formation of volatile fatty acids. This is supported by Ray et al. (2004) and Tan et al. (2011), who suggest that saponins have antibacterial, antimicrobial and anti-inflammatory activities. Hence the presence of the extract may

to some extent alter the rumen environment and affect its microflora behaviour i.e. fungi, protozoa and bacteria. Low volumes of gas release were at 10.00 and 12.00 mg/ml treatments with no significant difference (p<0.05). However, the ideal extract concentration is 8.00 mg/ml with total gas release of 8.00 ml per 250 mg feed dry matter, where the highest feed digestion efficiency was also observed *in vitro* (see chapter 4, section 4.4.1). This extensive decrease in total gas release also indicates substantial suppression of mechanisms for gas production by the rumen microflora. Furthermore, excess H_2 may be utilised by homoacetogens. This is also highlighted by Bodas et al. (2012), that the ideal way to deal with CO_2 and H_2 availability is to find alternative sinks instead of the conventional methanogenesis. Plant secondary products are associated with properties that promote a plant's defence mechanisms against pathogens and insects. These plant properties may induce the desired results when ingested by ruminants in varied proportions with their ordinary diet e.g. reduction in rate of methanogenesis and protozoal activity. It is evident that the crude saponin extract in the current investigation suppressed gas production. This is the ideal property of plant candidates that should be utilised to address ruminant methane emissions and improvement of feed utilisation. According to Tan et al. (2011) *Leucaena leucocephala* condensed tannin extracts reduced methane production by 33% at extract inclusion of 10 mg CT/500 mg dry material. Furthermore, at higher levels reductions were up to 63%. Supplementation (25 g/kg or 12.5 mg CT/500 mg dry material) with *Acacia mearnsii* reduced methane production by 12% in sheep. In other *in vitro* studies, various essential oils and essential oil-blends where compared for effects on rumen fermentation and dry material degradability. A decrease in methane and an increase in propionate production were observed although there was a decrease in dry material degradability in most cases. The essential oils were garlic oil (*Allium sativum* L., 0.25 and 1.00 g/l), lemon grass (*Cymbopogon citratus*, 833 µl/l), peppermint oil (*Mentha piperita* L., 0.10 and 0.25 g/l) as outlined by Cobellis et al. (2016). Based on the above mentioned *in vitro* observations, a reduction in methane and an increase in propionate (VFA) and dry material degradability is a challenge. In the case of saponins as mitigation strategy for methane emissions and feed utilisation improvements, there are numerous challenges too. The rumen microbial flora may easily adapt to ingested saponin extracts as highlighted by Cobellis et al. (2016), when *Quillaja* saponins were incubated with rumen microbes 50% of saponins were

degraded after 12 hours. Hart et al., (2008) conforms to the degradation of *Quillaja* saponins by rumen microbes but within a 24 hour period. Jouany and Morgavi (2007), suggest that saponins also may be degraded by unidentified components in rumen saliva and Gram-positive bacteria's cell wall structure can be altered which in turn may affect digestion. In the current study a decrease of 99% gas release was induced at extract inclusion of 200 mg methanolic crude saponin extract/250 mg dry material after 72 hours of incubation. This is substantially very large amounts of extract inclusion compared to feewdsawhat other studies have reported. This implies that there was substantial inhibition or alteration of rumen microflora activities responsible for gas production (methanogenesis and general enteric fermentation). However, feed digestion was significantly increased by 55% (see chapter 4 section 4.4.1). Therefore, the substantial inhibition of gas release did not suppress feed digestibility. Saponins are toxic to microbes i.e. protozoa, decrease ammonia and bacterial protein turnover in the rumen. The destruction of protozoal cell membranes by saponins is caused by forming complexes with sterols in the membrane. A grain-rich/starch based diet also aid in the increase in propionate production. The longer incubation periods (72 to 96 hours) in this study were ideal for observation of the crude saponin extract's activity, this was to quantify its effects during elongated time periods in the rumen (Figure 3.2). This is because Greathouse (1964) suggests feed remains in the rumen and reticulum for more than 24 hours to enable sufficient rumination.

3.5.1.2 *Effect of leaf powder on gas production*

The inclusion of leaf powder in various percentages had varied effects on total gas release. The lowest gas releases were observed in the 24 and 96 hours incubation periods (Figure 3.3). During this incubation periods, there was no significant variation of total gas releases in treatments of 0.25 to 4.00%, whereas a similar trend was evident in the control, 8.00 and 16.00% treatments. The addition of leaf powder had no effect within the early stages of ruminant digestion. However, as the incubation period increased to 48 and 72 hours a substantial increase was observed, it is evident that microbial/bacterial activity was at its optimum during these periods. Nevertheless, the activity is not sustained to an extent that total gas production at 96 hours is similar to that in the 24 hour stage of ruminant digestion. These findings suggest that although gas is produced in the various stages of rumination, it was

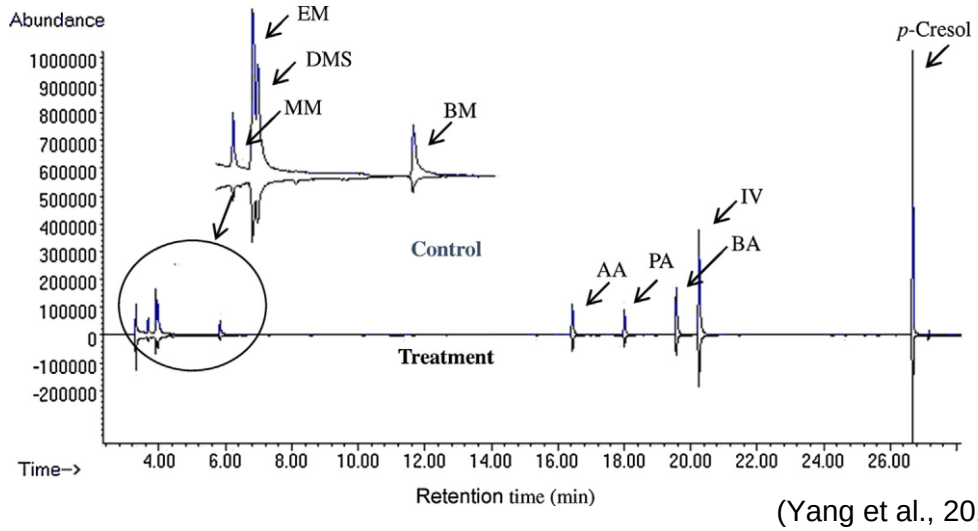
somehow consumed by various microbes within the system. They may in turn produce other useful products that may be utilised by the animal e.g. total volatile fatty acids. The gas accumulated as total gas within the 48 and 72 hour incubation periods may be dominated by H_2 and CO_2. According to Bodas et al. (2012), CH_4 is induced by methanogens with excess H_2 so if an alternative H_2 sink is found gas may be reduced within the rumen. Moreover, homoacetogen activity may be promoted, utilising available H_2 for volatile fatty acid production. At 96 hours incubation period lower gas releases are observed, this may be an indication that the leaf powder inclusion induced a substantial decrease in gas production during enteric fermentation. Gas chromatography analysis indicated methane gas concentrations were directly proportional to leaf powder inclusion. At lower or no leaf powder inclusion methane gas concentrations were high as indicated in Figure 3.5 and Table 3.8. However, the total gas volumes were of similar quantities as the 16% treatment as in Figure 3.3. The leaf powder may contain plant secondary metabolites which act on ruminal bacteria to provide an efficient sink for H_2 disposal. This was demonstrated by the sudden escalation then decrease in total gas release as the incubation period is increased. Feed digestion was minimum (see chapter 4 section 4.4.2) and total gas release was not substantially reduced across individual incubation periods. One of the characteristics of an ideal feed additive is that it should lower gas production with an increase in feed digestion (Cobellis et al., 2016).

3.5.2 Analysis of gas release by gas chromatography

3.5.2.1 *Effect of extract concentration on gas release*

The gas chromatography analysis indicated that high methane concentrations were recorded at the range 0.00 to 2.00 mg/ml treatments of extract inclusion. The highest peak areas (69.2, 41.0, 30.7 and 23.0 million area units, the area units are expressed this way as there was no GC calibration standard) were observed at this range and as extract concentrations increased the peak areas decreased across all treatments (Table 3.7 and Figure 3.4). The major peak observed at concentrations 0.00, 1.00, 8.00 and 12.00 mg/ml were postulated to be methane gas. This peak was similar to that in the study by Kamiński et al. (2003), in which CH_4 retention time was observed at ~2.20 minutes. However, an unidentified minor peak at retention time 6.57 minutes was also observed and increased as extract concentration was increased. The major peak suggests that methane gas was released in high

concentrations at treatment 0.00 to 2.00 mg/ml. This implies that methanogens were active at those extract concentrations. Their activity may be coupled with active enteric fermentation producing access CO_2 and H_2. Methanogens may utilise the available gas for methane production, homoacetogens may also be active at this stages in attempts to take advantage of gas availability. The use of H_2 by homoacetogens is beneficial to the animal. This is because they produce volatile fatty acids (acetate, propionate and butyrate) which may be absorbed by the animal (Buddle et al., 2011). This competitive interaction of methanogens and homoacetogens may be responsible to the unidentified minor peak at 6.57 minutes (retention time). Figure 3.6 shows a gas sample with retention times for various volatile organic compounds (Yang et al., 2015). However, these were not comparable to these in the current study as those were mercaptan derivatives.



(Yang et al., 2015)

Figure 3.6 Total ion chromatograph of a gas sample with a control sample from a standard gas mixture generation system (MM, methyl mercaptan; EM, ethyl mercaptan; DMS, dimethyl sulphide; BM, butyl mercaptan; AA, acetic acid, PA, propanoic acid; BA, butyric acid; IV, isovaleric acid), reproduced with permission from the correspondence author

3.5.2.2 *Effect of leaf powder on gas release*

The inclusion of leaf powder resulted in variable effects on gas emission without showing a clear trend during the different incubation periods. (Figure 3.3). The only clear trend is at 96 hours incubation where inclusion of leaf powder decreased total gas production to a constant level until at 8.0% powder where gas emission increased. The gas analysis indicated high peak areas (16.7 to 4.4 million area units

at ~2.63 retention time) with the leaf powder inclusion range of 0.00 to 4.00%, which is presumed to be methane gas. In that retention time a split in the peak was observed. The minor peak within the split was half the peak area of the major. This implies that these peaks may be of different gases, according Kamiński et al. (2003) carbon monoxide's retention time is prior but very close to that of methane.

3.6 CONCLUSION

The utilisation of the methanolic crude saponin extract and leaf powder yielded varied responses in *in vitro* ruminant enteric total gas release. The saponin extract was most active and at higher concentrations induced a substantial decrease in total gas release across all incubation periods compared to the control. The leaf powder was not effective in the reduction of total gas release. The results suggest that this methanolic saponin extract may be highly effective if utilised sufficiently and coupled with good feeds to promote digestion efficiency and minimise methane and total gas production.

3.7 REFERENCES

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CHAPTER 4

IN VITRO EFFECT OF *HELINUS INTEGRIFOLIUS* (LAM.) KUNTZE ON RUMINANT FODDER DIGESTIBILITY

4.1 INTRODUCTION

There are numerous strategies proposed to mitigate ruminant enteric methane (CH_4) emissions and improve feed digestibility. Although there are contradictions in some studies, the potential for the use of plant secondary metabolites to manipulate rumen digestion is demonstrated by Hart et al. (2008); Buddle et al. (2011) and Bodas et al. (2012) among others. Of the three ruminant methane mitigation strategies suggested by Knapp et al. (2014) feed, feed management and nutrition is the preferred strategy. As good-quality feeds may decrease acetate and increase propionate production, in turn decreasing hydrogen (H_2) availability for methane formation. The other two strategies are the use of rumen modifiers (use of plant secondary metabolites) and the increase of animal production through genetics.

Considerable attention has been placed on rumen modifiers (plant secondary metabolites) as a viable strategy for methane (CH_4) reduction and feed digestibility efficiency. Methane production in the rumen results from processes of utilising feed energy sources. A decrease in dry material (DM) degradability should result in reduced total gas emissions and methane production. Tan et al. (2011) noted a reduction in dry material degradability between 22 – 37% at condensed tannin (CT) levels of 20 – 30 mg but lower levels (15 mg) resulted in only 7% dry material degradability reduction. Patra and Sexena (2010) reported that numerous studies have demonstrated that similarly to saponins, tannin plant extracts (*Terminala balerica, Acacia mearnsii, Acacia magnum, Jatropha curcas* and *Biophytum petersianum*) did not affect digestibility but suppressed CH_4 production.

In vitro dry material degradability was either increased or decreased by the inclusion of essential oils, depending on dosages. However, there are contradictory reports of essential oils' effects on total volatile fatty acid (VFA) production e.g. propionate and acetate ratios. Studies also noted that there is a variation in volatile fatty acid production depending on rumen pH. It is proposed that essential oils have more impact at lower rumen pH. They may be responsible to primarily suppress methanogens and H_2 producing microorganisms at lower pH (Patra and Sexena, 2010). Plant secondary metabolites' potential has been demonstrated in wide array of studies but there is a need to further elucidate plant extracts and their bioactivities

against rumen microflora. This study investigated *in vitro* dry material degradability due to a methanolic crude saponin extract and leaf powder of *Helinus integrifolius*.

4.1.1 Motivation of the study

The reduction of methane contributed by livestock has recently received considerable attention. A lot of strategies to alleviate ruminant methane emissions have been proposed by various researchers. This is because methane has a much greater global warming potential than carbon dioxide. The ideal candidates to manipulate ruminant digestion, should decrease gas emission and increase fodder digestion efficiency. Studies have demonstrated that these candidates may be plants with high content of secondary products (Chanthakhoun et al., 2011).

It is evident that most plant secondary products do have a negative effect on ruminant digestion. Although in some cases, contradictory observations have been reported. Chanthakhoun et al. (2011) reported that hay supplemented with *Phaseolus calcaratus* resulted in increased efficiency of dry material intake and crude protein synthesis in buffalo (*Bubalus bubalis*). Nasser et al. (2009) concluded that fodder degradation (line straw) can be improved without the need for biological treatment, by mixing with high quality roughages. This study investigated viable options to manipulate enteric ruminant digestion promoting feed utilisation efficiency.

4.1.2 Aim of the study

The aim of this study was to investigate the effect of *Helinus integrifolius* (Lam.) Kuntze crude saponin extract and leaf powder on ruminant *in vitro* fodder digestibility.

4.1.3 The main objectives were:

i. To prepare and culture the rumen fluid for *in vitro* fodder digestion bioassay.
ii. To quantify ruminant fodder digestion in 24 hour intervals for 96 hours through a ruminant *in vitro* digestion bioassay.

4.2 LITERATURE REVIEW

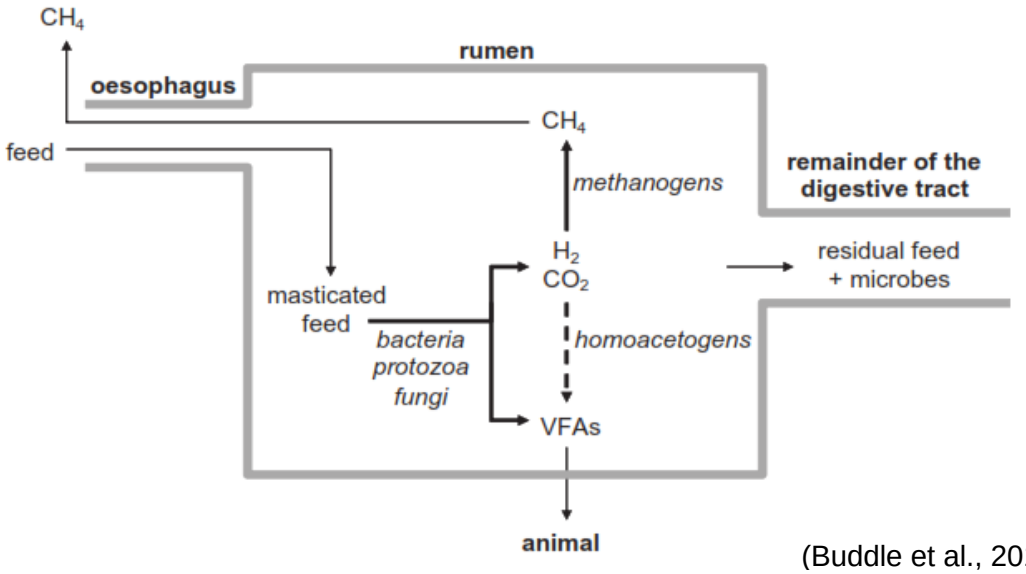
4.2.1 Ruminant digestion

Ruminants have very large, more efficient digestive system in comparison to monogastric animals. This digestive system enables ruminants to ingest enormous amounts of roughages with reasonable utilization of crude fibre and almost above 50% digestion rate. The digestive tract of a ruminant has three preliminary compartments (reticulum, rumen and omasum) before the true stomach (obamasum). The rumen has a very high population of microorganisms which are bacteria and protozoa; it is regarded as a large fermentation chamber. These bacteria secrete enzymes necessary for cellulose degradation which enables the ruminant to use roughages. It accounts for 70 – 85% of total utilization of digestible dry material (Dehority, 2003).

Greathouse (1964) asserts that the rumen maintains its contents at neutral pH 7.0 by constant flow of saliva, which has buffers to neutralise produced fatty acids. The saliva also controls absorption of end products by microbial action and general population of microorganisms depending on the type and amount of ingested feed. Feed material remains in the rumen and reticulum for a period of 24 hours or more, this enables ‘rumination’ which reduces the particle size of feeds. This in turn is responsible for efficient feed material fermentation and digestion. The rumination process allows the reticulum and omasum to assist in total feed utilization and digestion processes. The feed material in the rumen i.e. sugars, starches, cellulose, urea and proteins end up as VFA (acetate, butyrate and propionate), ammonia or bacterial proteins.

The fermentation of ingested feed by the animal generates methane in the rumen which is produced from H_2. Figure 4.1, illustrates an overview of rumen digestion. Bacteria, protozoa and fungi act on masticated feed for metabolic energy and the end products are volatile fatty acids along with carbon dioxide and hydrogen. Methanogens utilise hydrogen to reduce carbon dioxide to methane. When hydrogen concentrations are lowered in the rumen by H_2-consuming methanogens, this rapidly enhances primary fermentation of feed, promoting production of volatile fatty acids. Surveys of archaea in various ruminants have shown that varied methanogens belong to the genus *Methanobrevibacter* (Janssen and Kris, 2008). The different

methanogens include *M. ruminantium* and *M. gottschalki*. The genera *Methanosphaera, Methanosarcina, Methanomocrococcus, Mathenobacterium, Mathanoculleus* and *Methanomicrobium* also have a few rumen methanogenic species that have been identified (Buddle et al., 2011). These are primarily responsible for CH_4 formation during enteric fermentation in the rumen.



(Buddle et al., 2011)

Figure 4.1 A representation of some rumen processes, indicating microbial fermentation and production of volatile fatty acids, hydrogen, carbon dioxide and methane (reproduced with permission from the correspondence author)

4.2.2 Ruminant digestion disorders

Ruminant digestive disorders are due to changes in composition of ration. These changes should be made gradually to allow ruminal microorganisms to adopt to change in diet. Visser (2005) outlines three important disorders i.e. bloating, urea poisoning and acidosis. Bloat refers to the excessive accumulation of gas in the rumen, this may be acute or chronic. Furthermore, it may be induced by plants with high bloat potential such as clover (*Trifolium* sp) and lucerne (*Medicago sativa*) and animal feeding behaviour like greedy feeders. Roughages stimulate mobility in the rumen and belching efficiency but grain decreases it. Hence the type of feed is important in avoiding bloating in the animal.

According Visser (2005) metabolic disorders include urea poisoning, which is due to high levels of ammonia in the blood. The urea is converted to ammonia in the rumen. The increase in ammonia increases pH and absorption across the rumen that will be

converted back into urea in the liver. Which leads to secretion of excessive saliva, frequent urination and defaecation, this also induces bloating. A decrease in rumen pH is due to a sudden change in ration from predominately roughage to grains. This results from the rapid production of short chain fatty acids, increased activity of *Streptococcus bovis* which is a lactic acid producing species. It also results in increased histamine that may lead to laminitis. Rumen stasis, bloat, rumenitis, liver abscesses and dehydration are due to accumulation of lactic acid in the bloodstream (Beauchemin and McAllister, 2008).

4.2.3 Effects of plant secondary metabolites on ruminant enteric fermentation and fodder digestibility

The potential of plant secondary metabolites as organic agents to manipulate rumen fermentation and methane production has been demonstrated in numerous studies. This includes effects on ruminal microbiota, protozoa, fungi, bacteria and ruminal fermentation in general. Bodas et al. (2012) suggest that there is a decrease in ruminal fungi populations in response to saponin supplementation. Reports also suggest that the growth of *Prevotella ruminicola* was stimulated by saponins, whereas *Butyrivibrio fibrisolvens* and *Streptococcus bovis* growth were inhibited. Membranolytic properties of saponins mediated the antibacterial effect which is more pronounced at low pH as similar to essential oils' activity also noted by Patra and Sexena (2010). Plant secondary metabolites have effects on protozoal (defaunation), fungi and bacterial populations which in turn affects formation of fermentation end products like volatile fatty acids and ammonia in the rumen.

According to Bodas et al. (2012) the effects of saponins on volatile fatty acids are varied i.e. the increase in proportion of propionate and reduction in branched-chain volatile fatty acids and acetate. Therefore, various doses of saponins inhibit Gram-positive bacteria and protozoa while promoting propionate producing bacterial species, which results in an increase in propionate production in the rumen. The pH dependency of saponin activity suggests that the diet or type of feed influences their activity. The inhibition of protozoa by saponins may indirectly decrease methanogenesis. Patra and Yu (2014) assert that a combination of nitrates, saponins and sulphates substantially reduces methane production, with no effect on feed digestibility or rumen fermentation. These findings suggest that saponins and

essential oils may potentially curb enteric fermentation towards reduced levels of CH_4 production.

Hart et al. (2008) conforms to reports that suggest that essential oil extract from *Rosmarinus officinalis* do reduce protozoal viability at high concentrations. However, saponin activity is contradictory in a few cases. In the rumen of sheep fed *Sesbania sesban* a reduction in protozoal number was not evident according to Teferedegne et al. (1999), contrary to findings that the same extract reduced protozoal number when fed to sheep (Newbold et al., 1997). However, Odenyo et al. (1997) agrees that *S. sesban* did not reduce protozoal number in the rumen of sheep. Thus, it is evident that various saponins may induce varied effects based on saponin type, concentrations and basal diet. The characterization and description of tested plant extracts coupled with molecular profiling techniques may be crucial in rumen microbial population behavioural patterns. This may aid in understanding direct effect of plant secondary metabolites on rumen microflora. Furthermore, the manipulation of enteric fermentation processes, improved digestibility efficiency and mitigation of CH_4 production in ruminants may be a prospective possibility.

4.3 MATERIALS AND METHODS

4.3.1 Plant extract preparation

A methanolic crude saponin extract and leaf powder were extracted and prepared respectively as in Chapter 2, section 2.3.2.

4.3.2 Rumen fluid inoculum preparation

Fresh cow rumen fluid was collected at Vencor Abattoir Polokwane, South Africa, immediately after the cow was slaughtered. The rumen fluid was cultured with buffers and substrates (*Zea mays* stover and *Medicago sativa* powder) as suggested by Fon and Nsahlai, (2012) and incubated at 38 °C for 72 hours before use. After incubation, the inoculum was used for *in vitro* ruminant fermentation and fodder digestibility bioassay.

4.3.3 Ruminant fodder digestibility bioassay

Ruminant fodder digestibility bioassay was done according to the method of Getachew et al. (2004). The reactants were added in the reaction mixture to a final volume of 25.0 ml in each serum/medicine bottle (50.0 ml), according to Table 3.1

and 3.2, in Chapter 3. All treatments were done in triplicates. The serum bottles were flushed with carbon dioxide gas to maintain an anaerobic condition in the reaction chamber. The bottles were incubated at 39 °C in a water-bath for 24, 48, 72 and 96 hours with occasional shaking. The ruminant digest was filtered through pre-weighed Whatman filter papers (12.5 cm), dried and weighed again to determine digested feed.

4.4 RESULTS

4.4.1 Effect of extract concentration on ruminant fodder digestion

In Figure 4.2, increases in methanolic crude saponin extract concentrations elevated ruminant fodder digestion. This was evident in the various incubation periods of 24 to 96 hours. The crude saponin extract inclusion of 1.00 and 2.00 mg/ml showed no significant variations in fodder digestibility compared to the control (5,67±1.52 and 6.00±1.00%) in the early stages of digestion (24 and 48 hours). As extract concentration increased digestion also increased by up to 4-fold compared to the control. The highest fodder digestion was recorded at extract concentration (12.00 mg/ml) during the 48 hour incubation period. As incubation periods increased a threshold was clearly observed in the longest incubation period (96 hours) at extract concentration 8.00 mg/ml with digestion of 93.67±2.31%. This was the point of highest digestion percentage (Figure 4.2) and low recorded gas releases (see Chapter 3 section 3.4.1.1, Figure 3.2). Fodder digestibility of 65.67±3.05 and 69.0±0.00% digestion was evident in 4.00 and 12.00 mg/ml treatments at the 96 hour incubation period.

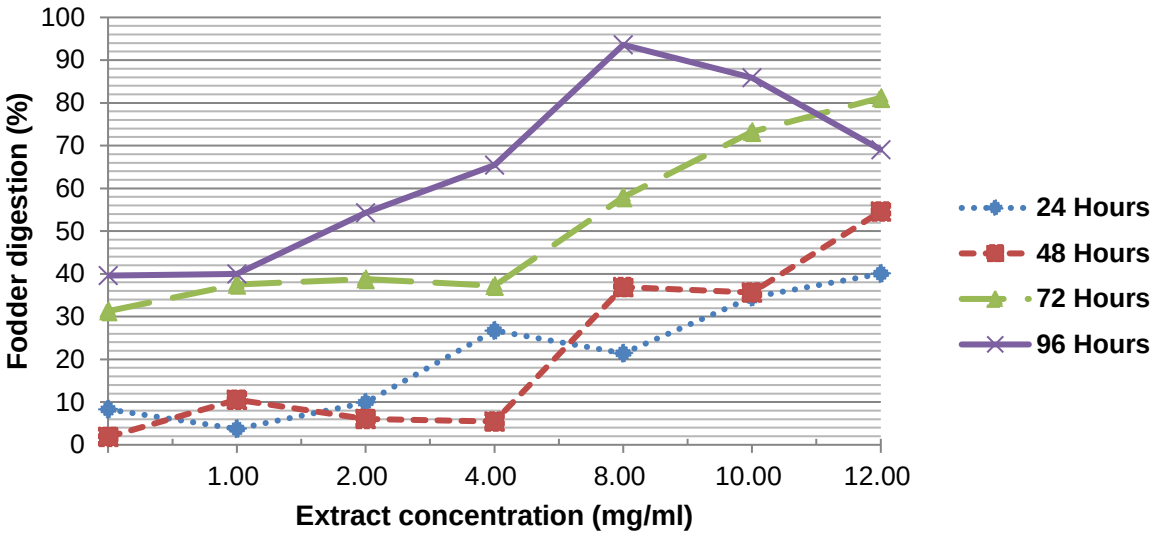


Figure 4.2 The effect of methanolic crude saponin extract on ruminant fodder digestion in 24 hour intervals

One-way *ANOVA* of fodder digestion due to methanolic crude saponin extract inclusion indicated that there were statistically significant differences (*F*(76.145)=152.290, *p* = 0.000 at 24 hours, *F*(130.198) = 260.396, *p* = 0.000 at 48 hours, *F*(75.381) = 150.761, *p* = 0.000 at 72 hours and *F*(64.430) = 128.876, *p* = 0.000 at 96 hours) between groups (Table 4.1). A Tukey post hoc test was done with the main focus on the 72 hour incubation period (Appendix F and G). This test revealed that fodder digestion percentage was lower at low and no (control) methanolic crude saponin extract inclusion (0.00, 1.00, 2.00 and 4.00 mg/ml) at which digestion was 31.0±2.64, 37.33±2.08, 39.67±2.65 and 38.67±2.65%, respectively with *p* = 0.054. It was also revealed that significantly high digestion was recorded at high crude extract inclusion (10.00 and 12.00 mg/ml) at which digestion was 73.33±2.64 and 81.33±2.08%, respectively with *p* = 1.000. There was statistically significant variation within high methanolic crude saponin extract inclusion (8.00, 10.00 and 12.00 mg/ml) and no significant difference within low extract inclusion of 1.00, 2.00 and 4.00 mg/ml. Low extract concentrations (0.00 to 4.00 mg/ml) induced low fodder digestion percentages at 24 and 48 hour incubation periods and high extract concentrations (8.00 to 12.00 mg/ml) substantially increased digestion at 72 and 96 hour incubation periods. The 8.00 mg/ml treatment was the lowest methanolic saponin extract concentration that induced substantially high digestion (93.67±2.3%) at 96 hours.

TABLE 4.1 ONE-WAY ANOVA OF INCUBATION PERIODS (24, 48, 72 AND 96 HOURS) VERSUS CONCENTRATIONS OF METHANOLIC CRUDE SAPONIN EXTRACT

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
Time_24H	Between Groups	3654.952	6	609.159	152.290	.000
	Within Groups	56.000	14	4.000		
	Total	3710.952	20			
Time_48H	Between Groups	7960.667	6	1326.778	260.396	.000
	Within Groups	71.333	14	5.095		
	Total	8032.000	20			
Time_72H	Between Groups	7021.143	6	1170.190	150.761	.000
	Within Groups	108.667	14	7.762		
	Total	7129.810	20			
Time_96H	Between Groups	7916.667	6	1319.444	128.876	.000
	Within Groups	143.333	14	10.238		
	Total	8060.000	20			

One-way *ANOVA* of fodder digestion due to methanolic crude saponin extraction inclusion indicated that there were statistically significant differences *(F*(239.814) = 479.628, *p* = 0.000 at 0.00 mg/ml, *F*(65.504) = 131.007, *p* = 0.000 at 1.00 mg/ml, *F*(234.334) = 468.667, *p* = 0.000 at 2.00 mg/ml, *F*(321.622) = 643.243, *p* = 0.000 at 4.00 mg/ml, *F*(138.226) = 276.452, *p* = 0.000 at 8.00 mg/ml, *F*(48.367) = 96.734, *p* = 0.118 at 10.00 mg/ml and *F*(103.573) = 207.145, *p* = 0.001 at 12.00 mg/ml) between groups (Table 4.2). The ideal methanolic crude saponin treatment was 8.00 mg/ml, this was the lowest treatment at which maximum digestion and minimum gas release was recorded. A Tukey post hoc test revealed that digestion percentages at 24, 48, 72 and 72 hour incubation period were 21.67±2.08, 37.00±3.61, 58.30±4.93 and 93.67±2.31% respectively with *p* = 1.00 (Appendix F and G). There was statistically significant difference between digestion percentages across all incubation periods (*p* = 1.00 at extract concentration 8.00 mg/ml.

TABLE 4.2 ONE-WAY ANOVA OF FODDER DIGESTION AT VARIOUS CONCENTRATIONS OF METHANOLIC CRUDE SAPONIN EXTRACT VERSUS INCUBATION PERIODS (24, 48, 72 AND 96 HOURS)

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
C0.00	Between Groups	3117.583	3	1039.194	479.628	.000
	Within Groups	17.333	8	2.167		
	Total	3134.917	11			
C1.00	Between Groups	2947.667	3	982.556	131.007	.000
	Within Groups	60.000	8	7.500		
	Total	3007.667	11			
C2.00	Between Groups	5155.333	3	1718.444	468.667	.000
	Within Groups	29.333	8	3.667		
	Total	5184.667	11			
C4.00	Between Groups	5950.000	3	1983.333	643.243	.000
	Within Groups	24.667	8	3.083		
	Total	5974.667	11			
C8.00	Between Groups	9744.917	3	3248.306	276.452	.000
	Within Groups	94.000	8	11.750		
	Total	9838.917	11			
C10.00	Between Groups	6094.250	3	2031.417	96.734	.000
	Within Groups	168.000	8	21.000		
	Total	6262.250	11			
C12.00	Between Groups	2848.250	3	949.417	207.145	.000
	Within Groups	36.667	8	4.583		
	Total	2884.917	11			

4.4.2 Effect of leaf powder on ruminant fodder digestion

The inclusion of leaf powder in various amounts showed varied fodder digestion percentages on dry material in 24 hour intervals of the 96 hour incubation period (Figure 4.3). All fodder digestion percentages recorded were below 50%. However, feed digestibility was at its highest at 1.00 and 2.00% (28.33±0.57 and 29.10±0.63%) with no significant difference in the two treatments after 96 hours. After 48 hours there was an increase in digestion then a decrease at 0.50% leaf powder inclusion. The decrease continued till it was the same level as the control (~20%). This further increased to the highest digestion percentage observed at 8.00% leaf powder inclusion. After 72 hours an increase due to leaf powder inclusion was observed till a

decrease after the 0.50% treatment was recorded. The decrease was significantly dropped by 5-fold at 1.00% treatment with a sudden increase of 5-fold at 4.00% treatment after which a decrease of up to 12-fold was observed (lowest digestion percentage).

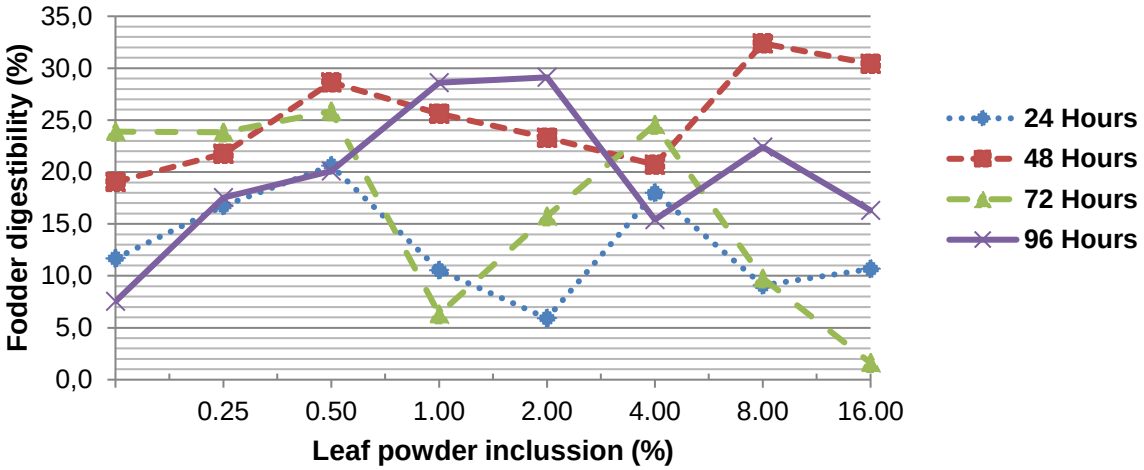


Figure 4.3 The effect of leaf powder on ruminant fodder digestion

One-way *ANOVA* of fodder digestion due to leaf powder inclusion indicated that there were statistically significant differences *(F*(181.586) = 363.171, *p* = 0.000 at 24 hours, *F*(276.548) = 553.095, *p* = 0.000 at 48 hours, *F*(122.91) = 245.820, *p* = 0.012 at 72 hours and *F*(104.568) = 209.136, *p* = 0.000 at 96 hours) between groups (Table 4.3). A Tukey post hoc test was done with the main focus on the 96 hour incubation period (Appendix H and I). This test revealed that fodder digestion was substantially low across all treatments with the highest recorded digestion being at 2.00 and 8.00% of leaf powder inclusion (29.10±0.63 and 30.07±1.53%, *p* = 0.105). This implies that there was no statistically significant difference in digestion due to leaf powder at 1.00 and 2.00% leaf powder inclusion. Although there was slight improvement in digestion compared to the control, this improvement did not reach 50% of fodder digestion. The ideal incubation period was 72 hours, which indicated no significant difference at leaf powder inclusion of 0.00, 0.25, 0.50 and 4.00% (24.00±1.73, 23.07±1.16, 26.00±1.00 and 24.67±1.16%, *p* = 0.194). However, those were not the highest digestion percentages observed, as stated above.

TABLE 4.3 ONE-WAY ANOVA OF INCUBATION PERIODS (24, 48, 72 AND 96 HOURS) VERSUS PERCENTAGES OF LEAF POWDER INCLUSION

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
Time_24H	Between Groups	529.625	7	75.661	363.171	.000
	Within Groups	3.333	16	.208		
	Total	532.958	23			
Time_48H	Between Groups	483.958	7	69.137	553.095	.000
	Within Groups	2.000	16	.125		
	Total	485.958	23			
Time_72H	Between Groups	1935.833	7	276.548	245.820	.000
	Within Groups	18.000	16	1.125		
	Total	1953.833	23			
Time_96H	Between Groups	1280.958	7	182.994	209.136	.000
	Within Groups	14.000	16	.875		
	Total	1294.958	23			

One-way *ANOVA* of fodder digestion due to leaf powder inclusion indicated that there were statistically significant differences *(F*(96.606) = 193.212, *p* = 0.000 at 0.00%, *F*(12.375) = 24.750, *p* = 0.000 at 0.25%, *F*(70.534) = 141.067, *p* = 0.000 at 0.50%, *F*(532.792) = 1065.583, *p* = 0.000 at 1.00%, *F*(249.084) = 498.167, *p* = 0.000 at 2.00 %, *F*(22.667) = 45.333, *p* = 0.000 at 4.00 %, *F*(407.639) = 815.278, *p* = 0.00 at 8.00 % and *F*(869.945) = 1739.889, *p* = 0.001 at 16.00 %) between groups (Table 4.4). A Tukey post hoc test revealed that fodder digestion at the highest percentage (16.00%) of leaf powder inclusion was 30.33±0.57, *p* = 1.000 at 48 hours of incubation (Appendix H and I). There was no statistically significant difference in digestion within all groups of incubation periods at the 16.00% leaf inclusion treatment. However, the highest digestion percentage was observed at 48 hour incubation period and the lowest was after 24 hours (10.33±0.57%, *p* = 1.000).

TABLE 4.4 ONE WAY ANOVA FODDER DIGESTION AT VARIOUS PERCENTAGES OF LEAF POWDER INCLUSION VERSUS INCUBATION PERIODS (24, 48, 72 AND 96 HOURS)

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
P0.00	Between Groups	531.333	3	177.111	193.212	.000
	Within Groups	7.333	8	.917		
	Total	538.667	11			
P0.25	Between Groups	99.000	3	33.000	24.750	.000
	Within Groups	10.667	8	1.333		
	Total	109.667	11			
P0.50	Between Groups	176.333	3	58.778	141.067	.000
	Within Groups	3.333	8	.417		
	Total	179.667	11			
P1.00	Between Groups	1065.583	3	355.194	1065.583	.000
	Within Groups	2.667	8	.333		
	Total	1068.250	11			
P2.00	Between Groups	996.333	3	332.111	498.167	.000
	Within Groups	5.333	8	.667		
	Total	1001.667	11			
P4.00	Between Groups	68.000	3	22.667	45.333	.000
	Within Groups	4.000	8	.500		
	Total	72.000	11			
P8.00	Between Groups	1222.917	3	407.639	815.278	.000
	Within Groups	4.000	8	.500		
	Total	1226.917	11			
P16.00	*Between Groups*	*1304.917*	*3*	*434.972*	*1739.889*	*.000*
	Within Groups	*2.000*	*8*	*.250*		
	Total	*1306.917*	*11*			

4.5 DISCUSSION

Various studies have attempted and demonstrated that plant secondary metabolites manipulate enteric fermentation with varying effects on both *in vitro* and *in vivo* ruminant gas production and dry material (DM) degradability. This may be due to plant extracts or supplements/additives' varied effects on enteric microflora populations, activities and their interactions thereof (Tan et al., 2011; Patra, 2016).

4.5.1 Effect of extract concentration on ruminant fodder digestion

In the current research effects on *in vitro* dry material degradability of methanolic crude saponin extract samples were investigated with varying observations. According to Sparg et al. (2004) saponins have antimicrobial and antifungal properties, which imply that when they are ingested either as methanolic crude saponin extract or leaf powder they are bound to change the microbial population in the rumen. This action should result in varying effects, predominantly decreased feed digestibility depending on dosages as suggested by Patra and Sexena (2010). At the 24 hours incubation period of digestion, the extract inclusion of 2.00 mg/ml showed no significant variations in fodder digestibility compared to the control (9.0±0.5 and 8.0±1.0%). However, digestion is stimulated at concentrations above 4.00 mg/ml for incubation period 24, 48 and 72 hours, but at 96 hours there is a gradual increase in digestion from 1.00 mg/ml. This implies that the rumen has ideal conditions for fermentation, the feed material remains in the rumen and reticulum for 24 hours or more to allow sufficient rumination. This time period also enable the microbial population to adapt to the current conditions in the rumen in relation to feed type, microbial fermentation and digestion will only be evident ideally after a 24 hour period and/or longer. Thus, this time period has allowed microbes and bacteria to establish themselves, efficiently act on feed as they go through their log phase of growth and releasing by-products in the process. It was noted that after a 96 hour incubation period, 65.67±3.05 and 69.00±0.00% digestion was evident in 4.00 and 12.00 mg/ml treatments. Furthermore, a higher digestion was recorded at 8.00 and 10.00 mg/ml treatments which were 93.67±2.31 and 85.67±7.09%, respectively, which is approximately twofold compared to the control treatment. This suggests that extract concentration is directly proportional to digestibility efficiency till a threshold is reached at 8.00 mg/ml. This is the maximum concentration of efficient digestion. Other concentrations beyond the threshold showed a decrease in digestion rate by 6 to 17%. These findings suggest a positive relationship among rumen microbial population with extract inclusion in various concentrations. This interaction substantially increased fodder digestibility by about 40 to 56% in comparison to the control treatment without the extract. A study presented by Tan et al. (2011) indicates that low level (15mg/500 mg DM) of condensed tannins (*Leucaena leucocephala*) reduced methanogen and protozoal populations. However dry

material (DM) digestibility was decreased by 7%. There are reports that the inclusion of saponins decreased feed intake in dairy cows (Lovett et al., 2006) in contrast to some studies observing an increase (Holtshausen et al., 2009, Patra and Sexena, 2010). According to Patra and Sexena (2009), a decrease in digestibility may be induced by the inhibition of microbial growth such as fungi and cellulolytic bacteria. Methanolic extract of *Sapindus rarak* reduced *Ruminococcus* species when administered in high concentrations. Saponins impose an inhibitory effect on protozoa. This effect is implicated to reduce digestibility efficiency. However, no effect on organic matter degradability was observed when *Sapindus saponaria* extract was administered despite recording a 54% decrease in protozoal population. It is evident that the inclusion of saponins or saponin extracts in ruminant diets has an effect on microbiota and organic matter degradability. These effects may induce rumination and enteric fermentation efficiency in turn influencing both total gas production and feed digestibility.

4.5.2 Effect of leaf powder on ruminant fodder digestion

There are numerous similarities in digestion quantities of leaf powder inclusion after a 96 hour incubation period to those of saponin extract inclusion. Although, leaf powder inclusion induced substantially low fodder digestion. In leaf powder percentages 0.25, 4.00 and 16.00 similar quantities of digestion (17.00±1.73, 15.48±5.57 and 16.33±0.57%) were observed (Figure 4.3), at 96 hours incubation period. At 1.00 and 2.00% leaf powder inclusion feed digestibility was at its highest (28.60±4.40 and 29.10±1.89) after 96 hours of incubation. This indicates that the plant material established its interaction with the microflora, enhancing digestion by just 3.7% compared to the standard treatment (0.00%) without the leaf powder. Although the digestibility efficiency is not as high as with the inclusion of crude saponin extract. The leaf powder has saponins and *Medicago sativa*, which is part of the substrate also possess some amounts of saponins. This may be one of the factors that contribute to observed digestion. This suggests that the utilisation of leaf powder may be a viable, easy and cost effective as a dietary feed additive. Although the digestion efficiency is not as high as when the crude saponin extract is utilized, it serves as a suitable alternative. The utilisation of plant secondary metabolites in ruminant gas mitigation and digestibility efficiency has yielded positive findings *in vitro* (Hart et al., 2008; Buddle et al., 2011; Tan et al., 2011; Bodas et al., 2012;

Bueno et al., 2015). It may be a viable option to use plant powder as an addition to ruminant feed, as this makes it possible for easy utilisation and administration. However, leaf powder does not have significant effect on the increase of fodder digestion and the decrease in gas released.

4.6 CONCLUSION

Ruminant enteric fermentation and digestion may be affected by various activities, this may result in different effects such as gas production, volatile fatty acid production and feed digestibility efficiency. It is evident that the addition of crude saponin extract shows desirable findings in both gas production and feed digestibility in high extract concentrations. Leaf powder inclusion induces low but improved digestibility efficiency compared to its control treatment. The low activity may be due to the need to extract the plant secondary metabolites in the plant material within the enteric system. This suggests that the saponins were only attained in small quantities, which in turn results in substantially low activity in contrast to the use of crude saponin extract.

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CHAPTER 5
GENERAL CONCLUSIONS

5.1 INTRODUCTION

The application of plant secondary metabolites as feed additives/supplements was prompted by the ever escalating methane production contributed by livestock production in commercial and subsistence farming. It is one of the innovative strategies to curbing greenhouse gas contributions to global warming by anthropogenic activities. Enteric methane accounts for 80 Mt of methane contribution to the atmosphere on a global scale per annum. It is evident that this needs to be reduced as the environmental impacts of global warming are very dire to the livelihood of mankind. This research focused on the use of *Helinus integrifolius* methanolic crude extract in *in vitro* ruminant gas production and fodder digestion. The major phytochemical group targeted were saponins, in which a crude extract was utilised. A crude extract was chosen in contrast to a pure compounds in recognition of synergistic relations within various phytochemicals to impose certain bioactivities. These plant secondary metabolites' bioactivities were exploited to reduce production of total gas release and methane in ruminants. Furthermore, feed digestibility should be improved.

5.2 KEY FINDINGS

The key findings of the research were:

- The crude saponin extract was found to contain 286.0±0.60 mg saponins/g of dry material and no condensed tannins were detected.
- The crude saponin extract reduced total gas release by 99% and fodder digestion was substantially improved by up to 55% compared to the control.
- The leaf powder inclusion did not induce a decrease in total gas release and fodder digestion was not significantly improved.

5.3 CONCLUSIONS

This research aimed at exploring the use of a methanolic crude saponin extract and leaf powder as supplements to mitigate enteric methane production by ruminants. The findings indicated that this may be a viable option such that total gas release was reduced and fodder digestibility was substantially improved. This implies that the methanolic crude saponins of the plant tested have great potential against

methanogens and may promote homoacetogen activity. Furthermore, the synergistic nature of phytochemicals to impose certain bioactivities has to be taken into consideration. These bioactivities that induce digestion and suppress gas production may be in association with other phytochemicals such as phenolics/flavonoids present in the plant. Although total gas release was reduced *in vitro*, *in vivo* trials are necessary to establish their effects for application in livestock production. This may aid in the decrease of methane gas production by the agriculture sector which contributes to atmospheric contamination by greenhouse gases.

5.4 RECOMMENDATIONS

The research was aimed at exploring the use of a crude extract and leaf powder. The plants in the plant family Rhamnaceae are known to have triterpenoid saponins, and have anti-inflammatory effects. It is recommended that the phytochemical elucidation of this plant be conducted. This will establish a phytochemical profile for the plant only documented to contain saponins and was used as substitute for soap among other uses. The identification of its active phytochemicals will aid in establishing further uses besides the ones tested in this research. Identification of a more bioactive phytochemical fraction will enable its use as a supplement/feed additive. This will further allow its use in the smallest quantities for adequate application in livestock production. Its potential as a rumen enteric fermentation modifier paves further investigations on both its effect on certain type of enteric microorganisms. The type and ratio of total volatile fatty acids production, effects on protozoal populations, overall impact on meat/milk yield production and the well-being of animals have to be investigated.

APPENDICES

APPENDIX A: Thin layer chromatography spray reagents

1. Natural products-polyethylene glycol (NP/PEG or NEU-) reagent

The plate is sprayed with 1% methanolic diphenylboric acid-B-ethylamino ester (=diphenylboryxyethylamine, NP), followed by 5% ethanolic polyethylene glycol-4000 (PEG) (10 ml and 8 ml, respectively). The plate is evaluated in UV-365 nm.

Detection of flavonoids and aloin.

2. Anisaldehyde-sulphuric acid (AS) reagent

0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid, in that order. The TLC plate is sprayed with about 10 ml, heated at 100 °C for 5-10 minutes and then evaluated in vis. or UV-365 nm.

Detection of terpenoids, propylpropanoids, pungent and principles, saponins.

3. Vanillin-phosphoric acid (VPA) reagent

Vanillin of 1.0 g was dissolved in 100 ml of 50% phosphoric acid. The TLC plate is sprayed, heated at 100 °C for 5-10 minutes and then evaluated in vis. or UV-365 nm.

Detection of terpenoids, cucurbitacins and lignanes.

APPENDIX B: Total gas release (methanolic crude saponin extract) descriptive statistics table

Descriptives									
						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
24H	0.00	3	10.6667	1.15470	.66667	7.7982	13.5351	10.00	12.00
	1.00	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	2.00	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	4.00	3	9.3333	.57735	.33333	7.8991	10.7676	9.00	10.00
	8.00	3	2.6667	.28868	.16667	1.9496	3.3838	2.50	3.00
	10.00	3	2.9000	.36056	.20817	2.0043	3.7957	2.50	3.20
	12.00	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	Total	21	7.5333	4.23950	.92513	5.6035	9.4631	2.50	13.00
48H	0.00	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	1.00	3	25.9333	1.79258	1.03494	21.4803	30.3863	24.80	28.00
	2.00	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	4.00	3	17.3333	1.15470	.66667	14.4649	20.2018	16.00	18.00
	8.00	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	10.00	3	3.0000	.20000	.11547	2.5032	3.4968	2.80	3.20
	12.00	3	3.5000	.30000	.17321	2.7548	4.2452	3.20	3.80
	Total	21	13.9667	9.95396	2.17213	9.4357	18.4977	2.80	28.00
72H	0.00	3	32.3333	1.15470	.66667	29.4649	35.2018	31.00	33.00
	1.00	3	36.6667	1.15470	.66667	33.7982	39.5351	36.00	38.00
	2.00	3	37.3333	1.15470	.66667	34.4649	40.2018	36.00	38.00
	4.00	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	8.00	3	3.3333	.57735	.33333	1.8991	4.7676	3.00	4.00
	10.00	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	12.00	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	Total	21	20.0238	15.50361	3.38316	12.9667	27.0810	3.00	38.00
96H	0.00	3	27.8333	.76376	.44096	25.9360	29.7306	27.00	28.50
	1.00	3	30.0000	.50000	.28868	28.7579	31.2421	29.50	30.50
	2.00	3	31.0667	.11547	.06667	30.7798	31.3535	31.00	31.20
	4.00	3	20.8333	.28868	.16667	20.1162	21.5504	20.50	21.00
	8.00	3	8.0000	.50000	.28868	6.7579	9.2421	7.50	8.50
	10.00	3	2.3333	.57735	.33333	.8991	3.7676	2.00	3.00
	12.00	3	1.7333	.46188	.26667	.5860	2.8807	1.20	2.00
	Total	21	17.4000	12.41857	2.70996	11.7471	23.0529	1.20	31.20

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
C0.00	24 hours	3	10.6667	1.15470	.66667	7.7982	13.5351	10.00	12.00
	48 hours	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	72 hours	3	32.3333	1.15470	.66667	29.4649	35.2018	31.00	33.00
	96 hours	3	27.8333	.76376	.44096	25.9360	29.7306	27.00	28.50
	Total	12	22.8750	8.58679	2.47879	17.4192	28.3308	10.00	33.00
C1.00	24 hours	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	48 hours	3	25.5000	1.32288	.76376	22.2138	28.7862	24.50	27.00
	72 hours	3	36.6667	1.15470	.66667	33.7982	39.5351	36.00	38.00
	96 hours	3	30.0000	.50000	.28868	28.7579	31.2421	29.50	30.50
	Total	12	26.0417	9.47115	2.73409	20.0240	32.0593	11.00	38.00
C2.00	24 hours	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	48 hours	3	24.6667	.76376	.44096	22.7694	26.5640	24.00	25.50
	72 hours	3	38.0000	.00000	.00000	38.0000	38.0000	38.00	38.00
	96 hours	3	31.3333	.57735	.33333	29.8991	32.7676	31.00	32.00
	Total	12	26.5000	10.05214	2.90180	20.1132	32.8868	11.00	38.00
C4.00	24 hours	3	9.3333	.57735	.33333	7.8991	10.7676	9.00	10.00
	48 hours	3	17.5000	1.32288	.76376	14.2138	20.7862	16.00	18.50
	72 hours	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	96 hours	3	20.8333	.28868	.16667	20.1162	21.5504	20.50	21.00
	Total	12	18.0000	5.84263	1.68662	14.2878	21.7122	9.00	25.00

C8.00	24 hours	3	2.6667	.28868	.16667	1.9496	3.3838	2.50	3.00
	48 hours	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	72 hours	3	3.3333	.57735	.33333	1.8991	4.7676	3.00	4.00
	96 hours	3	8.0000	.50000	.28868	6.7579	9.2421	7.50	8.50
	Total	12	4.2500	2.30119	.66430	2.7879	5.7121	2.50	8.50
C10.00	24 hours	3	2.9000	.36056	.20817	2.0043	3.7957	2.50	3.20
	48 hours	3	3.0000	.20000	.11547	2.5032	3.4968	2.80	3.20
	72 hours	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	96 hours	3	2.3333	.57735	.33333	.8991	3.7676	2.00	3.00
	Total	12	2.8500	.46221	.13343	2.5563	3.1437	2.00	3.50
C12.00	24 hours	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	48 hours	3	3.5000	.30000	.17321	2.7548	4.2452	3.20	3.80
	72 hours	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	96 hours	3	1.7333	.46188	.26667	.5860	2.8807	1.20	2.00
	Total	12	2.8500	.74772	.21585	2.3749	3.3251	1.20	3.80

APPENDIX C: Total gas release (methanolic crude saponin extract) multiple comparison statistics

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) mg/ml	(J) mg/ml	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
24H	0.00	1.00	-1.33333	.61438	.368	-3.4312	.7645
		2.00	-1.33333	.61438	.368	-3.4312	.7645
		4.00	1.33333	.61438	.368	-.7645	3.4312
		8.00	8.00000*	.61438	.000	5.9022	10.0978
		10.00	7.76667*	.61438	.000	5.6688	9.8645
		12.00	7.50000*	.61438	.000	5.4022	9.5978
	1.00	0.00	1.33333	.61438	.368	-.7645	3.4312
		2.00	.00000	.61438	1.000	-2.0978	2.0978
		4.00	2.66667*	.61438	.009	.5688	4.7645
		8.00	9.33333*	.61438	.000	7.2355	11.4312
		10.00	9.10000*	.61438	.000	7.0022	11.1978
		12.00	8.83333*	.61438	.000	6.7355	10.9312
	2.00	0.00	1.33333	.61438	.368	-.7645	3.4312
		1.00	.00000	.61438	1.000	-2.0978	2.0978
		4.00	2.66667*	.61438	.009	.5688	4.7645
		8.00	9.33333*	.61438	.000	7.2355	11.4312
		10.00	9.10000*	.61438	.000	7.0022	11.1978
		12.00	8.83333*	.61438	.000	6.7355	10.9312
	4.00	0.00	-1.33333	.61438	.368	-3.4312	.7645
		1.00	-2.66667*	.61438	.009	-4.7645	-.5688
		2.00	-2.66667*	.61438	.009	-4.7645	-.5688
		8.00	6.66667*	.61438	.000	4.5688	8.7645
		10.00	6.43333*	.61438	.000	4.3355	8.5312
		12.00	6.16667*	.61438	.000	4.0688	8.2645
	8.00	0.00	-8.00000*	.61438	.000	-10.0978	-5.9022
		1.00	-9.33333*	.61438	.000	-11.4312	-7.2355
		2.00	-9.33333*	.61438	.000	-11.4312	-7.2355
		4.00	-6.66667*	.61438	.000	-8.7645	-4.5688
		10.00	-.23333	.61438	1.000	-2.3312	1.8645
		12.00	-.50000	.61438	.979	-2.5978	1.5978
	10.00	0.00	-7.76667*	.61438	.000	-9.8645	-5.6688
		1.00	-9.10000*	.61438	.000	-11.1978	-7.0022

		2.00	-9.10000*	.61438	.000	-11.1978	-7.0022
		4.00	-6.43333*	.61438	.000	-8.5312	-4.3355
		8.00	.23333	.61438	1.000	-1.8645	2.3312
		12.00	-.26667	.61438	.999	-2.3645	1.8312
	12.00	0.00	-7.50000*	.61438	.000	-9.5978	-5.4022
		1.00	-8.83333*	.61438	.000	-10.9312	-6.7355
		2.00	-8.83333*	.61438	.000	-10.9312	-6.7355
		4.00	-6.16667*	.61438	.000	-8.2645	-4.0688
		8.00	.50000	.61438	.979	-1.5978	2.5978
		10.00	.26667	.61438	.999	-1.8312	2.3645
48H	0.00	1.00	-5.26667*	.71336	.000	-7.7025	-2.8308
		2.00	-3.66667*	.71336	.002	-6.1025	-1.2308
		4.00	3.33333*	.71336	.005	.8975	5.7692
		8.00	17.66667*	.71336	.000	15.2308	20.1025
		10.00	17.66667*	.71336	.000	15.2308	20.1025
		12.00	17.16667*	.71336	.000	14.7308	19.6025
	1.00	0.00	5.26667*	.71336	.000	2.8308	7.7025
		2.00	1.60000	.71336	.334	-.8358	4.0358
		4.00	8.60000*	.71336	.000	6.1642	11.0358
		8.00	22.93333*	.71336	.000	20.4975	25.3692
		10.00	22.93333*	.71336	.000	20.4975	25.3692
		12.00	22.43333*	.71336	.000	19.9975	24.8692
	2.00	0.00	3.66667*	.71336	.002	1.2308	6.1025
		1.00	-1.60000	.71336	.334	-4.0358	.8358
		4.00	7.00000*	.71336	.000	4.5642	9.4358
		8.00	21.33333*	.71336	.000	18.8975	23.7692
		10.00	21.33333*	.71336	.000	18.8975	23.7692
		12.00	20.83333*	.71336	.000	18.3975	23.2692
	4.00	0.00	-3.33333*	.71336	.005	-5.7692	-.8975
		1.00	-8.60000*	.71336	.000	-11.0358	-6.1642
		2.00	-7.00000*	.71336	.000	-9.4358	-4.5642
		8.00	14.33333*	.71336	.000	11.8975	16.7692
		10.00	14.33333*	.71336	.000	11.8975	16.7692
		12.00	13.83333*	.71336	.000	11.3975	16.2692
	8.00	0.00	-17.66667*	.71336	.000	-20.1025	-15.2308
		1.00	-22.93333*	.71336	.000	-25.3692	-20.4975
		2.00	-21.33333*	.71336	.000	-23.7692	-18.8975
		4.00	-14.33333*	.71336	.000	-16.7692	-11.8975
		10.00	.00000	.71336	1.000	-2.4358	2.4358
		12.00	-.50000	.71336	.990	-2.9358	1.9358

	10.00	0.00	-17.66667*	.71336	.000	-20.1025	-15.2308
		1.00	-22.93333*	.71336	.000	-25.3692	-20.4975
		2.00	-21.33333*	.71336	.000	-23.7692	-18.8975
		4.00	-14.33333*	.71336	.000	-16.7692	-11.8975
		8.00	.00000	.71336	1.000	-2.4358	2.4358
		12.00	-.50000	.71336	.990	-2.9358	1.9358
	12.00	0.00	-17.16667*	.71336	.000	-19.6025	-14.7308
		1.00	-22.43333*	.71336	.000	-24.8692	-19.9975
		2.00	-20.83333*	.71336	.000	-23.2692	-18.3975
		4.00	-13.83333*	.71336	.000	-16.2692	-11.3975
		8.00	.50000	.71336	.990	-1.9358	2.9358
		10.00	.50000	.71336	.990	-1.9358	2.9358
72H	0.00	1.00	-4.33333*	.67259	.000	-6.6300	-2.0367
		2.00	-5.00000*	.67259	.000	-7.2966	-2.7034
		4.00	8.00000*	.67259	.000	5.7034	10.2966
		8.00	29.00000*	.67259	.000	26.7034	31.2966
		10.00	29.16667*	.67259	.000	26.8700	31.4633
		12.00	29.33333*	.67259	.000	27.0367	31.6300
	1.00	0.00	4.33333*	.67259	.000	2.0367	6.6300
		2.00	-.66667	.67259	.948	-2.9633	1.6300
		4.00	12.33333*	.67259	.000	10.0367	14.6300
		8.00	33.33333*	.67259	.000	31.0367	35.6300
		10.00	33.50000*	.67259	.000	31.2034	35.7966
		12.00	33.66667*	.67259	.000	31.3700	35.9633
	2.00	0.00	5.00000*	.67259	.000	2.7034	7.2966
		1.00	.66667	.67259	.948	-1.6300	2.9633
		4.00	13.00000*	.67259	.000	10.7034	15.2966
		8.00	34.00000*	.67259	.000	31.7034	36.2966
		10.00	34.16667*	.67259	.000	31.8700	36.4633
		12.00	34.33333*	.67259	.000	32.0367	36.6300
	4.00	0.00	-8.00000*	.67259	.000	-10.2966	-5.7034
		1.00	-12.33333*	.67259	.000	-14.6300	-10.0367
		2.00	-13.00000*	.67259	.000	-15.2966	-10.7034
		8.00	21.00000*	.67259	.000	18.7034	23.2966
		10.00	21.16667*	.67259	.000	18.8700	23.4633
		12.00	21.33333*	.67259	.000	19.0367	23.6300
	8.00	0.00	-29.00000*	.67259	.000	-31.2966	-26.7034
		1.00	-33.33333*	.67259	.000	-35.6300	-31.0367
		2.00	-34.00000*	.67259	.000	-36.2966	-31.7034
		4.00	-21.00000*	.67259	.000	-23.2966	-18.7034

		10.00	.16667	.67259	1.000	-2.1300	2.4633
		12.00	.33333	.67259	.999	-1.9633	2.6300
	10.00	0.00	-29.16667*	.67259	.000	-31.4633	-26.8700
		1.00	-33.50000*	.67259	.000	-35.7966	-31.2034
		2.00	-34.16667*	.67259	.000	-36.4633	-31.8700
		4.00	-21.16667*	.67259	.000	-23.4633	-18.8700
		8.00	-.16667	.67259	1.000	-2.4633	2.1300
		12.00	.16667	.67259	1.000	-2.1300	2.4633
	12.00	0.00	-29.33333*	.67259	.000	-31.6300	-27.0367
		1.00	-33.66667*	.67259	.000	-35.9633	-31.3700
		2.00	-34.33333*	.67259	.000	-36.6300	-32.0367
		4.00	-21.33333*	.67259	.000	-23.6300	-19.0367
		8.00	-.33333	.67259	.999	-2.6300	1.9633
		10.00	-.16667	.67259	1.000	-2.4633	2.1300
96H	0.00	1.00	-2.16667*	.40552	.002	-3.5513	-.7820
		2.00	-3.23333*	.40552	.000	-4.6180	-1.8487
		4.00	7.00000*	.40552	.000	5.6153	8.3847
		8.00	19.83333*	.40552	.000	18.4487	21.2180
		10.00	25.50000*	.40552	.000	24.1153	26.8847
		12.00	26.10000*	.40552	.000	24.7153	27.4847
	1.00	0.00	2.16667*	.40552	.002	.7820	3.5513
		2.00	-1.06667	.40552	.189	-2.4513	.3180
		4.00	9.16667*	.40552	.000	7.7820	10.5513
		8.00	22.00000*	.40552	.000	20.6153	23.3847
		10.00	27.66667*	.40552	.000	26.2820	29.0513
		12.00	28.26667*	.40552	.000	26.8820	29.6513
	2.00	0.00	3.23333*	.40552	.000	1.8487	4.6180
		1.00	1.06667	.40552	.189	-.3180	2.4513
		4.00	10.23333*	.40552	.000	8.8487	11.6180
		8.00	23.06667*	.40552	.000	21.6820	24.4513
		10.00	28.73333*	.40552	.000	27.3487	30.1180
		12.00	29.33333*	.40552	.000	27.9487	30.7180
	4.00	0.00	-7.00000*	.40552	.000	-8.3847	-5.6153
		1.00	-9.16667*	.40552	.000	-10.5513	-7.7820
		2.00	-10.23333*	.40552	.000	-11.6180	-8.8487
		8.00	12.83333*	.40552	.000	11.4487	14.2180
		10.00	18.50000*	.40552	.000	17.1153	19.8847
		12.00	19.10000*	.40552	.000	17.7153	20.4847
	8.00	0.00	-19.83333*	.40552	.000	-21.2180	-18.4487
		1.00	-22.00000*	.40552	.000	-23.3847	-20.6153

		2.00	-23.06667*	.40552	.000	-24.4513	-21.6820
		4.00	-12.83333*	.40552	.000	-14.2180	-11.4487
		10.00	5.66667*	.40552	.000	4.2820	7.0513
		12.00	6.26667*	.40552	.000	4.8820	7.6513
	10.00	0.00	-25.50000*	.40552	.000	-26.8847	-24.1153
		1.00	-27.66667*	.40552	.000	-29.0513	-26.2820
		2.00	-28.73333*	.40552	.000	-30.1180	-27.3487
		4.00	-18.50000*	.40552	.000	-19.8847	-17.1153
		8.00	-5.66667*	.40552	.000	-7.0513	-4.2820
		12.00	.60000	.40552	.752	-.7847	1.9847
	12.00	0.00	-26.10000*	.40552	.000	-27.4847	-24.7153
		1.00	-28.26667*	.40552	.000	-29.6513	-26.8820
		2.00	-29.33333*	.40552	.000	-30.7180	-27.9487
		4.00	-19.10000*	.40552	.000	-20.4847	-17.7153
		8.00	-6.26667*	.40552	.000	-7.6513	-4.8820
		10.00	-.60000	.40552	.752	-1.9847	.7847

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
C0.00	24 hours	48 hours	-10.00000*	.77280	.000	-12.4748	-7.5252
		72 hours	-21.66667*	.77280	.000	-24.1414	-19.1919
		96 hours	-17.16667*	.77280	.000	-19.6414	-14.6919
	48 hours	24 hours	10.00000*	.77280	.000	7.5252	12.4748
		72 hours	-11.66667*	.77280	.000	-14.1414	-9.1919
		96 hours	-7.16667*	.77280	.000	-9.6414	-4.6919
	72 hours	24 hours	21.66667*	.77280	.000	19.1919	24.1414
		48 hours	11.66667*	.77280	.000	9.1919	14.1414
		96 hours	4.50000*	.77280	.002	2.0252	6.9748
	96 hours	24 hours	17.16667*	.77280	.000	14.6919	19.6414
		48 hours	7.16667*	.77280	.000	4.6919	9.6414
		72 hours	-4.50000*	.77280	.002	-6.9748	-2.0252
C1.00	24 hours	48 hours	-13.50000*	.84984	.000	-16.2215	-10.7785
		72 hours	-24.66667*	.84984	.000	-27.3881	-21.9452
		96 hours	-18.00000*	.84984	.000	-20.7215	-15.2785
	48 hours	24 hours	13.50000*	.84984	.000	10.7785	16.2215

		72 hours	-11.16667*	.84984	.000	-13.8881	-8.4452
		96 hours	-4.50000*	.84984	.003	-7.2215	-1.7785
	72 hours	24 hours	24.66667*	.84984	.000	21.9452	27.3881
		48 hours	11.16667*	.84984	.000	8.4452	13.8881
		96 hours	6.66667*	.84984	.000	3.9452	9.3881
	96 hours	24 hours	18.00000*	.84984	.000	15.2785	20.7215
		48 hours	4.50000*	.84984	.003	1.7785	7.2215
		72 hours	-6.66667*	.84984	.000	-9.3881	-3.9452
C2.00	24 hours	48 hours	-12.66667*	.56519	.000	-14.4766	-10.8567
		72 hours	-26.00000*	.56519	.000	-27.8100	-24.1900
		96 hours	-19.33333*	.56519	.000	-21.1433	-17.5234
	48 hours	24 hours	12.66667*	.56519	.000	10.8567	14.4766
		72 hours	-13.33333*	.56519	.000	-15.1433	-11.5234
		96 hours	-6.66667*	.56519	.000	-8.4766	-4.8567
	72 hours	24 hours	26.00000*	.56519	.000	24.1900	27.8100
		48 hours	13.33333*	.56519	.000	11.5234	15.1433
		96 hours	6.66667*	.56519	.000	4.8567	8.4766
	96 hours	24 hours	19.33333*	.56519	.000	17.5234	21.1433
		48 hours	6.66667*	.56519	.000	4.8567	8.4766
		72 hours	-6.66667*	.56519	.000	-8.4766	-4.8567
C4.00	24 hours	48 hours	-8.16667*	.64550	.000	-10.2338	-6.0996
		72 hours	-15.00000*	.64550	.000	-17.0671	-12.9329
		96 hours	-11.50000*	.64550	.000	-13.5671	-9.4329
	48 hours	24 hours	8.16667*	.64550	.000	6.0996	10.2338
		72 hours	-6.83333*	.64550	.000	-8.9004	-4.7662
		96 hours	-3.33333*	.64550	.004	-5.4004	-1.2662
	72 hours	24 hours	15.00000*	.64550	.000	12.9329	17.0671
		48 hours	6.83333*	.64550	.000	4.7662	8.9004
		96 hours	3.50000*	.64550	.003	1.4329	5.5671
	96 hours	24 hours	11.50000*	.64550	.000	9.4329	13.5671
		48 hours	3.33333*	.64550	.004	1.2662	5.4004
		72 hours	-3.50000*	.64550	.003	-5.5671	-1.4329
C8.00	24 hours	48 hours	-.33333	.33333	.754	-1.4008	.7341
		72 hours	-.66667	.33333	.264	-1.7341	.4008
		96 hours	-5.33333*	.33333	.000	-6.4008	-4.2659
	48 hours	24 hours	.33333	.33333	.754	-.7341	1.4008
		72 hours	-.33333	.33333	.754	-1.4008	.7341
		96 hours	-5.00000*	.33333	.000	-6.0675	-3.9325
	72 hours	24 hours	.66667	.33333	.264	-.4008	1.7341
		48 hours	.33333	.33333	.754	-.7341	1.4008
		96 hours	-4.66667*	.33333	.000	-5.7341	-3.5992

	96 hours	24 hours	5.33333*	.33333	.000	4.2659	6.4008
		48 hours	5.00000*	.33333	.000	3.9325	6.0675
		72 hours	4.66667*	.33333	.000	3.5992	5.7341
C10.00	24 hours	48 hours	-.10000	.31269	.988	-1.1014	.9014
		72 hours	-.26667	.31269	.828	-1.2680	.7347
		96 hours	.56667	.31269	.335	-.4347	1.5680
	48 hours	24 hours	.10000	.31269	.988	-.9014	1.1014
		72 hours	-.16667	.31269	.949	-1.1680	.8347
		96 hours	.66667	.31269	.222	-.3347	1.6680
	72 hours	24 hours	.26667	.31269	.828	-.7347	1.2680
		48 hours	.16667	.31269	.949	-.8347	1.1680
		96 hours	.83333	.31269	.107	-.1680	1.8347
	96 hours	24 hours	-.56667	.31269	.335	-1.5680	.4347
		48 hours	-.66667	.31269	.222	-1.6680	.3347
		72 hours	-.83333	.31269	.107	-1.8347	.1680
C12.00	24 hours	48 hours	-.33333	.25386	.580	-1.1463	.4796
		72 hours	.16667	.25386	.910	-.6463	.9796
		96 hours	1.43333*	.25386	.002	.6204	2.2463
	48 hours	24 hours	.33333	.25386	.580	-.4796	1.1463
		72 hours	.50000	.25386	.275	-.3129	1.3129
		96 hours	1.76667*	.25386	.001	.9537	2.5796
	72 hours	24 hours	-.16667	.25386	.910	-.9796	.6463
		48 hours	-.50000	.25386	.275	-1.3129	.3129
		96 hours	1.26667*	.25386	.005	.4537	2.0796
	96 hours	24 hours	-1.43333*	.25386	.002	-2.2463	-.6204
		48 hours	-1.76667*	.25386	.001	-2.5796	-.9537
		72 hours	-1.26667*	.25386	.005	-2.0796	-.4537

*. The mean difference is significant at the 0.05 level.

APPENDIX D: Total gas release (leaf powder) descriptive statistics table

Descriptives									
						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
24H	0.00	3	10.6667	1.15470	.66667	7.7982	13.5351	10.00	12.00
	1.00	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	2.00	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	4.00	3	9.3333	.57735	.33333	7.8991	10.7676	9.00	10.00
	8.00	3	2.6667	.28868	.16667	1.9496	3.3838	2.50	3.00
	10.00	3	2.9000	.36056	.20817	2.0043	3.7957	2.50	3.20
	12.00	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	Total	21	7.5333	4.23950	.92513	5.6035	9.4631	2.50	13.00
48H	0.00	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	1.00	3	25.9333	1.79258	1.03494	21.4803	30.3863	24.80	28.00
	2.00	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	4.00	3	17.3333	1.15470	.66667	14.4649	20.2018	16.00	18.00
	8.00	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	10.00	3	3.0000	.20000	.11547	2.5032	3.4968	2.80	3.20
	12.00	3	3.5000	.30000	.17321	2.7548	4.2452	3.20	3.80
	Total	21	13.9667	9.95396	2.17213	9.4357	18.4977	2.80	28.00
72H	0.00	3	32.3333	1.15470	.66667	29.4649	35.2018	31.00	33.00
	1.00	3	36.6667	1.15470	.66667	33.7982	39.5351	36.00	38.00
	2.00	3	37.3333	1.15470	.66667	34.4649	40.2018	36.00	38.00
	4.00	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	8.00	3	3.3333	.57735	.33333	1.8991	4.7676	3.00	4.00
	10.00	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	12.00	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	Total	21	20.0238	15.50361	3.38316	12.9667	27.0810	3.00	38.00
96H	0.00	3	27.8333	.76376	.44096	25.9360	29.7306	27.00	28.50
	1.00	3	30.0000	.50000	.28868	28.7579	31.2421	29.50	30.50
	2.00	3	31.0667	.11547	.06667	30.7798	31.3535	31.00	31.20
	4.00	3	20.8333	.28868	.16667	20.1162	21.5504	20.50	21.00
	8.00	3	8.0000	.50000	.28868	6.7579	9.2421	7.50	8.50
	10.00	3	2.3333	.57735	.33333	.8991	3.7676	2.00	3.00
	12.00	3	1.7333	.46188	.26667	.5860	2.8807	1.20	2.00
	Total	21	17.4000	12.41857	2.70996	11.7471	23.0529	1.20	31.20

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
C0.00	24 hours	3	10.6667	1.15470	.66667	7.7982	13.5351	10.00	12.00
	48 hours	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	72 hours	3	32.3333	1.15470	.66667	29.4649	35.2018	31.00	33.00
	96 hours	3	27.8333	.76376	.44096	25.9360	29.7306	27.00	28.50
	Total	12	22.8750	8.58679	2.47879	17.4192	28.3308	10.00	33.00
C1.00	24 hours	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	48 hours	3	25.5000	1.32288	.76376	22.2138	28.7862	24.50	27.00
	72 hours	3	36.6667	1.15470	.66667	33.7982	39.5351	36.00	38.00
	96 hours	3	30.0000	.50000	.28868	28.7579	31.2421	29.50	30.50
	Total	12	26.0417	9.47115	2.73409	20.0240	32.0593	11.00	38.00
C2.00	24 hours	3	12.0000	1.00000	.57735	9.5159	14.4841	11.00	13.00
	48 hours	3	24.6667	.76376	.44096	22.7694	26.5640	24.00	25.50
	72 hours	3	38.0000	.00000	.00000	38.0000	38.0000	38.00	38.00
	96 hours	3	31.3333	.57735	.33333	29.8991	32.7676	31.00	32.00
	Total	12	26.5000	10.05214	2.90180	20.1132	32.8868	11.00	38.00
C4.00	24 hours	3	9.3333	.57735	.33333	7.8991	10.7676	9.00	10.00
	48 hours	3	17.5000	1.32288	.76376	14.2138	20.7862	16.00	18.50
	72 hours	3	24.3333	.57735	.33333	22.8991	25.7676	24.00	25.00
	96 hours	3	20.8333	.28868	.16667	20.1162	21.5504	20.50	21.00
	Total	12	18.0000	5.84263	1.68662	14.2878	21.7122	9.00	25.00

C8.00	24 hours	3	2.6667	.28868	.16667	1.9496	3.3838	2.50	3.00
	48 hours	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	72 hours	3	3.3333	.57735	.33333	1.8991	4.7676	3.00	4.00
	96 hours	3	8.0000	.50000	.28868	6.7579	9.2421	7.50	8.50
	Total	12	4.2500	2.30119	.66430	2.7879	5.7121	2.50	8.50
C10.00	24 hours	3	2.9000	.36056	.20817	2.0043	3.7957	2.50	3.20
	48 hours	3	3.0000	.20000	.11547	2.5032	3.4968	2.80	3.20
	72 hours	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	96 hours	3	2.3333	.57735	.33333	.8991	3.7676	2.00	3.00
	Total	12	2.8500	.46221	.13343	2.5563	3.1437	2.00	3.50
C12.00	24 hours	3	3.1667	.28868	.16667	2.4496	3.8838	3.00	3.50
	48 hours	3	3.5000	.30000	.17321	2.7548	4.2452	3.20	3.80
	72 hours	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
	96 hours	3	1.7333	.46188	.26667	.5860	2.8807	1.20	2.00
	Total	12	2.8500	.74772	.21585	2.3749	3.3251	1.20	3.80

APPENDIX E: Total gas release (leaf powder) multiple comparison statistics

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) mg/ml	(J) mg/ml	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
24H	0.00	1.00	-1.33333	.61438	.368	-3.4312	.7645
		2.00	-1.33333	.61438	.368	-3.4312	.7645
		4.00	1.33333	.61438	.368	-.7645	3.4312
		8.00	8.00000*	.61438	.000	5.9022	10.0978

		10.00	7.76667*	.61438	.000	5.6688	9.8645
		12.00	7.50000*	.61438	.000	5.4022	9.5978
	1.00	0.00	1.33333	.61438	.368	-.7645	3.4312
		2.00	.00000	.61438	1.000	-2.0978	2.0978
		4.00	2.66667*	.61438	.009	.5688	4.7645
		8.00	9.33333*	.61438	.000	7.2355	11.4312
		10.00	9.10000*	.61438	.000	7.0022	11.1978
		12.00	8.83333*	.61438	.000	6.7355	10.9312
	2.00	0.00	1.33333	.61438	.368	-.7645	3.4312
		1.00	.00000	.61438	1.000	-2.0978	2.0978
		4.00	2.66667*	.61438	.009	.5688	4.7645
		8.00	9.33333*	.61438	.000	7.2355	11.4312
		10.00	9.10000*	.61438	.000	7.0022	11.1978
		12.00	8.83333*	.61438	.000	6.7355	10.9312
	4.00	0.00	-1.33333	.61438	.368	-3.4312	.7645
		1.00	-2.66667*	.61438	.009	-4.7645	-.5688
		2.00	-2.66667*	.61438	.009	-4.7645	-.5688
		8.00	6.66667*	.61438	.000	4.5688	8.7645
		10.00	6.43333*	.61438	.000	4.3355	8.5312
		12.00	6.16667*	.61438	.000	4.0688	8.2645
	8.00	0.00	-8.00000*	.61438	.000	-10.0978	-5.9022
		1.00	-9.33333*	.61438	.000	-11.4312	-7.2355
		2.00	-9.33333*	.61438	.000	-11.4312	-7.2355
		4.00	-6.66667*	.61438	.000	-8.7645	-4.5688
		10.00	-.23333	.61438	1.000	-2.3312	1.8645
		12.00	-.50000	.61438	.979	-2.5978	1.5978
	10.00	0.00	-7.76667*	.61438	.000	-9.8645	-5.6688
		1.00	-9.10000*	.61438	.000	-11.1978	-7.0022
		2.00	-9.10000*	.61438	.000	-11.1978	-7.0022
		4.00	-6.43333*	.61438	.000	-8.5312	-4.3355
		8.00	.23333	.61438	1.000	-1.8645	2.3312
		12.00	-.26667	.61438	.999	-2.3645	1.8312
	12.00	0.00	-7.50000*	.61438	.000	-9.5978	-5.4022
		1.00	-8.83333*	.61438	.000	-10.9312	-6.7355
		2.00	-8.83333*	.61438	.000	-10.9312	-6.7355
		4.00	-6.16667*	.61438	.000	-8.2645	-4.0688
		8.00	.50000	.61438	.979	-1.5978	2.5978
		10.00	.26667	.61438	.999	-1.8312	2.3645
48H	0.00	1.00	-5.26667*	.71336	.000	-7.7025	-2.8308
		2.00	-3.66667*	.71336	.002	-6.1025	-1.2308

		4.00	3.33333*	.71336	.005	.8975	5.7692
		8.00	17.66667*	.71336	.000	15.2308	20.1025
		10.00	17.66667*	.71336	.000	15.2308	20.1025
		12.00	17.16667*	.71336	.000	14.7308	19.6025
	1.00	0.00	5.26667*	.71336	.000	2.8308	7.7025
		2.00	1.60000	.71336	.334	-.8358	4.0358
		4.00	8.60000*	.71336	.000	6.1642	11.0358
		8.00	22.93333*	.71336	.000	20.4975	25.3692
		10.00	22.93333*	.71336	.000	20.4975	25.3692
		12.00	22.43333*	.71336	.000	19.9975	24.8692
	2.00	0.00	3.66667*	.71336	.002	1.2308	6.1025
		1.00	-1.60000	.71336	.334	-4.0358	.8358
		4.00	7.00000*	.71336	.000	4.5642	9.4358
		8.00	21.33333*	.71336	.000	18.8975	23.7692
		10.00	21.33333*	.71336	.000	18.8975	23.7692
		12.00	20.83333*	.71336	.000	18.3975	23.2692
	4.00	0.00	-3.33333*	.71336	.005	-5.7692	-.8975
		1.00	-8.60000*	.71336	.000	-11.0358	-6.1642
		2.00	-7.00000*	.71336	.000	-9.4358	-4.5642
		8.00	14.33333*	.71336	.000	11.8975	16.7692
		10.00	14.33333*	.71336	.000	11.8975	16.7692
		12.00	13.83333*	.71336	.000	11.3975	16.2692
	8.00	0.00	-17.66667*	.71336	.000	-20.1025	-15.2308
		1.00	-22.93333*	.71336	.000	-25.3692	-20.4975
		2.00	-21.33333*	.71336	.000	-23.7692	-18.8975
		4.00	-14.33333*	.71336	.000	-16.7692	-11.8975
		10.00	.00000	.71336	1.000	-2.4358	2.4358
		12.00	-.50000	.71336	.990	-2.9358	1.9358
	10.00	0.00	-17.66667*	.71336	.000	-20.1025	-15.2308
		1.00	-22.93333*	.71336	.000	-25.3692	-20.4975
		2.00	-21.33333*	.71336	.000	-23.7692	-18.8975
		4.00	-14.33333*	.71336	.000	-16.7692	-11.8975
		8.00	.00000	.71336	1.000	-2.4358	2.4358
		12.00	-.50000	.71336	.990	-2.9358	1.9358
	12.00	0.00	-17.16667*	.71336	.000	-19.6025	-14.7308
		1.00	-22.43333*	.71336	.000	-24.8692	-19.9975
		2.00	-20.83333*	.71336	.000	-23.2692	-18.3975
		4.00	-13.83333*	.71336	.000	-16.2692	-11.3975
		8.00	.50000	.71336	.990	-1.9358	2.9358
		10.00	.50000	.71336	.990	-1.9358	2.9358

72H	0.00	1.00	-4.33333*	.67259	.000	-6.6300	-2.0367
		2.00	-5.00000*	.67259	.000	-7.2966	-2.7034
		4.00	8.00000*	.67259	.000	5.7034	10.2966
		8.00	29.00000*	.67259	.000	26.7034	31.2966
		10.00	29.16667*	.67259	.000	26.8700	31.4633
		12.00	29.33333*	.67259	.000	27.0367	31.6300
	1.00	0.00	4.33333*	.67259	.000	2.0367	6.6300
		2.00	-.66667	.67259	.948	-2.9633	1.6300
		4.00	12.33333*	.67259	.000	10.0367	14.6300
		8.00	33.33333*	.67259	.000	31.0367	35.6300
		10.00	33.50000*	.67259	.000	31.2034	35.7966
		12.00	33.66667*	.67259	.000	31.3700	35.9633
	2.00	0.00	5.00000*	.67259	.000	2.7034	7.2966
		1.00	.66667	.67259	.948	-1.6300	2.9633
		4.00	13.00000*	.67259	.000	10.7034	15.2966
		8.00	34.00000*	.67259	.000	31.7034	36.2966
		10.00	34.16667*	.67259	.000	31.8700	36.4633
		12.00	34.33333*	.67259	.000	32.0367	36.6300
	4.00	0.00	-8.00000*	.67259	.000	-10.2966	-5.7034
		1.00	-12.33333*	.67259	.000	-14.6300	-10.0367
		2.00	-13.00000*	.67259	.000	-15.2966	-10.7034
		8.00	21.00000*	.67259	.000	18.7034	23.2966
		10.00	21.16667*	.67259	.000	18.8700	23.4633
		12.00	21.33333*	.67259	.000	19.0367	23.6300
	8.00	0.00	-29.00000*	.67259	.000	-31.2966	-26.7034
		1.00	-33.33333*	.67259	.000	-35.6300	-31.0367
		2.00	-34.00000*	.67259	.000	-36.2966	-31.7034
		4.00	-21.00000*	.67259	.000	-23.2966	-18.7034
		10.00	.16667	.67259	1.000	-2.1300	2.4633
		12.00	.33333	.67259	.999	-1.9633	2.6300
	10.00	0.00	-29.16667*	.67259	.000	-31.4633	-26.8700
		1.00	-33.50000*	.67259	.000	-35.7966	-31.2034
		2.00	-34.16667*	.67259	.000	-36.4633	-31.8700
		4.00	-21.16667*	.67259	.000	-23.4633	-18.8700
		8.00	-.16667	.67259	1.000	-2.4633	2.1300
		12.00	.16667	.67259	1.000	-2.1300	2.4633
	12.00	0.00	-29.33333*	.67259	.000	-31.6300	-27.0367
		1.00	-33.66667*	.67259	.000	-35.9633	-31.3700
		2.00	-34.33333*	.67259	.000	-36.6300	-32.0367
		4.00	-21.33333*	.67259	.000	-23.6300	-19.0367

		8.00	-.33333	.67259	.999	-2.6300	1.9633
		10.00	-.16667	.67259	1.000	-2.4633	2.1300
96H	0.00	1.00	-2.16667*	.40552	.002	-3.5513	-.7820
		2.00	-3.23333*	.40552	.000	-4.6180	-1.8487
		4.00	7.00000*	.40552	.000	5.6153	8.3847
		8.00	19.83333*	.40552	.000	18.4487	21.2180
		10.00	25.50000*	.40552	.000	24.1153	26.8847
		12.00	26.10000*	.40552	.000	24.7153	27.4847
	1.00	0.00	2.16667*	.40552	.002	.7820	3.5513
		2.00	-1.06667	.40552	.189	-2.4513	.3180
		4.00	9.16667*	.40552	.000	7.7820	10.5513
		8.00	22.00000*	.40552	.000	20.6153	23.3847
		10.00	27.66667*	.40552	.000	26.2820	29.0513
		12.00	28.26667*	.40552	.000	26.8820	29.6513
	2.00	0.00	3.23333*	.40552	.000	1.8487	4.6180
		1.00	1.06667	.40552	.189	-.3180	2.4513
		4.00	10.23333*	.40552	.000	8.8487	11.6180
		8.00	23.06667*	.40552	.000	21.6820	24.4513
		10.00	28.73333*	.40552	.000	27.3487	30.1180
		12.00	29.33333*	.40552	.000	27.9487	30.7180
	4.00	0.00	-7.00000*	.40552	.000	-8.3847	-5.6153
		1.00	-9.16667*	.40552	.000	-10.5513	-7.7820
		2.00	-10.23333*	.40552	.000	-11.6180	-8.8487
		8.00	12.83333*	.40552	.000	11.4487	14.2180
		10.00	18.50000*	.40552	.000	17.1153	19.8847
		12.00	19.10000*	.40552	.000	17.7153	20.4847
	8.00	0.00	-19.83333*	.40552	.000	-21.2180	-18.4487
		1.00	-22.00000*	.40552	.000	-23.3847	-20.6153
		2.00	-23.06667*	.40552	.000	-24.4513	-21.6820
		4.00	-12.83333*	.40552	.000	-14.2180	-11.4487
		10.00	5.66667*	.40552	.000	4.2820	7.0513
		12.00	6.26667*	.40552	.000	4.8820	7.6513
	10.00	0.00	-25.50000*	.40552	.000	-26.8847	-24.1153
		1.00	-27.66667*	.40552	.000	-29.0513	-26.2820
		2.00	-28.73333*	.40552	.000	-30.1180	-27.3487
		4.00	-18.50000*	.40552	.000	-19.8847	-17.1153
		8.00	-5.66667*	.40552	.000	-7.0513	-4.2820
		12.00	.60000	.40552	.752	-.7847	1.9847
	12.00	0.00	-26.10000*	.40552	.000	-27.4847	-24.7153
		1.00	-28.26667*	.40552	.000	-29.6513	-26.8820

	2.00	-29.33333*	.40552	.000	-30.7180	-27.9487
	4.00	-19.10000*	.40552	.000	-20.4847	-17.7153
	8.00	-6.26667*	.40552	.000	-7.6513	-4.8820
	10.00	-.60000	.40552	.752	-1.9847	.7847

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
P0.00	24 hours	48 hours	-15.1000*	.5370	.000	-16.820	-13.380
		72 hours	-19.2667*	.5370	.000	-20.986	-17.547
		96 hours	-6.9333*	.5370	.000	-8.653	-5.214
	48 hours	24 hours	15.1000*	.5370	.000	13.380	16.820
		72 hours	-4.1667*	.5370	.000	-5.886	-2.447
		96 hours	8.1667*	.5370	.000	6.447	9.886
	72 hours	24 hours	19.2667*	.5370	.000	17.547	20.986
		48 hours	4.1667*	.5370	.000	2.447	5.886
		96 hours	12.3333*	.5370	.000	10.614	14.053
	96 hours	24 hours	6.9333*	.5370	.000	5.214	8.653
		48 hours	-8.1667*	.5370	.000	-9.886	-6.447
		72 hours	-12.3333*	.5370	.000	-14.053	-10.614
P0.25	24 hours	48 hours	-12.6667*	.9354	.000	-15.662	-9.671
		72 hours	-13.3333*	.9354	.000	-16.329	-10.338
		96 hours	-.3333	.9354	.983	-3.329	2.662
	48 hours	24 hours	12.6667*	.9354	.000	9.671	15.662
		72 hours	-.6667	.9354	.889	-3.662	2.329
		96 hours	12.3333*	.9354	.000	9.338	15.329
	72 hours	24 hours	13.3333*	.9354	.000	10.338	16.329
		48 hours	.6667	.9354	.889	-2.329	3.662
		96 hours	13.0000*	.9354	.000	10.004	15.996
	96 hours	24 hours	.3333	.9354	.983	-2.662	3.329
		48 hours	-12.3333*	.9354	.000	-15.329	-9.338
		72 hours	-13.0000*	.9354	.000	-15.996	-10.004
P0.50	24 hours	48 hours	-14.1667*	.9936	.000	-17.348	-10.985
		72 hours	-18.0000*	.9936	.000	-21.182	-14.818
		96 hours	.3333	.9936	.986	-2.848	3.515
	48 hours	24 hours	14.1667*	.9936	.000	10.985	17.348
		72 hours	-3.8333*	.9936	.020	-7.015	-.652

		96 hours	14.5000*	.9936	.000	11.318	17.682
	72 hours	24 hours	18.0000*	.9936	.000	14.818	21.182
		48 hours	3.8333*	.9936	.020	.652	7.015
		96 hours	18.3333*	.9936	.000	15.152	21.515
	96 hours	24 hours	-.3333	.9936	.986	-3.515	2.848
		48 hours	-14.5000*	.9936	.000	-17.682	-11.318
		72 hours	-18.3333*	.9936	.000	-21.515	-15.152
P1.00	24 hours	48 hours	-15.0000*	.6745	.000	-17.160	-12.840
		72 hours	-16.5000*	.6745	.000	-18.660	-14.340
		96 hours	.9333	.6745	.542	-1.227	3.093
	48 hours	24 hours	15.0000*	.6745	.000	12.840	17.160
		72 hours	-1.5000	.6745	.196	-3.660	.660
		96 hours	15.9333*	.6745	.000	13.773	18.093
	72 hours	24 hours	16.5000*	.6745	.000	14.340	18.660
		48 hours	1.5000	.6745	.196	-.660	3.660
		96 hours	17.4333*	.6745	.000	15.273	19.593
	96 hours	24 hours	-.9333	.6745	.542	-3.093	1.227
		48 hours	-15.9333*	.6745	.000	-18.093	-13.773
		72 hours	-17.4333*	.6745	.000	-19.593	-15.273
P2.00	24 hours	48 hours	-15.5000*	.7431	.000	-17.880	-13.120
		72 hours	-17.3333*	.7431	.000	-19.713	-14.954
		96 hours	.1000	.7431	.999	-2.280	2.480
	48 hours	24 hours	15.5000*	.7431	.000	13.120	17.880
		72 hours	-1.8333	.7431	.141	-4.213	.546
		96 hours	15.6000*	.7431	.000	13.220	17.980
	72 hours	24 hours	17.3333*	.7431	.000	14.954	19.713
		48 hours	1.8333	.7431	.141	-.546	4.213
		96 hours	17.4333*	.7431	.000	15.054	19.813
	96 hours	24 hours	-.1000	.7431	.999	-2.480	2.280
		48 hours	-15.6000*	.7431	.000	-17.980	-13.220
		72 hours	-17.4333*	.7431	.000	-19.813	-15.054
P4.00	24 hours	48 hours	-17.3333*	.6346	.000	-19.366	-15.301
		72 hours	-18.6667*	.6346	.000	-20.699	-16.634
		96 hours	.3333	.6346	.951	-1.699	2.366
	48 hours	24 hours	17.3333*	.6346	.000	15.301	19.366
		72 hours	-1.3333	.6346	.231	-3.366	.699
		96 hours	17.6667*	.6346	.000	15.634	19.699
	72 hours	24 hours	18.6667*	.6346	.000	16.634	20.699
		48 hours	1.3333	.6346	.231	-.699	3.366
		96 hours	19.0000*	.6346	.000	16.968	21.032

	96 hours	24 hours	-.3333	.6346	.951	-2.366	1.699
		48 hours	-17.6667*	.6346	.000	-19.699	-15.634
		72 hours	-19.0000*	.6346	.000	-21.032	-16.968
P8.00	24 hours	48 hours	-17.5000*	1.5811	.000	-22.563	-12.437
		72 hours	-15.8333*	1.5811	.000	-20.897	-10.770
		96 hours	-7.6667*	1.5811	.006	-12.730	-2.603
	48 hours	24 hours	17.5000*	1.5811	.000	12.437	22.563
		72 hours	1.6667	1.5811	.725	-3.397	6.730
		96 hours	9.8333*	1.5811	.001	4.770	14.897
	72 hours	24 hours	15.8333*	1.5811	.000	10.770	20.897
		48 hours	-1.6667	1.5811	.725	-6.730	3.397
		96 hours	8.1667*	1.5811	.004	3.103	13.230
	96 hours	24 hours	7.6667*	1.5811	.006	2.603	12.730
		48 hours	-9.8333*	1.5811	.001	-14.897	-4.770
		72 hours	-8.1667*	1.5811	.004	-13.230	-3.103
P16.00	24 hours	48 hours	-16.5000*	3.0713	.003	-26.335	-6.665
		72 hours	-17.1667*	3.0713	.002	-27.002	-7.331
		96 hours	-4.4333	3.0713	.510	-14.269	5.402
	48 hours	24 hours	16.5000*	3.0713	.003	6.665	26.335
		72 hours	-.6667	3.0713	.996	-10.502	9.169
		96 hours	12.0667*	3.0713	.018	2.231	21.902
	72 hours	24 hours	17.1667*	3.0713	.002	7.331	27.002
		48 hours	.6667	3.0713	.996	-9.169	10.502
		96 hours	12.7333*	3.0713	.014	2.898	22.569
	96 hours	24 hours	4.4333	3.0713	.510	-5.402	14.269
		48 hours	-12.0667*	3.0713	.018	-21.902	-2.231
		72 hours	-12.7333*	3.0713	.014	-22.569	-2.898

*. The mean difference is significant at the 0.05 level.

APPENDIX F: Fodder digestion (methanolic crude saponin extract) descriptive statistics table

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Time_24H	0.00	3	6.000	1.0000	.5774	3.516	8.484	5.0	7.0
	1.00	3	4.667	.5774	.3333	3.232	6.101	4.0	5.0

	2.00	3	7.333	.5774	.3333	5.899	8.768	7.0	8.0
	4.00	3	20.667	.5774	.3333	19.232	22.101	20.0	21.0
	8.00	3	16.667	2.0817	1.2019	11.496	21.838	15.0	19.0
	10.00	3	34.667	4.5092	2.6034	23.465	45.868	30.0	39.0
	12.00	3	40.333	1.1547	.6667	37.465	43.202	39.0	41.0
	Total	21	18.619	13.6216	2.9725	12.419	24.820	4.0	41.0
Time_48H	0.00	3	1.667	.5774	.3333	.232	3.101	1.0	2.0
	1.00	3	6.333	.5774	.3333	4.899	7.768	6.0	7.0
	2.00	3	6.000	1.0000	.5774	3.516	8.484	5.0	7.0
	4.00	3	5.667	1.5275	.8819	1.872	9.461	4.0	7.0
	8.00	3	37.000	3.6056	2.0817	28.043	45.957	33.0	40.0
	10.00	3	35.667	2.5166	1.4530	29.415	41.918	33.0	38.0
	12.00	3	54.667	3.5119	2.0276	45.943	63.391	51.0	58.0
	Total	21	21.000	20.0400	4.3731	11.878	30.122	1.0	58.0
Time_72H	0.00	3	31.000	2.6458	1.5275	24.428	37.572	28.0	33.0
	1.00	3	38.667	2.0817	1.2019	33.496	43.838	37.0	41.0
	2.00	3	39.000	2.6458	1.5275	32.428	45.572	37.0	42.0
	4.00	3	37.333	.5774	.3333	35.899	38.768	37.0	38.0
	8.00	3	58.333	4.9329	2.8480	46.079	70.587	55.0	64.0
	10.00	3	73.000	2.6458	1.5275	66.428	79.572	71.0	76.0
	12.00	3	81.333	2.0817	1.2019	76.162	86.504	79.0	83.0
	Total	21	51.238	18.8810	4.1202	42.644	59.833	28.0	83.0
Time_96H	0.00	3	39.667	.5774	.3333	38.232	41.101	39.0	40.0
	1.00	3	40.000	.0000	.0000	40.000	40.000	40.0	40.0
	2.00	3	54.333	2.5166	1.4530	48.082	60.585	52.0	57.0
	4.00	3	65.667	3.0551	1.7638	58.078	73.256	63.0	69.0
	8.00	3	93.667	2.3094	1.3333	87.930	99.404	91.0	95.0
	10.00	3	85.667	7.0946	4.0961	68.043	103.291	78.0	92.0
	12.00	3	69.000	.0000	.0000	69.000	69.000	69.0	69.0
	Total	21	64.000	20.0749	4.3807	54.862	73.138	39.0	95.0

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
C0.00	24 hours	3	6.000	1.0000	.5774	3.516	8.484	5.0	7.0

	48 hours	3	1.667	.5774	.3333	.232	3.101	1.0	2.0
	72 hours	3	31.000	2.6458	1.5275	24.428	37.572	28.0	33.0
	96 hours	3	39.667	.5774	.3333	38.232	41.101	39.0	40.0
	Total	12	19.583	16.8817	4.8733	8.857	30.309	1.0	40.0
C1.00	24 hours	3	4.667	.5774	.3333	3.232	6.101	4.0	5.0
	48 hours	3	10.667	4.1633	2.4037	.324	21.009	6.0	14.0
	72 hours	3	37.333	3.5119	2.0276	28.609	46.057	34.0	41.0
	96 hours	3	40.000	.0000	.0000	40.000	40.000	40.0	40.0
	Total	12	23.167	16.5355	4.7734	12.660	33.673	4.0	41.0
C2.00	24 hours	3	7.333	.5774	.3333	5.899	8.768	7.0	8.0
	48 hours	3	6.000	1.0000	.5774	3.516	8.484	5.0	7.0
	72 hours	3	39.000	2.6458	1.5275	32.428	45.572	37.0	42.0
	96 hours	3	54.333	2.5166	1.4530	48.082	60.585	52.0	57.0
	Total	12	26.667	21.7102	6.2672	12.873	40.461	5.0	57.0
C4.00	24 hours	3	20.667	.5774	.3333	19.232	22.101	20.0	21.0
	48 hours	3	5.667	1.5275	.8819	1.872	9.461	4.0	7.0
	72 hours	3	37.333	.5774	.3333	35.899	38.768	37.0	38.0
	96 hours	3	65.667	3.0551	1.7638	58.078	73.256	63.0	69.0
	Total	12	32.333	23.3056	6.7278	17.526	47.141	4.0	69.0
C8.00	24 hours	3	16.667	2.0817	1.2019	11.496	21.838	15.0	19.0
	48 hours	3	37.000	3.6056	2.0817	28.043	45.957	33.0	40.0
	72 hours	3	58.333	4.9329	2.8480	46.079	70.587	55.0	64.0
	96 hours	3	93.667	2.3094	1.3333	87.930	99.404	91.0	95.0

	Total	12	51.417	29.9073	8.6335	32.414	70.419	15.0	95.0
C10.00	24 hours	3	34.667	4.5092	2.6034	23.465	45.868	30.0	39.0
	48 hours	3	35.667	2.5166	1.4530	29.415	41.918	33.0	38.0
	72 hours	3	73.000	2.6458	1.5275	66.428	79.572	71.0	76.0
	96 hours	3	85.667	7.0946	4.0961	68.043	103.291	78.0	92.0
	Total	12	57.250	23.8599	6.8878	42.090	72.410	30.0	92.0
C12.00	24 hours	3	40.333	1.1547	.6667	37.465	43.202	39.0	41.0
	48 hours	3	54.667	3.5119	2.0276	45.943	63.391	51.0	58.0
	72 hours	3	81.333	2.0817	1.2019	76.162	86.504	79.0	83.0
	96 hours	3	69.333	.5774	.3333	67.899	70.768	69.0	70.0
	Total	12	61.417	16.1946	4.6750	51.127	71.706	39.0	83.0

APPENDIX G: Fodder digestion (methanolic crude saponin extract) multiple comparison statistics

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Concentration (mg/ml)	(J) Concentration (mg/ml)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Time_24H	0.00	1.00	1.3333	1.6330	.979	-4.243	6.909
		2.00	-1.3333	1.6330	.979	-6.909	4.243
		4.00	-14.6667*	1.6330	.000	-20.243	-9.091
		8.00	-10.6667*	1.6330	.000	-16.243	-5.091
		10.00	-28.6667*	1.6330	.000	-34.243	-23.091
		12.00	-34.3333*	1.6330	.000	-39.909	-28.757
	1.00	0.00	-1.3333	1.6330	.979	-6.909	4.243
		2.00	-2.6667	1.6330	.666	-8.243	2.909
		4.00	-16.0000*	1.6330	.000	-21.576	-10.424
		8.00	-12.0000*	1.6330	.000	-17.576	-6.424

		10.00	-30.0000*	1.6330	.000	-35.576	-24.424
		12.00	-35.6667*	1.6330	.000	-41.243	-30.091
	2.00	0.00	1.3333	1.6330	.979	-4.243	6.909
		1.00	2.6667	1.6330	.666	-2.909	8.243
		4.00	-13.3333*	1.6330	.000	-18.909	-7.757
		8.00	-9.3333*	1.6330	.001	-14.909	-3.757
		10.00	-27.3333*	1.6330	.000	-32.909	-21.757
		12.00	-33.0000*	1.6330	.000	-38.576	-27.424
	4.00	0.00	14.6667*	1.6330	.000	9.091	20.243
		1.00	16.0000*	1.6330	.000	10.424	21.576
		2.00	13.3333*	1.6330	.000	7.757	18.909
		8.00	4.0000	1.6330	.249	-1.576	9.576
		10.00	-14.0000*	1.6330	.000	-19.576	-8.424
		12.00	-19.6667*	1.6330	.000	-25.243	-14.091
	8.00	0.00	10.6667*	1.6330	.000	5.091	16.243
		1.00	12.0000*	1.6330	.000	6.424	17.576
		2.00	9.3333*	1.6330	.001	3.757	14.909
		4.00	-4.0000	1.6330	.249	-9.576	1.576
		10.00	-18.0000*	1.6330	.000	-23.576	-12.424
		12.00	-23.6667*	1.6330	.000	-29.243	-18.091
	10.00	0.00	28.6667*	1.6330	.000	23.091	34.243
		1.00	30.0000*	1.6330	.000	24.424	35.576
		2.00	27.3333*	1.6330	.000	21.757	32.909
		4.00	14.0000*	1.6330	.000	8.424	19.576
		8.00	18.0000*	1.6330	.000	12.424	23.576
		12.00	-5.6667*	1.6330	.045	-11.243	-.091
	12.00	0.00	34.3333*	1.6330	.000	28.757	39.909
		1.00	35.6667*	1.6330	.000	30.091	41.243
		2.00	33.0000*	1.6330	.000	27.424	38.576
		4.00	19.6667*	1.6330	.000	14.091	25.243
		8.00	23.6667*	1.6330	.000	18.091	29.243
		10.00	5.6667*	1.6330	.045	.091	11.243
Time_48H	0.00	1.00	-4.6667	1.8430	.220	-10.960	1.627
		2.00	-4.3333	1.8430	.287	-10.627	1.960
		4.00	-4.0000	1.8430	.368	-10.293	2.293
		8.00	-35.3333*	1.8430	.000	-41.627	-29.040
		10.00	-34.0000*	1.8430	.000	-40.293	-27.707
		12.00	-53.0000*	1.8430	.000	-59.293	-46.707
	1.00	0.00	4.6667	1.8430	.220	-1.627	10.960
		2.00	.3333	1.8430	1.000	-5.960	6.627

		4.00	.6667	1.8430	1.000	-5.627	6.960
		8.00	-30.6667*	1.8430	.000	-36.960	-24.373
		10.00	-29.3333*	1.8430	.000	-35.627	-23.040
		12.00	-48.3333*	1.8430	.000	-54.627	-42.040
	2.00	0.00	4.3333	1.8430	.287	-1.960	10.627
		1.00	-.3333	1.8430	1.000	-6.627	5.960
		4.00	.3333	1.8430	1.000	-5.960	6.627
		8.00	-31.0000*	1.8430	.000	-37.293	-24.707
		10.00	-29.6667*	1.8430	.000	-35.960	-23.373
		12.00	-48.6667*	1.8430	.000	-54.960	-42.373
	4.00	0.00	4.0000	1.8430	.368	-2.293	10.293
		1.00	-.6667	1.8430	1.000	-6.960	5.627
		2.00	-.3333	1.8430	1.000	-6.627	5.960
		8.00	-31.3333*	1.8430	.000	-37.627	-25.040
		10.00	-30.0000*	1.8430	.000	-36.293	-23.707
		12.00	-49.0000*	1.8430	.000	-55.293	-42.707
	8.00	0.00	35.3333*	1.8430	.000	29.040	41.627
		1.00	30.6667*	1.8430	.000	24.373	36.960
		2.00	31.0000*	1.8430	.000	24.707	37.293
		4.00	31.3333*	1.8430	.000	25.040	37.627
		10.00	1.3333	1.8430	.989	-4.960	7.627
		12.00	-17.6667*	1.8430	.000	-23.960	-11.373
	10.00	0.00	34.0000*	1.8430	.000	27.707	40.293
		1.00	29.3333*	1.8430	.000	23.040	35.627
		2.00	29.6667*	1.8430	.000	23.373	35.960
		4.00	30.0000*	1.8430	.000	23.707	36.293
		8.00	-1.3333	1.8430	.989	-7.627	4.960
		12.00	-19.0000*	1.8430	.000	-25.293	-12.707
	12.00	0.00	53.0000*	1.8430	.000	46.707	59.293
		1.00	48.3333*	1.8430	.000	42.040	54.627
		2.00	48.6667*	1.8430	.000	42.373	54.960
		4.00	49.0000*	1.8430	.000	42.707	55.293
		8.00	17.6667*	1.8430	.000	11.373	23.960
		10.00	19.0000*	1.8430	.000	12.707	25.293
Time_72H	0.00	1.00	-7.6667	2.2748	.054	-15.434	.101
		2.00	-8.0000*	2.2748	.042	-15.767	-.233
		4.00	-6.3333	2.2748	.147	-14.101	1.434
		8.00	-27.3333*	2.2748	.000	-35.101	-19.566
		10.00	-42.0000*	2.2748	.000	-49.767	-34.233
		12.00	-50.3333*	2.2748	.000	-58.101	-42.566

	1.00	0.00	7.6667	2.2748	.054	-.101	15.434
		2.00	-.3333	2.2748	1.000	-8.101	7.434
		4.00	1.3333	2.2748	.996	-6.434	9.101
		8.00	-19.6667*	2.2748	.000	-27.434	-11.899
		10.00	-34.3333*	2.2748	.000	-42.101	-26.566
		12.00	-42.6667*	2.2748	.000	-50.434	-34.899
	2.00	0.00	8.0000*	2.2748	.042	.233	15.767
		1.00	.3333	2.2748	1.000	-7.434	8.101
		4.00	1.6667	2.2748	.988	-6.101	9.434
		8.00	-19.3333*	2.2748	.000	-27.101	-11.566
		10.00	-34.0000*	2.2748	.000	-41.767	-26.233
		12.00	-42.3333*	2.2748	.000	-50.101	-34.566
	4.00	0.00	6.3333	2.2748	.147	-1.434	14.101
		1.00	-1.3333	2.2748	.996	-9.101	6.434
		2.00	-1.6667	2.2748	.988	-9.434	6.101
		8.00	-21.0000*	2.2748	.000	-28.767	-13.233
		10.00	-35.6667*	2.2748	.000	-43.434	-27.899
		12.00	-44.0000*	2.2748	.000	-51.767	-36.233
	8.00	0.00	27.3333*	2.2748	.000	19.566	35.101
		1.00	19.6667*	2.2748	.000	11.899	27.434
		2.00	19.3333*	2.2748	.000	11.566	27.101
		4.00	21.0000*	2.2748	.000	13.233	28.767
		10.00	-14.6667*	2.2748	.000	-22.434	-6.899
		12.00	-23.0000*	2.2748	.000	-30.767	-15.233
	10.00	0.00	42.0000*	2.2748	.000	34.233	49.767
		1.00	34.3333*	2.2748	.000	26.566	42.101
		2.00	34.0000*	2.2748	.000	26.233	41.767
		4.00	35.6667*	2.2748	.000	27.899	43.434
		8.00	14.6667*	2.2748	.000	6.899	22.434
		12.00	-8.3333*	2.2748	.032	-16.101	-.566
	12.00	0.00	50.3333*	2.2748	.000	42.566	58.101
		1.00	42.6667*	2.2748	.000	34.899	50.434
		2.00	42.3333*	2.2748	.000	34.566	50.101
		4.00	44.0000*	2.2748	.000	36.233	51.767
		8.00	23.0000*	2.2748	.000	15.233	30.767
		10.00	8.3333*	2.2748	.032	.566	16.101
Time_96H	0.00	1.00	-.3333	2.6125	1.000	-9.254	8.587
		2.00	-14.6667*	2.6125	.001	-23.587	-5.746
		4.00	-26.0000*	2.6125	.000	-34.921	-17.079
		8.00	-54.0000*	2.6125	.000	-62.921	-45.079

	10.00	-46.0000*	2.6125	.000	-54.921	-37.079
	12.00	-29.3333*	2.6125	.000	-38.254	-20.413
1.00	0.00	.3333	2.6125	1.000	-8.587	9.254
	2.00	-14.3333*	2.6125	.001	-23.254	-5.413
	4.00	-25.6667*	2.6125	.000	-34.587	-16.746
	8.00	-53.6667*	2.6125	.000	-62.587	-44.746
	10.00	-45.6667*	2.6125	.000	-54.587	-36.746
	12.00	-29.0000*	2.6125	.000	-37.921	-20.079
2.00	0.00	14.6667*	2.6125	.001	5.746	23.587
	1.00	14.3333*	2.6125	.001	5.413	23.254
	4.00	-11.3333*	2.6125	.009	-20.254	-2.413
	8.00	-39.3333*	2.6125	.000	-48.254	-30.413
	10.00	-31.3333*	2.6125	.000	-40.254	-22.413
	12.00	-14.6667*	2.6125	.001	-23.587	-5.746
4.00	0.00	26.0000*	2.6125	.000	17.079	34.921
	1.00	25.6667*	2.6125	.000	16.746	34.587
	2.00	11.3333*	2.6125	.009	2.413	20.254
	8.00	-28.0000*	2.6125	.000	-36.921	-19.079
	10.00	-20.0000*	2.6125	.000	-28.921	-11.079
	12.00	-3.3333	2.6125	.852	-12.254	5.587
8.00	0.00	54.0000*	2.6125	.000	45.079	62.921
	1.00	53.6667*	2.6125	.000	44.746	62.587
	2.00	39.3333*	2.6125	.000	30.413	48.254
	4.00	28.0000*	2.6125	.000	19.079	36.921
	10.00	8.0000	2.6125	.093	-.921	16.921
	12.00	24.6667*	2.6125	.000	15.746	33.587
10.00	0.00	46.0000*	2.6125	.000	37.079	54.921
	1.00	45.6667*	2.6125	.000	36.746	54.587
	2.00	31.3333*	2.6125	.000	22.413	40.254
	4.00	20.0000*	2.6125	.000	11.079	28.921
	8.00	-8.0000	2.6125	.093	-16.921	.921
	12.00	16.6667*	2.6125	.000	7.746	25.587
12.00	0.00	29.3333*	2.6125	.000	20.413	38.254
	1.00	29.0000*	2.6125	.000	20.079	37.921
	2.00	14.6667*	2.6125	.001	5.746	23.587
	4.00	3.3333	2.6125	.852	-5.587	12.254
	8.00	-24.6667*	2.6125	.000	-33.587	-15.746
	10.00	-16.6667*	2.6125	.000	-25.587	-7.746

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
C0.00	24 hours	48 hours	4.3333*	1.2019	.028	.485	8.182
		72 hours	-25.0000*	1.2019	.000	-28.849	-21.151
		96 hours	-33.6667*	1.2019	.000	-37.515	-29.818
	48 hours	24 hours	-4.3333*	1.2019	.028	-8.182	-.485
		72 hours	-29.3333*	1.2019	.000	-33.182	-25.485
		96 hours	-38.0000*	1.2019	.000	-41.849	-34.151
	72 hours	24 hours	25.0000*	1.2019	.000	21.151	28.849
		48 hours	29.3333*	1.2019	.000	25.485	33.182
		96 hours	-8.6667*	1.2019	.000	-12.515	-4.818
	96 hours	24 hours	33.6667*	1.2019	.000	29.818	37.515
		48 hours	38.0000*	1.2019	.000	34.151	41.849
		72 hours	8.6667*	1.2019	.000	4.818	12.515
C1.00	24 hours	48 hours	-6.0000	2.2361	.104	-13.161	1.161
		72 hours	-32.6667*	2.2361	.000	-39.827	-25.506
		96 hours	-35.3333*	2.2361	.000	-42.494	-28.173
	48 hours	24 hours	6.0000	2.2361	.104	-1.161	13.161
		72 hours	-26.6667*	2.2361	.000	-33.827	-19.506
		96 hours	-29.3333*	2.2361	.000	-36.494	-22.173
	72 hours	24 hours	32.6667*	2.2361	.000	25.506	39.827
		48 hours	26.6667*	2.2361	.000	19.506	33.827
		96 hours	-2.6667	2.2361	.648	-9.827	4.494
	96 hours	24 hours	35.3333*	2.2361	.000	28.173	42.494
		48 hours	29.3333*	2.2361	.000	22.173	36.494
		72 hours	2.6667	2.2361	.648	-4.494	9.827
C2.00	24 hours	48 hours	1.3333	1.5635	.828	-3.673	6.340
		72 hours	-31.6667*	1.5635	.000	-36.673	-26.660
		96 hours	-47.0000*	1.5635	.000	-52.007	-41.993
	48 hours	24 hours	-1.3333	1.5635	.828	-6.340	3.673
		72 hours	-33.0000*	1.5635	.000	-38.007	-27.993
		96 hours	-48.3333*	1.5635	.000	-53.340	-43.327
	72 hours	24 hours	31.6667*	1.5635	.000	26.660	36.673
		48 hours	33.0000*	1.5635	.000	27.993	38.007
		96 hours	-15.3333*	1.5635	.000	-20.340	-10.327
	96 hours	24 hours	47.0000*	1.5635	.000	41.993	52.007
		48 hours	48.3333*	1.5635	.000	43.327	53.340
		72 hours	15.3333*	1.5635	.000	10.327	20.340

C4.00	24 hours	48 hours	15.0000*	1.4337	.000	10.409	19.591
		72 hours	-16.6667*	1.4337	.000	-21.258	-12.075
		96 hours	-45.0000*	1.4337	.000	-49.591	-40.409
	48 hours	24 hours	-15.0000*	1.4337	.000	-19.591	-10.409
		72 hours	-31.6667*	1.4337	.000	-36.258	-27.075
		96 hours	-60.0000*	1.4337	.000	-64.591	-55.409
	72 hours	24 hours	16.6667*	1.4337	.000	12.075	21.258
		48 hours	31.6667*	1.4337	.000	27.075	36.258
		96 hours	-28.3333*	1.4337	.000	-32.925	-23.742
	96 hours	24 hours	45.0000*	1.4337	.000	40.409	49.591
		48 hours	60.0000*	1.4337	.000	55.409	64.591
		72 hours	28.3333*	1.4337	.000	23.742	32.925
C8.00	24 hours	48 hours	-20.3333*	2.7988	.000	-29.296	-11.371
		72 hours	-41.6667*	2.7988	.000	-50.629	-32.704
		96 hours	-77.0000*	2.7988	.000	-85.963	-68.037
	48 hours	24 hours	20.3333*	2.7988	.000	11.371	29.296
		72 hours	-21.3333*	2.7988	.000	-30.296	-12.371
		96 hours	-56.6667*	2.7988	.000	-65.629	-47.704
	72 hours	24 hours	41.6667*	2.7988	.000	32.704	50.629
		48 hours	21.3333*	2.7988	.000	12.371	30.296
		96 hours	-35.3333*	2.7988	.000	-44.296	-26.371
	96 hours	24 hours	77.0000*	2.7988	.000	68.037	85.963
		48 hours	56.6667*	2.7988	.000	47.704	65.629
		72 hours	35.3333*	2.7988	.000	26.371	44.296
C10.00	24 hours	48 hours	-1.0000	3.7417	.993	-12.982	10.982
		72 hours	-38.3333*	3.7417	.000	-50.315	-26.351
		96 hours	-51.0000*	3.7417	.000	-62.982	-39.018
	48 hours	24 hours	1.0000	3.7417	.993	-10.982	12.982
		72 hours	-37.3333*	3.7417	.000	-49.315	-25.351
		96 hours	-50.0000*	3.7417	.000	-61.982	-38.018
	72 hours	24 hours	38.3333*	3.7417	.000	26.351	50.315
		48 hours	37.3333*	3.7417	.000	25.351	49.315
		96 hours	-12.6667*	3.7417	.039	-24.649	-.685
	96 hours	24 hours	51.0000*	3.7417	.000	39.018	62.982
		48 hours	50.0000*	3.7417	.000	38.018	61.982
		72 hours	12.6667*	3.7417	.039	.685	24.649
C12.00	24 hours	48 hours	-14.3333*	1.7480	.000	-19.931	-8.736
		72 hours	-41.0000*	1.7480	.000	-46.598	-35.402
		96 hours	-29.0000*	1.7480	.000	-34.598	-23.402
	48 hours	24 hours	14.3333*	1.7480	.000	8.736	19.931

		72 hours	-26.6667*	1.7480	.000	-32.264	-21.069
		96 hours	-14.6667*	1.7480	.000	-20.264	-9.069
	72 hours	24 hours	41.0000*	1.7480	.000	35.402	46.598
		48 hours	26.6667*	1.7480	.000	21.069	32.264
		96 hours	12.0000*	1.7480	.001	6.402	17.598
	96 hours	24 hours	29.0000*	1.7480	.000	23.402	34.598
		48 hours	14.6667*	1.7480	.000	9.069	20.264
		72 hours	-12.0000*	1.7480	.001	-17.598	-6.402

*. The mean difference is significant at the 0.05 level.

APPENDIX H: Fodder digestion (leaf powder) descriptive statistics table

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Time_24H	0.00	3	11.67	.577	.333	10.23	13.10	11	12
	0.25	3	17.00	.000	.000	17.00	17.00	17	17
	0.50	3	20.67	.577	.333	19.23	22.10	20	21
	1.00	3	10.67	.577	.333	9.23	12.10	10	11
	2.00	3	6.00	.000	.000	6.00	6.00	6	6
	4.00	3	18.00	.000	.000	18.00	18.00	18	18
	8.00	3	9.33	.577	.333	7.90	10.77	9	10
	16.00	3	10.33	.577	.333	8.90	11.77	10	11
	Total	24	12.96	4.814	.983	10.93	14.99	6	21
Time_48H	0.00	3	19.00	.000	.000	19.00	19.00	19	19
	0.25	3	22.00	.000	.000	22.00	22.00	22	22
	0.50	3	29.00	.000	.000	29.00	29.00	29	29
	1.00	3	25.67	.577	.333	24.23	27.10	25	26
	2.00	3	23.00	.000	.000	23.00	23.00	23	23
	4.00	3	20.67	.577	.333	19.23	22.10	20	21
	8.00	3	32.00	.000	.000	32.00	32.00	32	32
	16.00	3	30.33	.577	.333	28.90	31.77	30	31
	Total	24	25.21	4.597	.938	23.27	27.15	19	32
Time_72H	0.00	3	24.00	1.732	1.000	19.70	28.30	22	25
	0.25	3	23.67	1.155	.667	20.80	26.54	23	25
	0.50	3	26.00	1.000	.577	23.52	28.48	25	27
	1.00	3	6.33	.577	.333	4.90	7.77	6	7

	2.00	3	15.67	.577	.333	14.23	17.10	15	16
	4.00	3	24.67	1.155	.667	21.80	27.54	24	26
	8.00	3	9.67	1.155	.667	6.80	12.54	9	11
	16.00	3	1.33	.577	.333	-.10	2.77	1	2
	Total	24	16.42	9.217	1.881	12.52	20.31	1	27
Time_96H	0.00	3	6.67	.577	.333	5.23	8.10	6	7
	0.25	3	17.00	1.732	1.000	12.70	21.30	16	19
	0.50	3	19.67	.577	.333	18.23	21.10	19	20
	1.00	3	28.33	.577	.333	26.90	29.77	28	29
	2.00	3	30.67	1.528	.882	26.87	34.46	29	32
	4.00	3	20.67	.577	.333	19.23	22.10	20	21
	8.00	3	26.67	.577	.333	25.23	28.10	26	27
	16.00	3	16.00	.000	.000	16.00	16.00	16	16
	Total	24	20.71	7.504	1.532	17.54	23.88	6	32

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P0.00	24 hours	3	11.6667	.57735	.33333	10.2324	13.1009	11.00	12.00
	48 hours	3	19.0000	.00000	.00000	19.0000	19.0000	19.00	19.00
	72 hours	3	24.0000	1.73205	1.00000	19.6973	28.3027	22.00	25.00
	96 hours	3	6.6667	.57735	.33333	5.2324	8.1009	6.00	7.00
	Total	12	15.3333	6.99784	2.02010	10.8871	19.7795	6.00	25.00
P0.25	24 hours	3	17.0000	.00000	.00000	17.0000	17.0000	17.00	17.00
	48 hours	3	22.0000	.00000	.00000	22.0000	22.0000	22.00	22.00
	72 hours	3	23.3333	1.52753	.88192	19.5388	27.1279	22.00	25.00
	96 hours	3	17.0000	1.73205	1.00000	12.6973	21.3027	16.00	19.00
	Total	12	19.8333	3.15748	.91149	17.8272	21.8395	16.00	25.00
P0.50	24 hours	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00

	48 hours	3	29.0000	.00000	.00000	29.0000	29.0000	29.00	29.00
	72 hours	3	26.0000	1.00000	.57735	23.5159	28.4841	25.00	27.00
	96 hours	3	19.6667	.57735	.33333	18.2324	21.1009	19.00	20.00
	Total	12	23.8333	4.04145	1.16667	21.2655	26.4011	19.00	29.00
P1.00	24 hours	3	10.6667	.57735	.33333	9.2324	12.1009	10.00	11.00
	48 hours	3	25.6667	.57735	.33333	24.2324	27.1009	25.00	26.00
	72 hours	3	6.3333	.57735	.33333	4.8991	7.7676	6.00	7.00
	96 hours	3	28.3333	.57735	.33333	26.8991	29.7676	28.00	29.00
	Total	12	17.7500	9.85463	2.84479	11.4887	24.0113	6.00	29.00
P2.00	24 hours	3	6.0000	.00000	.00000	6.0000	6.0000	6.00	6.00
	48 hours	3	23.0000	.00000	.00000	23.0000	23.0000	23.00	23.00
	72 hours	3	15.6667	.57735	.33333	14.2324	17.1009	15.00	16.00
	96 hours	3	30.6667	1.52753	.88192	26.8721	34.4612	29.00	32.00
	Total	12	18.8333	9.54257	2.75470	12.7703	24.8964	6.00	32.00
P4.00	24 hours	3	18.0000	.00000	.00000	18.0000	18.0000	18.00	18.00
	48 hours	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	72 hours	3	24.6667	1.15470	.66667	21.7982	27.5351	24.00	26.00
	96 hours	3	20.6667	.57735	.33333	19.2324	22.1009	20.00	21.00
	Total	12	21.0000	2.55841	.73855	19.3745	22.6255	18.00	26.00
P8.00	24 hours	3	9.3333	.57735	.33333	7.8991	10.7676	9.00	10.00
	48 hours	3	32.0000	.00000	.00000	32.0000	32.0000	32.00	32.00
	72 hours	3	9.6667	1.15470	.66667	6.7982	12.5351	9.00	11.00
	96 hours	3	26.6667	.57735	.33333	25.2324	28.1009	26.00	27.00

	Total	12	19.4167	10.56115	3.04874	12.7064	26.1269	9.00	32.00
P16.00	24 hours	3	10.3333	.57735	.33333	8.8991	11.7676	10.00	11.00
	48 hours	3	30.3333	.57735	.33333	28.8991	31.7676	30.00	31.00
	72 hours	3	1.6667	.57735	.33333	.2324	3.1009	1.00	2.00
	96 hours	3	16.0000	.00000	.00000	16.0000	16.0000	16.00	16.00
	Total	12	14.5833	10.90003	3.14657	7.6578	21.5089	1.00	31.00

APPENDIX I: Fodder digestion (leaf powder) multiple comparison statistics

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Plant Material %	(J) Plant Material %	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Time_24H	0.00	0.25	-5.333*	.373	.000	-6.62	-4.04
		0.50	-9.000*	.373	.000	-10.29	-7.71
		1.00	1.000	.373	.197	-.29	2.29
		2.00	5.667*	.373	.000	4.38	6.96
		4.00	-6.333*	.373	.000	-7.62	-5.04
		8.00	2.333*	.373	.000	1.04	3.62
		16.00	1.333*	.373	.040	.04	2.62
	0.25	0.00	5.333*	.373	.000	4.04	6.62
		0.50	-3.667*	.373	.000	-4.96	-2.38
		1.00	6.333*	.373	.000	5.04	7.62
		2.00	11.000*	.373	.000	9.71	12.29
		4.00	-1.000	.373	.197	-2.29	.29
		8.00	7.667*	.373	.000	6.38	8.96
		16.00	6.667*	.373	.000	5.38	7.96
	0.50	0.00	9.000*	.373	.000	7.71	10.29
		0.25	3.667*	.373	.000	2.38	4.96
		1.00	10.000*	.373	.000	8.71	11.29
		2.00	14.667*	.373	.000	13.38	15.96
		4.00	2.667*	.373	.000	1.38	3.96
		8.00	11.333*	.373	.000	10.04	12.62
		16.00	10.333*	.373	.000	9.04	11.62

	1.00	0.00	-1.000	.373	.197	-2.29	.29
		0.25	-6.333*	.373	.000	-7.62	-5.04
		0.50	-10.000*	.373	.000	-11.29	-8.71
		2.00	4.667*	.373	.000	3.38	5.96
		4.00	-7.333*	.373	.000	-8.62	-6.04
		8.00	1.333*	.373	.040	.04	2.62
		16.00	.333	.373	.982	-.96	1.62
	2.00	0.00	-5.667*	.373	.000	-6.96	-4.38
		0.25	-11.000*	.373	.000	-12.29	-9.71
		0.50	-14.667*	.373	.000	-15.96	-13.38
		1.00	-4.667*	.373	.000	-5.96	-3.38
		4.00	-12.000*	.373	.000	-13.29	-10.71
		8.00	-3.333*	.373	.000	-4.62	-2.04
		16.00	-4.333*	.373	.000	-5.62	-3.04
	4.00	0.00	6.333*	.373	.000	5.04	7.62
		0.25	1.000	.373	.197	-.29	2.29
		0.50	-2.667*	.373	.000	-3.96	-1.38
		1.00	7.333*	.373	.000	6.04	8.62
		2.00	12.000*	.373	.000	10.71	13.29
		8.00	8.667*	.373	.000	7.38	9.96
		16.00	7.667*	.373	.000	6.38	8.96
	8.00	0.00	-2.333*	.373	.000	-3.62	-1.04
		0.25	-7.667*	.373	.000	-8.96	-6.38
		0.50	-11.333*	.373	.000	-12.62	-10.04
		1.00	-1.333*	.373	.040	-2.62	-.04
		2.00	3.333*	.373	.000	2.04	4.62
		4.00	-8.667*	.373	.000	-9.96	-7.38
		16.00	-1.000	.373	.197	-2.29	.29
	16.00	0.00	-1.333*	.373	.040	-2.62	-.04
		0.25	-6.667*	.373	.000	-7.96	-5.38
		0.50	-10.333*	.373	.000	-11.62	-9.04
		1.00	-.333	.373	.982	-1.62	.96
		2.00	4.333*	.373	.000	3.04	5.62
		4.00	-7.667*	.373	.000	-8.96	-6.38
		8.00	1.000	.373	.197	-.29	2.29
Time_48H	0.00	0.25	-3.000*	.289	.000	-4.00	-2.00
		0.50	-10.000*	.289	.000	-11.00	-9.00
		1.00	-6.667*	.289	.000	-7.67	-5.67
		2.00	-4.000*	.289	.000	-5.00	-3.00
		4.00	-1.667*	.289	.001	-2.67	-.67

	8.00	-13.000*	.289	.000	-14.00	-12.00
	16.00	-11.333*	.289	.000	-12.33	-10.33
0.25	0.00	3.000*	.289	.000	2.00	4.00
	0.50	-7.000*	.289	.000	-8.00	-6.00
	1.00	-3.667*	.289	.000	-4.67	-2.67
	2.00	-1.000*	.289	.050	-2.00	.00
	4.00	1.333*	.289	.005	.33	2.33
	8.00	-10.000*	.289	.000	-11.00	-9.00
	16.00	-8.333*	.289	.000	-9.33	-7.33
0.50	0.00	10.000*	.289	.000	9.00	11.00
	0.25	7.000*	.289	.000	6.00	8.00
	1.00	3.333*	.289	.000	2.33	4.33
	2.00	6.000*	.289	.000	5.00	7.00
	4.00	8.333*	.289	.000	7.33	9.33
	8.00	-3.000*	.289	.000	-4.00	-2.00
	16.00	-1.333*	.289	.005	-2.33	-.33
1.00	0.00	6.667*	.289	.000	5.67	7.67
	0.25	3.667*	.289	.000	2.67	4.67
	0.50	-3.333*	.289	.000	-4.33	-2.33
	2.00	2.667*	.289	.000	1.67	3.67
	4.00	5.000*	.289	.000	4.00	6.00
	8.00	-6.333*	.289	.000	-7.33	-5.33
	16.00	-4.667*	.289	.000	-5.67	-3.67
2.00	0.00	4.000*	.289	.000	3.00	5.00
	0.25	1.000*	.289	.050	.00	2.00
	0.50	-6.000*	.289	.000	-7.00	-5.00
	1.00	-2.667*	.289	.000	-3.67	-1.67
	4.00	2.333*	.289	.000	1.33	3.33
	8.00	-9.000*	.289	.000	-10.00	-8.00
	16.00	-7.333*	.289	.000	-8.33	-6.33
4.00	0.00	1.667*	.289	.001	.67	2.67
	0.25	-1.333*	.289	.005	-2.33	-.33
	0.50	-8.333*	.289	.000	-9.33	-7.33
	1.00	-5.000*	.289	.000	-6.00	-4.00
	2.00	-2.333*	.289	.000	-3.33	-1.33
	8.00	-11.333*	.289	.000	-12.33	-10.33
	16.00	-9.667*	.289	.000	-10.67	-8.67
8.00	0.00	13.000*	.289	.000	12.00	14.00
	0.25	10.000*	.289	.000	9.00	11.00
	0.50	3.000*	.289	.000	2.00	4.00

		1.00	6.333*	.289	.000	5.33	7.33
		2.00	9.000*	.289	.000	8.00	10.00
		4.00	11.333*	.289	.000	10.33	12.33
		16.00	1.667*	.289	.001	.67	2.67
	16.00	0.00	11.333*	.289	.000	10.33	12.33
		0.25	8.333*	.289	.000	7.33	9.33
		0.50	1.333*	.289	.005	.33	2.33
		1.00	4.667*	.289	.000	3.67	5.67
		2.00	7.333*	.289	.000	6.33	8.33
		4.00	9.667*	.289	.000	8.67	10.67
		8.00	-1.667*	.289	.001	-2.67	-.67
Time_72H	0.00	0.25	.333	.866	1.000	-2.66	3.33
		0.50	-2.000	.866	.346	-5.00	1.00
		1.00	17.667*	.866	.000	14.67	20.66
		2.00	8.333*	.866	.000	5.34	11.33
		4.00	-.667	.866	.993	-3.66	2.33
		8.00	14.333*	.866	.000	11.34	17.33
		16.00	22.667*	.866	.000	19.67	25.66
	0.25	0.00	-.333	.866	1.000	-3.33	2.66
		0.50	-2.333	.866	.194	-5.33	.66
		1.00	17.333*	.866	.000	14.34	20.33
		2.00	8.000*	.866	.000	5.00	11.00
		4.00	-1.000	.866	.934	-4.00	2.00
		8.00	14.000*	.866	.000	11.00	17.00
		16.00	22.333*	.866	.000	19.34	25.33
	0.50	0.00	2.000	.866	.346	-1.00	5.00
		0.25	2.333	.866	.194	-.66	5.33
		1.00	19.667*	.866	.000	16.67	22.66
		2.00	10.333*	.866	.000	7.34	13.33
		4.00	1.333	.866	.777	-1.66	4.33
		8.00	16.333*	.866	.000	13.34	19.33
		16.00	24.667*	.866	.000	21.67	27.66
	1.00	0.00	-17.667*	.866	.000	-20.66	-14.67
		0.25	-17.333*	.866	.000	-20.33	-14.34
		0.50	-19.667*	.866	.000	-22.66	-16.67
		2.00	-9.333*	.866	.000	-12.33	-6.34
		4.00	-18.333*	.866	.000	-21.33	-15.34
		8.00	-3.333*	.866	.024	-6.33	-.34
		16.00	5.000*	.866	.001	2.00	8.00
	2.00	0.00	-8.333*	.866	.000	-11.33	-5.34

		0.25	-8.000*	.866	.000	-11.00	-5.00
		0.50	-10.333*	.866	.000	-13.33	-7.34
		1.00	9.333*	.866	.000	6.34	12.33
		4.00	-9.000*	.866	.000	-12.00	-6.00
		8.00	6.000*	.866	.000	3.00	9.00
		16.00	14.333*	.866	.000	11.34	17.33
	4.00	0.00	.667	.866	.993	-2.33	3.66
		0.25	1.000	.866	.934	-2.00	4.00
		0.50	-1.333	.866	.777	-4.33	1.66
		1.00	18.333*	.866	.000	15.34	21.33
		2.00	9.000*	.866	.000	6.00	12.00
		8.00	15.000*	.866	.000	12.00	18.00
		16.00	23.333*	.866	.000	20.34	26.33
	8.00	0.00	-14.333*	.866	.000	-17.33	-11.34
		0.25	-14.000*	.866	.000	-17.00	-11.00
		0.50	-16.333*	.866	.000	-19.33	-13.34
		1.00	3.333*	.866	.024	.34	6.33
		2.00	-6.000*	.866	.000	-9.00	-3.00
		4.00	-15.000*	.866	.000	-18.00	-12.00
		16.00	8.333*	.866	.000	5.34	11.33
	16.00	0.00	-22.667*	.866	.000	-25.66	-19.67
		0.25	-22.333*	.866	.000	-25.33	-19.34
		0.50	-24.667*	.866	.000	-27.66	-21.67
		1.00	-5.000*	.866	.001	-8.00	-2.00
		2.00	-14.333*	.866	.000	-17.33	-11.34
		4.00	-23.333*	.866	.000	-26.33	-20.34
		8.00	-8.333*	.866	.000	-11.33	-5.34
Time_96H	0.00	0.25	-10.333*	.764	.000	-12.98	-7.69
		0.50	-13.000*	.764	.000	-15.64	-10.36
		1.00	-21.667*	.764	.000	-24.31	-19.02
		2.00	-24.000*	.764	.000	-26.64	-21.36
		4.00	-14.000*	.764	.000	-16.64	-11.36
		8.00	-20.000*	.764	.000	-22.64	-17.36
		16.00	-9.333*	.764	.000	-11.98	-6.69
	0.25	0.00	10.333*	.764	.000	7.69	12.98
		0.50	-2.667*	.764	.047	-5.31	-.02
		1.00	-11.333*	.764	.000	-13.98	-8.69
		2.00	-13.667*	.764	.000	-16.31	-11.02
		4.00	-3.667*	.764	.004	-6.31	-1.02
		8.00	-9.667*	.764	.000	-12.31	-7.02

	16.00	1.000	.764	.883	-1.64	3.64
0.50	0.00	13.000*	.764	.000	10.36	15.64
	0.25	2.667*	.764	.047	.02	5.31
	1.00	-8.667*	.764	.000	-11.31	-6.02
	2.00	-11.000*	.764	.000	-13.64	-8.36
	4.00	-1.000	.764	.883	-3.64	1.64
	8.00	-7.000*	.764	.000	-9.64	-4.36
	16.00	3.667*	.764	.004	1.02	6.31
1.00	0.00	21.667*	.764	.000	19.02	24.31
	0.25	11.333*	.764	.000	8.69	13.98
	0.50	8.667*	.764	.000	6.02	11.31
	2.00	-2.333	.764	.105	-4.98	.31
	4.00	7.667*	.764	.000	5.02	10.31
	8.00	1.667	.764	.410	-.98	4.31
	16.00	12.333*	.764	.000	9.69	14.98
2.00	0.00	24.000*	.764	.000	21.36	26.64
	0.25	13.667*	.764	.000	11.02	16.31
	0.50	11.000*	.764	.000	8.36	13.64
	1.00	2.333	.764	.105	-.31	4.98
	4.00	10.000*	.764	.000	7.36	12.64
	8.00	4.000*	.764	.002	1.36	6.64
	16.00	14.667*	.764	.000	12.02	17.31
4.00	0.00	14.000*	.764	.000	11.36	16.64
	0.25	3.667*	.764	.004	1.02	6.31
	0.50	1.000	.764	.883	-1.64	3.64
	1.00	-7.667*	.764	.000	-10.31	-5.02
	2.00	-10.000*	.764	.000	-12.64	-7.36
	8.00	-6.000*	.764	.000	-8.64	-3.36
	16.00	4.667*	.764	.000	2.02	7.31
8.00	0.00	20.000*	.764	.000	17.36	22.64
	0.25	9.667*	.764	.000	7.02	12.31
	0.50	7.000*	.764	.000	4.36	9.64
	1.00	-1.667	.764	.410	-4.31	.98
	2.00	-4.000*	.764	.002	-6.64	-1.36
	4.00	6.000*	.764	.000	3.36	8.64
	16.00	10.667*	.764	.000	8.02	13.31
16.00	0.00	9.333*	.764	.000	6.69	11.98
	0.25	-1.000	.764	.883	-3.64	1.64
	0.50	-3.667*	.764	.004	-6.31	-1.02
	1.00	-12.333*	.764	.000	-14.98	-9.69

	2.00	-14.667*	.764	.000	-17.31	-12.02
	4.00	-4.667*	.764	.000	-7.31	-2.02
	8.00	-10.667*	.764	.000	-13.31	-8.02

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
P0.00	24 hours	48 hours	-7.33333*	.78174	.000	-9.8367	-4.8299
		72 hours	-12.33333*	.78174	.000	-14.8367	-9.8299
		96 hours	5.00000*	.78174	.001	2.4966	7.5034
	48 hours	24 hours	7.33333*	.78174	.000	4.8299	9.8367
		72 hours	-5.00000*	.78174	.001	-7.5034	-2.4966
		96 hours	12.33333*	.78174	.000	9.8299	14.8367
	72 hours	24 hours	12.33333*	.78174	.000	9.8299	14.8367
		48 hours	5.00000*	.78174	.001	2.4966	7.5034
		96 hours	17.33333*	.78174	.000	14.8299	19.8367
	96 hours	24 hours	-5.00000*	.78174	.001	-7.5034	-2.4966
		48 hours	-12.33333*	.78174	.000	-14.8367	-9.8299
		72 hours	-17.33333*	.78174	.000	-19.8367	-14.8299
P0.25	24 hours	48 hours	-5.00000*	.94281	.003	-8.0192	-1.9808
		72 hours	-6.33333*	.94281	.001	-9.3525	-3.3141
		96 hours	.00000	.94281	1.000	-3.0192	3.0192
	48 hours	24 hours	5.00000*	.94281	.003	1.9808	8.0192
		72 hours	-1.33333	.94281	.525	-4.3525	1.6859
		96 hours	5.00000*	.94281	.003	1.9808	8.0192
	72 hours	24 hours	6.33333*	.94281	.001	3.3141	9.3525
		48 hours	1.33333	.94281	.525	-1.6859	4.3525
		96 hours	6.33333*	.94281	.001	3.3141	9.3525
	96 hours	24 hours	.00000	.94281	1.000	-3.0192	3.0192
		48 hours	-5.00000*	.94281	.003	-8.0192	-1.9808
		72 hours	-6.33333*	.94281	.001	-9.3525	-3.3141
P0.50	24 hours	48 hours	-8.33333*	.52705	.000	-10.0211	-6.6455
		72 hours	-5.33333*	.52705	.000	-7.0211	-3.6455
		96 hours	1.00000	.52705	.301	-.6878	2.6878
	48 hours	24 hours	8.33333*	.52705	.000	6.6455	10.0211
		72 hours	3.00000*	.52705	.002	1.3122	4.6878
		96 hours	9.33333*	.52705	.000	7.6455	11.0211

	72 hours	24 hours	5.33333*	.52705	.000	3.6455	7.0211
		48 hours	-3.00000*	.52705	.002	-4.6878	-1.3122
		96 hours	6.33333*	.52705	.000	4.6455	8.0211
	96 hours	24 hours	-1.00000	.52705	.301	-2.6878	.6878
		48 hours	-9.33333*	.52705	.000	-11.0211	-7.6455
		72 hours	-6.33333*	.52705	.000	-8.0211	-4.6455
P1.00	24 hours	48 hours	-15.00000*	.47140	.000	-16.5096	-13.4904
		72 hours	4.33333*	.47140	.000	2.8237	5.8429
		96 hours	-17.66667*	.47140	.000	-19.1763	-16.1571
	48 hours	24 hours	15.00000*	.47140	.000	13.4904	16.5096
		72 hours	19.33333*	.47140	.000	17.8237	20.8429
		96 hours	-2.66667*	.47140	.002	-4.1763	-1.1571
	72 hours	24 hours	-4.33333*	.47140	.000	-5.8429	-2.8237
		48 hours	-19.33333*	.47140	.000	-20.8429	-17.8237
		96 hours	-22.00000*	.47140	.000	-23.5096	-20.4904
	96 hours	24 hours	17.66667*	.47140	.000	16.1571	19.1763
		48 hours	2.66667*	.47140	.002	1.1571	4.1763
		72 hours	22.00000*	.47140	.000	20.4904	23.5096
P2.00	24 hours	48 hours	-17.00000*	.66667	.000	-19.1349	-14.8651
		72 hours	-9.66667*	.66667	.000	-11.8016	-7.5318
		96 hours	-24.66667*	.66667	.000	-26.8016	-22.5318
	48 hours	24 hours	17.00000*	.66667	.000	14.8651	19.1349
		72 hours	7.33333*	.66667	.000	5.1984	9.4682
		96 hours	-7.66667*	.66667	.000	-9.8016	-5.5318
	72 hours	24 hours	9.66667*	.66667	.000	7.5318	11.8016
		48 hours	-7.33333*	.66667	.000	-9.4682	-5.1984
		96 hours	-15.00000*	.66667	.000	-17.1349	-12.8651
	96 hours	24 hours	24.66667*	.66667	.000	22.5318	26.8016
		48 hours	7.66667*	.66667	.000	5.5318	9.8016
		72 hours	15.00000*	.66667	.000	12.8651	17.1349
P4.00	24 hours	48 hours	-2.66667*	.57735	.007	-4.5155	-.8178
		72 hours	-6.66667*	.57735	.000	-8.5155	-4.8178
		96 hours	-2.66667*	.57735	.007	-4.5155	-.8178
	48 hours	24 hours	2.66667*	.57735	.007	.8178	4.5155
		72 hours	-4.00000*	.57735	.001	-5.8489	-2.1511
		96 hours	.00000	.57735	1.000	-1.8489	1.8489
	72 hours	24 hours	6.66667*	.57735	.000	4.8178	8.5155
		48 hours	4.00000*	.57735	.001	2.1511	5.8489
		96 hours	4.00000*	.57735	.001	2.1511	5.8489
	96 hours	24 hours	2.66667*	.57735	.007	.8178	4.5155

		48 hours	.00000	.57735	1.000	-1.8489	1.8489
		72 hours	-4.00000*	.57735	.001	-5.8489	-2.1511
P8.00	24 hours	48 hours	-22.66667*	.57735	.000	-24.5155	-20.8178
		72 hours	-.33333	.57735	.936	-2.1822	1.5155
		96 hours	-17.33333*	.57735	.000	-19.1822	-15.4845
	48 hours	24 hours	22.66667*	.57735	.000	20.8178	24.5155
		72 hours	22.33333*	.57735	.000	20.4845	24.1822
		96 hours	5.33333*	.57735	.000	3.4845	7.1822
	72 hours	24 hours	.33333	.57735	.936	-1.5155	2.1822
		48 hours	-22.33333*	.57735	.000	-24.1822	-20.4845
		96 hours	-17.00000*	.57735	.000	-18.8489	-15.1511
	96 hours	24 hours	17.33333*	.57735	.000	15.4845	19.1822
		48 hours	-5.33333*	.57735	.000	-7.1822	-3.4845
		72 hours	17.00000*	.57735	.000	15.1511	18.8489
P16.00	24 hours	48 hours	-20.00000*	.40825	.000	-21.3074	-18.6926
		72 hours	8.66667*	.40825	.000	7.3593	9.9740
		96 hours	-5.66667*	.40825	.000	-6.9740	-4.3593
	48 hours	24 hours	20.00000*	.40825	.000	18.6926	21.3074
		72 hours	28.66667*	.40825	.000	27.3593	29.9740
		96 hours	14.33333*	.40825	.000	13.0260	15.6407
	72 hours	24 hours	-8.66667*	.40825	.000	-9.9740	-7.3593
		48 hours	-28.66667*	.40825	.000	-29.9740	-27.3593
		96 hours	-14.33333*	.40825	.000	-15.6407	-13.0260
	96 hours	24 hours	5.66667*	.40825	.000	4.3593	6.9740
		48 hours	-14.33333*	.40825	.000	-15.6407	-13.0260
		72 hours	14.33333*	.40825	.000	13.0260	15.6407

*. The mean difference is significant at the 0.05 level.