

***In vitro* assessment of selected ethno-medicinal plants as potential alternatives for the control of gastrointestinal nematodes in sheep and goats**

By

Mhlongo Lindokuhle Christopher

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School of Agricultural, Earth and Environmental Science

College of Agriculture, Engineering and Science

University of KwaZulu-Natal



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Supervisor: Professor Ignatius Verla Nsahlai

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Declaration

I, **Lindokuhle Christopher Mhlongo** declare that this dissertation is my own original work conducted under the supervision of Professor Ignatius Verla Nsahlai. This dissertation has not previously been submitted for any purposes to any other institution. All the information obtained from other authors have been cited and acknowledged accordingly.

Mhlongo, L.C.(Author)..... Date.....

Prof. Nsahlai, I.V.(Supervisor)..... Date.....

List of Abbreviations

Control <i>n</i>	Number of gastrointestinal nematodes in controls (0% v/v)
CPE	Crude plant extract
CV	Coefficient of variance
g	Grams
GIN	Gastrointestinal nematodes
GLM	Generalised linear model
IC ₅₀	Half maximal inhibitory concentration
LSM	Least Square Mean
L3	Stage 3 infective larvae of gastrointestinal nematodes
mg	Milligram
ml	Millilitre
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay
NRF	National Research Foundation
P	Probability value
SAS	Statistical Analysis Systems
SE	Standard error
Treated <i>n</i>	Number of gastrointestinal nematodes in treated faecal samples
UKZN	University of KwaZulu-Natal
V/V	Volume per Volume
W.A.A.V.P	World Association for Advancement of Veterinary Parasitology
WFPM	Webb's fractional product method

General abstract

Commercial anthelmintics are becoming ineffective against gastrointestinal nematodes (GIN) of ruminants due to development of resistant parasites. Research is exploiting anthelmintic ethno-medicinal plants for an alternative remedy. This study assessed the *in vitro*: (1) dose activity at different concentrations, (2) combined synergistic activity of ethanolic crude plant extracts on mixed GIN of sheep and goats; and (3) cytotoxic activity of these extracts on kidney vero cells. During assessment of *in vitro* dose activity, faecal samples of sheep and goats that were grazing on contaminated pasture were collected, cultured (12 days) to L₃ larvae stage, and treated with 40, 20, 10, 5, 2.5, 1.25 and 0.25% v/v of *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Crinum macowanii*, *Gunnera perpensa*, *Nicotiana tabacum*, *Ricinus communis*, *Sarcosterma viminalis*, *Trema orientalis*, *Urtica dioica*, *Vernonia amygdalina*, *Zanthoxylum capense*, *Zingiber officinale*, *Zizyphus mucronata* and *Aloe vanbalenii* extracts. Larvae were subjected to Baermann technique for isolation and later observed under a microscope (10x objective). During the assessment of synergism at 1.25% v/v concentration (1:1), 28 crude plant extract combinations from eight (8) mostly edible plants namely: *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Vernonia amygdalina*, *Zingiber officinale*, *Aloe vanbalenii* and *Nicotiana tabacum* (inedible) were tested for their synergistic activity. The simple and Webb's fractional product method were used to compute interactions of crude plant extract combinations. During assessment of cytotoxic activity MTT assay was used to assess effect of 16 individual plant extracts mentioned above on vero kidney cells.

Results revealed that goats had a significantly higher efficacy than sheep at 40% ($P=0.0253$) and 20% ($P=0.038$) concentration (v/v); but goats had significantly lower efficacy at concentration (v/v) 1.25% ($P= 0.0305$) and 0.625% ($P= 0.0158$) relative to sheep. On the other hand, both goats and sheep had insignificant ($P>0.05$) efficacy for CPEs concentration (v/v) 10%, 5% and 2.5%. Plant species had no effect on efficacy at concentration (v/v) 40%, 20%, 10%, 5%, 2.5%, but had significant effect at lowest concentration (v/v) of 1.25 % ($P=0.0085\%$) and 0.625 ($P=0.0234\%$) which was not dose-dependent. Few plants had high activities at the lowest tested concentration (0.625% v/v). In goats it was *Gunnera perpensa* (89.47%±12.40), while in sheep *Gunnera perpensa* (100%±12.40), *Urtica dioica* (95.24%±12.40), *Zizyphus mucronata* (90.47%±12.40), *Allium cepa* (90.47%±12.40), *Aloe vanbalenii*

(85.71%±12.40) and *Bidens pilosa* (80.95%±12.40). Interactions following Webb's fractional product method were antagonistic and synergistic, whereas those following simple method yielded synergistic interactions only. In goats, *V. amygdalina* + *Z. officinale* (100%) was the most efficacious, while in sheep, *A. cepa* + *C. papaya* (100%), *V. amygdalina* + *Z. officinale* (100%), *V. amygdalina* + *Z. officinale* (100%) and *A. comosus* + *N. tabacum* (100%) were most efficacious. Animal species had a significant effect ($P<0.001$) on efficacy of combinations, efficacy was lower in goats (89.16%±0.95) relative to sheep (95.45%±0.095). Plant species did not affect ($P>0.05$) the efficacy of crude plant extract combinations. *Vernonia amygdalina* ($IC_{50} = 0.01$ mg/ml) followed by *Zingiber officinale* ($IC_{50} = 0.02$ mg/ml) were the most cytotoxic crude extracts, while *Allium cepa* ($IC_{50} = 0.27$) and *Aloe vanbalenii* ($IC_{50} = 0.22$ mg/ml) were the least cytotoxic crude extracts. Cytotoxicity increased in a dose dependent manner. The concentration-cell viability relationship was negative linear in most crude plant extracts. While it was negative quadratic for *Gunnera perpensa*, *Zingiber officinale* and *Vernonia amygdalina*. Anthelmintic crude plant extracts are efficacious against GIN of sheep and goats. Although they are mostly harmless minimum effective concentration should be used. Crude plant extracts that were efficacious at the lowest concentration and observed synergistic crude plant extract combinations should be tested *in vivo*.

Keywords: Anthelmintics, Animal species, Activity, Cytotoxic, Crude plant extract(s), Concentration, Ethno-medicinal, Gastrointestinal nematodes, Goats, *In vitro*, Plant species, Resistant, Sheep

Thesis output

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Dedication

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Chapter 1

1.1 Background

Ruminant production provides manure and alternative food when there is crop failure, to ensure food security mainly for communal farmers (Kosgey *et al.*, 2008). Farmers mainly keep sheep and goats for cash (Mapiye *et al.*, 2009). Gastrointestinal nematodes (GIN) are some of the major constraints to ruminant production as they suck blood within the gastrointestinal tract of the host animal (Mapiye *et al.*, 2009). This depresses milk production, meat quality, health and fertility (Ahmed, 2010). Common GIN affecting ruminants are *Haemonchus contortus*, *Nematodirus spp.*, *Trichostongylus spp.*, *Cooperia spp.*, *Oesophagostomum spp.* and *Trichuris spp.*

GIN infestations are mainly controlled by commercial anthelmintics namely: Benzimidazoles, imidazothiazoles, praziquantel, benzimidazoles, levamisole, ivermectin, doramectin and moxidectin (Shalaby, 2013). These anthelmintics have lost efficacy due to the development of resistant GIN. Furthermore, these anthelmintics are expensive for communal farmers, none biodegradable, meat products contaminants and difficult to use by communal farmers due to their educational background (Houghton *et al.*, 2007).

Communal farmers use ethno-medicinal plants to control GIN (Kunene *et al.*, 2003; Maphosa & Masika, 2010; Sanhokwe *et al.*, 2016). Some anthelmintic plants that are used are namely: *Crinium macowani*, *Discostachys cineria*, *Erythrina caffra*, *Ficus ingens*, *Ginidia kraussiana*, *Gunnera perpensa*, *Kigelia africana*, *Laporttea perduncularis*, *Ricinus communis*, *Vernonia neocorymbosa* and *Ziziphus mucronata* (Kunene *et al.*, 2003). Ethnoveterinary medicine is defined as an act of controlling diseases in animals without the use of conventional knowledge (Maphosa & Masika, 2010; Luseba & Tshisikhawe, 2013). Identification of plants that are used in this practice necessitates assessment of *in vitro* dose, synergistic and cytotoxic activity of these plants before they could be used in ruminants. Such studies could lead to identification of effective safe doses of ethno-medicinal plants against resistant GIN. In addition, ethno-medicinal plants unlike commercial anthelmintics are environmentally friendly as they are a natural control and affordable (Maphosa & Masika, 2010). These plants have been used for generations effectively to control GIN. Unlike commercial anthelmintics, these plants as anthelmintics do not lose efficacy or leave residues in meat products (Maphosa & Masika, 2010; Sanhokwe, 2015; Sanhokwe *et al.*, 2016).

1.2 Problem statement

Small ruminant production is being limited by GIN (Akingbade *et al.*, 2001). Commercial anthelmintics that are used to control GIN are losing efficacy due to resistant parasites development (Shalaby, 2013). Due to this, there is a search for an alternative in ethno-medicinal plants, but these plants cannot be readily used in animal. Since, *in vitro* dose, synergistic and cytotoxic activity of these plants is limited. Hence, these plants should be evaluated against mixed parasites of sheep and goats for *in vitro* cytotoxicity, individual and synergistic activity, using a better solvent to identify safe and effective crude plant extracts.

1.3 Justification

Unlike commercial anthelmintics, the potential alternative must not taint meat products and pollute environment (Sanhokwe, 2015). Ethno-medicinal plants used in ethnoveterinary medicine have a potential of producing natural ingredients for commercial anthelmintics. Before these plants could be accepted for use in animals they should be tested *in vitro*. Few plant species used in ethnoveterinary medicine have ever been validated for their *in vitro* cytotoxic, individual and synergistic activity. Different solvents also produce crude plant extracts of different efficacies. Water extracts produce less efficacies relative to ethanolic extracts (Ahmed *et al.*, 2013). Mixture of water with ethanol can be used for extraction of an efficacious crude extract. Aqueous-ethanol (70% ethanol) is a better solvent for extraction of polar and non-polar phytochemicals (Bimakr *et al.*, 2011).

In vitro dose activity of these plants extracts needs to be evaluated solely on GIN of both sheep and goats. Foraging habit of ruminants is classified as grazers and browsers (Gordon, 2003). Sheep are grazers while goats are browsers (Yisehak, *et al.*, 2016). Unlike the former the latter grazes closer to the ground (Duval, J., 1994). Hence, they are infested with GIN of different resistances (Papadopoulos, 2008; Fomum & Nsahlai, 2017a, b). Therefore, it is essential to study effect of crude plant extracts on sheep and goats, as they are representing ruminants of different foraging habits. This could help identify crude plant extracts that are effective against GIN which are foraging habit specific (Grazers and browsers). This study can also potentially identify plants that could treat linearly the GIN of both grazers and browsers.

Further assessment of synergism from a combination of different crude plant extracts against sheep and goats' parasites is vital. Some ethno-medicinal plants are ineffective on their own

unless they are mixed with other ethno-medicinal plants (Ferreira, 2017). Hence, some ethno-medicinal plants activate other anthelmintic ethno-medicinal plants causing synergism to develop (Chartier *et al.*, 2001). Synergism is when a combination of anthelmintics produces a higher activity compared to the sole activities of anthelmintics involved in the combination (Williams *et al.*, 2012). Therefore, it is essential to identify plants that produce synergism when combined.

The study of synergism in these plants could also discover crude plant extracts which deter development of resistant GIN because crude plant extracts are composed of different phytochemicals. Hence, parasites cannot develop resistance towards all phytochemicals in a combination at once (Hoste *et al.*, 2009; Chou, 2010). Different ethno-medicinal plants control different nematode species and stages (Hounzangbe-Adote *et al.*, 2005). A combination of crude plant extracts can increase chances of developing an anthelmintic that can control different species of nematodes with one concoction, since ruminants are affected by mixed nematode species and stages. Crude plant extracts also use different modes of action to control GIN (Egualé *et al.*, 2007). This increases the efficacy of anthelmintics when different plants are combined. Hence, necessitates use of a minimum dose for maximum activity. Since toxicity increases with dose increase (Chou, 2006, 2010), study of synergism in these plants might discover a safe minimum effective dose.

Previous research has mostly focused on effect of plant extracts on GIN and rarely on the effect of plant extracts on the host animals (Fomum & Nsahlai, 2017b). There is an assumption that ethno-medicinal plants are nontoxic which is not substantiated (Street *et al.*, 2008). It has been proven that some plants have poisonous defensive phytochemicals against herbivores (Street *et al.*, 2008). Some plants are linked to damage of heart, gastrointestinal tract and kidneys (Kudumela *et al.*, 2018). Determining crude plant extract(s) cytotoxicity effects is important for the discovery of effective ethno-medicinal plants that can be safe enough to be used on animals.

1.4. Objectives

1.4.1 To determine *in vitro* activity of crude plant extracts against mixed GIN of sheep and goats at different concentration levels.

1.4.2. To determine *in vitro* synergistic activity of crude plant extract combinations from mainly edible plants on mixed GIN of sheep and goats.

1.4.3. To determine *in vitro* cytotoxic activity of anthelmintic crude plant extracts on kidney vero cells.

1.5. Hypotheses

1.5.1. Crude plant extracts control mixed GIN of sheep and goats in a dose dependent manner.

1.5.2. Combination of crude plant extracts have a synergistic activity against mixed GIN of sheep and goats.

1.5.3. Crude plant extracts are not cytotoxic on kidney vero cells.

Chapter 2

Literature Review

2.1 Introduction

Small ruminant production is important for food security to resource-poor farmers as they are sold for cash, serve as source of food, generate much needed income for medical needs, contribute to off-farm investments and generates income for the purchase of additional stock (Kosgey *et al.*, 2008). Gastrointestinal nematodes (GIN) infestation is a common constraint of small ruminant production. Small stock farmers are the ones that are affected the most by this constraint. Unlike commercial farmers, most small stock owners do not have access to commercial anthelmintics; and do not have sufficient information to help manage this challenge. Even when they have access to these anthelmintics, they cannot administer them correctly due to inadequate knowledge, resulting in wrong dosing (Houghton *et al.*, 2007). These farmers are constrained to using ethnoveterinary medicinal plants exerting anthelmintic activities to control GIN in their stock. These plants are locally available to them. Ethnoveterinary medicine is a practice of controlling diseases in animals using indigenous knowledge (Maphosa & Masika, 2010).

Worldwide, commercial farmers use commercial anthelmintics, some of which include benzimidazoles, imidazothiazoles, praziquantel, levamisole, ivermectin, doramectin and moxidectin to control GIN in ruminants. However, with widespread development of drug resistance, these anthelmintics are becoming less effective. Resistance is defined by lack of susceptibility to anthelmintics by GIN causing lack of activity (Shalaby, 2013). This has left the animal production industry with a need to look for potential alternative anthelmintics. Unlike commercial anthelmintics, potential anthelmintic alternatives should be biodegradable, have no contaminants for meat and highly effective.

Consequently, research is exploring plants with potential anthelmintic activities used by resource poor farmers. These plants have not been sufficiently evaluated *in vitro* and *in vivo* for their toxicity and residual effects in hosts animal(s). Hence, traditional practice needs to be improved so that active natural chemicals can be identified. It is therefore essential to understand common GIN that affect sheep and how resistance develops. The objective of this review is to evaluate ethno-medicinal plants with anthelmintic properties. This review

discusses how parasitized animals are identified, identification of ethno-medicinal plants, collection time, preparation method, plant parts used, dosage, activity improvements and limitations.

2.2 Gastrointestinal nematodes of ruminants

GIN cause loss of productivity, loss of appetite, low body condition score, and loss of profit in most farming communities. This results to food insecurity in the communal areas, where they depend on small ruminants in times of crop failure because of drought or inclement weather conditions. Resistant GIN are more common in small ruminants (Table 2.1).

Table 2. 1 Different gastrointestinal nematodes which affect ruminants

Oesophagus and Omasum	Abomasum	Small intestines	Large intestines	Reference
<i>Cotylophoron spp.</i> , <i>Gongylonema pulchrum</i> , and <i>Paramphistomum spp</i>	<i>Haemonchus contortus</i> , <i>Teladorsagia circumcincta</i> , <i>Teladorsagia trifurcata</i> , <i>Parabonema spp.</i> , and <i>Trichostrongylus axei</i>	<i>Avitellina centripunctata</i> , <i>Bunostomum trigonocephalum</i> , <i>Cooperia curticei</i> , <i>Cooperia surnabada</i> , <i>Gaigeria pachyscelis</i> , <i>Moniezia expansa</i> , <i>Nematodirus battus</i> , <i>Nematodirus filicollis</i> , <i>Nematodirus spathiger</i> , <i>Strongyloids papillosus</i> , <i>Trichostrongylus capricola</i> and <i>Trichostrongylus vitirinus</i>	<i>Chabertia ovina</i> , <i>Oesophagostomum Columbianum</i> , <i>Oesophagostomum Venulosum</i> , <i>Skjabinema Ovis</i> , <i>Trichuris ovis</i> and <i>Trichuris skrjabini</i>	Roeber <i>et al.</i> (2013)

Most common helminths that affect ruminants belong to nemathelminths phylum, and include *Trichostrongyloidea*, *Strongyloidea*, *Metastrongyloidea*, *Ancylostomatoidea*, *Rhaditoidea*, *Trichuroidea*, *Filarioidea*, *Oxyliroidea*, *Ascaridoidea* and *Spiruoidea* (Sissay, 2007). Predisposing factors of ruminants to GIN include low immunity, contaminated pastures, highly humid and wet areas, genetic make-up, overstocking of pastures, and resistance to drugs (Maria, 2006).

Ruminants are born without infestation but get infested through grazing contaminated pasture with infective larvae (L₃) (Roeber *et al.*, 2013). Infective larvae migrate to the specific part of the gastrointestinal tract where it grows through a pre-adult larvae (L₄) to a developed adult form (L₅). Adult nematodes (male and female) live in the target site of host (Table 2.1). Adult

female nematodes lay 5000-10000 eggs/day which are passed onto faeces to contaminate pastures (Roeber *et al.*, 2013). Under favourable conditions such as warm and moist environment, eggs hatch into larvae (L₁). Thereafter, L₁ larvae moult into L₂ and L₃ stages and accumulate in the pasture. GIN mainly feed on erythrocytes of host causing compromised productivity (Tables 2.1 and 2.2), and anaemia which sometimes leads to death (Maria, 2006).

2.3 Anthelmintic resistance by gastrointestinal nematodes

Resistance poses a huge threat to the economic returns of ruminant farming. This is because almost all major broad spectrum commercial anthelmintics are now ineffective against GIN (Kaplan, 2004). GIN are usually controlled by different broad spectrum commercial anthelmintics. These drugs include benzimidazoles, imidazothiazoles, praziquantel, levamisole, ivermectin, doramectin and moxidectin (Shalaby, 2013). Resistance occurs when animals exposed to GIN show a decreased response towards an anthelmintic drug. Similarly, resistance results when a certain population of GIN possess a gene associated with resistance (Prichard, 2007). This can be due to genetic disorders such as mutation, deletion or amplification. Furthermore, a result of epigenetics through methylation promoter regions or promoter regions reduces GIN susceptibility to anthelmintic (Shalaby, 2013). Full drug resistance is confirmed when maximum dosage shows less efficacy (Coles, 2006).

Table 2. 2 Characteristics of different gastrointestinal nematodes

Nematode (Scientific name/Common name)	Morphology	Life cycle	Signs	Reference
<i>Haemonchus contortus</i> (Barber-pole)	Males have shorter length than females (10-20 vs 18-30 mm). White uteri and ovaries have a barber-pole look.	Direct (intermediate host absent).	Acute anaemia, intense blood loss, bottle jaw, stool, pale gums and inner eyelids.	Roeber <i>et al.</i> (2013)
<i>Nematodirus spp</i> (Thread necked strongyle)	10-30 mm length, thin exterior and swollen head.	Direct. 15-28 days.	Inappetence, Stool, loss of weight and wool.	
<i>Trichostongylus spp.</i> (Bankrupt worm/stomach hair worm)	No filament	Direct. Prepatent period of 20- 25 days.	Weight loss, reduced growth rate, wart like inflammations, stool and inappetence	
<i>Cooperia spp.</i> (Small intestine worm)	Brownish-red, 4-6 mm long.	Direct. Prepatent period of 15-20 days	Inappetence, stool and weight loss.	
<i>Oesophagostomum spp.</i> (<i>Nodular Worm</i>)	20 mm long, thin front.	Direct. 6-7 days.	Stool, swelling large intestinal wall, mucus covered faeces,	
<i>Trichuris spp.</i> Whipworm)	Thin neck, thick hind end, males (5080 mm long) with a curved tail. Females are 35-70 mm long.	Prepatent period of 1-3 months.	Caecal wall swelling and stool.	

Resistance manifests in two ways; decreased efficacy and delayed effectiveness of the anthelmintic. Host animals infested with drug resistant GIN, need frequent dosing compared to hosts without drug resistant counterparts. As a result, this can increase drug residues in meat products. Persistent drug resistance in ruminants is a major challenge. Hence, anthelmintic plants used in ethnoveterinary medicine are a potential alternative. This is because ethno-medicinal plants have been used for years in controlling GIN with less reports on inefficacy. Belina *et al.* (2017) reported that 79 % Ethiopian communal farmers noticed no resistance while 21 % noticed drug resistance in their anthelmintic medicinal plants. The lack of resistance in anthelmintic plants might be due to vast diversity in chemical composition as compared to chemical anthelmintics (Hammond *et al.*, 1997).

2.4 Reasons why farmers prefer ethno-medicinal plants

Different plants are used by communal farmers to combat GIN burden in ruminants. Ethnoveterinary medicine is orally passed on from one generation to the next. Therefore, this might influence acceptance by communal farmers. Eighty percent of Africans depend on ethnoveterinary medicine to control and treat diseases in ruminants (Luseba & Tshisikhawe, 2013). Different tribes use different ethnoveterinary medicines to treat diseases. Thus, there are a lot of anthelmintic plants available as alternatives when others become ineffective due to resistance. Communal farmers preference of ethnoveterinary medicine over anthelmintic drugs might be because they cannot afford commercial anthelmintics (Belina *et al.*, 2017), uncertain advantage over anthelmintic plants, lack of side effects, high efficacy, easy accessibility and usage, and lack of veterinarians in communal areas (Sanhokwe *et al.*, 2016).

A majority of communal farmers depend on animal products including milk and meat but are ignorant of drug residues in these products. Anthelmintic residues in meat and related products are a huge challenge. Thus, anthelmintic remedies used to treat GIN are passed onto consumers, and can be potentially harmful (Hammond *et al.*, 1997; Radhakrishnan *et al.*, 2010; Cooper *et al.*, 2012). Synthetic drugs leave residues in hair, skin and subcutaneous adipose (Lespine *et al.*, 2005).

Belina *et al.* (2017) reported that 77% of communal farmers in Ethiopia had no knowledge about commercial anthelmintics withdrawal period compared to 23% who did. Hence, this suggests that, unlike commercial anthelmintics, ethnoveterinary practices might be beneficial to communal farmers as it does not contaminate meat products. This is because most of the medicinal plants used to treat GIN including *C. papaya*, *A. vanbalenii*, *A. comosus*, *A. sativum* and *A. cepa* are edible (Fomum & Nsahlai, 2017). Luseba & Van der Merwe (2006) reported that communal farmers prefer ethnoveterinary medicine to treat GIN in ruminants because they do not taint the meat products. Other factors that might have influenced preference of ethnoveterinary medicine by communal farmers are that these remedies are effective against them, and they think that they are superior to synthetic drugs. Additionally, it may be because they do not pollute the environment and are biologically degradable.

2.5 Diagnosis of gastrointestinal nematode infestation

Diagnosis of GIN in ruminants by communal farmers is sometimes carried out using supernatural methods, some of which include consulting spirits and divination (Ngeh *et al.*, 2007). Senses of taste, touch, smell and sight have also been used (Ngeh *et al.*, 2007; Maroyi, 2012). Common signs for monitoring nematode infestation are loss of body condition, loss of appetite and rubbing against poles (Djoueche *et al.*, 2011). Nevertheless, there are limitations that come with common signs, since helminthiasis can be confused with other diseases which have similar signs such as fluke (Luseba & Van der Merwe, 2006) and coccidiosis (Maroyi, 2012). Therefore, using common signs such as body condition score can be limiting because low body weight is not a distinct sign of GIN infestation (Kenyon *et al.*, 2009). Hence, there is little correlation between body condition score and faecal egg count in terms of accurately detecting GIN infection in ruminants (Molento *et al.*, 2011).

Adoption of these diagnostic symptoms can affect the efficacy of anthelmintic, dosage, and validity in the anthelmintic ability of the plant (Sanhokwe *et al.*, 2016). Distinct signs can be used to make accurate diagnosis because communal farmers cannot afford accurate modern methods such as McMaster Technique. One of such distinct signs of gastrointestinal parasitism is bottle jaw. This condition is caused by depletion of blood protein when GIN suck blood in the host (Coleman, 2012).

Clinical signs are not enough to diagnose GIN. Hence, more reliable techniques have been developed to detect these parasites with accuracy. One of such methods for accurate diagnosis of GIN burden in ruminants is the FAMACHA chart (Molento *et al.*, 2011). This method identifies animals suffering from anaemia, which is a common GIN infestation symptom by checking the eye colour (Zajac, 2016). It compares eye colour of the membrane with that on the chart showing 5 levels of anaemia. Level 1 signifies the absence of anaemia, while level 5 represents highly anaemic condition (Zajac, 2016). Anaemia is a sign of severe GIN infestation by *Haemonchus contortus*. The disadvantage of this method is that anaemia may be due to non-parasitic infection (Molento *et al.*, 2011).

Presently, faecal egg count is the commonly used method (Molento *et al.*, 2011). This method uses a microscope to evaluate GIN in faeces and is very accurate for detecting parasites within the host (Molento *et al.*, 2011). Animals with higher nematode egg shed or count have the

highest GIN burden. The main disadvantage of using faecal egg count method is that communal farmers cannot adopt it without the use of laboratory, which can be challenging for these farmers (Molento *et al.*, 2011). Also, this method does not identify types of GIN affecting the herd. For instance, *Trichostrongylus colubriformis*, *Cooperia spp.* and *Bunmtomum trigonocephalum* highly affect sheep, on the other hand *Oesophagostomum columbianum* and *Haemonchus contortus* affect goats (McCulloch & Kasimbala, 1968).

There are two types of faecal egg count tests, one of which is qualitative and the other is quantitative. Qualitative test is a floatation of contaminated faecal samples under a microscope to examine GIN (Molento *et al.*, 2011). The results are reported as positive or negative as proof of infection progress over time (Molento *et al.*, 2011). Quantitative test uses eggs per gram of known weight of sample of faeces, a McMaster slide and floatation solution. Two chambers of the slide are filled with faecal solution multiplied by dilution factor and the type of nematode eggs are identified under the microscope (Molento *et al.*, 2011). Quantitative evaluation technique is easier, inexpensive and reusable compared to qualitative faecal egg count method (Molento *et al.*, 2011).

2.6 Modes of action for anthelmintic ethno-medicinal plants

2.6.1 Phytochemicals and digestive enzymes

Different plants, used in ethnoveterinary medicine to control GIN, contain different anthelmintic phytochemicals and enzymes (Table 2.3). It is not fully known how all phytochemicals of different plants used by communal farmers control GIN except for a few like those mentioned in Table 2.4. Plants like Papaya and Fig trees have latex which contains a lot of proteolytic enzymes while Pineapples have cysteine proteinases. These enzymes digest nematodes. *Ficus spp.* have also been reported to have ficin (Steppek *et al.*, 2004). *Saba senegalensis* has compounds such as tannins, saponins, triter, pene glycoside and steroid. These compounds attach on to free proteins within tubes for larval nutrition thus kill the GIN (Wabo *et al.*, 2011). While commercial anthelmintics contain one molecule acting on parasite(s). Anthelmintic plants possess numerous active molecules which act together in synergy against gastrointestinal parasites. This increases efficiency and reduce development of resistant GIN (Fouche *et al.*, 2016). Aloe has amino acids such as sterols and pherols which negatively affect protein and body repair of nematodes (Fouche *et al.*, 2016). While ginger anthelmintic activity is due to gingerols, shogaols, zingerone and paradol (Ghayur & Gilani, 2005). These

phytochemicals activate cholinergic receptors. This causes contraction of gastrointestinal tract. Thus, parasites are expelled (Iqbal *et al.*, 2006).

Table 2. 3 Different anthelmintic phytochemicals found in plant extracts and their effect on parasites

Anthelmintic phytochemicals	Mode of action	Reference
Saponins	Targets the permeability of the cuticle of the parasites.	Bauri <i>et al.</i> (2015)
Benyl isocyanate	Paralyses the motor activity and metabolism of the parasite.	
Cysteine proteinases	Contains proteolytic chymopapain and Papain, which are responsible for the breakdown of the parasites' cuticle.	
Isoflavones	Affects the glycolysis and glycogenolysis activity enzymes and calcium ions of the parasite.	
Artemisinin	Causes the cleavage of endoperoxide bridges by iron producing free radicals. This Stresses the biological molecules of the parasite through oxidation.	
Phenolic compounds	Uncouple the oxidative phosphorylation mechanism and disturbs the glycoprotein of the cell surface, resulting in death of the parasite.	
Tannins	Uncouple the oxidative phosphorylation, attach to free glycoproteins of the gastrointestinal wall and attach to the glycoproteins of the parasites causing death to the parasite.	
Alkaloids	Paralyse the central nervous system, steroidal alkaloids and oligoglycosides which suppress sucrose from travelling from stomach to the small intestines; alkaloids act as an antioxidant, thus inhibiting homeostasis condition excellent for parasites development.	

2.6.2. Neurotransmitter control

Active anthelmintic plant extract is the one with ability to inhibit acetylcholinesterase of GIN (Korayem *et al.*, 1993; Lee, 1996). Acetylcholinesterase is a serine hydrolase that is responsible for the catalysis of a neurotransmitter called acetylcholine into acetate and choline. This results in the formation of a substrate-enzyme complex. This is followed by acetylation of the hydroxyl

group of the amino acid serine, which is present in the esteratic site that is finally deacetylated. Its inhibition leads to paralysis and death of the nematode (Begum *et al.*, 2010).

Korayem *et al.* (1993) reported that *Helicotylenchus dihystera* treated with *Punica granatum*, *Thymus vulgaris* and *Artemisia absinthium* extracts were able to suppress acetylcholine of nematodes. It was then concluded that the efficacy of these extracts shows a relation between nematode poisoning and the inhibition of acetylcholine. This suggests that the observed efficacy of the used plant extracts is partly due to inhibition of acetylcholine activity.

2.6.3. Entry route

Anthelmintic drugs control GIN via oral ingestion or by trans-cuticular diffusion. It is argued that the latter route is the most common way of entry for anthelmintic drugs in nematodes (Egualé *et al.*, 2007). Hence, effective extract type against GIN must have phytochemicals that can penetrate the cuticle of nematodes (Egualé *et al.*, 2007). Lipophilic anthelmintics exert their effect through trans-cuticular diffusion easily compared to hydrophobic ones (Geary *et al.*, 1999). This suggests that the extract type that is more effective might be containing more lipophilic than hydrophilic chemicals.

2.6.4. Trace minerals content

Supplementing with trace minerals (Selenium, zinc, copper, and iron) increases immunity. This alleviates the infestation especially during crucial physiological stages (Ferreira, 2017). There is a linear relationship between white blood cells and trace minerals (Ferreira, 2017). Supplementation with trace minerals can improve plant extracts efficacy against GIN (Coleman, 2012). Singh *et al.* (2016) reported that efficacious anthelmintic extracts showed high content of zinc, copper and protein in addition to flavonoids and tannins. Supplementation with copper kills GIN and decreases egg count (Chartier *et al.*, 2001). Hence, nutritious plants with high trace minerals and anthelmintic phytochemicals content are potential alternative pastures. Such type of pastures can eliminate the need for laborious vaccination and harvesting of anthelmintic plants.

2.7 Common plants that serve as ethno-medicinal anthelmintics

Table 2.4 shows that there is a wide range of plant families that are used for anthelmintic ethno-medicinal medicine. Githiori (2004); Githiori *et al.* (2006); Chinsebu *et al.* (2014) reported

that communal farmers predominantly use plants of *Fabaceae* family. While Sanhokwe *et al.* (2016) reported that *Asphodelaceae* was the most frequently used plant family by communal farmers in Kwezi and Ntambethemba villages in Eastern Cape province. Maroyi (2012) also noted that respondents in Nhema village, Zimbabwe, frequently used plants of the families *Fabaceae*, *Solanaceae* and *Asphodelaceae*.

Use of different families of plant species in various regions seems to be influenced by plant population distribution and their multiple biological activities (Maroyi, 2012). This is exemplified by plant species including *Clerodendrum glarum* that is used in treating helminths, diarrhoea, bile and cough, while *Gnidia kraussiana* is used in treating bile and cough in addition to its anthelmintic activity. *Laportea peduncularis* on the other hand is used to treat diarrhoea and cough; *Salvadora australis* to treat foams in cattle and *Ziziphus mucronata* also treats diarrhoea (Kunene *et al.*, 2003), beside their anthelmintic activities. Similarly, *Zingiber officinale* is used in ethnoveterinary medicine to treat arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, indigestion, nausea, hypertension, dementia, fever and GIN (Nadkarni, 1976). Plant species family that can treat human diseases in addition to GIN of livestock seem to be prioritised.

Table 2. 4 Plants species that are used in South Africa by communal farmers to control gastrointestinal nematodes in ruminants.

Plant family	Scientific name (common name)	Parts used/ Preparation	Reference
Apocynaceae	<i>Acokanthera oppositifolia</i> (Bushman's poison)	Leaves, Boiling	Maphosa & Masika (2010)
Apocynaceae	<i>Dischrostachys cinerea</i> (Sickle bush)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Apocynaceae	<i>Salvadora austral</i> (Mustard tree)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Agapanthaceae	<i>Agapanthus praecox</i> (African lily)	Leaves, Infusion	Maphosa & Masika (2010)
Amaryllidaceae	<i>Crinum macowanii</i> (Cape coast lily)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Anacardiaceae	<i>Harpephyllum caffrum</i> (Wild plum)	Bark, Boiling	Maphosa & Masika (2010)
Apiaceae	<i>Centella coriacea</i> (Swamp Pennywort)	Bark, Boiling	Maphosa & Masika (2010)
Araliaceae	<i>Cussonia spicata</i> (Natal cabbage tree)	Bark, Infusion	Maphosa & Masika (2010); Sanhokwe <i>et al.</i> (2016)
Asphodelaceae	<i>Aloe ferox</i> (Bitter aloe)	Leaves, Boiling	Maphosa & Masika (2010); Sanhokwe <i>et al.</i> (2016)
Asphodelaceae	<i>Gasteria bicolor</i> (Elephant's Foot)	Leaves, Infusion	Maphosa & Masika (2010)
Asphodelaceae	<i>Bulbine latifolia</i> (Broad leaved bulbine)	Leaves, Boiling	Maphosa & Masika (2010)
Asphodelaceae	<i>Bulbine frutescens</i> (Cat's tail)	Whole plant, Infusion	Maphosa & Masika (2010)
Asphodelaceae	<i>Bulbine abyssinica</i> (Snake flower)	Leaves, Boiling	Maphosa & Masika (2010)
Asphodelaceae	<i>Aloe arborescens</i> (Bitter aloe)	Leaves, Boiling	Maphosa & Masika (2010)
Asteraceae	<i>Vernonia neocorymbosa</i> (Vernonia)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Bignoniaceae	<i>Kigelia africana</i> (Sausage tree)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Capparidaceae	<i>Capparis sepiaria</i> (Caper bush)	Roots, Infusion	Maphosa & Masika (2010)
Euphorbiaceae	<i>Ricinus communis</i> (Castor bean)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Fabaceae	<i>Elephantorrhiza elephantina</i> (Elephant's root)	Roots, Boiling	Maphosa & Masika (2010); Sanhokwe <i>et al.</i> (2016)
Fabaceae	<i>Schotia latifolia</i> (Bush Boer bean)	Bark, Boiling	Maphosa & Masika (2010)
Fabaceae	<i>Erythrina caffra</i> (Coral tree)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Geraniaceae	<i>Pelargonium reniforme</i> (Pelargonium)	Tuber, Boiling	Maphosa & Masika (2010)
Gunneraceae	<i>Gunnera perpensa</i> (River pumpkin)	Tuber, Boiling	Maphosa & Masika (2010)
Hyacinthaceae	<i>Albuca setosa</i> (Soldier in the box)	Tuber, Boiling	Sanhokwe <i>et al.</i> (2016)
Hypoxidaceae	<i>Hypoxis argentea</i> (Yellow stars)	Tuber, Boiling	Maphosa & Masika (2010)
Lamiaceae	<i>Teucrium trifidum</i> (Dutchmen's fever plant)	Leaves, Infusion	Maphosa & Masika (2010)
Lamiaceae	<i>Leonotis leonurus</i> (Wild dagga)	Leaves, Boiling	Maphosa & Masika (2010)
Lamiaceae	<i>Ocotea bullata</i> (Black stinkwood)	Bark, Boiling	Maphosa & Masika (2010)
Loganiaceae	<i>Strychnos hemingsii</i> (Red bitter berry)	Bark, Boiling	Maphosa & Masika (2010)
Moraceae	<i>Ficus ingens</i> (Fig tree)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Pittosporaceae	<i>Pittosporum viridiflorum</i> (Cheese wood)	Bark, Infusion	Maphosa & Masika (2010)
Polygonaceae	<i>Rumex lanceolatus</i> (Common dock)	Roots, Boiling	Maphosa & Masika (2010)
Ptaeroxylaceae	<i>Ptaeroxylon obliquum</i> (Sneeze wood)	Leaves Boiling	Maphosa & Masika (2010)
Rhamnaceae	<i>Ziziphus mucronata</i> (Buffalo Thorn)	Leaves, Infusion	Maphosa & Masika (2010)
Rutaceae	<i>Zanthoxylum capense</i> (Small knob wood)	Roots, Boiling	Maphosa & Masika (2010)
Sterculiaceae	<i>Hermannia incana</i> (Sweet yellow bells)	Whole plant, Boiling	Maphosa & Masika (2010)
Thymelaeaceae	<i>Gnidia kraussiana</i> (Yellow heads)	Leaves, Boiling	Kunene <i>et al.</i> (2003)
Tiliaceae	<i>Grewia occidentalis</i> (Cross berry)	Bark, Boiling	Maphosa & Masika (2010)
Urticaceae	<i>Laportea peduncularis</i> (River nettle)	Leaves, Boiling	Kunene <i>et al.</i> (2003)

2.8 Harvest time, preparation methods and solvents used to prepare ethno-medicinal plants

Seeds can be collected during the ripening season of fruits. Roots can be collected in any season as they store nutrients of the plant. Barks should be collected when the sap is running. Fruits should be collected during early ripe season. Leaves are collected before flowering season, as plants use metabolites for flowering. Leaves are usually collected in summer (Tanzin *et al.*, 2010; Ngeh *et al.*, 2007) since there is supply of effective medicinal plants (Luseba & Van der Merwe, 2006), as phytochemicals peak at this time.

Harvest time might also be carefully chosen to prevent coincidence with peak infestation (Vercruysse, 1983). So that plant parts (stem, root, leaves, fruits and barks) that are used to make ethnoveterinary medicine cannot be contaminated with GIN. Furthermore, the time of collection may be influenced by peak of anthelmintic phytochemicals during this time. For example, Tannin which is the common phytochemical used to control GIN increases in winter and decreases in summer months (Max *et al.*, 2003).

Ethnoveterinary medicinal plants with anthelmintic activities can be prepared through boiling and infusion. Table 2.4 shows that boiling (aqueous solution) is the most commonly used method of preparation of anthelmintic plant species or their different parts by communal farmers (Kunene *et al.* 2003; Maphosa & Masika, 2010; Djoueche *et al.*, 2011; Sanhokwe *et al.*, 2016;). Boiling is suggested to either deactivate toxic thermolabile components of the plants that can be poisonous to the infested animal (Sanhokwe *et al.*, 2016) or negatively by deactivating some of the active anthelmintic phytochemicals that are thermolabile (Sanhokwe *et al.*, 2016). Water may dilute the concentration of plant extracts and render crude extracts less poisonous (Maphosa & Masika, 2010). Choice of aqueous extracts by communal farmers might be because water is easily accessible. This method of preparation is also easy to master because it only requires water to boil for a certain time.

Solvent used seems to influence the efficacy of anthelmintic plants extracts. Aqueous extracts, which is common extract type, have lower efficacy compared to ethanolic and methanolic extracts (Tariq *et al.*, 2008; Fouche *et al.*, 2016). This might be due to aqueous extract characteristics such as low anthelmintic activity, biological activity and type of phytochemicals (Bizimenyera *et al.*, 2006; Worku *et al.*, 2009). Differences between aqueous and other types of extracts might be attributed to different proportion of phytochemicals activated by solvent(s)

resulting in different effect on nematodes (Egualé *et al.*, 2011). Therefore, water might be activating lesser phytochemicals compared to other solvents such as ethanol, acetone, chloroform and methanol.

Khan *et al.* (2016) reported aqueous extracts of *Iris kashimiriana* in sheep showed superior *in vitro* efficacy against *Haemonchus contortus* compared to methanolic extracts (100 vs 85 %, respectively). In the same study using the *in vivo* method, the aqueous extract remained superior compared to methanolic extract (70.2 vs. 33.2 %, respectively). The superiority of aqueous extracts was explained to be due to high concentration of water-soluble active molecules within the extracts. Aqueous anthelmintic plant extracts have low shelf life because water allows microbial growth (Tiwari *et al.*, 2011). There is a need of a solvent that can extract polar and non-polar anthelmintic chemicals. This is because the plant cell contains water soluble and ethanol soluble bioactive chemicals. Therefore, mixture of water and other solvents can be used for extraction. For instance, aqueous ethanol (70%) is a better solvent than pure ethanol because the proportion of bioactive chemicals polar and none-polar can be extracted to increase efficacy (Bimakr *et al.*, 2011). Other types of solvents can be used for extraction, acetone which extracts both hydrophilic and lipophilic phytochemicals in plants is another example. It is useful especially when phenolic plants need to be extracted (Bimakr *et al.*, 2011). Also, ether can be used and is better suited as a solvent to extract fatty acids and coumarins compounds of the plant exercising anthelmintic activity (Bimakr *et al.*, 2011). Similarly, chloroform is also better at extracting terpenoids and lactones.

2.9 Commonly used plant parts as anthelmintics

Commonly used plant parts to control GIN are leaves of anthelmintic plants (Maroyi, 2012; Sanhokwe *et al.*, 2016), this can also be observed in Table 2.4. This might be because leaves are infective larvae free since they are at the top of trees and dry compared to other parts such as roots and barks which can be close to contaminated grass. Chinsemu *et al.* (2014) reported that communal farmers prefer using leaves because harvesting them is easier compared to collecting other plant parts. Sanhokwe *et al.* (2016) reported that one of the reasons communal farmers prefer leaves is because they want to conserve the plants to avoid extinction as opposed to using roots or stem (Maphosa & Masika, 2010). Consequently, picking leaves for ethnoveterinary medicine can kill the plant especially if the leaves picked are younger ones instead of old ones since leaves are biologically important for survival of plants (Gakuubi &

Wanzala, 2012). On the other hand, some communal farmers use the whole plant to prepare medicine because they believe that using individual plant parts weakens the efficacy of the medicine (Rodriguez-Fragoso *et al.*, 2008). Plant parts that have higher shelf life are highly preferred such as barks, bulbs, fruits and seeds (Cunningham, 1993), shrubs, tubers and whole plant (Maroyi, 2012).

Djoueche *et al.* (2011) reported that 58.3% farmers in Benoue region of Cameroon were commonly using stem and bark, perhaps the trees used are in abundance. This suggests that different plant parts have different levels of activities in combatting GIN in ruminants. These commonly used plant part(s) might be chosen based on relative efficacy to other plant parts. For instance, pineapple has more anthelmintic phytochemical(s) (Bromelain) in the stem compared to other parts (Stanger, 2013), and will most likely exert greater efficacy if used as source of extract.

Thus, this suggests that plant parts with higher proportion of phytochemicals than others should be isolated and used, to control GIN in small ruminants. This is because leaves are part of browse that goats feed on. Hence, GIN of goats might be adapted to phytochemicals within the browse. Goats also seem to acquire weak immune system towards GIN. Nematodes in goats develop resistance quicker compared to sheep (Worku *et al.*, 2009). Anthelmintic plants which are effective in goats are expected to be more effective in sheep.

2.10 Dosages of ethno-medicinal plant extracts

Ethnoveterinary medicine used to control GIN is measured using spoons, calabash, bottles, clay pots, hand palms and finger pinches (Ngeh *et al.*, 2007). Therefore, there is no exact amount of plants material per volume of water. Sometimes qualitative measures determine concentration such as colour change once the plant material is soaked in water (Ngeh *et al.*, 2007). As a result, most ethnoveterinary medicines may be toxic compared to modern anthelmintics (Hammond *et al.*, 1997). Ethnoveterinary medicine needs to be standardised for effective concentration. This can prevent under dosing and over dosing (Luseba & Van der Merwe, 2006). Hence, standardisation of concentration can limit death of ruminants from toxicity and residues in meat products.

2.11 Improvement of anthelmintic plant extract activity

Different anthelmintic plants are mixed by communal farmers with one another for synergistic purposes, or with other non-plant substances to increase the efficacy of treatments. These non-plant substances used include flour (laxative effect), butter (increase flavour), rock salt (emulsification), oil cake (labile secretion) and Epson salt (Sanhokwe *et al.*, 2016). Therefore, to prevent inconsistent anthelmintic activities studies need to determine whether plant extracts work best individually or in combination (McGaw & Eloff, 2010). Since, some plant combinations are synergistic when the dose ratio is different between plants involved in the combination. Klongsiriwet *et al.* (2015) found that synergistic effect tends to happen at lower concentration of tannins types in flavonoids and condensed tannins combination.

Javed & Akhtar (1990) reported that combination of *Vernonia anthelmintica* and *Embelia ribes* showed 83-93% efficacy in controlling the GIN. Synergism defines a condition where two or more agents are combined to result in an effect that is greater than that of a single agent (Klongsiriwet *et al.*, 2015). Synergistic effect is calculated by monitoring additive individual effects from treatments (Bliss, 1939). They are then compared to effects from combination of treatments with assumption that they have independent effects. The additive effect is compared to the combination effect of treatment. If additive effect is less than the combined effect, then there is synergism, while if it is more than combined effect then there is antagonism (Williams *et al.*, 2012). Synergism is advantageous because that is where a plant combination which is effective in both sheep and goats can be identified, since these plants produce different activities in these ruminants (Fomum & Nsahlai, 2017).

There is also an advantage of discovering combination of plants with phytochemicals that can combat resistance. Since, nematodes cannot be resistant to both plant extracts used in the combination quickly. Different plants extracts are nematode and parasite stage specific (Hoste *et al.*, 2009). There is also a possibility of targeting a nematode stage with a wrong plant extract. Hounzangbe-Adote *et al.* (2005) noted that *Fagara* extracts specifically affected eggs and adult GIN. While *Morinda lucida* extracts mainly affected eggs and larval stage. Combining different plant extracts can be advantageous in this instance since different plant target different nematode stages. Hence, further studies on combination of plant extracts needs to be performed. For identification of plant extract combination with ability to destroy all stages of nematodes. Since, the gut of small ruminants there are GIN of all stages.

2.12 Activity validation of ethno-medicinal plants

Out of 250 000 plant species in the world, only 4-5% have been studied for bioactive chemicals (McGaw & Eloff, 2010). Therefore, most anthelmintic plants still need to be discovered and studied for their anthelmintic activities (McGaw & Eloff, 2010). This is because these plants have different phytochemical compositions which produce different anthelmintic activities (McGaw & Eloff, 2010). Bioassays used for isolating plants with anthelmintic activities should be simple, accurate, affordable. This is vital for easy identification of small concentrations of effective and ineffective compounds (McGaw & Eloff, 2010). The *in vitro* method is the most used bioassay to isolate anthelmintic plants because it is ethical, less laborious, and cheap (McGaw & Eloff, 2010). This study is a laboratory imitation of the biological conditions without using an animal. *In vivo* studies involve feeding a parasitized host animal certain amount of anthelmintic plants (Githiori *et al.*, 2006). *In vivo* studies produce more accurate results than *in vitro* studies but due to animal welfare rules in many countries it has limited use (McGaw & Eloff, 2010).

In a previous study 60 plants evaluated using an *in vitro* method had only half of them influencing nematode load in parasite hosts (Bizimana, 1994). Similarly, Wattle plant extracts showed a significant *in vitro* effect on nematodes burden but a non-significant *in vivo* effect on GIN (Max *et al.*, 2003). This might be attributed to change of anthelmintic properties of the plant by the gut microorganisms in the gastrointestinal tract of the host (Ferreira *et al.*, 2013).

To counter *in vivo* inactivation of plant extracts communal farmers concentrate extracts. This might increase compounds prone to inactivation by microorganisms in the gastrointestinal tract (Houghton *et al.*, 2007). Thus, there is a need of identifying plant extracts with phytochemicals that are resistant to digestion by microflora of the gut. Hence, concentrating plant extracts can potentially lead to poisoning of ruminants. However, the disadvantage of *in vivo* bioassay is that it uses few control animals or none. Hence, this inhibits the analysis of data statistically, it is expensive and labour intensive. In addition, another limitation with the *in vivo* method is that a lot of animal welfare organisations are against the use of animals. *In vivo* methods have a lot of indirect and direct factors that influence affect results such as nutrition, age, season, and so forth. However, it is the most useful method of validating anthelmintic plant species.

2.13 Limitations of anthelmintic plants as alternatives to conventional products

Usage of plants as alternative control of GIN in ruminants comes with a couple of limitations. These plants contain compounds with unknown direct and indirect mechanism of action on parasites. This limits adoption of effective plants as alternatives (Hördegen *et al.*, 2003). There is also no accurate scientific acceptable method of preparing these plants (McGaw & Eloff, 2010). This because it is difficult to prepare ethno-medicine as communal farmers do (McGaw & Eloff, 2010). Hence, using bioassays such as *in vitro* and *in vivo* might exaggerate efficacy of ethno-medicinal plants (McGaw & Eloff, 2010). Thus, adoption of an incorrect dose is possible. As a result, this might increase toxicity of these plants. Toxicity increases with efficacy due to dose dependency. Hence, an efficacious dose might be too toxic to be used in animals (McGaw & Eloff, 2010) to obtain desired efficacy.

Other factors such as ease of plant cultivation, harvesting, supply and mode of administration can limit use of plant extracts. Palatability, stability, biodegradation of anthelmintic compounds within the plants, and lack of accurate dosage which can also lead to poisoning of animals (Waller *et al.*, 2001). Some plants such as *Lotus spp.* and *H. coronarium* are weak, cannot tolerate grazing and stamping by ruminants and die easily (Waller *et al.*, 2001). Furthermore, communal farmers tend to give a single collective name to a group of plants based on their resemblance or characteristics. Plants producing latex are collectively called *Mithuri* by Kenyan communal farmers regardless of family or medical purpose (Gakuubi & Wanzala, 2012). This makes identification of anthelmintic plants difficult.

Traditional healers are very secretive about ethnoveterinary medicine. This limits the identification of most anthelmintic plants (Hammond *et al.*, 1997). Communal farmers in Nhema, midlands of Zimbabwe explained that the reasons for secrecy about ethnoveterinary medicine knowledge is jealousy (Maroyi, 2012). Secrecy might also be because this knowledge is passed down orally strictly through family lineages (Gakuubi & Wanzala, 2012). Furthermore, herbalists use plants of different efficacies to make ethnoveterinary medicine. Customers tend to prefer the herbalist with most effective ethnoveterinary medicine. Another possible explanation for secrecy might be competition between herbalists to attract more customers. This limits discovery of a plants with effective compounds because full information is not given (McGaw & Eloff, 2010).

2.14 Summary

Ethno-medicinal plants are a potential source of ingredients to develop sustainable commercial anthelmintics. However, their activity is anecdotal as it is not standardised for scientific use. Therefore, to produce safe, effective and none contaminating anthelmintics, these plants need to be further evaluated scientifically following *in vitro* assay. Then, they can be used to treat animals. Studies should focus on their toxicity, dose response, chemical composition, mode of action and synergism to produce a reputable source of anthelmintics.

***In vitro* treatment of gastrointestinal nematodes of sheep and goats with different anthelmintic crude plant extracts at different concentrations**

Abstract

Currently used commercial anthelmintics are less sustainable due to development of resistant gastrointestinal nematodes (GIN). Research is taking advantage of ethno-medicinal plants with anthelmintic activities to produce a solution. This study assessed the *in vitro* activity of selected ethanolic crude plant extracts (CPEs) on mixed GIN of sheep and goats. Faecal samples of goats and sheep were collected and incubated (12 days) to culture L₃ stage larvae. They were then treated with 16 ethanolic CPEs of *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Crinum macowanii*, *Gunnera perpensa*, *Nicotiana tabacum*, *Ricinus communis*, *Sarcosterma viminale*, *Trema orientalis*, *Urtica dioica*, *Vernonia amygdalina*, *Zanthozylum capense*, *Zingiber officinale*, *Zizyphus mucronata* and *Aloe vanbalenii* (40, 20, 10, 5, 2.5, 1.25 and 0.625%, v/v). Isolation of L₃ larvae was done using Baermann technique. Objective of 10x was used to count GIN under a microscope. Goats had a significantly higher efficacy than sheep at 40% ($P=0.0253$) and 20% ($P=0.038$) concentration (v/v); but a significantly lower efficacy at concentration (v/v) 1.25% ($P=0.0305$) and 0.625% ($P=0.0158$) relative to sheep. On the other hand, both goats and sheep had insignificant ($P>0.05$) efficacies for CPEs concentration (v/v) 10%, 5% and 2.5%. Plant species had no effect on efficacy at concentration (v/v) 40, 20, 10, 5 and 2.5%, but had significant effect at lowest concentration (v/v) of 1.25% ($P=0.0085$) and 0.625% ($P=0.0234$) which was not dose-dependent. Few plants had high activity at the lowest tested concentration of 0.625% v/v. In goats it was *Gunnera perpensa* (89.47%±12.40), while in sheep it was *Gunnera perpensa* (100%±12.40), *Urtica dioica* (95.24%±12.40), *Zizyphus mucronata* (90.47%±12.40) *Allium cepa* (90.47%±12.40), *Aloe vanbalenii* (85.71%±12.40) and *Bidens pilosa* (80.95%±12.40). CPEs that were efficacious at the lowest concentration need to be tested *in vivo*.

Keywords: Animal species, Ethno-medicinal plants, Faecal samples, L₃ stage larvae, Plant species

3.1 Introduction

Ruminant production is important for milk, meat, leather, emergencies and manure that can be used to generate income for communal farmers (Devendra, 2005). However, GIN namely: *Haemonchus contortus* (Barber-pole), *Nematodirus spp.*, *Trichostongylus spp.*, *Cooperia spp.*, *Oesophagostomum spp.* (Nodular Worm) and *Trichuris spp.* (Whip worms) are major limiting constraints in ruminant production (Zajac, 2006). They cause loss in productivity, carcass quality, and blood protein (Zajac, 2006). Therefore, food security of different farmers who are dependent on ruminant production is at risk because of this challenge. Commercial anthelmintics (Benzimidazoles, imidazothiazoles, praziquantel, levamisole, ivermectin, doramectin and moxidectin) are getting ineffective due to resistant GIN; they also contaminate

meat products, pollute environment and are unaffordable for communal farmers (Maphosa & Masika, 2010; Shalaby, 2013). No effective alternative anthelmintic has been found without these mentioned disadvantages in commercial anthelmintics.

To sustain small ruminant production, research is exploiting ethno-medicinal plants with anthelmintic activity to produce potential alternatives. As bio-control remedies, they are naturally degradable, easily available and effective against GIN (Sanhokwe *et al.*, 2016). Ethno-medicinal plants with anthelmintic activities have little scientific acceptance (Maphosa & Masika, 2010; Luseba & Tshisikhawe, 2013; Sanhokwe *et al.*, 2016). Ethno-medicinal plants have no known minimum safe dosages or limits. To increase acceptance there is need to assess their unknown *in vitro* activities before recommending them for ruminants. To isolate effective doses of these plants that could be tested further for *in vivo* activities. Above all, to improve activities of ethno-medicinal plant, they need to be extracted with better solvent such ethanol and acetone (Bimakr *et al.*, 2011). Currently, common ethno-medicinal plants are extracted with water, which produces less effective CPE(s) relative to the above-mentioned solvents (Maphosa & Masika, 2010).

In vitro dose activity of these plants extracts needs to be evaluated solely on GIN of both sheep and goats. Foraging habit of ruminants is classified as grazers and browsers (Gordon, 2003). Sheep are grazers while goats are browsers (Yisehak, *et al.*, 2016). Unlike the former the latter grazes closer to the ground (Duval, J., 1994). Hence, they are infested with GIN of different resistances (Papadopoulos, 2008; Fomum & Nsahlai, 2017a, b). Therefore, it is essential to study effect of crude plant extracts on sheep and goats, as they are representing ruminants of different foraging habits. This could help identify crude plant extracts that are effective against GIN which are foraging habit specific (Grazers and browsers). This study can also potentially identify plants that could treat linearly the GIN of both grazers and browsers.

Sheep and goats are also more susceptible to GIN infestation than cattle. Cattle develop higher immunity against GIN as they grow, GIN of cattle are lesser in population compared to that of small ruminants and have higher water concentration which reduces survival of eggs than small ruminants (FDA's Centre for Veterinary Medicine Antiparasitic, n.d.). CPEs that are effective against resistant GIN of sheep and goats are expected to be more effective against less resistant GIN of cattle.

Ethno-medicinal plants have been previously reported to produce different activities in sheep and goats, which could be due to dosing goats with sheep doses and higher preference of

anthelmintic pasture by goats relative to sheep (Papadopoulos, 2008; Jackson *et al.*, 2012; Fomum & Nsahlai, 2017b). Notably, it is vital to test activities of CPEs on goats and sheep. The objective of the current study was to assess individual *in vitro* activities of different plants in sheep and goats. It was hypothesized that CPEs would control GIN of sheep and goats in a dose dependent manner.

3.2 Materials and Methods

This study was conducted with the approval of the University of KwaZulu-Natal Ethics Committee, the Animal Ethics Sub-Committee (ref. AREC/058/018M).

3.2.1 Collection of anthelmintic plants

Sixteen anthelmintic plant species namely: *Allium cepa* (Onion), *Ananas comosus* (Pineapple), *Bidens pilosa* (Black jack), *Carica papaya* (Paw paw), *Crinum macowanii* (Cape coast lily), *Gunnera perpensa* (River pumpkin), *Nicotiana tabacum* (Tobacco), *Ricinus communis* (Castor bean), *Sarcosterma viminale* (Caustic bush), *Trema orientalis* (Charcoal tree), *Urtica dioica* (Common nettle), *Vernonia amygdalina* (Bitter leaf), *Zanthoxylum capense* (Small knob wood), *Zingiber officinale* (Ginger), *Zizyphus mucronata* (Buffalo horn), *Aloe vanbalenii* (Aloe) commonly used in ethnoveterinary medicine in South Africa were collected. They were sourced from private gardens, University of KwaZulu-Natal (UKZN) Botanical gardens, and National botanical garden, Pietermaritzburg. Voucher samples were deposited at the UKZN Herbarium, Pietermaritzburg.

3.2.2 Extraction and dilution of CPEs to different concentrations

Fresh plant materials were washed, cut and oven dried (Oven mark; LABCON, Model 5SOEIB, Maraisburg 1700) at 50-60°C to constant weight. Oven dried plant samples were milled through a 1 mm sieve using an electric centrifuge mill (RETSCH, GmbH and Co.KG, 5657 HAANI, West Germany). Milled plant samples were stored in air tight labelled plastic containers and kept in a cool dry place at room temperature. For every plant species, 10 g was weighed into thimbles, fitted into distillation columns and extracted with 100ml of ethanol (70%) using Soxhlet's apparatus over a heating unit (RETSCH, GmbH and Co.KG, 5657 HAANI, West Germany). Completion of extraction was noted by absence of colour in the solvent within the thimble carrying section. For every plant species, the same procedure was followed. Extraction

was done 4 times for each plant species. CPEs were diluted to 40, 20, 10, 5, 2.5, 1.25 and 0.625% (v/v.) concentrations.

3.2.3 Collection of faecal samples and faecal culture

Faecal samples were collected from infested Nguni goats (4) and Merino sheep (5) which were selected regardless of sex, age and weight. Animals were grazing on contaminated pasture at Ukulinga research farm. Before the experiment started, infestation was confirmed by determining egg count using McMaster Technique. Faecal samples were collected from infested sheep and goats using labelled sealable bags. Faeces were thoroughly mixed and pooled for each species of goats and sheep.

Each CPEs was assigned labelled petri dishes, which contained 5g of faecal samples. This was done per animal species and the process was replicated three times. Twenty-one labelled petri dishes were assigned faecal samples (5g) as controls (0% v/v). These samples were incubated (MEMMERT, 854 Schwabach, West-Germany) for 12 days at 27°C. During incubation faecal samples were watered every day at 10:00 am to keep them moisturized.

3.2.4 Treatment, isolation and counting of larvae

Treatment of larvae from goats and sheep with 40, 20, 10, 5, 2.5, 1.25 and 0.625 %, v/v of 16 CPEs was done in this order: 5 ml of each concentration of 16 CPEs was dosed on 5 g of faecal culture except for controls (0% v/v). After treatment petri dishes were taken to the incubator maintained at 27°C and kept for 24 hours. A day after treatment, faecal cultures treated with CPEs were folded with a double cheese cloth. Cheese cloth containing faecal samples were put in a labelled funnel and filled with lukewarm water. The apparatus was left to stand for 24 hours; 15 ml liquid was drawn into blood test tubes and left to stand for 30 minutes. Larvae (L₃) were put in a McMaster slide and observed under 10x magnification. Data recorded on Excel (2013) were computed using the following formula described by Abbott (1925):

$$\text{Activity (\%)} = \left(1 - \left(\frac{\text{Treated } n}{\text{Control } n} \right) \right) \times 100$$

Where, treated *n* is the number of L₃ larvae found in the treated plates and control *n* is the number of L₃ larvae found in controls.

3.2.5 Statistical analysis

Data were statistically analysed using Generalised linear model (GLM) of SAS 9.4 (2013). The following model was used to statistically analyse the efficacy of treatments:

$$Y_{ijk} = \mu + S_i + C_j + X_k + e_{ijk}$$

Where, Y_{ijk} = Individual observation, μ = overall mean, S_i =Effect of plant species, C_j =Effect of animal species and X_k = Effect of concentration, and e_{ijk} = Error term.

3.3 Results

In Table 3.1, the *in vitro* activities of 16 CPEs tested under seven concentrations (40, 20, 10, 5, 2.5, 1.25 and 0.625% v/v) are shown. Plant species had no significant effect ($P>0.05$) on efficacy at concentration (v/v) 40, 20, 10, 5 and 2.5%, but had a significant effect at lowest concentration (v/v) of 1.25% ($P=0.0085$) and 0.625% ($P=0.0234$) which was not dose-dependent. The activities of CPEs ranged mainly from 80-100%. Few CPEs produced best activities even at lowest concentration tested (0.625 %, v/v). Hence, in goats *Gunnera perperna* (89.47%±12.40) was the most efficacious, while in sheep *Allium cepa* (90.47%±12.40), *Bidens pilosa* (80.95%±12.40), *Gunnera perperna* (100%±12.40), *Urtica dioica* (95.24%±12.40), *Zizyphus mucronata* (90.47%±12.40) and *Aloe vanbalenii* (85.71%±12.40) were the most efficacious CPEs.

Efficacy of CPEs was dose dependent in sheep and goats. Goats had a significantly higher efficacy than sheep at concentrations (v/v) of 40% ($P= 0.0253$), 20% and ($P= 0.038$); but there was a significantly lower activity in goats at concentration (v/v) 1.25% ($P= 0.0305$) and 0.625% ($P= 0.0158$) relative to sheep (Figure 3.1). Concentration of 3.8% v/v was the concentration which produced equal efficacies (82.05%) against parasites of sheep and goats.

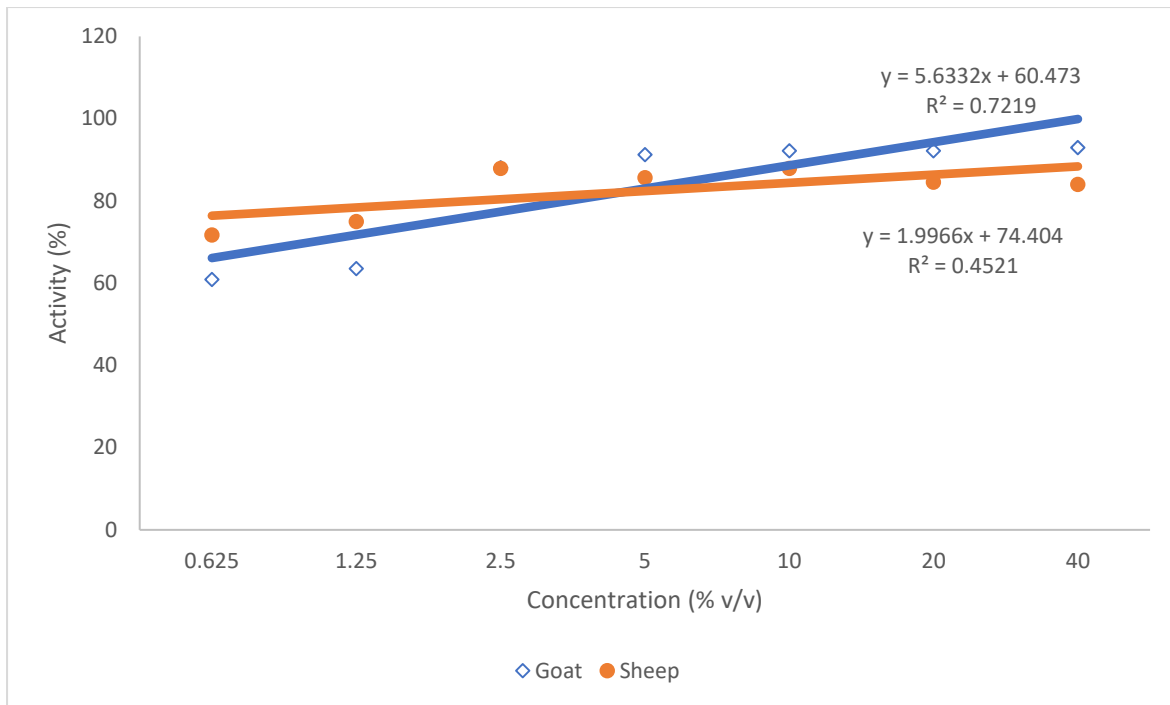


Figure 3. 1 Efficacy of ethanolic CPEs on Larvae of sheep and goats at different concentrations

Table 3. 1 *In vitro* activity (LSM±SE) of 16 ethanolic CPEs on larvae of sheep and goats at different concentrations

Plant	Animal	40% v/v	20% v/v	10% v/v	5% v/v	2.5% v/v	1.25% v/v	0.625% v/v
<i>Allium cepa</i>	Goat	88.3±11.03	96.1±10.21	96.1±9.10	96.1±9.8	88.3±9.59	100.0±14.64	63.2±12.40
<i>Ananas comosus</i>	Goat	96.1±11.03	92.2±10.21	88.3±9.10	92.2±9.8	88.3±9.59	50.0±14.64	73.7±12.40
<i>Bidens pilosa</i>	Goat	96.1±11.03	84.4±10.21	96.1±9.10	92.2±9.8	80.5±9.59	50.0±14.64	73.7±12.40
<i>Carica Papaya</i>	Goat	92.2±11.03	96.1±10.21	88.3±9.10	76.6±9.8	88.3±9.59	83.3±14.64	73.7±12.40
<i>Crinum macowanii</i>	Goat	96.1±11.03	96.1±10.21	88.3±9.10	92.2±9.8	76.6±9.59	66.7±14.64	52.6±12.40
<i>Gunnera perpensa</i>	Goat	96.1±11.03	88.3±10.21	96.1±9.10	84.4±9.8	96.1±9.59	83.3±14.64	89.5±12.40
<i>Nicotiana tabacum</i>	Goat	96.1±11.03	92.2±10.21	96.1±9.10	92.2±9.8	72.7±9.59	33.3±14.64	63.2±12.40
<i>Ricinus communis</i>	Goat	92.2±11.03	84.4±10.21	84.4±9.10	96.1±9.8	88.3±9.59	66.7±14.64	57.9±12.40
<i>Sarcosterma viminale</i>	Goat	96.1±11.03	80.5±10.21	96.1±9.10	96.1±9.8	96.1±9.59	66.7±14.64	42.1±12.40
<i>Trema orientalis</i>	Goat	96.1±11.03	96.1±10.21	88.3±9.10	88.3±9.8	96.1±9.59	100.0±14.64	47.4±12.40
<i>Urtica dioica</i>	Goat	92.2±11.03	92.2±10.21	88.3±9.10	96.1±9.8	88.3±9.59	66.7±14.64	78.1±12.40
<i>Vernonia amygdalina</i>	Goat	80.5±11.03	96.1±10.21	88.3±9.10	92.2±9.8	96.1±9.59	66.7±14.64	52.6±12.40
<i>Zanthozylum capense</i>	Goat	92.2±11.03	92.2±10.21	96.1±9.10	96.1±9.8	96.1±9.59	50.0±14.64	78.1±12.40
<i>Zingiber officinale</i>	Goat	84.4±11.03	96.1±10.21	96.1±9.10	88.3±9.8	88.3±9.59	66.7±14.64	52.6±12.40
<i>Zizyphus mucronata</i>	Goat	96.1±11.03	96.1±10.21	96.1±9.10	92.2±9.8	88.3±9.59	33.3±14.64	42.10±12.40
<i>Aloe vanbalenii</i>	Goat	96.1±11.03	96.1±10.21	96.1±9.10	88.3±9.8	80.5±9.59	100.0±14.64	31.6±12.40
<i>Allium cepa</i>	Sheep	82.3±11.03	91.2±10.21	82.4±9.10	91.2±9.8	60.0±9.59	50.0±14.64	90.5±12.40
<i>Ananas comosus</i>	Sheep	91.2±11.03	91.2±10.21	91.2±9.10	91.2±9.8	86.7±9.59	90.0±14.64	52.4±12.40
<i>Bidens pilosa</i>	Sheep	73.5±11.03	91.2±10.21	91.2±9.10	91.2±9.8	66.7±9.59	90.0±14.64	80.1±12.40
<i>Carica Papaya</i>	Sheep	91.2±11.03	82.4±10.21	91.2±9.10	82.4±9.8	100.0±9.59	50.0±14.64	61.9±12.40
<i>Crinum macowanii</i>	Sheep	91.2±11.03	82.4±10.21	91.2±9.10	91.2±9.8	93.3±9.59	60.0±14.64	52.4±12.40
<i>Gunnera perpensa</i>	Sheep	82.4±11.03	82.4±10.21	91.2±9.10	82.4±9.8	93.3±9.59	90.0±14.64	100.0±12.40
<i>Nicotiana tabacum</i>	Sheep	91.2±11.03	82.4±10.21	82.4±9.10	73.5±9.8	93.3±9.59	90.0±14.64	66.7±12.40
<i>Ricinus communis</i>	Sheep	73.5±11.03	64.7±10.21	82.4±9.10	91.2±9.8	86.7±9.59	100.±14.64	42.9±12.40
<i>Sarcosterma viminale</i>	Sheep	82.4±11.03	91.2±10.21	82.4±9.10	82.5±9.8	93.3±9.59	70.0±14.64	71.4±12.40
<i>Trema orientalis</i>	Sheep	73.5±11.03	91.2±10.21	91.2±9.10	82.4±9.8	86.7±9.59	60.0±14.64	71.4±12.40
<i>Urtica dioica</i>	Sheep	82.5±11.03	91.2±10.21	91.2±9.10	91.2±9.8	93.3±9.59	50.0±14.64	95.2±12.40
<i>Vernonia amygdalina</i>	Sheep	82.5±11.03	91.2±10.21	82.4±9.10	91.2±9.8	86.7±9.59	90.0±14.64	57.1±12.40
<i>Zanthozylum capense</i>	Sheep	82.5±11.03	82.4±10.21	91.2±9.10	82.4±9.8	93.3±9.59	70.0±14.64	66.7±12.40
<i>Zingiber officinale</i>	Sheep	82.4±11.03	82.4±10.21	91.2±9.10	91.2±9.8	86.7±9.59	100.0±14.64	61.9±12.40
<i>Zizyphus mucronata</i>	Sheep	91.2±11.03	91.2±10.21	82.4±9.10	82.4±9.8	100.0±9.59	50.0±14.64	90.5±12.40
<i>Aloe vanbalenii</i>	Sheep	91.2±11.03	64.7±10.21	91.2±9.10	73.5±9.8	86.7±9.59	90.00±14.64	85.7±12.40
CV %		21.59	20.08	17.52	19.2	18.89	36.63	32.39
RMSE		19.11	17.68	15.77	16.1	16.62	25.37	21.47

SE=Standard error, LSM; Least square mean

3.4 Discussion

CPEs tested in the current study produced mostly 80-100% activity regardless of concentration (Table 3.1). These CPEs could be recommended for potential anthelmintic solution. Efficacy of tested CPEs is according to World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) rule. This rule states that an effective CPE produces 90-100% and moderate CPE produces 80-90% activity (Coles *et al.*, 1992). Digestion of phytochemicals by gut microflora causes *in vivo* and *in vitro* results to be different (Hoste *et al.*, 2009). These CPEs are recommended for further *in vivo* study.

Cytotoxic profile of CPEs decreases with concentration reduction (Chou, 2006; Maphosa & Masika, 2010). Thus, CPEs which produced the best (80-100%) activity at 0.625 % (v/v) are mainly recommended for *in vivo* study (Table 3.1). *Gunnera perpensa* (89.47%±12.40) could be recommended for goat parasites and *Allium cepa* (90.47%±12.40), *Bidens pilosa* (80.95%±12.40), *Gunnera perpensa* (100%±12.40), *Urtica dioica* (95.24%±12.40), *Zizyphus mucronata* (100%±14.64), *Zizyphus mucronata* (90.47%±12.40) and *Aloe vanbalenii* (85.71%±12.40) could be recommended for sheep parasites. The above mentioned CPEs must be safest to host animals at this concentration.

It was observed that, at high concentration (2.5%-40%) all tested anthelmintic CPEs controlled GIN without dose dependency regardless of animal species (Table 3.1). Lack of dose-dependency has been reported in previous studies (Hounzangbe-Adote *et al.*, 2005). Storage conditions of CPEs used in the current study might have induced toxigenic fungi development. This type of fungi deactivates phytochemicals (Horie *et al.*, 1979). This might have prevented the even disperse of phytochemicals during dilution. CPEs used in the current study are of different plant families. Different plant families possess different phytochemical compositions (Hounzangbe-Adote *et al.*, 2005). The explanation for significant plant species effect at low concentration (1.25% and 0.625% v/v) in Table 5.1 could be attributed to different CPE phytochemicals content. Phytochemicals at these concentrations must have been significantly low and allowed different efficacies per CPE to be visible.

Ahmed (2017) reported that sheep and goats at Ukulinga research farm were infected with *Trichostrongylus spp.* (22-24.5%) > *Strongyloids spp.* (19-21%) > *Haemonchus contortus* (14.5-16%) > *Nematodirus spp.* (13-16%). Goats develop GIN infections of distinct species relative to sheep (Almalaik *et al.*, 2008). GIN species have different CPEs susceptibilities from

one another (Asadi Sardari *et al.*, 2015). This limits uniform treatment of different GIN species. During the current study nematode species must have fluctuated in sheep and goats. In Figure 3.1, beyond 2.5% (v/v) concentration, goats nematodes species must have had high nematodes species that are susceptible to CPEs than sheep. Also, below 2.5% (v/v) concentration, sheep nematode species must have been more susceptible to CPEs than that of goats. This could explain, the significant effect of animal species at 40, 20, 1.25 and 0.625% v/v, respectively. It is difficult to explain why treating with 10, 5, 2.5% v/v of extract failed to elicit an effect, except that 2.5% v/v of the extract was the cross-over point.

CPEs exert different efficacies in sheep and goats (Papadopoulos, 2008; Kumar *et al.*, 2013; Fomum & Nsahlai, 2017b, a). Goats and sheep tend to consume different anthelmintic plants (Villalba *et al.*, 2014). This renders different resistances in these animal species as goats are browsers while sheep are grazers. Observed significant effect of animal species at 40, 20% v/v might have also been due to treating with effective concentration in goats relative to sheep. These results (Figure 3.1) also suggest that mixture of these extracts would reveal if their effect is synergistic or antagonistic when these concentrations are within the range 1.25 - 0.625 % v/v where efficacies are below 75%. They also imply that to prevent wastage of CPEs it better to treat parasites of sheep and goats with 3.83% v/v concentration, for 82.05% efficacy which is efficacious for crude anthelmintics.

In addition, using single CPEs dosage should always be higher than 2.5% v/v of CPE to combat new ingested L₃ larvae in both sheep and goats. Comparatively, this might have been due to ethno-medicinal plants being comprised of portions of feeds normally given and largely preferred by goats. Hence, at low concentrations the proportion of phytochemicals which GIN are exposed to might have been higher in goats relative to sheep. At higher concentrations, these phytochemicals in CPEs might have high anthelmintic activity narrowing down differences between plants to negligible extent.

3.5 Conclusions

CPEs were highly efficacious against GIN of sheep and goats and could be considered as anthelmintic sources for small ruminants. Results showed that CPEs efficacy was high in all tested against nematodes of sheep and goats. Dose independency towards different concentrations tested was observed. Efficacy of CPEs differs between sheep and goats. Few CPEs were effective at the lowest concentration namely: *Gunnera perpensa* was the best treat

ment of goat nematodes while, while *Gunnera perpensa*, *Urtica dioica*, *Zizyphus mucronata*, *Allium cepa*, *Aloe vanbalenii* and *Bidens pilosa* were the best treatment for sheep nematodes. CPEs that were effective mainly at 0.625% v/v concentration can be recommended for *in vivo* test.

***In vitro* treatment of gastrointestinal nematodes of sheep and goats with crude plant extract combinations from edible anthelmintic plants**

Abstract

Current commercial anthelmintics that are used to control gastrointestinal nematodes (GIN) in small ruminants are less sustainable due to the development of resistant parasites. Combinations of ethno-medicinal plants possessing anthelmintic activity is a solution to deter resistance. This study evaluated the *in vitro* synergistic interaction of 28 combinations at 1.25% v/v (1:1) from eight (8) mainly edible ethanolic crude plant extracts (CPEs) (*Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Nicotiana tabacum*, *Vernonia amygdalina*, *Zingiber officinale* and *Aloe vanbalenii*) against mixed GIN of sheep and goats. Rectal faecal sample grabs containing helminth eggs from sheep (5) and goats (4) grazing contaminated pasture were cultured for 12 days. Samples were then treated with 28 combinations, followed by use of Baermann technique to isolate surviving L₃ larvae. Larvae were observed and counted under light microscope at 10x resolution to compute synergism from combinations. Webb's fractional product method (WFPM) and simple method (SM) for computing synergy were used to determine interactions. CPE combinations demonstrated high activity against GIN (80%-100%). Interactions following WFPM were antagonistic and synergistic, whereas those following SM yielded synergistic interactions only. In goats, a combination of *V. amygdalina* + *Z. officinale* (100%) was the most efficacious, while in sheep, combinations *A. cepa* + *C. papaya* (100%), *V. amygdalina* + *Z. officinale* (100%), *V. amygdalina* + *Z. officinale* (100%) and *A. comosus* + *N. tabacum* (100%) were most efficacious. Animal species had significant effects ($P < 0.001$) on efficacy of combinations: efficacy was lower in goats (89.16%±0.95) relative to sheep (95.45%±0.95). Plant species effect was not significant ($P > 0.05$). It is recommended that combination of CPEs be further studied *in vivo* to ascertain *in vitro* outcome in the present study.

Keywords: Alternative, Commercial Anthelmintics, Faecal culture, Goats, L₃, Resistant, Sheep

4.1 Introduction

Gastrointestinal nematodes (GIN) infestation is one of the main constraints of small ruminant production. Chronic infestation results to poor productivity, poor health, reduced feed intake and death of ruminants (Sykes, 1987; Szyszka *et al.*, 2013). GIN are mainly controlled using commercial anthelmintics including benzimidazoles, imidazothiazoles, praziquantel, levamisole, ivermectins, doramectin and moxidectin. These anthelmintics are no longer sustainable because these parasites have developed resistance (Shalaby, 2013). Development of resistance is facilitated by most commercial anthelmintics tending to have one or relatively fewer active chemical(s) that control GIN. As a result, nematodes easily develop resistance. There is a need therefore to explore combinations of CPEs for alternative remedies that will be hard for

resistance to be developed by nematodes. Thus, study of the use of combined anthelmintic CPEs is essential since they potentially contain numerous active phytochemicals, and GIN cannot develop resistance towards all phytochemicals combined at once (Hoste *et al.*, 2009).

Research has now taken advantage of CPEs from ethno-medicinal plants with anthelmintic properties to evaluate synergistic interactions of combinations. Synergism is when the efficacy of combined anthelmintics is more than individual activities of combined anthelmintics (Bliss, 1939; Williams *et al.*, 2012). On the other hand, when the individual efficacies of combined anthelmintics is more than the efficacy of their combination, there is said to be antagonism (Bliss, 1939; Williams *et al.*, 2012). Combination of phytochemicals within these extracts may target multiple GIN types simultaneously because small ruminants are affected by diverse types of nematodes which may be susceptible to different active phytochemicals (Hounzangbe-Adote *et al.*, 2005; Hoste *et al.*, 2009). Synergism also tends to increase efficacy and lower the dosage of CPEs while sustaining high efficacy. Additionally, synergism lowers dose administered and avoid toxicity that may arise from high dosage (Chou, 2006). Since there has been observed lower efficacy of the same CPEs in goats relative to sheep (Papadopoulos, 2008; Fomum & Nsahlai, 2017), synergism assessment might bring about CPE combinations that are equally effective in both sheep and goats.

Currently, adopted commercial anthelmintics leave residues on meat products, which might endanger health of consumers. It is recommended that synergistic interaction of combinations of CPEs from edible plants be tested, as they naturally do not have any harmful effects. Though these combinations taint meat products, they might not be toxic to both host animal and consumers. Thus, use of edible plants as a solution might result in easily sourced, available, acceptable and affordable, anthelmintic resources. For reasons mentioned above, edible plants with anthelmintic activities are the potential solution to the present challenge. The objective of this study was to assess *in vitro* synergistic activity of CPE combinations (28) from eight (8) mainly edible CPEs on mixed GIN of sheep and goats. It was hypothesized that combination of CPEs would produce synergistic interactions at treatment of GIN of sheep and goats.

4.2 Materials and Methods

This study was conducted with the approval of the University of KwaZulu-Natal Ethics Committee, the Animal Ethics Sub-Committee (ref. AREC/058/018M).

4.2.1 Collection of anthelmintic plants

Seven (7) edible anthelmintic plant species namely: *Allium cepa* (Onion), *Ananas comosus* (Pineapple), *Bidens Pilosa* (Black jack), *Carica Papaya* (Pawpaw), *Vernonia amygdalina* (Bitter leaf), *Zingiber officinale* (Ginger) and *Aloe vanbalenii* (Aloe); and one (1) inedible plant species, *Nicotiana tabacum* (Tobacco) commonly used in ethnoveterinary medicine by South Africans were collected. Some were sourced from University of KwaZulu-Natal (UKZN) Botanical garden, and others harvested from the National botanical garden, Pietermaritzburg. Voucher samples were deposited at the UKZN Herbarium, Pietermaritzburg.

4.2.2 Ethno-medicinal plants extraction, dilution of CPEs to tested concentration, faecal samples collection and their culture

Using method described in Chapter 3: Selected plants were extracted; CPEs were diluted to tested concentrations of 0.625 % v/v, while faecal samples were collected and cultured.

4.2.3 Combination of CPEs and treatment of gastrointestinal nematodes

The experiment was replicated three times. Selected plant species were combined following permutation and combinations resulting in 28 pairs. CPEs combinations were composed by measuring 0.625% v/v of each CPE involved in the combination and combining them at 1:1 ratio. Each of 28 combinations used contained 1.25% v/v. Cultured faecal material of 5 g was treated with 5 ml of 1.25% v/v of combined CPEs. For each treatment, three samples were allotted and treated on the thirteenth day and three negative controls (0% v/v) included for each animal species. The process was repeated three times, giving rise to three replicates per animal species. After treatment, samples were further incubated at 27 °C for 24 hours.

4.2.4 Isolation of treated samples and counting of larvae

Baermann technique was used to isolate Larvae while counting of Larvae was performed using McMaster slide as described in Chapter 3.

4.2.5 Counting of activity and interactions of combined CPEs

Efficacy of tested CPE combinations was calculated following Abbott (1925) using the following formula described by Abbott (1925):

$$Activity (\%) = \left(1 - \left(\frac{Treated\ n}{Control\ n} \right) \right) \times 100$$

Where, treated n is the number of L₃ larvae found in the treated plates and control n is the number of L₃ larvae found in controls.

For resulting interactions (ab) from combinations relative to additive effect (a+b) from interaction, it was computed using, Webb's fractional product method below:

$$Additive\ effect\ (a + b) = (1 - (1 - a) * (1 - b))$$

Alternatively, interactions were computed using SM, whereby the mean of individual plant species efficacy were used in the process as presented below:

$$Additive\ effect\ (a + b) = \frac{a + b}{2}$$

Whereby, a=individual activity of CPE A in the combination, b=Individual activity of CPE B in the combination. Hence, ab (combined activity of CPE A and B) is synergistic and antagonistic where $ab > a + b$, $ab < a+b$, respectively.

4.2.6 Statistical analysis

Collected data were statistically analyzed using the general linear model (GLM) of SAS 9.4 (2013) software. The following statistical model was used to analyze combined efficacy of CPEs on GIN larvae (L₃):

$$Y_{ijk} = \mu + A_j + B_k + e_{jk}$$

Where, Y_{jk} = individual observation, μ = overall mean, A_j = effect of animal species, B_k = effect of plant species and e_{ijk} = error term.

4.3 Results

Tables 4.1 and 4.2 show interaction of CPE combinations against GIN of sheep and goats using Webb's fractional product method (WFPM) and Simple method (SM), respectively. All tested

CPE combinations demonstrated high anthelmintic activity (80-100%) against GIN of sheep and goats. However, most efficacious (100%) synergistic CPEs combination in goats was *V. amygdalina* + *Z. officinale*. In sheep, most efficacious (100%) combinations were *A. cepa* + *C. papaya*, *V. amygdalina* + *Z. officinale* and *A. comosus* + *N. tabacum*. Animal species had a significant effect on observed effect of combinations ($P < 0.001$), efficacy was lower in goats (89.16%±0.95) relative to sheep (95.45%±0.095). However, plant species had no significant effect on the observed effect of CPE combinations.

The simple method computed interactions produced no antagonistic interactions, while WFPM showed antagonistic combinations in sheep and goats (Table 4.1). Ten (10) combinations were antagonistic in goats (*B. pilosa* + *C. papaya*, *C. papaya* + *V. amygdalina*, *C. papaya* + *N. tabacum*, *A. cepa* + *N. tabacum*, *A. cepa* + *B. pilosa*, *A. comosus* + *A. cepa*, *A. comosus* + *Z. officinale*, *A. comosus* + *N. tabacum*, *B. pilosa* + *Z. officinale* and *B. pilosa* + *V. amygdalina*). Nine (9) combinations were antagonistic in sheep (*B. Pilosa* + *C. papaya*, *C. Papaya* + *A. vanbalenii*, *A. cepa* + *A. vanbalenii*, *A. cepa* + *Z. officinale*, *A. cepa* + *V. amygdalina*, *A. cepa* + *N. tabacum*, *A. cepa* + *B. pilosa*, *B. pilosa* + *A. vanbalenii* and *A. comosus* + *Z. officinale*).

Table 4. 1 *In vitro* interactions of 28 combinations from mostly edible ethanolic CPEs (8) at 1.25 % v/v (1:1) against gastrointestinal nematodes of sheep and goats (LSM%±SE) using Webb's fractional product method (WFPM).

Plants (1:1) (A+B respectively)	Goats					Sheep				
	A	B	AB	A+B Effect	Interaction	A	B	AB	A+B Effect	Interaction
<i>B. Pilosa</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	96.4±5.1	95.5±7.40	S	80.1±13.5	66.7±14.6	97.5±5.1	95.9±7.40	S
<i>B. Pilosa</i> + <i>C. papaya</i>	73.7±13.5	73.7±14.6	89.3±5.1	90.9±7.40	A	80.1±13.5	61.9±14.6	87.5±5.1	92.5±7.40	A
<i>C. Papaya</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	89.3±5.1	79.2±7.40	S	61.9±13.5	85.7±14.6	92.5±5.1	95.6±7.40	A
<i>C. papaya</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	92.9±5.1	87.5±7.40	S	61.9±13.5	61.9±14.6	97.5±5.1	84.4±7.40	S
<i>C. papaya</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	85.7±5.1	89.2±7.40	A	61.9±13.5	57.1±14.6	92.5±5.1	85.7±7.40	S
<i>C. papaya</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	92.9±5.1	94.2±7.40	A	61.9±13.5	66.7±14.6	97.5±5.1	87.8±7.40	S
<i>N. tabacum</i> + <i>A. vanbalenii</i>	63.2±13.5	31.6±14.6	85.7±5.1	77.6±7.40	S	66.7±13.5	85.7±14.6	97.5±5.1	93.9±7.40	S
<i>N. tabacum</i> + <i>Z. officinale</i>	63.2±13.5	31.6±14.6	92.9±5.1	77.6±7.40	S	66.7±13.5	61.9±14.6	97.5±5.1	89.1±7.40	S
<i>N. tabacum</i> + <i>V. amygdalina</i>	63.2±13.5	52.6±14.6	89.3±5.1	80.9±7.40	S	66.7±13.5	57.1±14.6	97.5±5.1	85.7±7.40	S
<i>V. amygdalina</i> + <i>Z. officinale</i>	52.6±13.5	52.6±14.6	100.0±5.1	80.9±7.40	S	57.1±13.5	61.9±14.6	100.0±5.1	87.8±7.40	S
<i>V. amygdalina</i> + <i>A. vanbalenii</i>	52.6±13.5	31.6±14.6	92.9±5.1	80.9±7.40	S	57.1±13.5	61.9±14.6	97.5±5.1	87.8±7.40	S
<i>Z. officinale</i> + <i>A. vanbalenii</i>	52.6±13.5	31.6±14.6	89.3±5.1	80.9±7.40	S	61.9±13.5	85.7±14.6	97.5±5.1	95.2±7.40	S
<i>A. cepa</i> + <i>A. vanbalenii</i>	63.2±13.5	31.6±14.6	92.9±5.1	69.3±7.40	S	90.5±13.5	85.7±14.6	90.0±5.1	98.6±7.40	A
<i>A. cepa</i> + <i>Z. officinale</i>	63.2±13.5	52.6±14.6	85.7±5.1	65.9±7.40	S	90.5±13.5	61.9±14.6	95.0±5.1	95.2±7.40	A
<i>A. cepa</i> + <i>V. amygdalina</i>	63.2±13.5	52.6±14.6	89.3±5.1	85.0±7.40	S	90.5±13.5	57.1±14.6	97.5±5.1	97.1±7.40	A
<i>A. cepa</i> + <i>N. tabacum</i>	63.2±13.5	63.2±14.6	71.4±5.1	88.4±7.40	A	90.5±13.5	66.7±14.6	90.0±5.1	97.3±7.40	A
<i>A. cepa</i> + <i>C. papaya</i>	63.5±13.5	73.7±14.6	92.9±5.1	88.4±7.40	S	90.5±13.5	61.9±14.6	100.0±5.1	95.9±7.40	S
<i>A. cepa</i> + <i>B. pilosa</i>	63.2±13.5	73.7±14.6	89.3±5.1	90.9±7.40	A	90.5±13.5	80.1±14.6	95.0±5.1	97.1±7.40	A
<i>A. comosus</i> + <i>A. cepa</i>	73.7±13.5	63.2±14.6	85.7±5.1	90.0±7.40	A	52.4±13.5	90.5±14.6	97.5±5.1	94.6±7.40	S
<i>A. comosus</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	89.3±5.1	79.2±7.40	S	52.7±13.5	85.7±14.6	97.5±5.1	94.6±7.40	S
<i>A. comosus</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	82.1±5.1	91.7±7.40	A	52.7±13.5	61.9±14.6	95.0±5.1	78.9±7.40	A
<i>A. comosus</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	89.3±5.1	88.4±7.40	S	52.4±13.5	57.1±14.6	87.5±5.1	81.6±7.40	S
<i>A. comosus</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	92.9±5.1	95.0±7.40	A	52.7±13.5	66.7±14.6	100.0±5.1	86.4±7.40	S
<i>A. comosus</i> + <i>C. papaya</i>	73.7±13.5	52.6±14.6	96.4±5.1	91.7±7.40	S	52.4±13.5	61.9±14.6	97.5±5.1	78.9±7.40	S
<i>A. comosus</i> + <i>B. pilosa</i>	73.7±13.5	73.7±14.6	89.3±5.1	89.2±7.40	S	52.4±13.5	80.1±14.6	97.5±5.1	88.4±7.40	S
<i>B. pilosa</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	92.9±5.1	80.9±7.40	S	80.1±13.5	85.7±14.6	87.5±5.1	99.3±7.40	A
<i>B. pilosa</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	82.1±5.1	90.9±7.40	A	80.1±13.5	61.9±14.6	97.5±5.1	91.2±7.40	S
<i>B. pilosa</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	82.1±5.1	87.5±7.40	A	80.1±13.5	57.1±14.6	95.0±5.1	89.8±7.40	S
CV %	33.94	41.76	9.51	14.60		33.94	41.76	9.51	14.60	
RMSE	23.30	25.20	8.78	12.83		23.30	25.20	8.78	12.83	

A, Antagonism (AB<A+B); S, Synergism (AB>A+B); AB, Combination efficacy; A+B; Additive efficacy; CV%, Coefficient of variation; RMSE, Root Mean Square Error, LSM; Least square mean, SE; Standard error

Table 4. 2 *In vitro* interactions of 28 combinations from mostly edible ethanolic CPEs (8) at 1.25% v/v (1:1) against gastrointestinal nematodes of sheep and goats (LSM %±SE) using Simple method (SM).

Plants (1:1) (A+B respectively)	Goats					Sheep				
	A	B	AB	A+B average	Interaction	A	B	AB	A+B average	Interaction
<i>B. Pilosa</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	96.4±5.1	68.4±9.39	S	80.1±13.5	66.7±14.6	97.5±5.1	73.8±9.39	S
<i>B. Pilosa</i> + <i>C. papaya</i>	73.7±13.5	73.7±14.6	89.3±5.1	73.7±9.39	S	80.1±13.5	61.9±14.6	87.5±5.1	71.4±9.39	S
<i>C. Papaya</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	89.3±5.1	52.6±9.39	S	61.9±13.5	85.7±14.6	92.5±5.1	73.8±9.39	S
<i>C. papaya</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	92.9±5.1	63.2±9.39	S	61.9±13.5	61.9±14.6	97.5±5.1	61.9±9.39	S
<i>C. papaya</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	85.7±5.1	63.2±9.39	S	61.9±13.5	57.1±14.6	92.5±5.1	59.5±9.39	S
<i>C. papaya</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	92.9±5.1	68.4±9.39	S	61.9±13.5	66.7±14.6	97.5±5.1	64.3±9.39	S
<i>N. tabacum</i> + <i>A. vanbalenii</i>	63.2±13.5	31.6±14.6	85.7±5.1	47.4±9.39	S	66.7±13.5	85.7±14.6	97.5±5.1	76.2±9.39	S
<i>N. tabacum</i> + <i>Z. officinale</i>	63.2±13.5	31.6±14.6	92.9±5.1	47.4±9.39	S	66.7±13.5	61.9±14.6	97.5±5.1	64.3±9.39	S
<i>N. tabacum</i> + <i>V. amygdalina</i>	63.2±13.5	52.6±14.6	89.3±5.1	57.9±9.39	S	66.7±13.5	57.1±14.6	97.5±5.1	61.9±9.39	S
<i>V. amygdalina</i> + <i>Z. officinale</i>	52.6±13.5	52.6±14.6	100.0±5.1	52.6±9.39	S	57.1±13.5	61.9±14.6	100.0±5.1	59.5±9.39	S
<i>V. amygdalina</i> + <i>A. vanbalenii</i>	52.6±13.5	31.6±14.6	92.9±5.1	42.1±9.39	S	57.1±13.5	61.9±14.6	97.5±5.1	59.5±9.39	S
<i>Z. officinale</i> + <i>A. vanbalenii</i>	52.6±13.5	31.6±14.6	89.3±5.1	42.1±9.39	S	61.9±13.5	85.7±14.6	97.5±5.1	73.8±9.39	S
<i>A. cepa</i> + <i>A. vanbalenii</i>	63.2±13.5	31.6±14.6	92.9±5.1	47.4±9.39	S	90.5±13.5	85.7±14.6	90.0±5.1	88.1±9.39	S
<i>A. cepa</i> + <i>Z. officinale</i>	63.2±13.5	52.6±14.6	85.7±5.1	57.9±9.39	S	90.5±13.5	61.9±14.6	95.0±5.1	76.2±9.39	S
<i>A. cepa</i> + <i>V. amygdalina</i>	63.2±13.5	52.6±14.6	89.3±5.1	57.9±9.39	S	90.5±13.5	57.1±14.6	97.5±5.1	73.8±9.39	S
<i>A. cepa</i> + <i>N. tabacum</i>	63.2±13.5	63.2±14.6	71.4±5.1	63.2±9.39	S	90.5±13.5	66.7±14.6	90.0±5.1	78.6±9.39	S
<i>A. cepa</i> + <i>C. papaya</i>	63.5±13.5	73.7±14.6	92.9±5.1	68.4±9.39	S	90.5±13.5	61.9±14.6	100.0±5.1	76.2±9.39	S
<i>A. cepa</i> + <i>B. pilosa</i>	63.2±13.5	73.7±14.6	89.3±5.1	68.4±9.39	S	90.5±13.5	80.1±14.6	95.0±5.1	85.7±9.39	S
<i>A. comosus</i> + <i>A. cepa</i>	73.7±13.5	63.2±14.6	85.7±5.1	68.4±9.39	S	52.4±13.5	90.5±14.6	97.5±5.1	71.4±9.39	S
<i>A. comosus</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	89.3±5.1	52.6±9.39	S	52.7±13.5	85.7±14.6	97.5±5.1	69.1±9.39	S
<i>A. comosus</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	82.1±5.1	63.2±9.39	S	52.7±13.5	61.9±14.6	95.0±5.1	57.1±9.39	S
<i>A. comosus</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	89.3±5.1	63.2±9.39	S	52.4±13.5	57.1±14.6	87.5±5.1	54.8±9.39	S
<i>A. comosus</i> + <i>N. tabacum</i>	73.7±13.5	63.2±14.6	92.9±5.1	68.4±9.39	S	52.7±13.5	66.7±14.6	100.0±5.1	59.5±9.39	S
<i>A. comosus</i> + <i>C. papaya</i>	73.7±13.5	52.6±14.6	96.4±5.1	63.2±9.39	S	52.4±13.5	61.9±14.6	97.5±5.1	57.1±9.39	S
<i>A. comosus</i> + <i>B. pilosa</i>	73.7±13.5	73.7±14.6	89.3±5.1	73.7±9.39	S	52.4±13.5	80.1±14.6	97.5±5.1	66.7±9.39	S
<i>B. pilosa</i> + <i>A. vanbalenii</i>	73.7±13.5	31.6±14.6	92.9±5.1	52.6±9.39	S	80.1±13.5	85.7±14.6	87.5±5.1	83.3±9.39	S
<i>B. pilosa</i> + <i>Z. officinale</i>	73.7±13.5	52.6±14.6	82.1±5.1	63.2±9.39	S	80.1±13.5	61.9±14.6	97.5±5.1	71.4±9.39	S
<i>B. pilosa</i> + <i>V. amygdalina</i>	73.7±13.5	52.6±14.6	82.1±5.1	63.2±9.39	S	80.1±13.5	57.1±14.6	95.0±5.1	69.1±9.39	S
CV %	33.94	41.76	9.51	14.60		33.94	41.76	9.51	14.60	
RMSE	23.30	25.20	8.78	12.83		23.30	25.20	8.78	12.83	

A, Antagonism (AB<A+B); S, Synergism (AB>A+B); AB, Combination efficacy; A+B; Additive efficacy; CV%, Coefficient of variation; RMSE, Root Mean Square Error, LSM; Least square mean, SE; Standard error

4.4 Discussion

It is evident in the results that the Simple method (SM) showed only synergistic interactions compared to the Webb's fractional product (WFPM) which produced a synergism and antagonistic interactions at tandem (Tables 4.1 and 4.2). WFPM exaggerates antagonism relative to synergism (Chou, 2010). This method is suitable for mutually non-exclusive CPEs. This method also considers the efficacy of the CPE combination but ignores the curves of each CPEs in combinations. Combinations tested with this method should only produce a hyperbolic curve, not sigmoidal curves. Biological chemicals only produce sigmoidal curves (Chou & Talalay, 1984; Chou, 2010). This could be the explanation for observed synergistic and antagonistic interactions by WFPM (Table 4.1) relative to the SM method, which produced synergistic interactions only.

Effective synergistic combination(s) were *V. amygdalina* + *Z. officinale* for goats, and *A. cepa* + *C. papaya*, *V. amygdalina* + *Z. officinale*, *V. amygdalina* + *Z. officinale* and *A. comosus* + *N. tabacum* (Tables 4.1 and 4.2) for sheep. The World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) states that an anthelmintic that produces 80-90% and 90-100%, is moderate and effective, respectively (Ferreira *et al.*, 2013). The efficacy of these CPE combinations is in accordance with W.A.A.V.P. rule. These CPE combinations should be tested *in vivo* as they have high efficacies. *In vitro* efficacy of these CPE combinations might be lowered *in vivo*. The difference is attributed to digestion of phytochemicals by gut microbes (Houghton *et al.*, 2007; Ferreira *et al.*, 2013). CPE combinations tested in the current study are mainly edible. It would be vital to test their residues in meat products.

Synergism manifests when combined CPEs produce an efficacy that is higher than their individual efficacy (Williams *et al.*, 2012). Anthelmintics control GIN via oral entry and transcuticular diffusion (Egualé *et al.*, 2007). There is limited knowledge on modes of action of synergistic CPE combinations. However, it is known that CPEs are composed of phytochemicals which exert their effects through different modes of action (Bauri *et al.*, 2015). Thus, CPEs that have different modes of action increase efficacy of CPE combination (Chou, 2006, 2010). Observed synergistic CPE combinations must have been composed of CPEs that have different modes of action (Tables 4.1 and 4.2).

Antagonism is when the combination effect is lesser than the additive effect (Chou, 2006). Observed antagonistic CPE combinations in Table 5.1 might have been caused by buffering. Buffering is when activity of a CPE involved in the CPEs combination is masked by another CPE (Yeh *et al.*, 2009). Antagonism also manifests through activity suppression between CPEs involved in the combinations, whereby a weaker CPE in the combination is suppressed by a stronger CPE (Yeh *et al.*, 2009). Antagonistic CPE combinations should be perceived as a third CPE with its own dose-response relation (Chou, 2010). Klongsiriwet *et al.* (2015) found that a synergistic effect would tend to happen when the dose ratio of combined CPEs is different. Ratio of 1:1 used in the current study must have been limiting in observed antagonistic combinations (Table 4.1). This suggests that observed antagonistic combinations in the current study should be further tested for a synergistic dose ratio. One CPE in combination should be fixed while increasing the dose ratio of the other CPE in the combination (Chou & Talalay, 1984).

The observed significant effect of animal species on efficacy of CPE combinations, could be explained by the fact that CPE efficacy differs with animal species (Fomum & Nsahlai, 2017). This could be attributed to the fact that sheep and goats can self-medicate with anthelmintic plants when parasitized and goats tend to consume more anthelmintic feed than sheep (Villalba *et al.*, 1999; Gradé *et al.*, 2009; Lisonbee *et al.*, 2009; Landau *et al.*, 2010). This might render different resistance(s) in sheep and goats as observed in these results.

Goats should be dosed with twice the dosage for sheep for anthelmintics to exert similar effects in both sheep and goat, because goats house GIN of higher resistance relative to sheep (Papadopoulos, 2008). To make control of sheep and goat GIN easier, anthelmintics which produce similar activity in both sheep and goats should be identified. *B. pilosa* + *N. tabacum*, *C. papaya* + *Z. officinale*, *N. tabacum* + *Z. officinale*, *V. amygdalina* + *Z. officinale*, *V. amygdalina* + *A. vanbalenii*, *A. cepa* + *C. papaya* and *A. comosus* + *C. papaya* combinations produced 90-100% efficacy regardless of animal species. Hence, the above-mentioned CPE combinations have high broad-spectrum efficacy against nematodes of sheep and goats, thus should be recommended for GIN of both sheep and goats.

CPEs are mostly dose dependent in nature (Ahmed *et al.*, 2013). Concentration used in the current study might have rendered low proportions of phytochemicals in the combined CPEs for plant species to exert significant effects. CPEs also have polar and non-polar

phytochemicals which can be specifically extracted by solvent of similar polarity. Ethanol can extract polar and non-polar phytochemicals (Bimakr *et al.*, 2011). Thus, combination of ethanolic CPE in the current study likely homogenized proportion of phytochemicals. This might have rendered plant species insignificant effect of efficacy of CPE combinations.

4.5 Conclusions

CPEs are synergistically efficacious based on animal species. *V. amygdalina* + *Z. officinale* (100%) combination is the best treatment for goat nematodes. While *A. cepa* + *C. papaya* (100%), *V. amygdalina* + *Z. officinale* (100%), *V. amygdalina* + *Z. officinale* (100%) and *A. comosus* + *N. tabacum* (100%) combinations are the best treatments for sheep nematodes. Synergism differed with computation method, SM showed only synergistic interaction while WFPM produced mostly synergistic and few antagonistic interactions. Plant species had no significant effect on observed effect of CPE combinations. Efficacious synergistic CPE combinations should be evaluated *in vivo*. Antagonistic combinations should be studied under different dose ratios.

***In vitro* cytotoxic activity of ethno-medicinal plants with anthelmintic properties against gastrointestinal nematodes of ruminants**

Abstract

Research is adopting ethno-medicinal plants with anthelmintic properties against gastrointestinal nematodes (GIN), as a potential solution for unsustainable commercial anthelmintics. However, studies on the toxicity activity of these plants are limited, thus they must be tested for cytotoxicity activity to develop a safe anthelmintic remedy. This study assessed the *in vitro* cytotoxic activity of 16 plant species crude extracts exerting anthelmintic activity on GIN of sheep and goats. Tested crude plant extracts (CPEs) were from *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica Papaya*, *Crinum macowanii*, *Gunnera perpensa*, *Nicotiana tabacum*, *Ricinus communis*, *Sarcosterma viminale*, *Trema orientalis*, *Urtica dioica*, *Vernonia amygdalina*, *Zanthoxylum capense*, *Zingiber officinale*, *Zizyphus mucronata* and *Aloe vanbalenii*. Cytotoxicity was assessed using an MTT assay on kidney vero cells. *Vernonia amygdalina* (IC₅₀=0.01 mg/ml) followed by *Zingiber officinale* (IC₅₀ =0.02 mg/ml) were the most cytotoxic CPEs, while *Allium cepa* (IC₅₀ =0.27 mg/ml) and *Aloe vanbalenii* (IC₅₀ =0.22 mg/ml) were the least cytotoxic CPEs. Cytotoxicity increased in a dose dependent manner. The concentration-cell viability relationship was negative linear in most CPEs. While it was negative quadratic for *Gunnera perpensa*, *Zingiber officinale* and *Vernonia amygdalina*. Both CPEs which were deemed safe and unsafe can be purified to re-evaluate their cytotoxic effects *in vitro* prior to *in vivo* tests.

Keywords: Commercial anthelmintics, Goats, IC₅₀, Resistance, Sheep, Small ruminant production

5.1 Introduction

Small ruminant production is largely a primary source of income for small-scale livestock farmers in South Africa, Africa, Asia and Latin America, although some commercial operations exist around the world (McDermott *et al.*, 2010). This area of production helps in income generation, food security and economic status (Kosgey *et al.*, 2008). However, GIN remain one of the biggest constraints that limit goats and sheep production. GIN constitute an important global constraint to small ruminant production. Consequently, these nematodes cause loss of productivity, animal products quality and health of ruminants. As a result, food security of farmers whose livelihoods depend on small ruminant production becomes unsustainable.

Currently, commercial anthelmintics are used to control GIN but are no longer very effective as GIN have developed resistance towards these anthelmintics (Shalaby, 2013). These

anthelmintics leave residues in meat and other products, are not naturally degradable, thus polluting the environment and are economically expensive for small scale livestock farmers relative to commercial farmers (Hammond *et al.*, 1997).

To seek alternatives to these unsustainable commercial anthelmintics, research is exploring ethno-medicinal plants exerting anthelmintic properties. These plants are naturally degradable and difficult for GIN to develop resistance against, as they are composed of different phytochemicals (Kaiser *et al.*, 2009; Arora *et al.*, 2017). These plants are also locally available and cheap anthelmintic remedies (Maphosa & Masika, 2010). For the reasons, these plants can constitute potential anthelmintic remedies. Most studies on nematocidal CPEs focus on effect of extracts on parasite (Amin *et al.*, 2009; Fomum & Nsahlai, 2017b) and rarely on toxic effects of extracts on the host animal(s). Additionally, most of these plants have not been standardized scientifically, as a result, they might be too toxic to be used on animals (McGaw & Eloff, 2010). The use of ethno-medicinal plants is also linked to damage of heart, kidneys and gastrointestinal tract irritation (Kudumela *et al.*, 2018).

The assumption that ethno-medicinal plants are not toxic is unsubstantiated (Street *et al.*, 2008). Naturally, plants have evolved poisonous phytochemicals as defence mechanism against herbivory. So, it would be dangerous to use these plants without screening them for their toxicities (Street *et al.*, 2008). Studying their cytotoxic effects, will aid to identify plants that are safe enough as anthelmintic remedies for small ruminants. These plants need to be assessed for their *in vitro* cytotoxic activities on kidneys, because kidneys are responsible for excretion of both toxic or none toxic substances (Monteiro-Riviere *et al.*, 2015). The objective of this study was to assess *in vitro* cytotoxicity activity of sixteen anthelmintic CPEs on vero kidney cells. It was hypothesized that anthelmintic CPEs would not be cytotoxic to kidney vero cells.

5.2 Materials and Methods

This study was conducted with the approval of the University of KwaZulu-Natal Ethics Committee, the Animal Ethics Sub-Committee (ref. AREC/058/018M).

5.2.1 Ethno-medicinal plants collection and extraction

Using ethno-medicinal plants and method described in Chapter 3, ethno-medicinal plants were collected and extracted.

5.2.2 Cytotoxicity assay

Viable cell growth after incubation of African green monkey cells with test compound were evaluated using tetrazolium based colorimetric (MTT) assay previously used by Mosmann (1983). Cells of subconfluent culture were harvested and centrifuged at 200 x g for 5 minutes and resuspended in growth medium to 5×10^4 cells / ml. The growth medium that was used is minimum essential medium (MEM, Whitehead scientific) supplemented with 0.1 % gentamicin (virbac) and 5% foetal calf serum (FCS, Highveld Biological). A total of 200 μ l of cell suspension was pipetted into each well column 2 to 11 of a sterile 96 microtitre plate. MEM of 200ul was added into wells of columns 1 and 12 to minimise edge effect and maintain humidity. Plates were incubated for 24 hours at 37°C in 5% CO₂ incubator until cells were in an exponential growth phase.

The MEM was removed and replaced with test compounds. Serial dilutions of test extracts were prepared in MEM. Cells were as little as possible disturbed during aspiration of medium and addition of combined CPEs. Microtitre plates were incubated at 37°C with test compound for 48 hours in 5% CO₂ incubator. Untreated cells and control were included (doxorubicin, Pfizer laboratories).

After incubation cells were washed with phosphate buffer saline (PBS, Whitehead Scientific) and fresh MEM (200 μ l) was added to each well. 30ul MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide, sigma stock solution of 5mg/ml in PBS) was added to each well and incubated for further 4 hours. After incubation with MTT the medium was removed in each well without disturbing the crystals in these wells. MTT formazan crystals were dissolved by adding 50 μ l DMSO to each well after which plates were shaken gently until the MTT formazan solution was dissolved. The amount of MTT reduction was measured by immediately observing absorbance in a microplate reader (Chromate 4300) at a wavelength of 540 nm and reference wavelength of 630 nm. Wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The IC₅₀ was calculated as concentration of test compound resulting in 50% reduction of absorbance compared to untreated cells, IC₅₀<0.02 mg/ml=Cytotoxic (Mahavorasirikul *et al.*, 2010; Kuete *et al.*, 2011; Abdel-Hameed *et al.*, 2012). The experiment was triplicated.

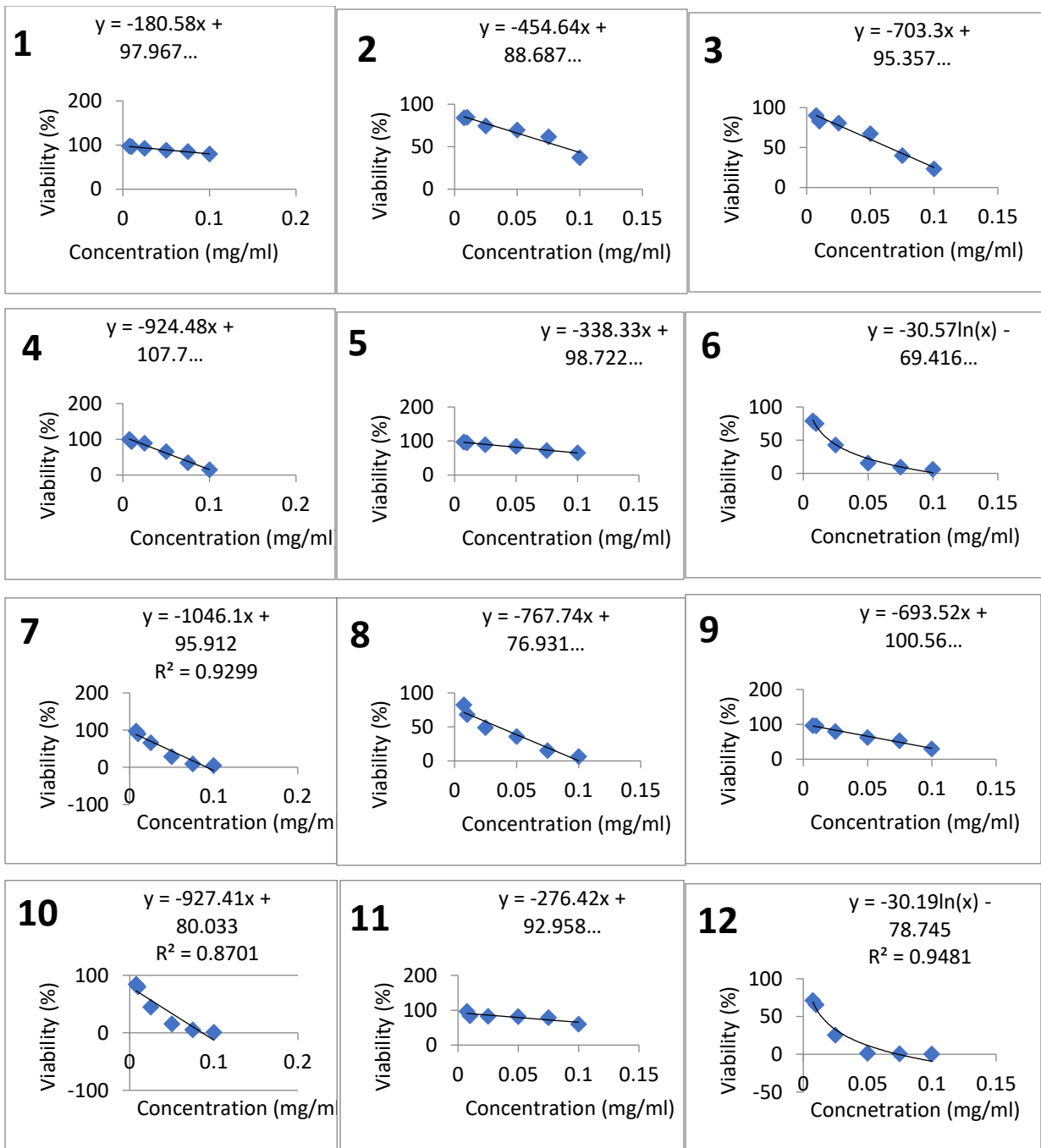
5.3 Results

The cytotoxicity of tested CPEs was determined using IC_{50} , this value determines concentration of CPEs that can kill 50% of vero kidney cells. Table 5.1 shows *in vitro* cytotoxicity activity (IC_{50}) of sixteen CPEs with anthelmintic properties against GIN of small ruminants. *Vernonia amygdalina* showed the highest cytotoxicity while *Allium cepa* the least cytotoxicity. The order of cytotoxicity from the most cytotoxic to the least cytotoxic CPE was thus: *Vernonia amygdalina* < *Zingiber officinale* < *Gunnera perpensa* < *Trema orientalis* < *Ricinus communis* < *Nicotiana tabacum* < *Zanthoxylum capense* < *Carica Papaya* < *Bidens pilosa* < *Zizyphus mucronata* < *Sarcosterna viminale* < *Ananas comosus* < *Crinium macowanii* < *Urtica dioica* < *Aloe vanbalenii* < *Allium cepa*.

Figure 5.1 shows relationship between dose of all tested crude plant extracts and cell viability. Cell viability showed a concentration dependent manner, most cells showed a negative linear relationship with the concentration of tested CPEs. However, cell viability showed a negative quadratic relationship with concentration of *Gunnera perpensa*, *Vernonia amygdalina* and *Zingiber officinale* extract

Table 5. 1 Inhibitory concentration-50 (IC₅₀) in mg/ml of sixteen different ethanolic CPEs with anthelmintic properties against gastrointestinal nematodes of small ruminants.

Ethno-medicinal plants	IC₅₀ (mg/ml)
<i>Allium cepa</i>	0.27
<i>Ananas comosus</i>	0.09
<i>Bidens pilosa</i>	0.06
<i>Carica Papaya</i>	0.06
<i>Crinum macowanii</i>	0.14
<i>Gunnera perpensa</i>	0.02
<i>Nicotiana tabacum</i>	0.04
<i>Ricinus communis</i>	0.04
<i>Sarcosterma viminale</i>	0.07
<i>Trema orientalis</i>	0.03
<i>Urtica dioica</i>	0.16
<i>Vernonia amygdalina</i>	0.01
<i>Zanthoxylum capense</i>	0.05
<i>Zingiber officinale</i>	0.02
<i>Zizyphus mucronata</i>	0.07
<i>Aloe vanbalenii</i>	0.22
Doxorubicin(μM)	0.01



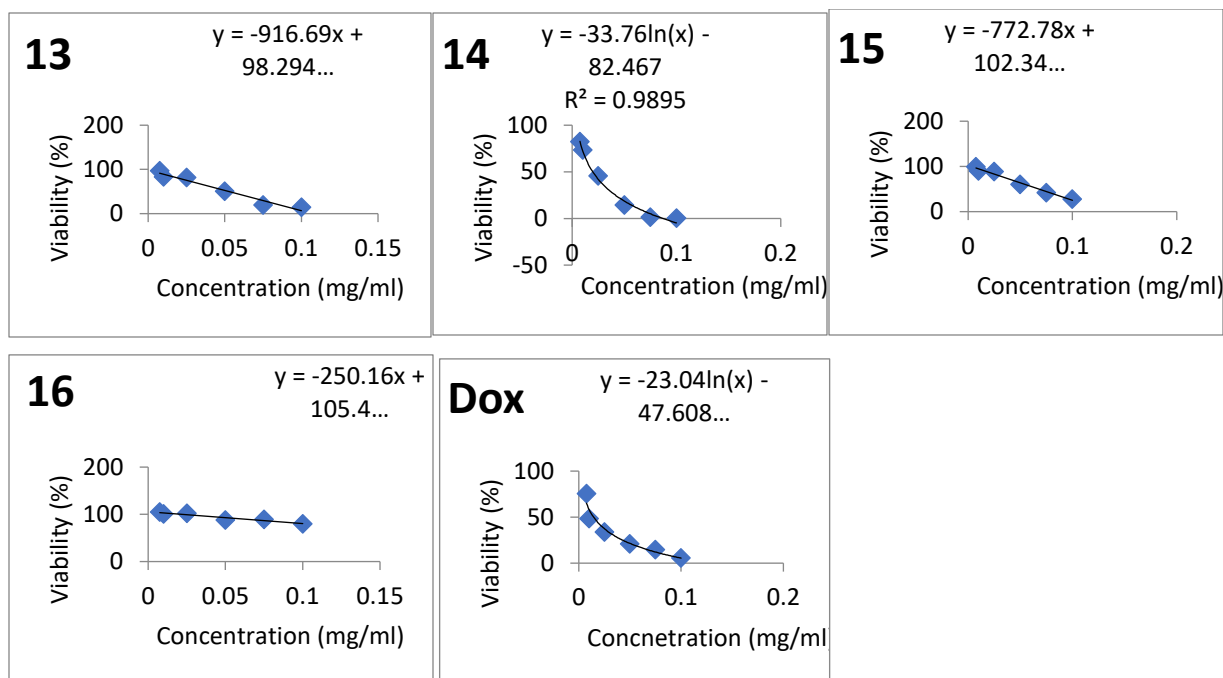


Figure 5. 1 Relationship between cell viability (%) of vero kidney cells and concentration of 1) *Allium cepa*; 2) *Ananas comosus*; 3) *Bidens pilosa* ; 4) *Carica papaya*; 5) *Crinum macowanii*; 6) *Gunnera perpensa*; 7) *Nicotiana tabacum* 8); *Ricinus communis* 9); *Sarcosterma viminale*;10) *Trema orientalis* ; 11) *Urtica dioica*; 12) *Vernonia amygdalina* 13); *Zanthoizylum capense*; 14); *Zingiber officinale*; 15) *Zizyphus mucronata* and 16); *Aloe vanbalenii* ethanolic extracts.

5.4 Discussions

Crude plant extract (CPE) is considered cytotoxic if it produces an IC_{50} value that is less than 0.02 mg/ml (Mahavorasirikul *et al.*, 2010; Kuete *et al.*, 2011; Abdel-Hameed *et al.*, 2012). Based on this rule harmless CPEs can be recommended as anthelmintics for sheep and goats are mainly *Allium cepa* and *Aloe vanbalenii* which have IC_{50} of 0.27 and 0.22 mg/ml, respectively (Table 5.1). This rule also suggests that *Vernonia amygdalina* was the only cytotoxic CPE in the current study, it should be discouraged as an anthelmintic. In the current study phytochemical composition of extracts was not determined. However, *Vernonia amygdalina* extract consists of alkaloids, saponins, tannins, cardiac glycosides, terpenes, steroids and resin (Bonsi *et al.*, 1995; Gazuwa *et al.*, 2013; Arora *et al.*, 2017). Some CPEs are more cytotoxic on normal cells (Nondo *et al.*, 2015) while some are more toxic to their target cell than kidney vero cells (Sserunkuma *et al.*, 2017). *Vernonia amygdalina* also possesses; anti-inflammatory phytochemical such as vernoamyosides (Quasie *et al.*, 2016) and anti-cancer phytochemical such as vernodalinol (Luo *et al.*, 2011) and epivernodalol (Owoeye *et al.*, 2010). Nevertheless, activity of these phytochemicals could have been neutralised by presence of relatively higher concentration of anthelmintic phytochemicals in this extract. Observed high cytotoxicity of *Vernonia amygdalina* might also explain the reason this plant is boiled before being fed to livestock (Bonsi *et al.*, 1995) as a protein supplement (Haile & Tolemariam, 2008; Daodu & Babayemi, 2009; Nampanzira *et al.*, 2015). Boiling of CPEs is associated degradation cytotoxic phytochemicals (Sanhokwe *et al.*, 2016).

Knowledge on relationship of phytochemical composition-extraction duration of tested CPE is limited. However, phytochemicals are degraded beyond 20 hours of extraction (Spigno *et al.*, 2007). The extraction duration (24 hrs) employed in the current study must have degraded phytochemicals of most CPEs tested. This could have influenced observed harmlessness of most of CPEs tested (Table 5.1). Another explanation for majority CPEs (15/16) being harmless could be attributed to the fact that they are edible in addition to their anthelmintic properties (Table 5.1) (Fomum & Nsahlai, 2017b, a). Sheep and goats tend to self-medicate with edible anthelmintic plants when infected with worms (Villalba *et al.*, 2014). Hence, CPEs used in the current study could be of plants that sheep and goats might prefer. For instance, *Allium cepa* (Ed DePeters, 2013), *Ananas comosus* (Lima *et al.*, 2015), *Bidens pilosa* (Hernández-Calva *et al.*, 2011), *Carica papaya* (Jafari *et al.*, 2018), *Zingiber officinale* (Kholif

et al., 2012), and *Zizyphus mucronata* (Osuga *et al.*, 2008) are preferred by goats and sheep as feed.

Anti-inflammatory and anti-cancer properties of harmless extracts might have contributed to their observed harmless activity (Table 5.1). *Allium cepa* partly contributes quercetin with anti-inflammatory properties (Kaiser *et al.*, 2009). This phytochemical is associated with prevention of chronic kidney disease and renal tubular damage (Yang *et al.*, 2018). Aloe contributes anti-inflammatory phytochemicals such as campesterol, β -sitosterol, lupeol, and cholesterol (Kar & Bera, 2018) and is associated with prevention of kidney cell(s) death (Kang *et al.*, 2014). These types of phytochemicals must have been present in all other tested CPEs but at higher proportion in *Allium cepa* and *Aloe vanbalenii* extracts. Cell viability decreased with increasing CPE concentration for tested plants; the decrease being negative linear in all CPEs except for *Gunnera perpensa*, *Vernonia amygdalina* and *Zingiber officinale* extracts where it was negatively quadratic (Figure 5.1). This is probably due to increase in phytochemicals proportion with an increase in concentration. Thus, increases cytotoxicity of the tested CPEs with increasing concentration. It is suggested that the concentration of these tested CPEs be maintained at minimum concentration for maximum safety.

Communal farmers boil CPEs (Kunene *et al.*, 2003; Maphosa & Masika, 2010) which takes a very short time, thus allowing extraction of minimum proportion of phytochemicals. Hence, it is likely that if the same method was adopted all tested CPEs might have been rendered harmless. Omoregie *et al.* (2011) reported that ethanolic *Vernonia amygdalina* extract was more cytotoxic than aqueous extract of the same plant ($IC_{50}=0.00982$ and 0.44 mg/ml, respectively). Communal farmers also use water CPEs to treat parasites in small stock (Kunene *et al.*, 2003; Luseba & Van der Merwe, 2006; Maphosa & Masika, 2010; Sanhokwe *et al.*, 2016). This solvent has a lower extraction ability relative to ethanol (Bimakr *et al.*, 2011; Ahmed *et al.*, 2013). Ferreira *et al.* (2013) reported presence of more phytochemicals in ethanol *Annona muricata* extract mainly than in the aqueous extract of the same plant.

In vivo CPEs are rendered less cytotoxic due to microbial metabolism inactivation of some phytochemicals (Nchu *et al.*, 2011; Adamu *et al.*, 2013). Therefore, CPEs for *Vernonia amygdalina* should be tested *in vivo* because its cytotoxic effect might be reduced. Alternatively, these extracts should be further subjected to chemical fractionation. This process would reduce cytotoxicity through isolation of cytotoxic phytochemicals. However, during this

process high anthelmintic activity of CPEs should be maintained. This could be done by maintaining anthelmintic phytochemicals of the tested CPEs. *In vivo* assessment of CPEs could assess whether these extracts possess other types of toxicities in the form of disorders. For instance, *Bidens pilosa* causes toxicities which are manifested as respiratory distress caused by its high nitrates content (Simmonds *et al.*, 2000). Similarly, *Ricinus communis* causes dullness, discomfort, inappetence and diarrhoea due to its high content of protein ricin (Simmonds *et al.*, 2000). Likewise, *Allium spp.* causes weakness and blood-stained urine (Simmonds *et al.*, 2000). Moreover, *Sarcosterma viminale* causes convulsions due to its strychnine content (Burkill, 1985).

5.5 Conclusions

Majority of tested CPEs were safe for use as anthelmintics for sheep and goats, *Allium cepa* was the safest CPE. *Vernonia amygdalina* CPE was the most cytotoxic CPE and should be used with caution. Cell viability was dose dependent on concentration of CPEs. Concentration of tested CPE should be kept at a minimum in *in vivo* studies. Safe CPEs need to be purified, reassessed *in vitro* and further assessed *in vivo*.

General discussion, conclusions, recommendations and further research

6.1 General discussion

The main hypothesis tested was that anthelmintic crude plant extracts (CPEs) are not cytotoxic on kidney vero cells, are dose dependent and synergistic against mixed gastrointestinal nematodes (GIN) of sheep and goats. Communal farmers depend on small ruminant production for food security in developing countries (Koné *et al.*, 2005). Small ruminant production is important for provision of money to buy food, medical insurance, pay for school fees and restocking animals (Kosgey *et al.*, 2008). GIN are one of the main constraints to small ruminant production by reducing productivity and meat product quality (Shalaby, 2013). These parasites cause lethargy, dullness, appetite loss, poor body condition, poor hair coat, weight loss, pallor of visible mucous membrane, depression, anaemia, hypoproteinemia and gastroenteritis (Okewole & Oduye, 2001).

Commercial anthelmintics are used to control GIN. However, they are becoming ineffective because of resistant parasites (Shalaby, 2013). These anthelmintics leave residues in meat products, pollute the environment since they are not biologically degradable, and resource limited farmers cannot afford them (Hammond *et al.*, 1997; Sanhokwe, 2015). Research is currently taking advantage of ethno-medicinal plants with anthelmintic properties to develop an anthelmintic remedy (Fomum & Nsahlai, 2017b). The reason for this approach is that ethno-medical plants have been used for years by communal farmers with little report of resistant parasites. Some of these plants are edible. Hence, there is limited chance of passing lethal residues on to meat products. Additionally, these plants can degrade in the environment biologically (Sanhokwe, 2015). These plants have been barely studied for their *in vitro* activities. Their dose-response relationship information is limited and must be studied to discover effective doses that can be tested further *in vivo*.

Anthelmintic plants have been reported for having higher activities in sheep than goats (Fomum & Nsahlai, 2017, Papadopoulos, 2008). Hence, it is vital to test efficacy of these

plants on sheep and goat nematodes. Discovery of an anthelmintic that can withstand resistant parasites could be done by also studying anthelmintic plant combinations. GIN cannot develop resistance towards all different phytochemicals of plants combined at the same time (Chou, 2006; Hoste *et al.*, 2009). Synergism study can also deter toxicity and increase efficacy of CPEs by using low concentration and combining CPEs, respectively, of different modes of action (Chou, 2006), though it equally possess potential dangers that needs *in vivo* testing.

Numerous studies mostly focus on the effect of CPEs on GIN and barely on their effects on the parasitized host animals (Fomum & Nsahlai, 2017b). Plants have evolved poisonous phytochemicals as defense mechanism against herbivores. It would be dangerous to use these plants without screening them for their cytotoxic effects (Street *et al.*, 2008). People deem medicinal plants as nontoxic which is unsubstantiated (Street *et al.*, 2008). Whereas, ethno-medicinal plants might be too toxic to kidneys, gastrointestinal tract and heart (Kudumela *et al.*, 2018) to be used on small ruminants (Sanhokwe, 2015). Studying cytotoxicity effects is important for discovery of safe effective CPEs that could be used on small ruminants.

Chapter 3 assessed the *in vitro* dose activity of 16 selected CPEs (40, 20, 10, 5, 2.5, 1.25 and 0.625% v/v) on mixed GIN of sheep and goats. The selected CPEs were from; *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Crinum macowanii*, *Gunnera perpersa*, *Nicotiana tabacum*, *Ricinus communis*, *Sarcosterma viminalis*, *Trema orientalis*, *Urtica dioica*, *Vernonia amygdalina*, *Zanthoxylum capense*, *Zingiber officinale*, *Zizyphus mucronata* and *Aloe vanbalenii*. Faecal samples of goats and sheep were collected and incubated for 12 days to culture L₃ stage larvae. Isolation of L₃ larvae was done using Baermann technique. Objective of 10x was used to count GIN under a microscope using a McMaster slide.

Goats had a significantly higher responses to treatment to the various treatment than sheep at 40% ($P=0.0253$), 20% ($P=0.038$), and low response at 1.25% ($P= 0.0305$) and at 0.625% ($P= 0.0158$) efficacy was higher in goats relative to sheep. On the other hand, both goats and sheep had insignificant ($P>0.05$) efficacies for CPEs concentration (v/v) 10, 5 and

2.5%. Plant species had no effect on efficacy at concentration (v/v) 40, 20, 10, 5 and 2.5%, but had significant effect at lowest concentration (v/v) of 1.25 % ($P=0.0085$) and 0.625 ($P=0.0234$) which was not dose-dependent. Few plants had high activities at the lowest tested concentration of 0.625% v/v. In goats it was *Gunnera perpensa* (89.47%±12.40), while in sheep *Gunnera perpensa* (100%±12.40), *Urtica dioica* (95.24%±12.40), *Zizyphus mucronata* (90.47%±12.40) *Allium cepa* (90.47%±12.40), *Aloe vanbalenii* (85.71%±12.40) and *Bidens pilosa* (80.95%±12.40).

The observed significant plant effect on 1.25 and 0.625% v/v might have been due to difference in phytochemicals concentration of different plant families. The probable explanation for lack of dose dependency might have been due to failure of dilutions to occur linearly per concentrations. The possible explanation for significantly high activity of CPEs at 1.25% ($P= 0.0305$) and 0.625% ($P= 0.0158$); and the significantly high activity on goats relative to sheep at of 40% ($P=0.0253$), 20% ($P=0.038$) might have been due to fluctuations of nematodes species in goats and sheep, which must have caused susceptibility of nematodes to differ between sheep and goats. Based on the results, the hypothesis that CPEs control mixed GIN of sheep and goat in a dose dependent manner is rejected.

In the second experiment (Chapter 4), the synergistic activity of twenty-eight (28) CPE combinations from mainly edible plants were assessed at a concentration of 1.25% v/v (1:1). Webb's fractional product method (WFPM) and simple method (SM) were used to calculate the interaction of combinations. In goats, *V. amygdalina* + *Z. officinale* (100%) was the most effective combination. In sheep, *A. cepa* + *C. papaya* (100%), *V. amygdalina* + *Z. officinale* (100%), *V. amygdalina* + *Z. officinale* (100%) and *A. comosus* + *N. tabacum* (100%) were most effective combinations. The WFPM produced mostly synergistic interactions relative to antagonistic interactions. While the SM produced only synergistic interactions. For both WFPM and SM; animal species had a significant effect ($P<0.001$) on efficacy of combinations, while plant species effect was not significant ($P=0.3063$). For both WFPM and SM; animal species had a significant effect ($P<0.05$) on additive effect of plants in combinations, while plant species effect was not significant ($P>0.05$).

Probable explanation for different synergism within WFPM and SM might have been because, WFPM exaggerates antagonism more than synergism (Chou, 2006, 2010). While the SM method must have exaggerated antagonism. CPEs control GIN through two routes namely: trans-cuticular and oral route. The explanation of observed synergism per WFPM and SM might have been due to combination of CPEs that are containing phytochemicals which use different routes. Some CPEs combinations show synergism when one CPE involved in the combinations is higher in concentration than the other. The probable explanation for observed antagonistic interaction from WFPM might have been due to use of a ratio that disallowed synergism to occur. CPEs produce different activity in sheep and goats (Fomum & Nsahlai, 2017a, b). Extracts might have been composed of feed that goats prefer. Which might render different resistance (s), this might explain the observed significant effect of animal species. CPEs are composed of different phytochemicals (i.e. Alkaloids, Saponins, Tannins) which are mainly dose-dependent (Fomum & Nsahlai, 2017b). It is possible that the concentration used in the current study might have been very low for activity to be influenced by plant species effect. Based on the results, the hypothesis that CPE combinations are synergistic against mixed GIN of sheep and goats, is rejected for SM results but accepted for WFPM results.

In Chapter 5, the *in vitro* cytotoxic activity of sixteen selected CPEs on vero kidney cells was assessed. Selected ethanolic extracts were from; *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Crinum macowanii*, *Gunnera perpensa*, *Nicotiana tabacum*, *Ricinus communis*, *Sarcosterna viminalis*, *Trema orientalis*, *Urtica dioica*, *Vernonia amygdalina*, *Zanthoxylum capense*, *Zingiber officinale*, *Zizyphus mucronata* and *Aloe vanbalenii*. MTT assay was performed on kidney vero cells. *Vernonia amygdalina* ($IC_{50} = 0.01$ mg/ml) followed by *Zingiber officinale* ($IC_{50} = 0.02$ mg/ml) were the most cytotoxic CPEs, while *Allium cepa* ($IC_{50} = 0.27$ mg/ml) and *Aloe vanbalenii* ($IC_{50} = 0.22$ mg/ml) were the least cytotoxic CPEs. The cell viability was dose dependent, hence a minimum dose should be used. A CPE is considered safe if it produces IC_{50} of more than 0.02 mg/ml (Mahavorasirikul *et al.*, 2010; Abdel-Hameed *et al.*, 2012). The probable explanation for dose-dependency of cell viability might be due to the increase of phytochemicals proportion with an increase in concentration. While the safest CPEs might have contained

high anti-tumour and anti-cancer phytochemicals. Therefore, observed high cytotoxic effect of *Vernonia amygdalina* must have been due to high concentration of phytochemicals which are toxic to cells. Based on the results, the hypothesis that crude plant extracts are not cytotoxic to kidney vero cells is rejected.

6.2. Conclusions

Efficacy was high in all tested CPEs, and dose independent towards different concentrations tested and which showed efficacy of CPEs differ between sheep and goats. At a low concentration few plants were efficacious. Hence, *Gunnera perpensa* was the best treatment of goat nematodes while, while *Gunnera perpensa*, *Urtica dioica*, *Zizyphus mucronata*, *Allium cepa*, *Aloe vanbaleonii* and *Bidens pilosa* was the best treatment for sheep nematodes. Combination of CPEs produced synergistic and antagonistic interactions based on WFPM and only synergistic interactions based on SM; animal species had a significant effect on efficacy of combinations while plant species effect did not. *V. amygdalina* + *Z. officinale* is the best treatment for goat nematodes. While *A. cepa* + *C. papaya*, *V. amygdalina* + *Z. officinale*, *V. amygdalina* + *Z. officinale* and *A. comosus* + *N. tabacum* combinations were the best treatment of sheep nematodes. The cytotoxicity of CPEs increased with an increase in concentration. Tested CPEs were not cytotoxic except for *Vernonia amygdalina* extract.

6.3. Recommendations and further research

In vitro and *in vivo* activities of CPEs do not correlate, due to gut microflora digestion of phytochemicals. During *in vitro* individual and synergistic activity test, CPEs that produced an efficacy of 80-100% were recommended for further *in vivo* tests. The dose of these extracts should be minimum efficacious concentration to prevent cytotoxicity. Since, results revealed that cytotoxic effect of the tested CPEs increases with an increase in concentration of extracts. CPEs that produced high activity at the lowest tested concentration (0.625% v/v) are recommended. *Gunnera perpensa* is recommended for treatment of goat nematodes while, while *Gunnera perpensa*, *Urtica dioica*, *Zizyphus*

mucronata, *Allium cepa*, *Aloe vanbalenii* and *Bidens pilosa* are recommended for treatment of sheep nematodes.

Efficacious combinations tested are also recommended. Since they were composed of 0.625 v/v from each CPEs involved in combinations. Hence, *V. amygdalina* + *Z. officinale* are recommended for treatment of goat nematodes. While *A. cepa* + *C. papaya*, *V. amygdalina* + *Z. officinale*, *V. amygdalina* + *Z. officinale* and *A. comosus* + *N. tabacum* combinations are recommended for treatment of sheep nematodes. Although, all CPEs were all safe except for *Vernonia amygdalina* extract, which should be discouraged as an anthelmintic or used with caution. *In vivo* cytotoxicity test of these CPEs is recommended, as it could render them safe. Since, microbial digestion of phytochemicals could reduce cytotoxic profile of tested CPEs. CPEs that had antagonistic interaction should be tested at different dose ratios. Since, they might be synergistic at a different dose ration instead of 1:1 ratio used in the current study.

Further research should assess:

1. *In vivo* activity of crude plant extracts on goats and sheep nematodes;
2. *In vivo* activity of crude plant extract combinations on goats and sheep nematodes;
3. Residuals of edible crude plant extract combinations in meat products;
4. *In vitro* activity of crude plant extracts on different nematode species; and
5. *In vitro* and *in vivo* cytotoxicity of CPEs and of combinations of CPEs.

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Appendix 1 Ethical approval to conduct animal study



06 October 2018

Mr Lindokuhle Mhlongo (213520360)
School of Agricultural, Earth & Environmental Sciences
Pietermaritzburg Campus

Dear Mr Mhlongo,

Protocol reference number: AREC/058/D18M

Project title: Improving the use of ethnobotanical anthelmintic remedies in control of nematode parasites of Small Ruminants in South Africa

Full Approval – Research Application

With regards to your revised application received on 29 August 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 October 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Professor S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor Ignatius Verla Nsahai
Cc Registrar: Mr Simon Mokoena

Cc Academic Leader Research: Professor Hussein Shimelis
Cc Ukulinga Research Farm

Animal Research Ethics Committee (AREC)

Ms Mariette Snyman (Administrator)

Westville Campus, Govan Mbeki Building

Postal Address: Private Bag X54001, Durban 4000

Telephone: +27 (0) 31 260 8360 Facsimile: +27 (0) 31 260 4609 Email: animalethics@ukzn.ac.za

Website: <http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx>



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