RELATIONSHIP BETWEEN TANNIN CONTENTS USING DIFFERENT TANNIN ASSAYS AND SHORT-TERM BIOLOGICAL RESPONSES IN RABBITS SUPPLEMENTED WITH LEAVES OF DIFFERENT ACACIA SPECIES

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BY

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Declaration

I hereby declare that this dissertation submitted to the University of the North for the degree of Master of Science in Agriculture is the result of my own work and investigation, and that it has neither wholly nor partially been presented as a dissertation for this university or elsewhere.

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Above all, I am most thankful to God Almighty for His grace upon my life, strength, comfort and wisdom He blessed me with. "In the Name of the Father, the Son and the Holy Spirit, Amen"

Dedication

This dissertation is dedicated to my dearly loved parents Daddy Nthedi and Mommy Mateisane and my sisters (adored angels) Lehlogonolo, Lebogang and Lefentse.

I, also, dedicate to Aunt Namunana Maja, who passed away during the writing of this dissertation "Robala ka khutso Mamogolo"

Abstract

Acacia trees form a large plant family in Southern Africa and are an important ecological component of the bushveld vegetation that is prevalent in the Limpopo Province of South Africa. The leaves from these trees are high in protein but their use has been limited by the scarcity of information relating the amounts and types of tannins that they contain to effect on animal performance. The main objective of this study was to determine the relationship between tannin contents by different chemical analytical techniques and short-term biological responses (intake, digestibility and nitrogen retention) in rabbits supplemented with tanniniferous leaves from different Acacia species found in the Capricorn region of Limpopo Province.

Acacia nilotica had higher total phenolics (2.04 % dry matter (DM)) than both *A. karoo* (1.51 % DM) and *A. tortilis* (1.25 % DM) ($P < 0.05$). Similarly, *A. nilotica* had the highest amount of simple phenolics and phenolics that bind to proteins. However, it had the lowest amount of extracted (0.37 % DM) and unextracted (1.38 % DM) condensed tannins. *Acacia karoo* had the highest amounts of condensed tannins, both extracted (4.52 % DM) and unextracted (3.72 % DM).

Eight New Zealand white male rabbits with a mean liveweight of 0.68 ± 0.05 kg were randomly assigned to metabolic crates in a two 4 x 4 Latin Square design. The four dietary treatments were a control diet (ground rabbit feed), control diet

mixed with *A. karoo* leaf meal (4 % of diet), control diet mixed with *A. nilotica* leaf meal (4 % of diet), and control diet mixed with *A. tortilis* leaf meal (4 %of diet). Results indicated that there were no differences in diet intakes and digestibilities between the control and those supplemented with 4 % leaf meal of *A. karoo*, *A. nilotica* and *A. tortilis* ($P > 0.05$). However, rabbits on diet supplemented with leaf meal from *A. tortilis* drank more water than those on the control, *A. karoo* and *A. nilotica* ($P < 0.05$). Faecal DM and nitrogen outputs, urine output, urine nitrogen output and nitrogen retention in rabbits were not significantly affected by supplementation with 4 % leaf meal of *A. karoo*, *A.nilotica* and *A. tortilis* ($P > 0.05$). The amount of tannins from the browses (4 % of the feed mixture) used in the present study were not likely large enough to adversely affect diet intake, digestibility and nitrogen retention in rabbits.

Negative correlation coefficients were observed between nitrogen digestibility and nitrogen retention (% of total N intake) in rabbits and total phenolics (r = - 0.9317 and - 0.9963, respectively), precipitable phenolics by filter paper (r = - 0.9295 and - 0.9724, respectively), radial diffusion (r = - 0.9570 and - 0. 9955, respectively), extracted condensed tannins (r = - 0.1308 and - 0.2285, respectively) and polyethylene glycol (r = - 0.9126 and - 0.9101, respectively) contents in leaves of Acacia species. Nitrogen digestibility and retention were most accurately predicted by contents of total phenolics (TP), radial diffusion (RD), precipitable phenolics by filter paper (PPFP) and polyethylene glycol (PEG) in the leaves. However, extracted condensed tannin contents in leaves of Acacia

browse supplements provided no reliable indication of diet dry matter and nitrogen digestibilities (r = -0.2083 and -0.1308, respectively), faecal nitrogen output (r = -0.2285) in rabbits. Poor to moderate relationships were observed between diet DM, nitrogen and water intakes in rabbits and tannin contents in Acacia leaves determined by different tannin assays. It is concluded that for the prediction of nitrogen digestibility and retention in rabbits, RD, TP, PPFP and PEG contents in Acacia leaves were most accurate. These are worth further study with the aim of confirming present results and developing accurate prediction equations for use with tanniniferous browses.

Table of Contents

Contents	Page
Declaration	i
Acknowledgement	ii
Dedication	iii
Abstract	iv
Table of Contents	vii
List of Tables	iv
List of Figures	v
List of Appendices	vi
Chapter 1	
1.0 Introduction	1
1.1 Background	2
1.2 Motivation	7
1.3 Objectives	8
1.3.1 Main Objective	8
1.3.2 Specific objectives	8
1.4 Hypotheses	9

Chapter 2

2.0 Literature Review 11

2.1 Introduction 12

2.2 Acacia Trees 12

2.2.1 *Acacia nilotica* 12

2.2.2 *Acacia karoo* 14

2.2.3 *Acacia tortilis* 15

2.3 Nutritive value of Acacia species 16

2.3.1 Protein 16

2.3.2 Digestibility 17

2.3.3 Intake 18

2.4 Tannins 18

2.4.1 Hydrolysable tannins 23

2.4.2 Condensed tannins 25

2.5 Analytical methods 29

2.5.1 Coloremetric methods 29

2.5.1.1 Folin Ciocaltaeu 29

2.5.1.2 Butanol HCl assays 30

2.5.2 Gravimetric methods 31

2.5.2.1 Polyvinyl Polypyrrolidone (PVPP) 31

2.5.2.2 PEG (Polyethyele Glycol) 31

2.5.3 Protein precipitate assay 33

2.5.3.1 Protein –binding capacity by filter paper 33

2.5.3.2 Radial Diffusion assays 38

2.6 Correlation between tannins and biological responses of livestock 34

2.7 Summary 37

Chapter 3

3.0 Material and Methods 39

3.1 Study site 40

3.2 Animals, management, diet and experimental design 40

3.3 Collection, drying and storage of plant material 42

3.4 Faecal and urine collection 42

3.5 Sampling and sample preparation 43

3.6 Chemical analyses 43

3.6.1. Dry matter determination 43

3.6.2. Crude protein determination 43

3.6.3. Feed, urine and feacal analyses 44

3.6.4. Extraction 44

3.6.5. Measurement of total phenolics and tannins using Folin-Ciocalteu method 45

3.6.6. Determination of simple phenolics using PVPP (Removal of tannin from the tannin-containing extract.) 46

3.6.7. Determination of condensed tannins 47

3.6.7.1. Extracted condensed tannins 47

3.6.7.2. Unextracted condensed tannins 47

3.6.8. Protein binding capacity by filter paper assays 48
3.6.9. Radial diffusion assays 49
3.6.9.1 Preparation of the plate 49
3.6.9.2 Assay procedure 50
3.6.10 Reaction of polyethylene glycol (PEG) with tannins 50
3.7 Statistical analyses 51

Chapter 4

4.0 Results 52

Chapter 5

5.0 Discussion, Conclusion and Recommendation 62
5.1. Discussion 63
5.1.1. Effect of browse supplementation on diet intake and digestibility in rabbits 63
5.1.2. Prediction of diet intake, digestibility and nitrogen retention in rabbits from different tannin assays 67
5.2 Conclusion 70
5.3 Recommendation 72

Chapter 6

6.0. References 73

Chapter 7

7.0. Appendices 98

LIST OF TABLES

TABLE	TITLE	PAGE
3.1.	The layout of two 4x4 Latin Square design	41
4.1.	Chemical composition of rabbit diet	56
4.2.	Tannin contents of Acacia leaves by methods of TP, PVPP, RD, EXCT, UNEXCT, PEG and PPFP	57
4.3.	Diet feed intake and digestibility in rabbits on control basal diet only and those supplemented with leaves from different Acacia species.	58
4.4.	Faecal output, urine nitrogen output and nitrogen retention in rabbits fed basal diet only and those supplemented with different Acacia species	59
4.5.	Prediction of intake in rabbits from different tannin assays	60
4.6.	Prediction of faecal, urine nitrogen output, nitrogen retention and digestibility in rabbits from different tannin assays.	61

LIST OF FIGURES

FIGURES	TITLE	PAGE
2.1.	Metabolism of gallic acid in hydrolysable tannins	24
2.2	The basic repeating unit in condensed tannins	27
2.3	Model structure for a Condensed tannin	27

LIST OF APPENDICES

APPENDICES	TITLE	PAGE
7.1	Analysis of variance for the effect of species on TP	99
7.2	Analysis of variance for the effect of species on PVPP	99
7.3	Analysis of variance for the effect of species on RD	99
7.4	Analysis of variance for the effect of species on ExCT	99
7.5	Analysis of variance for the effect of species on UnExCT	100
7.6	Analysis of variance for the effect of species on PEG	100
7.7	Analysis of variance for the effect of species on PPFP	100
7.8	Analysis of variance for the effect of square, rabbits, period and diet on DM faeces	100
7.9	Analysis of variance for the effect of square, rabbits, period and diet on water intake	101
7.10	Analysis of variance for the effect of square, rabbits, period and diet on DM intake	101
7.11	Analysis of variance for the effect of square, rabbits, period and diet on DM digestibility	101
7.12	Analysis of variance for the effect of square, rabbits, period and diet on nitrogen intake	102
7.13	Analysis of variance for the effect of square, rabbits, period and diet on faecal nitrogen output	102
7.14	Analysis of variance for the effect of square, rabbits,	

	period and diet on urine nitrogen output	102
7.15	Analysis of variance for the effect of square, rabbits, period and diet on nitrogen retention	103
7/16	Analysis of variance for the effect of square, rabbits, period and diet on nitrogen digestibility	103
7/17	Analysis of variance for the effect of square, rabbits, period and diet on nitrogen retention in % of total nitrogen intake	103

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Over the past years, there has been a significant increase of interest in the use of leaves of fodder trees and shrubs as sources of protein and other nutrients for non-ruminant animals. The main features of these browse plants are their high crude protein (CP) and mineral contents. The concentration of CP in the leaves and fruits of the majority of fodder trees and shrubs is above 10 % even in the dry season. (D'Mello *et al.*, 1987). Considerable attention has been given to the use of leaf meals derived from these trees and shrubs (Chen *et al.*, 1981). Much attention has been focused on leaf meals from *Leucaena leucocephala* and cassava, but more recently interest has turned to leaf meals derived from other sources such as Acacia species, pigeon pea, etc (Tangendjadja *et al.*, 1990).

Leguminous plants, which may be trees, or shrubs, are important components of the fodder resources for livestock and wildlife. The role and potential of tree legumes are being increasingly recognized for both intensive and extensive agroforestry systems, particularly in humid and semi-arid regions of the tropics (Skarpe and Bergstrom, 1986). Leguminous species have relatively high crude protein (CP) contents and they successfully survive in unfavourable soil and climatic conditions. Trees and shrubs have been used for generations as multipurpose resources (food, fodder, timber, wood, fiber and live fences) across all of the agro-ecological zones of the world.

The fodder value of the leaves and fruits of legumes is often superior to herbaceous plants. Fodder trees and shrubs constitute a vital component of livestock feeds in the arid and semi-arid zones (Von Kaufinann, 1986). They supply animals with the bulk of their nutrient requirements, particularly proteins (Von Kaufinann, 1986). In arid and semi arid zones, the legumes provide the largest part of the protein supply during the driest month, for example, it is estimated that in the Sahel up to 80% of the protein requirements are provided by plants of the *capparaceae* family during the three driest months of the year (Baumer, 1983). Tree fodder is richer in crude protein, minerals and digestible nutrients than grasses (Abdulrazak *et al.*, 2000). The dry matter intake and digestibility, which are related to nutrient composition, vary widely among tree and shrub species. The use of tree legume fodder as supplements is to improve feed intake, digestibility and animal performance (Norton, 1994).

The utility of the leaves, pods and edible twigs of shrubs and trees as animal feeds is limited due to the presence of anti-nutritional factors. An anti-nutritional factor in plants seems to be produced as a way of storing nutrients or as a means of defending their structure and reproductive elements (Harborne, 1989). In fact, plants contain thousands of such compounds which, depending upon the situation, can have beneficial or deleterious effects on organisms consuming them. These compounds, with the exception of nutrients, are referred to as allelochemicals (Abdulrazak *et al.*, 2000).

Low intake and digestibility of browse may have some connection with the harmful substances that it may contain, for instance, some browse such as *Acacia albida, Acacia tortilis*, etc, contain substances such as cyanogenic glycosides, fluoroacetate or tannins which may considerably reduce their nutritive value or even be toxic to animals. However, toxicity depends on the concentration of the deleterious compounds in the fodder and the rate at which the forage is eaten. An amount of the plant eaten quickly, say in one hour, could be fatal, whereas the same amount of plant material eaten slowly over, for example, a five hour period, would be harmless on the range (Storrs, 1982).

In the Limpopo Province, the tree fodders used are mostly from Acacia trees. They are among the most widely distributed plant species and are an integral part of both human and animal diets (Al Mamary *et al.*, 2001). Acacia trees dominate in many parts of arid and semi-arid areas of sub-Saharan Africa and have multiple uses. They provide food, medicine and fodder to livestock (Ben Salem et al., 1997). However, Acacia species have phenolic compounds. The presence of phenolic compounds in Acacia species has a negative effect on their intake and digestibility by livestock (D'Mello et al., 1987). Studies on some Acacia species have shown them to have either positive or negative effects on animal performance (Al Mamary et al., 2001).

Polyphenols have been traditionally considered as anti-nutrient by animal nutritionists because of the adverse effects of tannins (one type of the

polyphenol) on protein digestibility (Bravo, 1998). Tannins are found in grains, legumes, herbs and a variety of food tissues, including roots, bark, leaves, flowers, fruits and seeds (Foo and Porter, 1981).

The amount and type of tannins synthesized by plants vary considerably depending on the plant species, cultivars, tissues, stage of development and environmental conditions (Giner-chavez *et al.*, 1997). Therefore, the study of the nutritional effects of tannins on animals requires quantification of the tannins present in a particular diet. Due to the complexity of tannins, several methods have been developed for their quantification. For example, there are many methods used for sample preservation for condensed tannin analysis. These include drying in the sun, in the oven, in the shade or freeze-drying.

According to Dalzell and Shelton (1997), the field freezing of sampled material had no significant effect on the amount of extractable condensed tannins measured in any plant tissue. Even for protein bound condensed tannins extracted by this method there had been no effect on their amount. However, under most tropical conditions this method is not appropriate and drying under shade (in a room) is recommended (Mueller-Harvey, 2001) although most researchers use oven drying at temperatures below 60 °C (Hagerman, 1988; Makkar and Singh, 1991)

Similarly, there are a number of methods used to assay tannins, with each method measuring a different entity. The main methods commonly used can be divided into three major categories, colorimetric, gravimetric and protein precipitation assays. The colorimetric methods include Butanol-HCl, Folin-Ciocalteau, Vanillin HCl assay, Potassium iodate and Prussian blue assay. The gravimetric assays are Ytterbium precipitation assay, Polyvinylpolypyrrolidone assays, Rhodamine assays, Wilson and Hagerman Potassium Iodate assays and the Polyethylene Glycol (PEG) assay whilst the protein precipitation methods include Radial diffusion, Protein precipitation by filter paper and Protein phenolic precipitate. Colorimetric, protein precipitate and gravimetric tannin assays on alkali treated substrates are complicated by residual alkalinity in the extraction medium, which may cause an overestimation of the effectiveness of alkali (Makkar and Becker, 1996).

A major problem with the colorimetric methods is the requirement for a standard. Most of the commercially available standards do not reflect the biological activity of the tannins in plants used as feed. It is, therefore, important to use the right method for the right purpose. For example, Duarsa *et al.* (1992), with lotus species and Fursterburg and Van Hooven (1994), with *Acacia nigrescens* found that young and old premature leaves contained higher tannin contents than mature foliage. This was in contrast with Tangendjaja *et al.* (1986), who observed no changes in tannin contents in *Leucaena leucocephala* with increasing leaf maturity. This may be because of differences in methods used to

measure condensed tannins. Tangendjaja *et al.* (1986), used a protein precipitation method that does not quantify absolute tannin content but measured the combined effect of the protein binding capacity and the amount of tannins present while others used colorimetric methods that do not measure bound tannins.

The lack of correlation between tannin content using different tannin assays and biological effect of tannins on animal performance has tended to prevent development of management strategies that minimize the negative effects of tannins whilst optimizing the positive effects. This is essential if we are to improve animal productivity using feeds that contain tannins. This research will test a number of currently available assays against the performance of rabbits with a view to recommending a method that reflects biological reactivity of tannins.

1.2 MOTIVATION

There are many methods that are used to determine either the type or structure of tannins. A major constraint to proper management of feeds that contain tannins has been that the assays are not well proven to correlate with the performance of the animal. It is, therefore, important to find the assays that will correlate positively with the performance of the animal. This will help the smallholder-farming community, as most of the plants that are readily available to

their livestock contain tannins. If the amount of tannins in plants is known and the effect of the tannins on the animals can be predicted without experimenting with the animal again, then management strategies that minimize the negative effects and enhance the positive effects of tannins can be devised.

1.3 OBJECTIVES

1.3.1 Main Objective

The main objective of this study was to determine the relationships between different chemical analytical techniques for tannins and short-term biological responses (intake, digestibility and nitrogen retention) in rabbits supplemented with tanniniferous leaves from different Acacia species found in the Capricorn region of Limpopo Province.

1.3.2 Specific Objectives

The specific objectives of this study were:

(a) To determine tannin contents of different Acacia species found in the Capricorn region of Limpopo Province using different tannin assays.

(b) To determine food intake, digestibility and nitrogen retention of rabbits supplemented with leaf meals from different Acacia species found in the Capricorn region of Limpopo Province.

(c) To determine the relationship between tannin contents of leaves of Acacia species found in the Capricorn region of Limpopo Province and biological responses of rabbits supplemented with such leaves.

1.4 HYPOTHESIS

The main hypothesis of the study was that there were no relationships between different chemical analytical techniques of tannins, tannin contents and short-term biological responses (intake, digestibility, and nitrogen retention) in rabbits supplemented with different tanniniferous leaves found in the Capricorn region of Limpopo Province.

The specific hypotheses of this study were:

(a) Leaves from different Acacia species in the Capricorn region of Limpopo province contain similar tannin contents.

(b) Different tannin assays give similar results

(c) Rabbits supplemented with leaf meals from different Acacia species found in the Capricorn region of Limpopo Province have similar feed intake, digestibility, and nitrogen retention.

(d) There are no relationships between tannin contents (by different tannin assays) of leaves of Acacia species found in the Capricorn region of Limpopo Province and biological responses of rabbits supplemented with such leaves.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Goats, sheep and rabbits are herbivores, but rabbits are non-ruminants unlike goats and sheep, which are ruminants. The common thing for both groups is that they are domestic animals and they eat almost the same feed when considering the trees and grasses that they feed on. Most of these domestic herbivore animals browse heavily on Acacia species in South Africa such as Acacia *karoo, nilotica* and *tortilis*. These species are widespread and common throughout the Limpopo Province. Acacia trees have polyphenolics that include tannins. Tannins are known as anti-nutritional protein binding secondary plant compounds, which reduce the availability of dietary protein to the animals. The ability of tannins to precipitate proteins has led to the development of tannin assay methods (Silanikove *et al.,* 2001).

2.2 ACACIA TREES

The leaves of most Acacia species are distinctively feathery and consist of many leaflets. The number, shape and size of leaf pinnae and leaflets are often distinctive for certain species. Acacia belongs to the family Leguminosae, many species of which are quite rich in proteins and minerals (Kumar, 1983). Acacia's leaves, twigs and pods contain fairly high concentrations of proteins (Kumar, 1983). The seeds, in particular, have a high protein content, but normally, pass through the herbivore's system without being digested due to the hard covering. In the 1980s, during the prolonged dry season of about 8 years in Kenya Acacia

species served as a source of much needed nutrients to the domestic herbivores and wildlife since the domestic ruminants were kept extensively on the range (Abdulrazak et al, 2001). *Acacia nilotica*, *A. karoo* and *A. tortilis* are widespread and common throughout the Limpopo Province and animals browse heavily on these Acacia species.

2.2.1 *Acacia nilotica*

Acacia nilotica is a species of thorny Acacia that spreads at an alarming rate. Large areas of formerly treeless, Mitchell grass plains in South Africa are being invaded by this species creating problems with management of sheep and cattle. Although it is a widely used species (e.g. fences and woods) it produces reasonable quality browse for animals, which is high in tannins. *Acacia nilotica* is a thorny wattle plant found naturally in Africa, India and Pakistan. This Acacia species is widely distributed in tropical and sub-tropical Africa. This species is commonly found in soils with high clay content, but may grow on deep sandy loam in areas with the highest rainfall. It commonly grows close to waterways on seasonally flooded river flats and tolerates salinity well. It will grow in areas receiving less than 350mm of rainfall to areas receiving more than 1,500 mm per annum. The species is reported to be very sensitive to frost, but in Africa it will grow in areas where the mean monthly temperature of the coldest month is 16 ^{o}C (Shetty, 1977).

Acacia nilotica is extensively used as a browse, timber and as fire wood (New, 1984). The bark, leaves and seeds are used as a source of tannins (Shetty, 1977). The species is also used for medical purposes, for example, bark of *A. nilotica* has been used for treating haemorrhages, colds, diarrhoea, tuberculosis and leprosy while the roots have been used as an aphrodisiac and the flowers also have been used for treating syphilis lesions (New, 1984). The species is suitable for the production of paper and has similar pulping properties to a range of other tropical timbers (Nasroun, 1979). *Acacia nilotica* has a higher concentration of total phenolics (A_{675} = 2.21) only and a very small portion of these polyphenols are condensed tannins compared to *Acacia karoo* (Kahiya *et al.*, 2003)

2.2.2 *Acacia karoo*

This species is widely found in the Limpopo Province. It tends to have variable amounts of tannins. For example, Acacia karoo was found to have a high content of condensed tannins (A_{550} = 2.011au/g DM) and *Acacia nilotica* tended to have a high amount of total phenolics (A_{675} = 5.261au/g DM) while *Acacia Senegal* had lesser condensed tannins and total phenolics (Dube *et al.*, 2001). However, analysis of condensed tannins is complicated by the diversity of structures found within this group of compounds (Schofield *et al.*, 2001).

Acacia karoo is the most widely spread Acacia species in Southern Africa and this has resulted in confusion and difference of opinion amongst botanists in the past. *Acacia karoo* is found in the driest part of the country in the western side of the Drankensberg. The same species is found also in the Loskopdam area in Mpumalanga on the hills surrounding the dam. It colonises and degrades land over an exceptionally wide range of climatic and edaphic conditions, fixes nitrogen and provides high quality fuel, animal fodder and a valuable gum.

2.2.3 *Acacia tortilis*

Acacia tortilis is characterized by a peculiar pattern of spine production. The stipular spines may either lignify prior to elongation, thus becoming short and hooked or grow into long straight spines. *Acacia tortilis* is a major source of forage (Bitende and Ledin, 1996) and is a common woody legume typical from east African savannah. The effects of wild ungulates on woody plant production in the African savannah ecosystem have received much attention (Bitende and Ledin, 1996)

Acacia tortilis is a medium umbrella-shaped tree, 4–15 meters tall, often with several trunks, reduced to a small wiry shrub less than 1 m tall under extremely arid conditions. Two types of thorns abound (1) long, straight, and white, and (2) small, hooked, and brownish. It has leaves up to 2.5 cm long with 4–10 pairs of

pinnae, each with 15 pairs of leaflets. Flowers are white, aromatic and in small clusters. It has pods that are flat, glabrose, coiled into a spring-like array.

Native to much of Africa and the Middle East, this species has been introduced in many arid parts of the world. Ironically, it grows faster in the Rajastan Desert of India, where it is used for charcoal, firewood and fodder, than in its native Israel (Kaplan, 1979). In Malawi, this species is already scorned by the rural public, because it is thorny and difficult to work with. It is being tried for fencings (Nkaonjo, 1980).

2.3 NUTRITIVE VALUE OF ACACIA SPECIES

Compared to grasses, fodder trees and shrubs have a relatively higher concentration of crude protein, minerals and neutral detergent fibre plus acid detergent fibre. However, their average dry matter digestibility is lower. These nutrient contents are subject to less variation than with grasses, and this particularly enhances their value as dry season feeds for livestock (Wilson, 1977).

2.3.1 Protein

A significant variation in crude protein (CP) content occurs between species of trees and shrubs and even between edible parts of the same plant. In general,

leaves are higher in CP than twigs, almost twice as much in the case of Southern African browse (Nitis, 1989). They also contain more crude protein on average than other parts but the latter were found with higher organic matter and digestibility. Leguminous species were found to contain 25 to 50 % more crude protein than non-leguminous plants (Nitis, 1989). Leaves of phylladenous Acacia tend to have higher crude fibre, lower crude protein, phosphorus, organic matter and digestibility than other Acacias species

2.3.2 Digestibility

Dry matter digestibility (DMD) varies widely among tree and shrub species. Skarpe and Bergstrom (1986), working in Botswana with Kalahari woody species reported a range in digestibility from 38 to 78 %, similar in findings reported by Wilson (1977). The digestibility of cellulose and cell walls decreases as the lignin to cellulose or lignin to acid detergent fibre ratio increases. The digestibility of crude protein does not always match with the high CP content, which characterizes fodder trees and shrubs. For example, Wilson (1977) found apparent nitrogen digestibility as low as 14 % for *Heterodendrum oleifolium* containing 12.5 % CP while *Atriplex vesicaria*, also with 12,5 % CP, had a nitrogen digestibility of 71.4 %. Also, there is not always a correlation between intake and digestibility, highly digestible stuff maybe poorly consumed and vice versa (Wilson, 1977).

2.3.3 Intake

Domestic ruminants utilize trees and shrubs both as browse *in situ* and in "cut and carry" production systems. On rangelands, animals have the advantage of selecting from a wide range of browse and obtaining a high quality feed. Consumption of various types of forage reduces chances of poisoning. However, the effective contribution of trees to the nutrition of livestock is not always realized due to inability to reach the browsable canopy of tall trees (Wilson, 1977). The cut and carry system obviates the limitation of inaccessibility of the browse. The system also facilitates rational usage of trees and shrubs, its disadvantages include damage to trees and the risk of poisoning (Wilson, 1977).

2.4 TANNINS

Tannins (commonly referred to as tannic acid) are complex water-soluble polyphenolic compounds with a molecular weight greater than 500. They contain sufficient hydroxyl and other suitable groups (i.e. carboxyl) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied (Horvath, 1981). They have the ability to precipitate protein from aqueous solution and are present in many plant foods. They have a great diversity, and in browse plants influence the digestibility of available protein. They occur almost in all vascular plants. Tannins act as a

chemical defense mechanism in plants against pathogens, herbivores and hostile environmental conditions; they can exert detrimental effects in a multitude of ways (Clausen *et al.*, 1990).

Generally, tannins induce a negative response when consumed by animals. These effects can be instantaneous like astringency or a bitter or unpleasant taste, or can have a delayed response related to anti-nutritional/toxic effects (Miller *et al.*, 1996). The anti-nutritional factors may be defined as those substances generated in natural feedstuff by the normal metabolism of species and by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process or metabolic utilization of feed), which exert effects contrary to optimum nutrition (Mekasha *et al*, 1996). Being an anti-nutritional factor is not an intrinsic characteristic of a compound but depends upon the digestive process of the ingesting animal (Norton, 1994). It is important that the tannin level of the browse plants be analysed to obtain an estimation of the inhibitory effect that would result after consumption of such feed (Norton, 1994; Mekasha *et al*, 1996)).

Tannins are known as anti-nutritional protein binding secondary plant compounds, which reduce availability of dietary protein. Tannins, characteristically, bind with protein, carbohydrates and minerals and dramatically inhibit digestive and absorptive processes in the gastrointestinal tract of grazing animals (Kumar and Singh, 1984). Livestock consuming tannin-rich diets

(>5%w/v tannins) usually develop a negative nitrogen balance and lose weight and body condition unless supplemented with non-protein nitrogen, carbohydrate and minerals. As said above tannins have growth depressant factors because of their inhibitory effect on protein digestion. This results from either direct digestive enzyme inhibition, or by formation of indigestible complexes with food protein (Martin-Tanguy *et al.*, 1977). Tannins have a large number of free phenolic hydroxyl groups that form strong hydrogen bonds with protein and carbohydrates (Haslam *et al.*, 1989). The ability of tannins to precipitate proteins has led to the development of tannin assay methods.

Tannins, because of their protein-binding properties, are known to be strongly astringent. This astringency appears to be the major cause of reduced food intake in mammalian herbivores. There is some controversy, however, over whether reduced food intake is a result of the toxic nature of tannins. Singleton (1981) considers it unfair to consider the effects of tannins on feed intake as toxicity, since the result could be due to a failure to consume, rather than to consumption itself. On the other hand, Provenza *et al.* (1991), suggest that mammals may reject tannin-containing plants because they cause internal malaise. Severe growth depression can be a consequence of reduced feed intake, and has been shown to occur in rats and chicks when fed tannin-containing diets (Alldredge, 1994). When tannins complex with protein in an animal's gut, they are believed to be responsible not only for growth depression, but also for low protein digestibility and increased faecal nitrogen concentrations.

Thus, once they have been consumed, their adverse effects, again, seem to be related to their binding of dietary protein (Alldredge, 1994). There is evidence to suggest that enzymatic protein, as well as other endogenous proteins, comprise a considerable portion of excreted nitrogen when animals are fed tannins (Alldredge, 1994). When endogenous proteins are lost in this manner, the animal may incur a deficiency in one or more essential amino acids.

Tannins are secondary metabolites with a high capacity to form complexes with proteins. The effect of both secondary compounds and mycotoxins vary with animal species. Non-ruminants are usually more susceptible to toxicity than ruminants which have the capacity to denature potential toxins in the rumen. The nature and action of toxins in the plants have been the subject of several reviews (Hegarty, 1982; Barry and Manley, 1986) in which attention was on pasture plants of commercial influence. Gartner and Hurwood (1976) subsequently reported that the presence of tannins (7-10%) reduced mineral availability by complexing them with leaf protein following maceration of the leaf tissue.

Tannins have growth depressant factors because of their inhibitory effects on protein digestion. This results from either direct digestive enzyme inhibition or by formation of indigestible complexes with food protein (Rickards, 1986). Tannins are divided into two major structural classes, hydrolysable and condensed tannins. Although they differ biosynthetically and chemically, they are both phenolics and can precipitate proteins (Rickards, 1986).

Tannins are considered to have both adverse and beneficial effects depending on their concentration and nature besides other factors such as animal species, physiological state of the animal and composition of the diet. Purified tannins from various sources decreased the rate of gas production for more than the ultimate plateau value, i.e, extent of gas production of the *in vitro* true digestibility (Makkar *et al.*, 1995a). Therefore, the threshold level at which the rate is affected will be lower as compared to the digestibility.

The lower intake of tannin-rich feeds is generally attached to their astringent taste. Besides this unpleasant taste, the lower rates of digestion (higher rumen fill) in the presence of tannins should also be responsible for the lower feed intake (Makkar *et al.*, 1995a).

Much recent work in the area of plant-herbivore interactions has focused on the inhibitory effects of condensed tannins on protein and fibre digestion in mammals. Tannins have previously been classified as a quantitative plant defence that reduces the digestibility of nutrients following their ingestion by herbivores (Feeny, 1976; Rhodes and Cates, 1976). Robbins *et al.* (1991) suggested that the defensive nature of tannins as digestion inhibitors or toxins is dependent on the molecular characteristics of the tannin as it interacts with the physiological capability of the animal.

2.4.1 Hydrolysable tannins

Hydrolysable tannins (HTs) are composed of gallic acid or condensation products of ellagic acid esterified to the hydroxyl groups of glucose (Dalzell and Kerven, 1998). A central carbohydrate core characterizes hydrolysable tannins with a number of phenolic carboxylic acids bound by esteric linkage. The molecules can be hydrolysed into simpler fragments. The hydrolysable tannins are present in many plants and have been shown to be responsible for growth inhibition of agriculturally important animal species (Dalzell and Kerven, 1998). They have very low bioavailability following oral ingestion, due to their poor lipid solubility and ability to bind and form complex as with proteins (Dalzell and Kerven, 1998).

Hydrolysable tannins are most likely to react with the extracting solvent than condensed tannins. For example, methanol cleaves the depside bonds in the gallotannins at neutral pH (Porter and Woodrulffe, 1989) but acidified methanol (pH>3) will not cleave these bonds (Haslam *et al.*, 1989). Large and complex tannins are easily degraded into smaller tannins by water or dilute acids especially at elevated temperatures in just 30 minutes (Beasley *et al.*, 1997). Water at 60 ^{0}C is likely to liberate gallic and form the anomeric C+1 position of glucose (Tedder *et al.*, 1972). Water at 100 ^{0}C may also release ellagic acid from ellagitannins (Nishimura *et al.*, 1986) and cleave the ether-bond in the valoneoyl group (Okuda *et al.*, 1990).

Fig 2.1 shows the biosynthetic relationship between different HT compounds. The central compound, pentagalloylglucose, is the starting point for many complex tannin structures. This compound belongs to the so-called gallotannins. These consist of a central polyol, such as glucose, which is surrounded by several gallic acid units. Further gallic acid units can be attached through a depside bond. A very large number of hydrolysable tannin molecules exist in nature. The structural variation amongst these compounds is caused by oxidative coupling of neighbouring gallic acid units or by oxidation of aromatic rings (Nonaka, 1989; Okuda *et al.*, 1990)

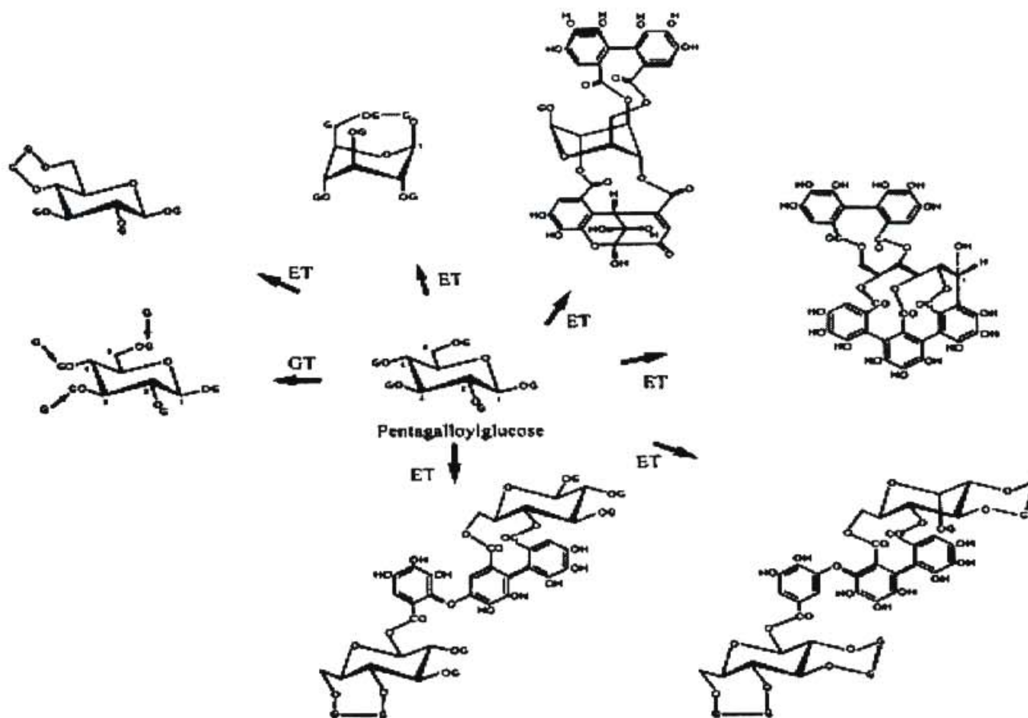


Fig. 2.1 Metabolism of gallic acid (G) in hydrolysable tannins (Self *et al.*, 1986); pentagalloylglocuse is the precursor of gallotannins (GT) and ellagitannins (ET).

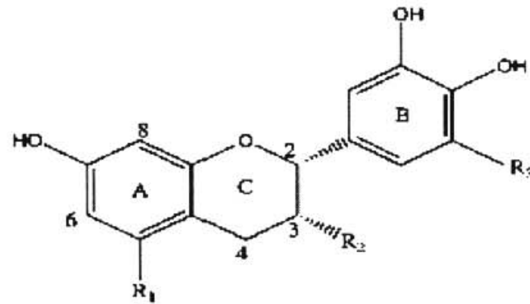
2.4.2 Condensed tannins

Condensed tannins are naturally-occurring compounds found in a number of different plants, including some pasture species. They are secondary carbon compounds produced in leaves that sometimes act to deter herbivorous insects. In New Zealand, the "legume lotus" is one of the primary sources of these substances. Sheep and cattle feeding on it reduce their methane emissions by as much as 16% (Idso and Idso, 2002).

Condensed tannins (CT) are polyphenolic secondary plant products that are widespread in the plant kingdom. They bind to protein and are regarded as anti-nutritional compounds, which reduce protein digestibility. Proanthocyanidins (PA), another name for condensed tannins, are abundant in vegetable food. Condensed tannins are derived from the condensation of flavonoid precursors without participation of enzymes (Schofield *et al.*, 2001).

Condensed tannins are more widely distributed in higher field species than hydrolysable variety and are thought to be more active in precipitating proteins. Their concentrations and chemical composition of condensed tannins change with physiological maturity of plants (Dalzell and Shelton, 1997). They are polymers of flavin-3-ols (gallocatechin) or flavan-3-4-diols (leucocyanidin-leucodelphinidin) linked through acid-labile carbon-carbon bonds (Rickards, 1986; Al-Mamary *et al.*, 2001). The catechin monomers have asymmetric

centers at positions 2 and 3 in ring C (Figure 2.2, adapted from Schofield *et al.*, 2001). There are variations that occur in natural tannins at this position and they were shown to have relatively little effect on most of the reactions used for tannin assays (Schofield *et al.*, 2001). Oxidative coupling between flavanol monomers occurs most commonly between positions 4 and 8, but may also involve positions 4 and 6 of the monomers (Figure 2.3) and other positions too (Schofield *et al.*, 2001). These variations in CT alter protein-binding capacities among polymers from different plant species and development stages (McMahon *et al.*, 1996). At low to moderate levels CT increase the quantity of dietary protein, especially essential amino acids, flowing to the small intestine (McMahon *et al.*, 1996). Tannins may benefit ruminants by protecting protein from bacterial deamination and preventing bloat (Burritt *et al.*, 1987). Thus, dietary CT may provide a means to beneficially manipulate protein digestion or prevent pasture bloat in ruminants.



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Fig.2.1. The basic repeating unit in condensed tannins. If R_1=R_2=OH, R_3=H, then the structure is that for (-)-epicatechin. The groups at R_1 and R_3 for other compounds are indicated below the structure. R_2=O-galloyl in the catechin gallates. (Schofield *et al.*, 2001)

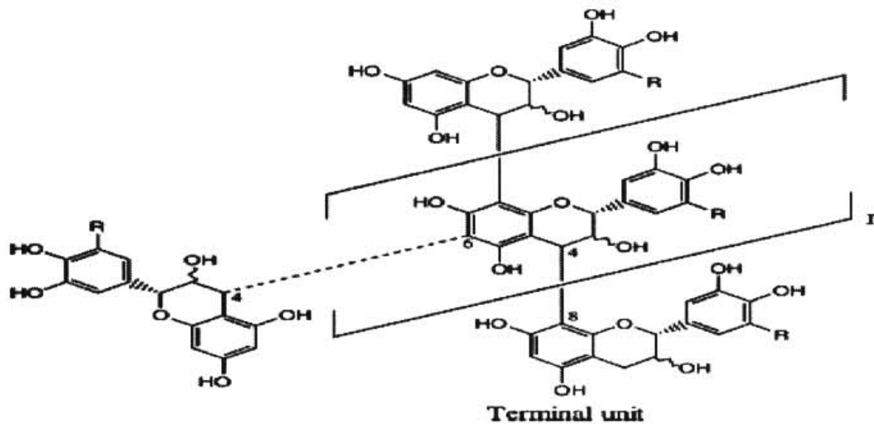


Fig. 2. Model structure for condensed tannin. If R=H or OH then the structure represents a procyanidin or prodelphinidin. The 4→6 linkage (dotted line) is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure. (Schofield *et al.*, 2001)

Proanthocyanidins (PA) can exert effects in organisms because of their ability to complex with protein. Aerts et al., (1999) found that forages containing moderate concentrations of PA (2 - 4% DM) can exert beneficial effects on protein metabolism in sheep, by slowing degradation of dietary protein to ammonia by rumen micro-organisms and increasing protein outflow from the rumen, thus increasing absorption of amino acids in the small intestine of the animal. They reported an increase in lactation, wool growth and live weight gain, while voluntary feed intake was not affected. In ruminants, condensed tannins improve digestion and reduce the occurrence of animal "bloat" by decreasing methane gas production, which represents a viable solution to reducing greenhouse gas in the environment (Aerts et al., 1999). These studies were carried out with ruminants and not yet with non-ruminant herbivores.

Condensed tannin polymerization and binding with cellular components (plant protein, polysaccharides, nucleic acids, metallic ions, saponins, alkaloid, and steroids) reduce their extractability and reactivity to a number of analytical assays (Dalzell and Shelton, 1997). Thus, it is essential to avoid sample processing procedures that unduly fracture cell structure.

2.5 ANALYTICAL METHODS

2.5.1 Colorimetric assays

The commonly used methods for determination of total phenolics are the Prussian blue assays and the Folin Ciocalteau. These methods entail oxidative reductions in which the phenolate ion is oxidized while the Fe $(CN)_6^{3-}$ ion (Prussian blue assays) or the phosphotungstic-phosphomolybdic compounds (Folin Ciocalteau) are reduced (Schofield *et al.*, 2001).

2.5.1.1 Folin- Ciocalteau

This method is a modification of the Folin Denis method. One of the weaknesses of the Folin Denis method is the formation of a precipitate that interferes with sprectrophotometry measurements (Singleton *et al.*, 1999). The *Folin Ciocalteau* reagent was designed to address this problem by using lithium sulfate. The reaction that will take place is the reduction of phosphomolybdic acid by phenol. The method is less prone to interference by non-phenolics than the original methods. The problem is that it does not differentiate between tannins and many phenolics that are not tannins. Interfering compounds such as ascorbic acid, tyrosine and possibly glucose are also measured (Schofield *et al.*, 2001)

2.5.1.2 Butanol HCl assays

This is specific for the condensed tannins. Hydrolysable tannins do not react in this assay. The acid butanol assay remains the most commonly used method for determination of condensed tannins in plant tissues. This is a colorimetric reaction that uses acid catalyzed oxidative depolymerasation of condensed tannins to yield red anthocyanidins. Schofield *et al.* (2001) described a procedure to use the reaction for quantitative analysis of condensed tannins. The problem is that not all red pigments would dissolve, resulting in tannin underestimation. The amount of water present may lead to variable colour development when differential plant species are used (Dalzell and Kerven, 1998). Colour yield is not always linear with the amount of tannin input. Large amounts may give a lower unit colour yield (Waterman and Mole, 1994). The butanol acid reaction should be used with caution as a quantitative assay. The assay's greater strength lies in its confirmation of the presence of a polymeric interflavon structure (Schofield *et al.*, 2001).

2.5.2 Gravimetric methods

2.5.2.1 Polyvinylpolypyrrolidone (PVPP)

It determines only the soluble tannins present in plant extracts; insoluble tannins are not measured because they are water- soluble. The PVPP irreversibly binds

to the tannins. The problem is that they bind less than the common colorimetric methods. It is not very sensitive and tends to underestimate tannins. A primary advantage of gravimetric method is that they do not require standards. For example, Makkar (1993) indicated that the method requires about 30 mg of PVPP to be completely bound to 2 mg of tannic acid. The percentage weight change in insoluble PVPP is thus quite small.

2.5.2.2 Polyethylene Glycol (PEG)

The quantification of tannins is important for predicting their negative effects. The methods described above are mainly colorimetric and these sometimes do not correlate to tannin effects because of variable structures of tannin polymers and lack of satisfactory standards. Protein precipitation is the major cause of nutritional and ecological effects of tannins. PEG (water- soluble) is a non-ionic detergent with a molecular weight of 4000, which forms insoluble complexes with condensed and hydrolysable tannins. The complexes are due to hydrogen bonding between oxygen and PEG and the hydroxyl groups in tannins and the complexation are similar to that of which occurs between tannins and proteins. PEG does not need tannins to be extracted from plant material before binding. This is a big advantage over other precipitation methods. Tannin extraction from plant tissues is affected by several conditions including drying method and temperature, content and characteristics of proteins in the plant and pH of extraction. Often, some tannin is not extracted and there is no easy way to

predict how much has not been extracted. Hence, tannin precipitation with PEG, which does not require extraction, is more repeatable than other precipitation (Silanikove *et al.*, 2001)

Tannins form insoluble complexes with PEG and many other dyes. An in situ method for determination of tannins in plant sample without extract using 14C-labelled PEG has been reported (Silankove *et al.*, 1997). Polyethylene glycol 4000 (PEG), which binds to a wide range of hydrolysable and condensed tannins formed a stable PEG tannin complex over a pH range of 2 to 8.5 (Schofield et al., 2001) and this tannin PEG complex was insoluble in boiling water, neutral and acid detergents and many organic solvents. This method is useful for plant material containing strong tannin protein complex in which extraction would give low tannin yields (Schofield et al., 2001). The PEG can disrupt these complexes because its affinity for tannin exceeds that of protein. The primary disadvantage of this method is safety issues and costs of working with and disposing of radioactive materials.

2.5.3 Protein precipitation assays

2.5.3.1 Protein-binding capacity by filter paper assays

Tannins are defined as protein-binding and precipitating agents and tannin assays used on protein precipitation have long been popular. The most

convenient form of this assay uses a protein, normally Bovine Serum Albumin (BSA), dyed with brilliant blue (Schofield *et al.*, 2001). Tannins and dyed protein are mixed under defined conditions of pH and ionic strength and allowed to precipitate. After centrifugation, the pellet is dissolved in an alkaline Sodium Diodecyl Sulfate (SDS) buffer for measurement of the dyed protein content. Protein precipitate can also be found in agarose plates. Although only semi-quantitative, the plate method does not require a dye-labeled protein and permits the simultaneous analysis of many samples (Schofield *et al.*, 2001).

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2.5.3.2 Radial diffusion assays

This method depends on the formation of complexes between tannins and bovine serum albumin embedded in agar. The plant extractions are placed in a well in the agar. They diffuse in the agar and they precipitate the albumin if tannins are present. In this assay, the amount of the tannin present in a crude plant extract is indirectly measured by the ability of the tannin to precipitate BSA. The tannin diffuses through a protein-containing gel, resulting in the formation of a tannin protein precipitate ring. The area of the precipitate is believed to be proportional to the amount of tannins in the extract. An advantage of this procedure is that many samples can be handled with limited laboratory facilities (FAO/IAEA, 2002).

2.6 CORRELATIONS BETWEEN TANNINS AND BIOLOGICAL RESPONSES OF LIVESTOCK.

Studies done mostly are about determining the tannin content of a particular plant or observing the biological responses of an animal. Few studies have been done correlating tannin content and biological response. Also not much research has been done using the Acacia species.

Mueller-Harvey and McAllan, (1992) fed cattle with 24 % peanut skin diet, which contained 6.2 % tannin, and found an apparent depression in intake, which they attributed to the decreased palatability. The lower intake of tannin-rich feeds may be attributed to astringent taste of tannins (Mueller-Harvey and McAllan, 1992). However, besides unpleasant taste, the adverse effects of tannins on the rate of digestion of feeds could be another mechanism by which feed intake is decreased (Makkar *et al.*, 1995b). When digestion rate is reduced, feed intake is also decreased (Van Soest, 1994).

Al-Mamary *et al.* (2001) observed that addition of low-tannin sorghum grains to animal's diet had no significant effect. However, addition of high- tannin sorghum grain significantly reduced rabbits' liveweight gain by 10 %, feed conversion ratio by 13 % and increased feed consumption by 3 % with respect to the control group. The increase in food consumption concomitantly with a decrease in body weight gain and feed conversion ratio in animals on high tannin sorghum grains

(2.02 % DM), was interpreted as a consequence of the reduced digestibility of most of the essential nutrients including minerals. There was, also, poorer feed efficiency than those fed the low tannin sorghum (Al-Mamary *et al.*, 2001). Consequently, the apparent nitrogen digestibility of sorghum grains was lower than those groups fed control diet or the low tannin diet. This reflects an increased nitrogen excretion with the high tannin diet (Al-Mamary *et al.,* 2001)

Goat production in the rangelands could be increased if pods of *Acacia tortilis* and *Acacia nilotica* are collected during periods of abundance, stored and used as supplements for growing goats (Sawe *et al.*, 1998). He also wrote that *Acacia tortilis* and *Acacia nilotica* were consumed in greater quantities among the supplements and goats fed on these had the best liveweight gains of 65.5 and 65.5 g/day, respectively. Supplementation with either pods or leaves did not affect forage intake (mean iintake of 6.62 g/day).

Black locust leaves contain primarily condensed tannins (Kumar and Horigome, 1986). Ayers *et al.* (1996) reported that the dietary inclusion of 25% Black locust reduced the growth rate of rabbits, as well as digestibilities of DM, CP, neutral detergent fibre (NDF), acid detergent fibre (ADF), calcium (Ca) and phosphorus (P). These effects are likely due to the presence of tannins in the Black locust leaves. Horton and Christensen (1981) found that when lambs were fed Black locust leaves as a sole feed, the digestibilities of organic matter, CP, ADF, NDF and P were less than half those of Alfalfa meal. Although, no significant

differences were seen between lambs fed Black locust leaves and Alfalfa meal in terms of average daily gain or DM intake. Addition of 1% PEG improved crude protein digestibility from 58.8 to 66.3% (Ayers *et al.*, 1996). Horigome *et al.* (1988), also, found that addition of 1.6 % PEG to rat diets containing Black locust leaves increased CP digestibility from 49.1 to 70.7%.

Pritchard *et al.* (1988) fed sheep *Acacia aneura* (contains 6.1% tannins) and found that drenching the sheep every day with PEG increased body weight and wool growth. These findings contrast with those of Nunez-Hernandez *et al.* (1991) who reported that addition of PEG to diets containing mountain Mahogany leaves, which have condensed tannins, did not improve nutrient digestion or affect nitrogen balance in goats and lambs. Garrido *et al.* (1991) found that the addition of PEG to Faba bean seed coat at a level of 200 mg of PEG/gram of seed coat increased crude protein digestibility *in vitro* from 52.4 % to 75.5%. Reductions of 97% of the tannins were observed by the addition of polyvinylpyrrolidone on leaves.

Most of the papers reviewed were interested in analyzing the difference between the tannin assays, however, not much was found in comparing the correlation or lack of difference between tannin analytical assays and animal performance. There is a therefore the need to investigate the existence or lack of such correlations

2.7 SUMMARY

Acacia trees are common in the Limpopo province. *Acacia nilotica, A. tortilis* and A. *karoo* are widespread and common throughout the province, and ruminant animals browse heavily on these Acacia species. Tannins in these trees induce a negative response when consumed by animals. They are, thus, considered as anti-nutritional. Tannins have been defined as any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyl and other suitable groups (i.e carboxylic) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions. The hydrolysable tannins are present in many plants and have been shown to be responsible for growth inhibition of agriculturally important animal species. They have low bioavailability following oral ingestion due to their poor lipid solubility, binding and formation of complex proteins.

The concentration and chemical composition of condensed tannins change with physiological maturity of plants. Condensed tannin polymerization and binding with cellular components (plant protein) reduce their extractability and reactivity to a number of analytical assays. A number of assays are used to analyse tannins in plants. Folin-ciocalteu, which determines the total phenol, does not differentiate the differences between tannins and other polyphenols. Butanol HCl assay is specific for condensed tannins, and potassium iodate assays are specifically for hydrolysable tannins.

In the studies done not much was done to compare the correlation between Acacia species and biological responses. Most of the studies concentrate on plants that contain tannins..

CHAPTER 3

MATERIAL AND METHODS

3.1 STUDY SITE

This study was conducted at the Experimental Farm of the University of the North, Limpopo Province, South Africa between July and November 2002. The University of the North is about 30km North of Polokwane, the Capital City of Limpopo Province. Mean minimum and maximum temperatures in winter (April to July) are 10.1 and 28.4 °C, and summer (August and March) are 18 and 36 °C, respectively. Mean annual rainfall is between 446.8 and 468.4 mm. The rainfall pattern of the area is unimodal with the dry season occurring between April and October and the rainy season occurring between November and March

3.2 ANIMALS, MANAGEMENT, DIETS AND EXPERIMENTAL DESIGN

Eight New Zealand White male rabbits with a mean liveweight of 0.68 ± 0.05 kg were used in this study. The rabbits were randomly assigned to metabolic crates in a two 4 x 4 Latin square design. The animals were housed in individual cages and given the experimental diets for an adaptation period of 10 days before the commencement of the actual experiment. Prior to this they had been kept in the cages for 8 weeks to get used to the metabolic cages and new environment. Rabbits were weighed at the beginning and end of each period. The experiment had four periods with 14 days for each period; the last four days were for collection of urine, water, leftovers and faeces. The leftovers were collected the following day before offering any feed and they were weighed and recorded on a daily basis for four days after the adjusting period. Intake was calculated by subtracting leftovers from the amount of feed given. Water intakes were

monitored by use of graduated water bottles, which indicated the amount of water consumed.

Experimental feeds included supplementation of *Acacia karoo*, *Acacia nilotica* and *Acacia tortilis* leaves to the basal feed. The Acacia leaves were collected from the University of the North during April and May. The leaves were harvested and dried in the shade then ground to a fine powder. The rabbits were given a basal diet (16 % CP and 12 ME MJ/kg DM) obtained from NTK, Polokwane. The diets were given *ad libitum*. Four percent of total feed offered were leaves of the Acacia species assigned to that rabbit. The ground basal diet was mixed with the ground leaf material (specifically for that treatment) and all the feeds were fed at the same time. The complete experimental layout is shown in Table 3.1.

Table 3.1. The lay out of two 4 x 4 Latin square design

Latin square design I

	Animal			
	R1	R2	R3	R4
Period 1	A	B	C	D
2	B	C	D	A
3	D	A	B	C
4	C	D	A	B

Latin square design II

	Animal			
	R1	R2	R3	R4
Period 1	A	B	C	D
2	B	C	D	A
3	D	A	B	C
4	C	D	A	B

Where A = Basal diet mixed with 4 % *A. karoo* leaves
B = Basal diet mixed with 4 % *A. nilotica* leaves
C = Basal diet mixed with 4 % *A. tortilis* leaves
D = Basal diet

3.3. COLLECTION, DRYING AND STORAGE OF PLANT MATERIALS (FAO, 2002)

Leaves were hand collected from the trees and put in brown paper bags. The leaves where dried in the laboratory under the shade for approximately three weeks. The leaves were then ground to pass through a 60 mm sieve using a laboratory grinding mill (Labotec, Pretoria, South Africa). The samples were kept in the cupboard until feeding or analysis.

3.4. FAECAL AND URINE COLLECTION

The amount of faeces voided daily was separated on a synthetic mesh, which covered the bottom of the individual pen for each rabbit. The contents were emptied into a plastic bottle labeled for each rabbit every 24 hours, weighed and recorded. The amount of urine voided by each animal was collected using a plastic bottle containing 50 ml of 10 % H_2SO_4. The volume of urine voided was measured using a graduated cylinder.

3.5 SAMPLING AND SAMPLE PREPARATION

The daily total faecal output of each animal was mixed thoroughly and 10 % of the material was sampled and placed in an air-tight bottle. The faeces for each period were bulked on an individual animal basis and kept frozen at –20 °C until

analysis (FAO/IAEA, 2002). Before they were frozen the faecal material of each animal was thoroughly mixed and sub-sampled. A portion of the bulked faecal sample of each animal was taken and oven dried at 105 °C for dry matter determination. Ten percent of the filtered daily urine output was sampled from each rabbit and bulked for the experimental period. The samples were kept frozen at –20 °C until analysis.

3.6 CHEMICAL ANALYSES

3.6.1. Dry matter determination

For dry matter determination, AOAC (1998) procedure was used. Air-dried plant material of 2.00 g was accurately weighed in duplicates into the pre-weighed beakers. The samples were put in the oven overnight at 105 °C to attain constant weight and calculated as follows:

$$DM\ (\%) = \frac{\text{(Weight of dry sample)}}{\text{(Weight of sample before drying)}} \times 100$$

3.6.2 Crude protein determination

Nitrogen content was determined using the Kjedahl method (AOAC, 1998). For crude protein determination of urine 6 ml was used and with faeces 2.00 g was weighed on a tarred-ashless filter paper. The folded filter paper was

quantitatively transferred into a Kjedahl flask with two Kjedahl catalyst tablets and 25 ml of sulphuric acid. The contents were digested for 2 hours or until clear. Sodium hydroxide was added and ammonia was distilled over into 50 ml of boric acid solution (40 g of boric acid per litre of distilled water). The solution was subsequently titrated with 0.1N hydrochloric acid using both bromothymol red and green mixture as an indicator. The observed end colour was pale pink. The calculations used were as follows:

N (%) = (ml acid titrated – ml blank titrated) x (acid N x 0.014 x 10) / Weight of sample in gram or volume of sample in ml

CP (%) = [N (%) x 6.25] therefore,

CP (as % DM) = (CP % / DM %) x 100

3.6.3 Feed, urine and faecal analyses

Feed, urine and faeces were analysed for nitrogen using the Kjeldahl method (AOAC, 1998). Nitrogen retention was calculated as the difference between amount of nitrogen eaten and the amount lost in faeces and urine. Dry matter in the faeces and feed samples were determined using the AOAC (1998) procedures. Digestibility was estimated as the amount of nutrients not lost in the faeces.

3.6.4. Extraction (FAO/IAEA, 2002)

The aim of extraction was to quantitatively diffuse phenolics present in the plant material to liquid phase. For every method done a freshly made extraction was done. The extraction was kept for less than 24 hours in the fridge at 4 °C before use. A finely ground sample of 0.200 g plant material was weighed in the beaker of approximately 25 ml capacity. To it 10 ml of 70 % aqueous acetone or 50 % methanol was added, according to the procedure given below. The beaker was suspended in an ultrasonic water bath (Branson 3210) and subjected to ultrasonic treatment for 20 minutes at room temperature (25 °C). The beaker was put on ice for a few minutes and the content was then transferred to the centrifuge tubes and centrifuged at 3000 **g** using an ordinary clinic centrifuge. The supernant was transferred to other tubes and used as the procedure required.

3.6.5. Measurement of total phenolics and tannins using Folin-Ciocalteu method (Makkar, 1993)

The reason for measuring total phenols was to know the efficiency of extraction of phenolics in solvents. This method can be coupled with the use of insoluble matrix, polyvinyl polypyrrolidone (PVPP, bind tannins-phenolics) for measurement of tannins. The nature of tannic acid varies from one commercial source to the other (Makkar, 1993)

Suitable aliquots of the tannin-containing an extract of 0.02 ml in test tubes were pipetted and the volume of 0.48 ml distilled water was added. Folin-Ciocalteu reagent of 0.25 ml and 1.25 ml of the sodium carbonate solution were added. The tubes were vortexed and then the absorbance at 725 nm was recorded after 40 minutes. The amount of total phenolics was calculated as tannic acid equivalent using a calibration curve. Total phenolics content was expressed on a dry matter basis (% DM tannic acid equivalent).

3.6.6. Determination of simple phenolics using polyvinylpolyprrolidone (PVPP) (removal of tannin from the tannin-containing extract) (Makkar, 1993)

A hundred milligram of PVPP was weighed into a 100 x 12 mm test tube, 1.0 ml distilled water and 1.0 ml of the tannin-containing extract were added. A hundred milligram of PVPP is sufficient to bind 2 mg of total phenols. If total phenolic content of the feed is more than 10 % on a dry matter basis, the extract was diluted appropriately. The tubes were vortexed and kept at 4 ^{0}C for 15 minutes. They were vortexed again, before they were centrifuged at 3000 **g** for 10 minutes, and the supernant was collected. This supernant had only simple phenolics other than tannins. The tannins had been precipitated along with the PVPP. The phenolic content of the supernant was measured by taking three times the volume used for total phenol estimation, because the extract was already diluted two-fold and was expected to lose tannin-phenols through binding with PVPP. The absorbance was recorded at 725 nm after 40 minutes. The

content of non-tannins phenols was expressed on a dry matter basis (% DM tannic acid equivalent).

3.6.7 Determination of condensed tannins (Porter et al, 1986)

3.6.7.1 Extracted condensed tannins

A 0.2 ml of the tannin extract diluted with 0.3 ml of 70 % acetone was pipetted into a 100 x 12 mm test tube, and 3.0 ml of the butanol HCL reagent and 0.1 ml of the ferric acid were added. The tube was vortexed with the mouth of each tube covered with a glass marble and put in the heating block adjusted at 97 to 100 °C for 60 minutes. The tube was cooled and absorbance was recorded at 550 nm. For the blank 0.5 ml of the extract 3 ml of butanol and 0.1 ml of ferric reagent were added. The formula for calculating percentage of condensed tannins as leucoanthocyanidin equivalent = (Absorbance 550 nm x 78.26 x dilution factor)/ (% DM)

3.6.7.2 Unextracted condensed tannins

A 0.01 g of the pellet from condensed tannins extract, 3.0 ml of the butanol HCL reagent and 0.1 ml of the ferric acid were added into a 100 x 12 mm glass test tube. The tube was vortexed with the mouth of the tube covered with a glass marble and put in the heating block adjusted at 97 to 100 °C for 60 minutes. The tube was cooled and absorbance was recorded at 550 nm. For the blank, 0.5 ml of the extract, 3 ml of butanol and 0.1 ml of ferric reagent were added. The

formula for calculating percentage of condensed tannins in a gram of sample was: (Absorbance 550 nm/ weight of sample used) x (1000 mg) / (% DM)

3.6.8. Protein binding capacity by filter paper assays (Dawra et al, 1988)

The extraction was done using 70 % of acetone, as acetone does not interfere in this method. A Whatman paper chromatography sheet of 1 mm was cut into an appropriate size of 60 cm x 15 cm. The squares of approximately 3 cm were drawn using a light lead pencil on the chromatography sheet. Different aliquots (5 to 25 μl containing tannic acid) were applied on the sheet; each aliquot was done in triplicate, (meaning on three different squares). Amounts of 50 μl of plant extract were applied on the middle of the squares on the chromatography sheet. The spots were allowed to dry and immediately BSA was used to spray the paper until it was wet. The paper was washed with acetate buffer (pH 5; 0.05 M) with three 10 minutes changes with slight shaking to remove the unbound BSA. The paper was stained with 0.2 % Ponceau S dye solution by keeping the strips dipped for 10 minutes in the stain solution. The stain strip was washed in 0.2 % acetic acid solution until no more colour was eluted from the strips.

The strips were air dried and the stained areas were cut in small pieces and put in the test tubes where the colour was eluted by adding 3 ml of 0.1N sodium hydroxide solution and it was vortexed, followed by addition of 0.3 ml of 10 % acetic acid and centrifugation at approximately 2500 **g**. The absorbance of the colour was recorded at 525 nm against corresponding blank, which was done this way: a plain chromatography sheet was stained simultaneously as the

sample chromatography and washed in the same manner similar to the samples. These absorbances were converted to protein content by using a standard curve. The standard curve was prepared by applying different concentrations of bovine serum albumin (BSA) (5 to 50 μl of 1 mg/ml BSA solution in the acetate buffer). This was applied as separate spots in triplicates for each concentration on a chromatography sheet and cut into strips. These strips were stained with the dye solution for 10 minutes, washed, dried and cut, and then absorbance was recorded the same way as for the samples. The calculations were done as tannic acid equivalent from the calibration curve and expressed as μg

3.6.9. Radial diffusion assay (Hagerman, 1987)

Acetone was used in the extract, as it does not interfere with the method.

3.6.9.1. Preparation of the plates

A 2.5 g of agarose was weighed into 250 ml of the acetate buffer and then boiled for approximately 15 minutes with continuous stirring on a magnetic stirrer until the agarose dissolved. The solution was cooled to 45 °C by keeping the vessel containing the agarose solution in a water bath set at approximately 45 °C. A 250 mg of BSA was dissolved in the agarose solution without allowing the solution to cool. Petri dishes used were about 8.5 cm in diameter. A glass pipette with a large tip opening of 10 ml was used to dispense approximately 10 ml of the solution into each petri plate kept on a flat surface. The bubbles were not introduced in the plates and the solution covered the whole surface of the

plate. The solution hardened and the plates were covered and sealed with the parafilm to prevent drying and cracking of the agarose layer. The plates were stored in a fridge for 24 hours

3.6.9.2. Assay procedure

On the day of performing the assays, the petri dishes were taken out of the refrigerator, brought to room temperature and then opened. A puncher was used to punch four wells, far apart, in the solidified agarose in petri dishes. In each well 15, 30, 45 and 65 μl of the extract were pipetted. The Petri dishes were covered and sealed using parafilm. The plates were placed in an oven adjusted at 30 °C. After 96 hours the petri dishes were removed, uncovered and the diameter of the ring was measured using a transparent millimeter ruler.

3.6.10. Reaction of Polyethylene glycol (PEG) with tannins (Silanikove et al, 1994)

A stock solution containing 100 g/l PEG 4000 (MW 4000) in a 0.5 m buffer Tris-BASE, pH 7.1 was prepared. A working solution was prepared by mixing 1 part of the stock solution and two parts of distilled water. The ratio between the plant sample weight and the working solution was 1:15. One gram of the sample was used. The reaction was carried out in 50 ml centrifuge tubes. After the samples had been mixed with the working solution or distilled water (in the case of those untreated or control), the tubes were left for 24 hours in a horizontal position, with

occasional mixing. The tubes were then centrifuged for 30 minutes at 2500 **g** and the supernant was collected.

Crucibles were used for the final part. They were initially dried in an oven to constant weights and then transferred into desiccators to cool down before weighing. A sample of 10 ml was poured in the crucible and then dried in the oven, weighted after drying. This procedure of weighing and drying was repeated three times after every 30 minutes and weights were recorded for each period. This was done for treated and untreated feed samples

3.7 STATISTICAL ANALYSIS

The effects of tannin level on feed and water intakes, digestibility and nitrogen retention in rabbits were assessed using analysis of variance as in a two replicates of a balanced 4 x 4 Latin square design. General Linear Models (GLM) procedures of SAS (1997) were used to analyse the data. Correlation analyses were used to relate tannin amounts determined by different methods to animal performance indices (intake, digestibility and nitrogen retention).

CHAPTER 4

RESULTS

Results of the chemical analysis of the basal diet (control) are given in Table 4.1. Rabbit diet was high in crude protein. Thus, there were 160g of crude protein per kilogram diet dry matter. It, also, had a metabolisable energy value of 12.86 MJ per kilogram dry matter.

Data on tannin contents of Acacia leaves are presented in Table 4.2 (details are indicated in the Appendices 7.1 to 7.7). The basal feed (control) is not included in this table because the analyses did not indicate any presence of tannins. Acacia nilotica had higher total phenolics (2.04% DM) than both Acacia karoo (1.51% DM) and Acacia tortilis (1.25% DM) ($P<0.05$). Similar differences were observed when tannins were measured by radial diffusion method. The analysis by polyvinylpolypyrrolidone method showed that A. karoo (0.57% DM), A. nilotica (1.22% DM) and A. tortilis (0.40 % DM) were significantly different ($P < 0.05$). Similar differences were observed when butanol HCl, polyethylene glycol and precipitable phenolics by filter paper methods were used.

Results of diet intakes and digestibility are given in Table 4.3 (details are indicated in the Appendices 7.8 to 7.12). There were no significant differences between treatments in diet DM intake, nitrogen intake, DM and nitrogen digestibilities ($P > 0.05$). Water intakes were 172, 185, 173 and 229g for rabbits on control, A. *karoo*, A. *nilotica* and A. *tortilis* treatments, respectively. Rabbits on Acacia *tortilis* diet drank more water than those on the other treatments ($P < 0.05$). However, there were no differences in water intake between rabbits on control, A. *karoo* and A. *nilotica* diets ($P > 0.05$).

Table 4.1. Chemical composition of rabbit diet

Composition	
Dry matter (g/kg)	970
Nutrients (g/kg DM)	
Crude Protein	160
Ash	120
Calcium	9
Phosphorus	4.8
Metabolisable Energy (MJ/kg DM)	12.86

Table 4.2. Tannin contents of Acacia leaves by methods of total phenolics (TP), polyvinylpolypyrrolidone (PVPP), radial diffusion (RD), extracted condensed tannin (ExCt), unextracted condensed tannin (UnExCt), polyethylene glycol (PEG) and precipitable phenolics by filter paper method (PPFP).

	Diet			
	Karoo	Nilotica	Tortilis	S.E
TP (%DM)*	1.51^{b}	2.04^{a}	1.25^{b}	0.079
PVPP (%DM)	0.57^{b}	1.22^{a}	0.40^{c}	0.044
RD (mm^2)	4.00^{b}	5.80^{a}	3.75^{b}	0.177
ExCT (%DM)**	4.52^{a}	0.37^{c}	4.07^{b}	0.068
UnExCT (%DM)**	3.72^{a}	1.83^{c}	2.60^{b}	0.104
PEG (mg/g)	0.39^{b}	0.48^{a}	0.47^{c}	0.002
PPFP (µg)	0.24^{b}	0.47^{a}	0.18^{c}	0.007

S.E : Standard error

* percentage DM tannic acid equivalent

** percentage DM Leucocyanidin equivalent

a,b,c : means within a row with different superscripts are significantly different at 5% level ($P<0.05$)

Table 4.3. Diet feed intake and digestibility in rabbits on control basal diet only and those supplemented with leaves from different Acacia species

	Diet			
	Control	A. karoo	A. nilotica	A. tortilis
Intake (g/rabbit/day)				
Diet DM	95.7±4.40	86.4±4.51	88.6±3.73	97.3±4.20
Nitrogen	2.4±0.12	2.3±0.12	2.3±0.10	2.6±0.11
Water	172^{b} ±9.9	185^{b}±10.2	173^{b} ±8.5	229^{a} ±9.5
Digestibility (%)				
Diet DM	71.1±5.89	73.2±6.04	62.7±6.99	64.1±5.63
Diet N	88.2±3.41	85.3±3.49	81.8±2.89	83.9±3.25

a,b: means with different superscripts within a row are significantly different at 5% level ($P < 0.05$)

± Standard error

Results of faecal output, urine nitrogen output and nitrogen retention in rabbits are given in Table 4.4 (details are indicated in the Appendices 7.13 to 7.17). There were no significant differences between treatments in faecal DM and nitrogen outputs ($P > 0.05$). Similarly, there were no differences between treatments in urine output, urine nitrogen output and nitrogen retention ($P > 0.05$).

A series of linear regressions that were used to predict intake in rabbits from total phenolics, extracted condensed tannins, polyethylene glycol, precipitable phenolics by filter paper and radial diffusion contents of Acacia browse species are given in Table 4.5. Dry matter intake was positively correlated with different tannin assays. These indicated that dry matter intake could be moderately predicted from total phenolics ($r = -0.6358$) and precipitable phenolics by filter paper ($r = -0.6429$) contents, but correlations with extracted condensed tannins ($r = -0.1315$), polyethylene glycol ($r = -0.3663$) and radial diffusion ($r = -0.5729$) contents were less accurate. Water intake by rabbits was poorly and positively correlated with total phenolics, extracted condensed tannins, polyethylene glycol and radial diffusion values. However, water intake by rabbits was poorly and negatively correlated with precipitable phenolics by filter paper. Water intake was positively correlated with extracted condensed tannins ($r = 0.679$). Nitrogen intake was poorly and positively correlated with extracted condensed tannins ($r = 0.3213$) and polyethylene glycol ($r = 0.0728$). However, negative and poor correlation coefficients were observed between nitrogen intake and total phenolics ($r = -0.2862$), precipitable phenolics by filter paper ($r = -0.4253$) and radial diffusion ($r = -0.2225$) contents of Acacia browses.

Table 4.4. Faecal out, urine nitrogen output and nitrogen retention (g/rabbit/day) in rabbits fed basal diet only and those supplemented with different Acacia leaves

	Diet			
	Control	A.karoo	A. nilotica	A. tortilis
Faecal output				
Dry matter	27.7±4.38	22.5±4.49	31.9±3.72	33.7±4.19
Nitrogen	0.3±0.07	0.3±0.07	0.4±0.06	0.4±0.06
Urine output	26.7±6.21	36.7±6.36	26.2±5.26	33.9±5.93
Urine N output	0.1±0.05	0.2±0.05	0.2±0.04	0.2±0.05
N retention	2.0±0.14	1.7±0.14	1.7±0.12	2.0±0.13
N retention (%)*	82.9±4.39	76.5±4.50	73.3±3.73	77.5±4.20

* Percentage of total nitrogen intake

± Standard error

Table 4.5. Prediction of intake (g/rabbit/day) in rabbits from different tannin assays

Factor	Y-variable	Formulae	r
Tp	DM intake	Y= -3.91X + 96	-0.636
ExCt	DM intake	Y= -0.29X + 92	-0.132
PEG	DM intake	Y= -8.6X + 94	-0.366
PPFP	DM intake	Y= -17.6X + 95	-0.643
RD	DM intake	Y= -1.25X + 96	-0.573
Tp	Water intake	Y= 3.38X + 186	0.109
ExCt	Water intake	Y= 7.63X + 173	0.679
PEG	Water intake	Y= 55.1X + 171	0.467
PPFP	Water intake	Y= -17.1X + 194	-0.124
RD	Water intake	Y= 1.73X + 134	0.158
Tp	N intake	Y= -3.01X + 71	-0.286
ExCt	N intake	Y= 0.45X + 66	0.321
PEG	N intake	Y= 0.05X + 2.4	0.073
PPFP	N intake	Y= -0.31X + 2.4	-0.425
RD	N intake	Y= -0.01X + 2.4	-0.223

Total phenolics (TP) = % DM tannic acid equivalent

Extracted condensed tannins (ExCt) = % DM leucocyanidin equivalent

Polyethylene glycol (PEG) = mg

Precipitable protein by filter paper (PPFP) = μg

Radial diffusion (RD) = mm^2

A series of linear regressions that predict faecal nitrogen output, digestibility and nitrogen retention in rabbits from total phenolics, extracted condensed tannins, polyethylene glycol precipitable phenolics by filter paper and radial diffusion contents of Acacia browse species are given in Table 4.6. Faecal nitrogen output in rabbits was poorly and negatively correlated with extracted condensed (r = -0.010) tannin contents. However, positive correlation coefficient was observed with total phenolics (r = 0.594), polyethylene glycol (r = 0.712), precipitable phenolics by filter paper (r = 0.610) and radial diffusion (r = 0.658) tannin contents.

Nitrogen digestibility was poorly and negatively correlated with extracted condensed tannin values of the browses (r = -0.1308). However, nitrogen digestibility was highly and negatively correlated with total phenolics (r = -0.9295) and radial diffusion (r = -0.9570) tannin contents of the browses.

Diet dry matter digestibility was positively correlated with different tannin assays. These indicated poor correlation coefficient with extracted condensed tannin contents (r = -0.2083). However, browse contents of total phenolics (r = -0.9317), polyethylene glycol (r = -0.5834), precipitable phenolics by filter paper (r = -0.5816), and radial diffusion (r = -0.5711) were moderately correlated with diet dry matter digestibility.

Nitrogen retention (percentage of total nitrogen intake) values were highly and negatively correlated with browse contents of total phenolics (r = -0.9963), polyethylene glycol (r = -0.9101), precipitable phenolics by filter paper (r = -0.9721) and radial diffusion (r = -0.9955).

Poor correlation coefficients were observed between nitrogen retention (percentage of total nitrogen intake) in rabbits and extracted condensed tannins (r = -0.2285) in the browses. The correlation coefficients between nitrogen retention (g/rabbit/day) and different tannin assays were good except with extracted condensed tannins (r = -0.2354), which, was less accurate.

Table 4.6. Prediction of faecal nitrogen output (g/rabbit/day), nitrogen retention (g/rabbit/day), digestibility (%), and nitrogen retention (percentage of nitrogen intake) in rabbits from different tannin assays

Factor	Y-variable	Formulae	R
Tp	N output	Y= 0.04X + 0.3	0.594
ExCt	N output	Y= 0.02X + 2.4	-0.010
PEG	N output	Y= 0.18X + 0.3	0.712
PPFP	N output	Y= 0.18X + 0.30	0.610
RD	N output	Y= 0.02X + 0.29	0.658
Tp	N digestibility	Y= -2.89X + 88	-0.932
ExCt	N digestibility	Y= -0.02X + 1.9	-0.131
PEG	N digestibility	Y= -10.8X + 88	-0.913
PPFP	N digestibility	Y= -12.2X + 87	-0.929
RD	N digestibility	Y= -1.05X + 88	-0.957
Tp	DM digestibility	Y= -0.05X + 2.5	-0.505
ExCt	DM digestibility	Y= -0.29X + 92	0.208
PEG	DM digestibility	Y= -13.2X + 72	-0.583
PPFP	DM digestibility	Y= -15.4X + 71	-0.582
RD	DM digestibility	Y= -1.23X + 71	-0.571
Tp	N retention*	Y= -4.59X + 83	-0.996
ExCt	N retention*	Y= -0.15X + 85	-0.229
PEG	N retention*	Y= -116X + 82	-0.910
PPFP	N retention*	Y= -20.3X + 82	-0.972
RD	N retention*	Y= -1.63X + 83	-0.996
Tp	N retention	Y= -0.21X + 2.1	-0.869
ExCt	N retention	Y= -0.02X + 0.35	-0.235
PEG	N retention	Y= -0.64X + 2.1	-0.666
PPFP	N retention	Y= -0.92X + 2.08	-0.848
RD	N retention	Y= -0.07X + 2.0	-0.828

* percentage of total nitrogen intake

Total phenolics (TP) = % DM tannic acid equivalent

Extracted condensed tannins (ExCt) = % DM leucocyanidin equivalent

Polyethylene glycol (PEG) = mg

Precipitable phenolics by filter paper (PPFP) = μg

Radial diffusion (RD) = mm^2

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1. Effect of browse supplementation on diet intake and digestibility in rabbits

Acacia trees are widespread and widely utilized by livestock in the Limpopo Province. However, Acacia trees have polyphenolics that include tannins. Tannins are a very complex group of plant secondary metabolites, which are soluble in polar solution and are distinguished from other polyphenolic compounds by their ability to precipitate proteins (Silanikove et al., 2001). Tannins are considered as anti-nutritive compounds and some are toxic to livestock, depending on quantity. Thus, tannins bind with proteins, carbohydrates and minerals and dramatically inhibit digestive and absorptive processes in the gastro intestinal tract of animals (Kumar and Singh, 1984; Kumar and Vaithiyanathan, 1990). However, when taken in low amounts, they have positive effects on diet intake, digestibility and nitrogen retention (Al-Mamary et al., 2001). Quantification of these anti-nutrients is necessary in order to predict their effect on animal performance.

The Acacia species used in this study contained different types of tannins in varying levels. Although *Acacia nilotica* had a highest concentration of total phenolics, simple phenolics, polyethylene glycol, precipitable phenolics by filter paper and radial diffusion, it had the lowest amounts of condensed tannins, both extracted and unextracted. Thus, few of the phenolics in *Acacia nilotica* were condensed tannins. Most of the phenolics in *A. nilotica* are catechin gallates (Mueller-Harvery et al., 1987), which may be absorbed by the gut, and may have toxic effects on the animal. *Acacia tortilis* had the lowest contents of total phenolics, simple phenolics, radial diffusion and

protein precipitation by filter paper. *Acacia karoo* was highest in condensed tannins. The condensed tannins are not absorbed into the blood streams, therefore, under normal physiological conditions, they are not likely to damage organs such as the liver, kidney, spleen etc., as has been the case for hydrolysable tannins (McSweeney et al., 1988). These results are consistent with the finding by Dube et al., 2001 where *A. nilotica* was reported to have the highest levels of total phenolics in a study that also included *A.albida, A. eriolaba* and *A.senegal.* Similar results have been reported elsewhere (Dube, 1993; Dube and Ndlovu, 1995; Kahiya et al., 2003).

Tannins have been reported to be responsible for decreases in intake and digestibility in experimental animals (Butler, 1992; Chang et al., 1998). Tannins have the ability to aggregate and precipitate proteins (Hagerman and Butler, 1989; Makkar et al., 1987; Butler and Bos, 1993; Jansman et al., 1994). They have been shown to impair macronutrient utilization by forming tannin-protein complexes with various digestive enzymes (Chang and fuller, 1964; Davis and Hoseney, 1979; Flores et al., 1994), precluding the formation of products absorbable by the small intestines. Results with rabbits reported here indicate that there were no significant differences in dry matter and nitrogen intakes and digestibilities between the control diet and those supplemented with tanniniferous browses of *A. karoo*, *A.nilotica* and *A. tortilis*. Results obtained are similar to those of Al-Mamary et al. (2001) who reported that addition of low- tannin (1.4% catechin equivalent) sorghum grains in the diets of rabbit did not significantly change growth rate, food intake or feed conversion ratio. However, they reported that the addition of high-tannin (3.5 % catechin equivalent) sorghum grains significantly reduced their live weight gain, feed conversion ratio, and slightly

increased food consumption with respect to the control diet. The authors suggested that lack of toxicity in rabbits fed low-tannin sorghum grain may indicate the existence of a threshold-limit. Similarly, McNabb et al. (1998) and Barry and Duncan (1984) showed that the effect of tannins depends on the type and level of tannin, and dietary nutrients involved. Therefore, in the study reported here, a possible explanation for the lack of significant differences may be that the rabbits did not take in sufficient amounts of tannins from the 4 % browse supplementation to cause problems in intake and digestibility. Since tannins form insoluble proteins and carbohydrate complexes, dry matter and nitrogen digestibilities are mostly to be affected by high concentrations of tannins in the diet (Barry and Duncan, 1984; Al-Mamary et al., 2001). The addition of 4 % *A. karoo*, *A. nilotica* or *A. tortilis* browses was not likely high enough to cause decreases in diet intakes and digestibilities.

Results from the literature regarding the intake and digestibility of tanniniferous browse species in ruminants are conflicting. Holocheck et al. (1990), using goats, found that diets containing browse species high in tannin compounds resulted in reduced intakes compared to diets with low levels of these substances. Nastis and Malechek (1981) reported that the inclusion of increasing amounts of immature *Quercus gambellii* in Alfalfa-based diets reduced dry matter intake compared to diets of alfalfa hay when both were fed to goats. Similarly, Sidahmed et al. (1981) reported a reduction in dry matter intake in goats given tanniniferous browses. In contrast, Nunez-Hernandez et al. (1991) reported that a *J. monosperma* diet fed to Angora goats resulted in a higher dry matter intake than an alfalfa diet only. Ramirez et al. (1992) reported that goats fed diets containing different levels of *Acacia farnesiana*

shrubs with high tannin contents (15.0 and 7.0 % DM, respectively) had the same intakes compared with goats fed on alfalfa diet only. These results suggest that a high tannin level does not always depress intake in ruminant animals. In ruminants, rumen microbes are capable of degrading hydrolysable tannins and higher load of phenols, beyond the capability of the liver to detoxify them. This cannot happen in non-ruminants, as they do not have rumen microbes, therefore, the degrading of hydrolysable tannins and a higher load of phenols may not take place. There was no literature found on non-ruminants that showed whether high or low tannin supplementations affect their intake or digestibility.

In the present study, rabbits on a diet supplemented with leaves from *A. tortilis* drank more water than those on the control, *A. karoo* and *A. nilotica*. It has been postulated that astringency, caused by tannins, may cause animals to drink more water than they would on low tannin diets (Hove et al., 2001). However, the mechanism of this is not clear. There were no differences between treatments in urine output, possibly indicating that substantial amount of water in rabbits on *A. tortilis* treatment was excreted in the faeces.

There were no significant differences between treatments in rabbit faecal nitrogen output, urine nitrogen output and nitrogen retention. However, the faecal nitrogen outputs of rabbits supplemented with Acacia browses tended to be higher than those on the control diet. It is likely that the amounts of tannins from the browses (4 % of the feed mixture) used in the present study were not large enough to significantly bind with proteins (dietary and enzymes) and consequently affect the faecal and urine

nitrogen outputs (Al-Mamary et al., 2001). The nutritional effects of tannins are associated with their ability to bind proteins, structural carbohydrate polymers found in plant cell walls and minerals with an overall effect of lowering the bio-availability of nutrients at specific sites in the gastro-intestinal tract (Davis and Hoseney, 1979; Butler, 1992; Chang et al., 1998; Al-Mamary et al., 2001). Al-Mamary et al. (2001) found that addition of low-tannin sorghum grains to the rabbit diet did not significantly have any effect on faecal nitrogen excretion and nitrogen retention with respect to the control diet. However, in their experiment, they also observed that the addition of high tannin sorghum grains significantly increased faecal nitrogen output and reduced nitrogen retention.

Rabbits eat their faeces and hence derive substantial amounts of microbial proteins from such diets (Cheeke, 1992). However, digestibility of the re-ingested tannin-complexes formed along the digestive tract is not known.

5.1.2. Prediction of diet intake, digestibility and nitrogen retention in rabbits from different tannin assays

A major constraint to proper management of tanniniferous feeds has been that the assays are not well proven to correlate with the performance of the animals that eat such feeds (Makkar, 2003). Such correlations would help in formulating management strategies that enhance the positive effects and reduce the negative effects that tanniniferous feeds cause to the productivity of animals that eat such feeds.

Diet dry matter intake in rabbits was negatively correlated with tannin contents in leaves. The negative correlation coefficients were expected since tannins tend to decrease diet digestibility through their ability to bind with proteins and other materials, resulting in decreased diet intake (Butler and Bos, 1993; Chang et al., 1998; Al-Mamary et al., 2001). The correlation coefficients between dry matter intake in rabbits and tannin assays ranged from low to moderate values. Such relationships have been reported elsewhere (Dube et al., 2001; Al Mamary et el., 2001). The correlations between dry matter intake in rabbits and tannin contents in leaves by different assays were negatively and well correlated but extracted condensed tannins and polyethylene glycol contents were less accurate. These negative correlation coefficients were expected since tannins have been reported to be responsible for decreases in feed intake (Butler and Bos, 1993; Chang et al., 1998; Ben Salem et al., 2000 and Al-Mamary et al., 2001). These indicate that the higher tannin content in a plant the less its dry matter intake.

Water intake in rabbits was moderately and positively correlated with extracted condensed tannins; but total phenolics, polyethylene glycol and radial diffusion contents were less accurate in predicting water intake. Water intake was negatively correlated with precipitable phenolics by filter paper. Positive correlation coefficients were expected since tannins dehydrate animals (Dube and Ndlovu, 1995), thus increasing water intake by the animal. The correlation coefficients between nitrogen intake and browse contents of total phenolics, polyethylene glycol, precipitable phenolics by filter paper and radial diffusion indicated that these different tannin assays provided no reliable indication of diet nitrogen intake in rabbits.

The correlation coefficients between faecal nitrogen output in rabbits and tannin contents in leaves by different assays were moderate and positive except with that of extracted condensed tannins, which was low and negative. The positive correlation coefficients were unexpected since tannins are expected to bind with proteins (dietary and enzymic) and consequently increase faecal nitrogen output in rabbits (Al- Mamary et al., 2001). Diet nitrogen digestibility in rabbits was highly and negatively correlated with total phenolics (r = -0.9317), polyethylene glycol (r = -0.9126), precipitable phenolics by filter paper (r = -0.9295) and radial diffusion (r = -0.9570) contents in browse supplements. These negative correlations were expected since tannins decrease nitrogen digestibility in experimental animals through their ability to aggregate and precipitate proteins and various digestive enzymes, thus rendering them unabsorbable (Makkar et al., 1987; Butler, 1992; Jansman et al., 1994; Flores et al., 1994; Chang et al., 1998; Al-Mamary et al., 2001). However, the predictions of diet dry matter digestibility from different assays for measuring tannin contents were less accurate. Extracted condensed tannins in leaves of Acacia species provided no reliable indication of diet dry matter and nitrogen digestibilities. There were no reports found on the relationships between diet digestibility in rabbits and tannin contents of leaves of Acacia species.

High and negative correlations were found between nitrogen retention in rabbits (percentage of total nitrogen intake) and total phenolics (r = -0.9963), polyethylene glycol (r = -0.9101), precipitable phenolics by filter paper (r = -0.9714) and radial diffusion (r = -0.9955) contents in leaves of Acacia species. Similar trends were observed when total phenolics, polyethylene glycol, precipitable phenolics by filter

paper and radial diffusion contents in leaves of Acacia species were used to predict nitrogen retention (g/rabbit/day). Prediction of nitrogen retentions (both in g/rabbit/day and nitrogen retention as a percentage of total nitrogen intake) by extracted condensed tannin contents in leaves of Acacia species was less accurate. The negative relationships can be explained by the detrimental effect of tannins on diet nitrogen digestibility, which eventually impacts negatively on nitrogen retention (Ben Salem et al., 2000).

Diet dry matter and nitrogen digestibilities, faecal nitrogen output and nitrogen retention in rabbits were accurately predicted by total phenolics, polyethylene glycol, precipitable phenolics by filter paper and radial diffusion contents in leaves of Acacia browse supplements. These different assays are worth further study with the aim of developing an accurate prediction equation for use with tanniniferous feeds. However, extracted condensed tannin contents in leaves of Acacia browse supplements provided no reliable indication of diet dry matter and nitrogen digestibilities, faecal nitrogen output and nitrogen retention in rabbits.

5.2. CONCLUSION

Acacia trees form a large plant family in Southern Africa and are an important ecological component of the bushveld vegetation that is prevalent in the Limpopo province. They have high protein contents but their use has been limited by the scarcity of information relating the amounts and types of tannins that they contain to effect on animal performance.

Tannin contents in leaves of different Acacia species found in the Capricorn region of the Limpopo Province were determined by different tannin assays. *Acacia nilotica* had the highest total phenolics, simple phenolics and phenolics that bind to proteins. However, it had the lowest amount of condensed tannins. *Acacia Karoo* had the highest amount of condensed tannins, both extracted and unextracted.

Results indicated that there were no significant differences in diet intakes and digestibilities between the control diet and those supplemented with 4 % leaf meal of *A. karoo, A. nilotica* and *A. tortilis.* However, rabbits on a diet supplemented with leaf meal from *A. tortilis* drank more water than those on the control, *A. karoo* and *A. nilotica* diets. Faecal dry matter and nitrogen output, urine nitrogen outputs and nitrogen retention in rabbits were not significantly affected by supplementation with 4 % leaf meal of *A. karoo*, *A. nilotica* or *A. tortilis*. The amount of tannins from the browses (4 % of the feed mixture) used in the present study were not likely large enough to significantly affect diet intake, digestibility and nitrogen retention in rabbits.

Relationships between tannin contents in Acacia leaves measured by different assays and biological responses in rabbits supplemented with such leaves were determined. Negative correlation coefficients were observed between nitrogen digestibility and nitrogen retention in rabbits and total phenolics (TP), polyvinylpolyprrolidone (PVPP), precipitable phenolics by filter paper (PPFP), radial diffusion (RD), extracted condensed tannin (EXCT) and polyethylene glycol (PEG) contents in leaves of Acacia species. Nitrogen digestibility and retention were most accurately predicted by contents of TP, RD, PPFP and PEG in the leaves. However, extracted condensed tannin contents in leaves of Acacia browse supplements provided no reliable indication of diet dry matter and nitrogen digestibilities, faecal nitrogen output and nitrogen retention in rabbits. Poor to moderate relationships were observed between diet dry matter, nitrogen and water intakes in rabbits and tannin contents in Acacia leaves determined by different tannin assays. It is concluded that for the prediction of nitrogen digestibility and retention in rabbits, RD, TP, PPFP and PEG contents in Acacia leaves were most accurate. These are worth further study with the aim of confirming present results and developing accurate prediction equations for use with tanniniferous browses.

5.3. RECOMMENDATIONS

Supplementation with Acacia leaf meals at 4% of the total diets did not adversely affect diet intake, digestibility and nitrogen retention in rabbits. An increase in the level of leaf meal supplementation may show some adverse effects.

A number of other biological responses, such as diet passage rate, growth and effects on organs, were not measured. Such responses can be measured in order to differentiate effect of the browses.

Blood profiles were not measured. These may be measured because they are affected by hydrolysable tannins.

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix 7.1 Analysis of Variance for the effect of species on Total phenolics (%DM)

Source	DF	Mean Square	F Value	Pr > F
Species	3	0.27552448	22.25	0.0059
Error	4	0.01238370		
R-Square	0.943460			

Appendix 7.2 Analysis of Variance for the effect of species on polyvinylpolyprrolidone (%DM)

Source	DF	Mean Square	F Value	Pr > F
Species	3	0.40456374	105.72	0.0003
Error	4	0.00382688		
R-Square	0.987545			

Appendix 7.3 Analysis of Variance for the effect of species on radial diffusion (mm^2)

Source	DF	Mean Square	F Value	Pr > F
Species	3	2.23666667	35.79	0.0024
Error	4	0.06250000		
R-Square	0.964080			

Appendix 7.4 Analysis of Variance for the effect of species on extracted condensed tannin (%DM)

Source	DF	Mean Square	F Value	Pr > F
Species	3	8.48754254	930.51	<.0001
Error	4	0.00912140		
R-Square	0.998569			

Appendix 7.5 Analysis of Variance for the effect of species on unextracted condensed tannin (%DM)

Source	DF	Mean Square	F Value	Pr > F
Species	3	1.72469161	79.69	0.0005
Error	4	0.02164238		
R-Square	0.983544			

Appendix 7.6 Analysis of Variance for the effect of species on Polyethylene glycol (mg)

Source	DF	Mean Square	F Value	Pr > F
Species	2	0.00412708	933.38	<.0001
Error	3	0.00000442		
R-Square	0.998396			

Appendix 7.7 Analysis of Variance for the effect of species on Precipitable phenolics by filter paper (µg)

Source	DF	Mean Square	F Value	Pr > F
Species	2	0.04779761	467.40	0.0002
Error	3	0.00010226		
R-Square	0.996801			

Appendix 7.8 Analysis of Variance for the effect of square, rabbit, period and diet on DM faeces (g/rabbit/day)

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.023295	0.00	0.9886
Rabbit (square)	6	131.611339	1.20	0.3702
Period (square)	6	201.906655	1.84	0.1737
Diet	3	203.648137	1.86	0.1910
Livewt	1	4.914054	0.04	0.8360
Error	12	109.765634		
R-Square	0.665194			

Appendix 7.9 Analysis of Variance for the effect of square, rabbit, period and diet on water intake (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	1725.20833	3.03	0.1074
Rabbit (square)	6	1955.98958	3.43	0.0328
Period (square)	6	2130.93171	3.74	0.0247
Diet	3	4789.96724	8.41	0.0028
livewt	1	263.77617	0.46	0.5091
Error	12	569.70508		
R-Square	0.856729			

Appendix 7.10 Analysis of Variance for the effect of square, rabbit, period and diet on DM intake (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	277.472746	2.51	0.1392
Rabbit (square)	6	259.456275	2.35	0.0984
Period (square)	6	172.286879	1.56	0.2415
Diet	3	137.844487	1.25	0.3362
Livewt	1	84.752875	0.77	0.3985
Error	12	110.587625		
R-Square	0.717244			

Appendix 7.11 Analysis of Variance for the effect of square, rabbit, period and diet on DM digestibility (%).

Source	DF	Mean Square	F Value	Pr > F
Square	1	30.707396	0.15	0.7009
Rabbit (square)	6	353.218147	1.78	0.1861
Period (square)	6	230.147955	1.16	0.3878
Diet	3	211.734852	1.07	0.3995
Livewt	1	8.218008	0.04	0.8422
Error	12	198.440946		
R-Square	0.636756			

Appendix 7.12 Analysis of Variance for the effect of square, rabbit, period and diet on nitrogen intake (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.17786041	2.34	0.1519
Rabbit (square)	6	0.18447045	2.43	0.0899
Period (square)	6	0.11892133	1.57	0.2392
Diet	3	0.10394747	1.37	0.2993
Livewt	1	0.05820065	0.77	0.3985
Error	12	0.07594363		
R-Square	0.722122			

Appendix 7.13 Analysis of Variance for the effect of square, rabbit, period and diet on faecal nitrogen output (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.00050380	0.02	0.8910
Rabbit (square)	6	0.02746534	1.07	0.4319
Period (square)	6	0.05450762	2.12	0.1261
Diet	3	0.02656070	1.03	0.4125
Livewt	1	0.00013347	0.01	0.9437
Error	12	0.02569477		
R-Square	0.649814			

Appendix 7.14 Analysis of Variance for the effect of square, rabbit, period and diet on Urine nitrogen output (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.00343126	0.26	0.6225
Rabbit (square)	6	0.02129822	1.58	0.2339
Period (square)	6	0.01306026	0.97	0.4838
Diet	3	0.00275926	0.21	0.8907
Livewt	1	0.00623169	0.46	0.5088
Error	12	0.01343914		
R-Square	0.581510			

Appendix 7.15 Analysis of Variance for the effect of square, rabbit, period and diet on nitrogen retention (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.11608499	1.03	0.3219
Rabbit (square)	6	0.32277839	2.88	0.0563
Period (square)	6	0.04155052	0.37	0.8840
Diet	3	0.12326139	1.10	0.3874
Livewt	1	0.11005279	0.98	0.3415
Error	12	0.11217310		
R-Square	0.673913			

Appendix 7.16 Analysis of Variance for the effect of square, rabbit, period and diet on nitrogen digestibility (g/rabbit/day).

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.7956796	0.01	0.9146
Rabbit (square)	6	97.0172959	1.46	0.2742
Period (square)	6	69.6457139	1.05	0.4414
Diet	3	46.8284474	0.71	0.5665
Livewt	1	10.0114596	0.15	0.7044
Error	12	66.301398		
R-Square	0.591339			

Appendix 7.17 Analysis of Variance for the effect of square, rabbit, period and diet on nitrogen retention in percentage of total intake.

Source	DF	Mean Square	F Value	Pr > F
Square	1	0.312645	0.00	0.9584
Rabbit (square)	6	167.302205	1.51	0.2541
Period (square)	6	14.758989	0.13	0.9892
Diet	3	85.839842	0.78	0.5290
Livewt	1	57.469553	0.52	0.4845
Error	12	110.448251		
R-Square	0.515055			

Appendix 7.18 Analysis of variance for the effect of total intake on N digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig
1	Regression	1	18.768	13.164	.068
	Residual	2	1.426		
	Total	3			

Appendix 7.19 Analysis of variance for the effect of total intake on N retention (g/rabbit/day)

Model		df	Mean Square	F	Sig
1	Regression	1	9.635	6.186	.131
	Residual	2	1.558		
	Total	3			

Appendix 7.20 Analysis of variance for the effect of total intake on nitrogen retention in percentage of total N intake

Model		df	Mean Square	F	Sig.
1	Regression	1	47.438	269.797	.004
	Residual	2	.176		
	Total	3			

Appendix 7.21 Analysis of variance for the effect of total intake on faecal nitrogen output (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	3.530	1.091	.406
	Residual	2	3.235		
	Total	3			

Appendix 7.22 Analysis of variance for the effect of PEG on DM intake (g/rabbit/day

Model		df	Mean Square	F	Sig.
1	Regression	1	11.363	.310	.634
	Residual	2	36.668		
	Total	3			

Appendix 7.23 Analysis of variance for the effect of PEG on N intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	3.172	.011	.927
	Residual	2	2.984		
	Total	3			

Appendix 7.24 Analysis of variance for the effect of PEG on water intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	469.565	.557	.533
	Residual	2	842.511		
	Total	3			

Appendix 7.25 Analysis of variance for the effect of PEG on DM digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	27.134	1.031	.417
	Residual	2	26.307		
	Total	3			

Appendix 7.26 Analysis of variance for the effect of PEG on N digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	18.008	9.971	.087
	Residual	2	1.806		
	Total	3			

Appendix 7.27 Analysis of variance for the effect of PEG on N retention (g/rabbi/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	5.658	1.596	.334
	Residual	2	3.546		
	Total	3			

Appendix 7.28 Analysis of variance for the effect of PEG on N retention (% of total N intake)

Model		df	Mean Square	F	Sig.
1	Regression	1	39.584	9.648	.090
	Residual	2	4.103		
	Total	3			

Appendix 7.29 Analysis of variance for the effect of PEG on feacal N output (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	5.074	2.060	.288
	Residual	2	2.463		
	Total	3			

Appendix 7.30 Analysis of variance for the effect of PPFP on DM intake (g/rabit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	35.013	1.409	.357
	Residual	2	24.843		
	Total	3			

Appendix 7.31 Analysis of variance for the effect of PPFP on N intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	1.085	.442	.575
	Residual	2	2.457		
	Total	3			

Appendix 7.32 Analysis of variance for the effect of PPFP on water intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	33.026	.031	.876
	Residual	2	1060.781		
	Total	3			

Appendix 7.33 Analysis of variance for the effect of PPFP on DM digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	26.969	1.022	.418
	Residual	2	26.389		
	Total	3			

Appendix 7.34 Analysis of variance for the effect of PPFP on N digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	18.678	12.699	.071
	Residual	2	1.471		
	Total	3			

Appendix 7.35 Analysis of variance for the effect of PPFP on N retention (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	9.172	5.127	.152
	Residual	2	1.789		
	Total	3			

Appendix 7.36 Analysis of variance for the effect of PPFP on N retention (% of total N intake)

Model		df	Mean Square	F	Sig.
1	Regression	1	45.190	34.762	.028
	Residual	2	1.300		
	Total	3			

Appendix 7.37 Analysis of variance for the effect of PPFP on faecal N output (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	3.723	1.186	.390
	Residual	2	3.138		
	Total	3			

Appendix 7.38 Analysis of variance for the effect of EXCT on DM intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	1.465	.035	.868
	Residual	2	41.618		
	Total	3			

Appendix 7.39 Analysis of variance for the effect of EXCT on N intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	6.191	.230	.679
	Residual	2	2.690		
	Total	3			

Appendix 7.40 Analysis of variance for the effect of EXCT on water intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	992.255	1.707	.321
	Residual	2	581.166		
	Total	3			

Appendix 7.41 Analysis of variance for the effect of EXCT on DM digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	3.462	.091	.792

		df	Mean Square	F	Sig.
	Residual	2	38.143		
	Total	3			

Appendix 7.42 Analysis of variance for the effect of EXCT on N digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	.370	.035	.869
	Residual	2	10.625		
	Total	3			

Appendix 7.43 Analysis of variance for the effect of EXCT on N retention (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	7.057	.117	.765
	Residual	2	6.022		
	Total	3			

Appendix 7.44 Analysis of variance for the effect of EXCT on N retention (% of total N intake)

Model		df	Mean Square	F	Sig.
1	Regression	1	2.493	.110	.772
	Residual	2	22.648		
	Total	3			

Appendix 7.45 Analysis of variance for the effect of EXCT on faecal N output (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	9.378	.000	.990
	Residual	2	5.000		
	Total	3			

Appendix 7.46 Analysis of variance for the effect of RD on DM intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	27.797	.977	.427
	Residual	2	28.451		
	Total	3			

Appendix 7.47 Analysis of variance for the effect of RD on N intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	2.972	.104	.777
	Residual	2	2.851		
	Total	3			

Appendix 7.48 Analysis of variance for the effect of RD on water intake (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	53.336	.051	.843
	Residual	2	1050.626		
	Total	3			

Appendix 7.49 Analysis of variance for the effect of RD on DM digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	26.006	.968	.429
	Residual	2	26.871		
	Total	3			

Appendix 7.50 Analysis of variance for the effect of RD on N digestibility (g/rabbit/day)

Model		df	Mean Square	F	Sig.
1	Regression	1	19.801	21.775	.043
	Residual	2	.909		
	Total	3			

Appendix 7.51 Analysis of variance for the effect of RD on N retention (% of total N intake)

Model		df	Mean Square	F	Sig.
1	Regression	1	47.364	222.615	.004
	Residual	2	.213		
	Total	3			