Plant-plant combination: an important option in the phase of failing anthelmintics to control nematodes in small ruminants

By

Sylvester Werekeh Fomum

B. Agric. Tech. Hons. Nigeria, MSc. Agric. Animal & Poultry Science

University of KwaZulu-Natal

A dissertation submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

in

Animal Science

Discipline of Animal and Poultry Sciences

School of Agriculture, Earth and Environmental Science

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

South Africa

2018

THESIS SUMMARY

The present study was designed to explore how combination phytoanthelmintic therapy can be employed to enhance livestock nematode control in goats and sheep. This was motivated by wide spread emergence of resistant varieties of nematodes and related helminths of livestock against chemical anthelmintics. Ongoing trend of selection for resistance by livestock nematodes has led to general anthelmintic failure, urging exploration and potential implementation of combination anthelmintic phytotherapy as an important option. Selected and tested plant species in the current study included *Allium cepa, Aloe van balenii, Ananas comosus, Bidens pilosa, Carica papaya, Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Ricinus communis, Sarcostema viminale, Trema orientalis, Urtica dioica, Vernonia amygdalina, Zanthozylum capense, Zingiber officinale* and *Zizyphus mucronata.*. From preceding studies, anthelmintic activity of plant species in the current project have been linked to some important macro anthelmintic biochemicals and grouped as such into sub-experiments (SEPs). These included alkaloids and condensed tannins in SEP 1, flavonoids in SEP 2 and, proteases and nitrogen compounds in SEP 3. Alkaloids and condensed tannins containing plant species (SEP 1), included *Aloe van balenii*, *Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Sarcostema viminale, Vernonia amygdalina*, *Zingiber officinale* and *Zizyphus mucronata*; flavonoid containing plant species (SEP 2) comprised of *Trema orientalis, Urtica dioica* and *Zanthozylum capense;* and proteases and nitrogen compound containing species (SEP 3), consisted of *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya,* and *Ricinus communis.*

In vitro studies initially tested oven-dried plant vegetative samples at 10g, 20g, and 40g equivalent crude extract in 70% ethanol, and concentrated to 100ml for efficacy on mixed nematode infected Nguni goats and Merino sheep in chapter 3. It sought to test effects of concentration, plant species, animal species, interaction between concentration and plant species, interaction between concentration and animal species, and interaction among plant species, animal species and concentration on efficacy. In SEP 1, animal species (P= 0.0107) and concentration (P= 0.0005) affected efficacy. Interaction between crude extract concentration and animal species affected efficacy (P= 0.0127). In SEP 2, concentration affected (P< 0.0001) efficacy. Animal species affected efficacy (P= 0.0046). Similarly, plant species affected efficacy (P= 0.0572). There were interactions between concentration and

animal species (P= 0.0010), concentration and plant species (P= 0.0123) and among concentration, animal and plant species (p= 0.0435). In sub-study (3), animal species affected (P= 0.0004) anthelmintic efficacy. Similarly, concentration affected (P= 0.0002) anthelmintic efficacy. Additionally, interaction between animal species and concentration also affected (P= 0.0015) anthelmintic efficacy. *Aloe van balenii* was confirmed to exert anthelmintic activity.

The following *in vitro* study in chapter 4 evaluated combined efficacy of plant species possessing similar macromolecule(s), in SEP 1, 2 and 3 on mixed nematode parasites of sheep. It was aimed at evaluating anthelmintic potency of plant species combination with similar macromolecules, and how these molecules relate with anthelmintic trait. Sub-experiment one had twenty one (21) combinations; SEP two, three (3) and SEP three, ten (10). Crude extract of each plant species was obtained by extracting 4 g dry matter (DM) in 70 % ethanol, and each experiment ran thrice. Expected combined efficacy computed as (a + b)/2, and simple synergy (differences between combined and expected efficacies) were also computed. Webb's synergy was computed using Webb's fractional product method. Alkaloids, condensed tannins and flavonoids contents were quantified and, simple and multiple regression analyses ran to determine their contribution to anthelmintic efficacy. High efficacies were observed for combined plant species of SEP 1, SEP 2, and SEP 3 but within sub-experiments were not different (P>0.05). Simple synergies were mostly positive, with means of 2.5 ± 0.67 % (SEP 1), 1.8 ± 1.19 % (SEP 2), and 2.8 ± 0.30 % (SEP 3). However, Webb's synergy were largely negative for SEP 1, SEP 2, and SEP 3, each being lower than zero. Among plant combinations, in SEP 1, condensed tannin and flavonoid contents were different (P< 0.0001), while alkaloid contents was similar (0.3037); in SEP 2 condensed tannin (P< 0.009) and flavonoid (P= 0.0211) contents were different but alkaloid contents were similar (P= 0.07); and in SEP 3, condensed tannin contents were not different (P= 0.4312), while the alkaloid (P= 0.0135) and flavonoid contents (P< 0.0001) were different. For all these macro-molecules, there was no discernible association with anthelmintic efficacy. There was potent activity arising from combinations as exemplified by high efficacy, which in the absence of any correlation is potentially attributed to activity of all macromolecules and bioactivity of other related phytochemicals. It is suggestive of a more complex and intricate macromolecular and biochemical interaction in combinations.

In the following trial in chapter 5, combinations were constituted across groups from the former. This was aimed at evaluating efficacy and synergistic effects, and additionally,

contribution of alkaloids, condensed tannins and flavonoids to these parameters in vitro in sheep. Intergroup combinations were thirty two (32) for condensed tannins/alkaloids and proteases/nitrogen compounds SEP 1; 13 combinations for flavonoids and alkaloids/tannin plant species in SEP 2; and 15 combinations for proteases/nitrogen compound and flavonoid containing plant species in SEP 3. Each experiment was run thrice. Extraction of plant species was done similarly to the former in chapter 3, and dosing mode also retained, but component plant species in combined pairs were from different SEPs'. Rectal faecal grabs from sheep collected and pooled to constitute test samples (chapter 4), incubated and cultured similarly. On day 13, dosing with combined plant species crude extract at 2.5 ml with double dose concentration of each constituting pair. While some controls were moistened and others treated with 70 % ethanol to eliminate potential solvent killing effect. Larval isolation was done following Baermann technique, counting using McMaster slide on day fourteenth. Corrected mortalities were evaluated following Abott's formula and adopted as indices of observed combined efficacies. Synergistic effects were computed following Webb's method and alternatively simple synergy from differences between observed and expected efficacies (a + b)/2. Data was analyzed following general linear model of SAS (2000). Combined efficacies of SEP 1 related species were not different, but high, mean (95.5 ± 0.12 %). Synergistic activities were similar (P= 0.3217), with mean (-4.0 ± 0.12 %). No association occurred between any of alkaloids, condensed tannins or flavonoids with observed efficacy for SEP 1, 2 and 3. Multiple regression analysis to seek any relationship among quantified macromolecules with efficacy was not useful either for SEP 1, 2 and 3. Efficacy of combinations SEP 2 were not different (p= 0.4318). Synergistic means were not different (P= 0.2685), but negative (-5.4 ± 0.34 %). Observed efficacy of combinations in SEP 3 were similar (P= 0.5968) and high, mean (95.8 ± 0.04 %). Webb's synergy was not different (P= 0.6264) and had mean (-3.8 ± 0.04 %). All synergistic means were negative. Crude extracts of all combinations exhibited anthelmintic activity, but could not be attributed to any specific macromolecule(s). Evidently, there is more to the active principles involved than has been examined in the current study, warranting a more detailed study in succeeding chapter 6.

All selected plant species were analyzed for phytochemical composition using GC/MS, in search of anthelmintic and other related biochemicals. Four grams (4 g) dry matter (DM) of each species vegetative material was extracted in 70 % ethanol, 2 µl injected into a chromatoprobe trap, and analysed for biochemical composition. Compound identification carried out using the NIST05 mass spectral library and comparisons with retention times of

chemical standards done. Where available, comparisons between calculated Kovats retention indices and those published in the literature were done. Clean chromatoprobe traps run in GC/MS as controls to identify background contamination. Compounds present at higher or similar percentages in controls were contaminants and excluded from analysis. For quantification, each peak area in each sample was quantified and converted to percentage of emission, and emitted mass in Nano grams. Phytochemicals identified belonged to aldehydes, amines, sulphur compounds, nitrogen compounds, Ketones, aliphatic acids, benzenoids, alcohols, lactones, amides, alkaloids, furans and esters. Means were determine, standard deviation, sum, minimum and maximum biochemical content in Nano grams. Reference to previous screening and related bodies of work identified and profiled phytochemicals with anthelmintic and other related biological activities. Fourty six phytochemicals had antibacterial activity, 42 antioxidant activity, 38 antifungal activity, 24 antiviral activity, and 13 anthelmintic activity. Allotment of thirteen anthelmintic related phytochemicals according to occurrence in selected plant species indicated that 2 plants had one, 6 plants had two, four plants had three phytochemicals, and 4 plants four phytochemicals. It is most plausible that anthelmintic and other related biological activities exerted by these plant species are closely linked to some phytochemical(s).

The following study in chapter 7 retained and analysed identified phytochemicals in chapter 6 for their relationship with observed anthelmintic efficacy, simple and Webb's synergies. Pearson correlation coefficient was run to explore association of phytochemical candidates with observed efficacy, simple and Webb's synergy. Multiple regressions (using a selection option stepwise) were run to explore the influence of various phytochemicals on efficacy, simple synergy and Webb's synergy, by conducting 10 searches to identify any of such influence. Some phytochemicals had positive influences including (benzofuran, 2,3-dihydro; pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-; 2 propenamide; phytol; 5-hydroxymethylfurfural; furfural) and others negative influences. Some phytochemicals selected to exerted positive influence, did so on both observed efficacy and simple synergy. Both correlation matrix and multiple regression relationships pointed to more consortia of phytochemical action.

In chapter 8, this study was designed to evaluate and identify the most effective combined dose of *Allium cepa* and *Vernonia amygdalina* (COMBP1)*;* and *Ananas comosus* and *Carica papaya* (COMBP2) at 50:50 weight for weight relative to positive control, "Zolvix" on natural nematode infected sheep *in vivo*. Sheep were fed 1.2 kg each of 4 % urea treated veld hay,

crushed yellow maize and milled Lucerne hay in ratio 1:1:1. Treatment doses included 5 g, 10 g, 15 g, and 20 g dry matter equivalent extract in 100 ml of 70 % ethanol. Two experiments; the first, evaluated egg per gram (epg) change post treatment and the second, egg hatch and larval recovery. Fifty six Merino ewes averaging 45.0 ± 0.09 kg were weighed and initial epg count done. Both parameters used as covariates at allotting sheep to treatment doses and control of experiment one. Effects of treatment over time, and interaction between treatment and time on epg were evaluated weekly for 4 weeks. In the second trial, faecal grabs from each treatment in the former were pooled, mixed and three sub-samples of 4 g incubated and cultured for egg hatch and larval recovery post treatment on days 1, 14 and 28, relative to negative control from untreated sheep. Effects of treatment, time and, interaction between treatment and time evaluated relative to egg hatch and larval recovery. In trial one, for COMBP1, initial sheep weight were similar, whereas final weight were higher ($P< 0.05$). Differences between initial and final weight were similar. Epg preceding dosing and others post treatment at end of weeks' 1, 2 and 3 were lower. Epg at end of week's 4 were higher ($P< 0.05$). For COMBP2, initial sheep weight preceding treatment were similar, whereas weight post treatment were mostly similar, but partially higher for treatments 1, (5 g DM equivalent crude extract), ($P< 0.05$). Initial epg pre-treatment for COMBP2 were similar ($P> 0.05$). Mean epgs post treatment at end of weeks' 1, 2 and 3 were lower, while that of week's 4 were higher ($P< 0.05$). In trial two, *e*gg hatch and larval recovery for COMBP1 were lower for all treatments on day 1 and 14, but oppositely higher for day 28 ($P< 0.05$). Mean egg hatch and larval recovery of COMBP2 for days 1 and 14 were lower, whereas that of 28 day post treatment were higher ($P< 0.05$). Egg hatch and larval recovery increased with time ($P< 0.0219$). Similarly, interaction of treatments and time resulted to higher egg hatch and larval recovery ($P= 0.0496$). Treatment trends for both combinations were seemingly consistent for the first two weeks post treatment.

During experiment one of this project, there were differences in plant species, animal species (goats and sheep), concentration of plant species crude extract and their interactions in relation to efficacy. *In vitro* combination phytotherapy, pairs of plant species carrying similar and different anthelmintic macromolecules exerted potent efficacy with little or no antagonism, whereas the same macromolecules did not associate with efficacy. Interaction of biochemicals in both combinations of plant species containing similar macromolecules, and combinations involving different macromolecules would most likely have been different though. Identification of various biochemicals from GC/MS analysis linked anthelmintic and other related activities to some biochemical candidates, some of which were not macromolecules

tipped initially to exert this activity. This was suggestive of a wide range of biochemical interactions among these phytochemicals leading to observed combined anthelmintic activity. Multiple regression analysis also failed to linked macromolecules to anthelmintic efficacy. In relation to efficacy vis a vis identified biochemicals, some related positively, while others related negatively, and such interactions have been associated with exercise of medicinal traits by plants. It was observed *in vivo* studies in sheep, that combination anthelmintic therapy of *Allium cepa* and *Vernonia amygdalina* (COMBP1); and *Ananas comosus* and *Carica papaya* (COMBP2) exerted anthelmintic activity, but most likely required a second dose following fast waning activity of the first. This will potentially sustain activity longer and improve control. It is recommended that this be done, as it is critical to advancing research in this area.

Declaration

I, Sylvester Werekeh Fomum declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original work.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from researchers. Where other written sources have been quoted, then:
   a) Their words have been rewritten but the general information attributed to them has been referenced;
   b) Where their exact words have been used, their writing has been placed inside quotation marks, and referenced.
5. Where I have produced a publication where I am an author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.
6. This thesis does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references section(s).

Adhering to the points above:

As the Researcher, I hereby submit this thesis for examination.

Signed:……

Date:……

Name: Sylvester Werekeh Fomum

As the Research Supervisor, I agree to the submission of this thesis for examination.

Signed:……

Date:……

Name: Prof. Ignatius Verla Nsahlai

Acknowledgements

The realization of this project is the collective efforts of a winning team comprising of people of good will in their majority. Irrespective of the order of acknowledgement, each and every contribution was critical to the accomplishment of this project and should be appreciated as such. I must acknowledge my divine blessing for ready service and assistance at request from people across the board in disciplines and institutions in and out of the university. The financial stretch of my beloved wife Mrs. Fomum Loveline Enyoh and care enabled me to settle, focus, think, work and write, as reflected in the outcome.

The expert conception, crafting, design and implementation of this work was closely directed and supervised by my academic mentor and coach Professor Ignatius Verla Nsahlai, who, I am indebted to and herein express my profound gratitude. My brother and friend Dr Yobo Kwasi was of immense material, academic and spiritual support to this project, as he had been in the previous, for these; I express my deep gratitude to him. Professor Akwumbuom Celestine Nse, my brother of the Federal University of Technology Owerri, Nigeria is deeply acknowledged for his financial, material, spiritual and moral support throughout my tertiary project in Nigeria and South Africa. Dr Fomum Athanasius Tita my beloved brother is herein appreciated and acknowledged for his academic, filial, moral, spiritual and financial support from the genesis of my post graduate project to date. My dear sister and friend Dr Bame Irene stands tall in her financial, material, spiritual, academic and moral support. In her, I found my most cherished and uncompromising principles of honesty, moral rectitude, fortitude, compassion and empathy. The academic, material, spiritual and financial support of Mrs. Janet Mah Ane Akob and Dr Christopher A. Akob was instrumental to this accomplishment. Dr. Nicky Tyler has consistently given opportunities for me to acquire experience and financial support; thanks and God bless you. Mr. Munteh Paul remained fateful and supportive as a brother and true friend, thank you. I wish to thank my brother Mr. Tah Cletus Acha for his much needed financial and spiritual support.

Technical and logistical support was received from Mrs. Alett Botha and Mr. Sam Khumalo of livestock section, Ukulinga Research farm. Mrs. Alison Young of the Discipline of Botany and University of KwaZulu-Natal Botanic Garden, Pietermaritzburg campus for identification and sourcing of some of the plant species used in the project. Dr Christina Potgiety of UKZN herbarium for identification, sourcing of some species and her directives on preparation and preservation of herbarium samples. Dr Suinyu Terence was helpful in sourcing of some plant

species samples, GC/MS analysis for biochemical composition and moral support. Mrs. Susan and Mr. Richard Bergdorf of plant Pathology. My colleagues Dr Nourou Abdul, Dr Mutimura Mupenzi and Mr Mehluli Moyo are all appreciated and acknowledged. The administrative and technical staff of the National Botanical Garden Pietermaritzburg, is deeply appreciated and acknowledged for their technical and material support.

The seed of development that was planted and nurtured by our late father, **Pa Fomum Joseph Monjah** has borne and yielded much fruit, and this is one of them; thank you Papa. My mother, Mrs. Fomum Celine Ako is heartily appreciated for her moral, spiritual and financial support. She has taken care of the family with and without me. My step Mother Mrs. Fomum Magdaline Anyi has been very supportive financially, spiritually and materially. The family team of brothers Mr. Fomum Athanasius Tekuh, Mr. Fomum Walters Gwana, Mr. Fomum Gilbert Nyong, Mr. Fomum Marcel Mbah, My sisters' Fomum Guilian Akoh and husband Mr. Charles Ndagha, Fomum Caroline Acha, Fomum Gwendoline Ako, Fomum Irene Assi have all supported me spiritually, financially morally and materially. My God given angels Werekeh Alistair Fomum, Werekeh Silgina Blessing Timben, Werekeh Wise Fomum Junior and the entire family.

To my fallen heroes, Mr. Fomum Christian Egwe, Mr. Fomum Sampson Enyong, my uncle and brother Ni Chistopher Tah, my beloved grandmother Mami Eboh Kuh Ako Lucy, my friend and brother Mr. Romanus Kwayeb, my God son and brother Mr. Fombang John Abang, Tanyi Ameh Cletus, etc, may God's perpetual light shine onto you all. See you someday.

To God be the glory.

DEDICATION

This work is dedicated to my wife Mrs. Fomum Loveline Enyoh, my children; Werekeh Wise Fomum Junior, Werekeh Alistair Fomum, Werekeh Silgina Timben and the entire Pa Fomum's family for their motivation and prayers during my studies away from home.

To God be the glory.

Thesis related and thesis based output
Published articles

1. **Fomum, S. W. and I. V Nsahlai, 2017.** *In vitro* control of parasitic nematodes of small ruminants using some plant species containing flavonoids. Tropical Animal Health and Production 49(2):375-382. DOI: 10.1007/s11250-016-1203-6.
2. **Fomum, S. W. and I. V. Nsahlai 2017.** *In vitro* evaluation of anthelmintic efficacy of some plant species possessing proteinases and/or other nitrogenous compounds in small ruminants. Journal of Alternative, Complementary and Integrative Medicine. DOI: 10.24966/ACIM-7562/100038
3. **Fomum, S. W. and I. V. Nsahlai, 2017.** *In vitro* nematicidal activity of plant species possessing alkaloids and tannins. Cogent Food and Agriculture. https://doi.org/10.1080/23311932.2017.1334295
4. **Fomum, S. W. and I. V. Nsahlai, 2015.** The buffalo co-infection conundrum. Spotlight. Trends in Parasitology 31(6), 230-231.

Conference presentation

1. **Fomum, S.** W**. and I. V. Nsahlai**, 2016. *In vitro* evaluation of anthelmintic efficacy of some plant species possessing proteinases and/or nitrogenous compounds in small ruminants. College of Agriculture, Engineering and Science Research day. Held at Howard College on 29th November 2016.
2. **Fomum S. W. and I. V. Nsahlai, 2015.** *In vitro* nematicidal activity of plant species possessing alkaloids and tannins. 48th annual congress of the South African Association of Animal Science (SASAS), held at in the University of Zululand from 21-23rd of September 2015.

Articles in preparation

1. **Fomum S. W. and I. V. Nsahlai.** Plant-plant combination: *in vitro* anthelmintic phytotherapy against mixed nematodes of sheep using species containing alkaloids and /or condensed tannins.
2. **Fomum S. W. and I. V. Nsahlai.** *In vitro* combined anthelmintic phytotherapy using plant species possessing flavonoids to control nematodes of sheep.

3. **Fomum S. W. and I. V. Nsahlai.** Combined anthelmintic phytotherapy using plant species containing proteases and /or nitrogen compounds in vitro to control mixed nematodes of sheep.
4. **Fomum S. W. and I. V. Nsahlai.** Constituting combinations of plant species containing different putative anthelmintic macromolecules; the case of alkaloids and/or condensed tannins, and proteases and/or nitrogen compounds in, *in vitro* control of mixed nematodes of sheep.
5. **Fomum S. W. and I. V. Nsahlai.** Use of plant species combinations containing flavonoids and others' containing proteases and/or nitrogen compounds to control nematodes of sheep in vitro.
6. **Fomum S. W. and I. V. Nsahlai.** *In vitro* combination phytoanthelmintic therapy using plant species possessing proteases and/or nitrogen compounds and flavonoids to control mixed nematodes of sheep.
7. **Fomum S. W. and I. V. Nsahlai.** Phytochemical anthelmintic, antibacterial, antifungal, antiviral and antioxidant profiling of selected plant species from GC/MS analysis.
8. **Fomum S. W. and I. V. Nsahlai.** Single and multiple relationships of identified phytochemicals of selected plant species with anthelmintic efficacy and synergistic effects from combination anthelmintic phytotherapy.

Table of Contents

List of Tables

List of Figures

Chapter 1

General introduction

Livestock parasites are a huge source of socio-economic losses to the industry worldwide as a result of their pathogenic activities (Zajac, 2006; Roeber *et al*., 2013). The impact of losses to stocks of global resource-limited farmers is unprecedented (Waller, 1999; Perry *et al*., 2002), and central to these, are gastrointestinal (GIT) nematodes (Perry and Randolph, 1999; Waller, 2004: Sargison, 2016a).

Most of these parasites were initially thought to be endemic in the warm wet regions of the world and in regions with a minimum monthly temperature of 18 º C and rainfall of 50 mm. These parasites of livestock now thrive even in the colder and drier parts of northern Europe (Waller and Chandrawathani, 2005). This spatial prevalence has partly been driven by climate change (Harvell *et al*., 2002, Van Dijk *et al*., 2010; Sargison, 2016a), that has resulted to rising temperatures that favour pre-infective nematode parasite survival and development. The spread might have also resulted from the evolution of more adapted nematode species to cold temperatures. Their occurrence is emphatically global (Torres-Acosta and Hoste, 2008), though climatic conditions impose various spans to viability of shed eggs and various larval stages during their external development (Nogareda *et al*, 2006; O'Connor *et al*., 2006; Roeber *et al*., 2013). The major difference lies in regular warm/hot temperatures and rainfall, favourable to chronic multiplication, survival and infection in the tropics (Barger, 1999) and subtropics (Banks *et al*., 1990) relative to temperate environments (Waller, 1997b). Some genera have adapted to different climatic regions of the world than others, and are relatively more prevalent (Nogareda *et al*., 2006). Nematode parasite of livestock at all levels of infection generally affect their health and productivity (Perry *et al*., 2002), impacting negatively on farm income and profit (Waller, 1997b; Zajac, 2006).

Their effects on livestock have been highly skewed towards grazing ruminants (Zajac, 2006; Jackson *et al*., 2012), in both developing (Perry *et al*., 2002) and developed economies (Perry and Randolph, 1999), with huge losses recorded (Sykes, 1994; Knox *et al*., 2006). Grazing/browsing ruminants are trapped in a cycle of infection, treatment and reinfection from

the range environment (Waller, 1997a). Their negative impact on efficient production of livestock at low burden is primarily by diverting nutrient resources from production to any of replacement, repair or outright losses (Coop and Kyriazakis, 2001). Additionally, host appetite is reduced, leading to insufficient nutrient intake to support production, reproduction, repair and other metabolic processes. Insufficient proteins ensuing from depressed appetite as a result of helminth infection, is further exacerbated by loss of endogenous proteins into the gastrointestinal tract by leakage of plasma proteins. There is also epithelial cell slough off, and huge quantities of mucoproteins being lost as well (Holmes, 1993; Coop and Kyriazakis, 2001). The collective pathogenic effects caused by nematode parasites on their host are referred to as parasitic gastroenteritis (PGE). The extent to which the infected host is affected depends on a number of factors.

1.1 Factors affecting pathogenicity of GIT nematode parasites of livestock

Generally, nematode parasite species challenge and size of infection are primary drivers of the type of disease potentially caused to the host animal. The level or magnitude of pathogenicity is a culmination of a number of secondary factors, some of which include diet, immunological status, stocking rate and stress, state of pasture and type, temperature and humidity, and management (Odoi *et al*., 2007).

1.1.1 Prevailing nematode parasite species in most pastoral ecosystems in the world

Nematode parasite species that are present within different agro-pastoral ecological zones of the world, are dependent on climatic conditions and their specific capacities to adapt to changes in temperature and moisture brought about by seasons (Anderson *et al*., 1978; O'Connor *et al*., 2006; Rose *et al*., 2016). Particular species can no longer be ascribed to specific regions of the world, because most of them have a global incidence, though with different levels of prevalence and are often mixed. Additionally, global changes in temperature have also aided the spread and prevalence of nematode species previously thought to be tropical or subtropical to temperate regions (Waller, 2004; Harvell *et al*., 2002; Sargison, 2016a).

Bunostomum species, Haemonchus contortus, Nematodirus battus, Oesophagostomum species, Teladorsagia circumcinta and *Strongyloides species* are some prevailing nematode parasite species of economic importance in temperate climates (Nogareda *et al*., 2006; Fox *et al*., 2012);

with *H. contortus*, *Teladorsagia colubriformis and Teladorsagia circumcincta* thriving best in tropical and subtropical regions (Krecek and Waller, 2006; O'Connor *et al*., 2006; Getachew *et al*., 2007). Under hot and dry weather conditions, all three species hardly survive out of their host in the process of external development (O'Connor *et al*., 2006).The main species of nematodes affecting small ruminants in the tropics and subtropics include *Haemonchus contortus*, *Teladorsagia circumcinta* and *Trichostrongylus* species, all of which belong to the order *Strongylida* (Anderson, 2000; Sutherland and Scott, 2010) and share common life cycle (Levine, 1968). Larval development of *Nematodirus species* takes place within the egg, setting it apart from other species of the same order (Roeber *et al*., 2013).

Haemonchus contortus with its wide distribution in small ruminant herds of the tropical, subtropical and regions with summer rainfall, is highly prolific and pathogenic (Sutherland and Scott, 2010). Disease caused to livestock results from L_4 larvae and adult worms, which feed on blood, eliciting anaemia and/or death depending on parasite burden, immunological and nutritional status of infected animals (Eysker and Ploeger, 2000). Another species of importance is *Teladorsagia species.*

Its multiplication is far lower than that of *Haemonchus species,* and pathogenic effects are different. Extensive damage is caused by L_4 larvae which burrow into parietal cells, forming nodules in abomasal mucosa. Hydrochloric acid from this site is reduced, resulting to accumulation of inactive plasma pepsinogen and reduced digestion of proteins. Its pathogenic effects are commonly seen in lambs, weaners and adults, and there is reduce wool growth in wool producing species (Donald *et al*., 1978). *Trichostrongylus* species is another genus of economic importance to grazing livestock.

This genus has three main species; *T. colubriformis*, *T. ragatus* and *T. vitrinus.* Pathogenic effects are caused by L_3 larvae which burrow between enterocytes of intestinal villi forming intra-epithelial tunnels (Beveridge *et al*., 1989) where they develop to young nematodes. Migrating growing worms further exert extensive damage to duodenal mucosa. General signs relating to *Trichostrongylus* infection include generalised enteritis, haemorrhage, oedema, plasma protein loss followed by hypoalbuminaemia and hypoproteinaemia (Barker, 1975; Barker and Titchen, 1982). Nematode parasites broadly either feed on blood or burrow into gastro intestinal mucosa; both pathogenic activities provoking gastrointestinal enteritis and relevant specific effects.

1.1.2 Larval/worm burden within host gastrointestinal tract and severity of pathogenesis

Larval/worm burden is critical to the magnitude of disease caused to the infected host (Jackson and Miller, 2006) and at clinical level of infection are associated with specific signs; though some of them may be common to most parasite species. *Haemonchus* species is usually linked to pathogenic signs including, haemorrhagic anaemia, dark coloured faeces, oedema, weakness, reduced production of wool and muscle tissue, and at times sudden death at acute infection. On the other hand, protracted infection with *Haemonchus contortus* will result to progression from depressed appetite, through weight loss and anaemia (Taylor *et al*., 2007).

Clinical infection with *Teladorsagia species* will cause diarrhoea, weight loss, low weight gain and decreased wool production (Taylor *et al*., 2007). Additionally, another important group of nematode parasites to grazing small ruminants is *Trichostrongylus species*, which mostly inhabit the small intestine.

Trichostrongylus species infection is usually associated with extensive damage to the small intestine, where some species establish, reduced nutrient intake and signs of malnourishment (Sykes and Coop, 1976). High parasite burden is characterized by prolonged watery diarrhoea, which stains hind quarters (Levine, 1968). *Trichostrongylus axei* establishes in the abomasum and is generally in small numbers. Interaction among nutrition, immune response and health are important elements that will either render animals susceptible or resilient/resistant to nematode infection and pathogenesis.

1.1.3 Nutritional status of animal host, immunological status and general health

Nutrition, immune expression and health are intricately connected (Coop and Holmes, 1996, Athanasiadou *et al*., 2008); with appropriate nutrition igniting the right link that enables proper function, integrity and productivity of animal organ system. Livestock in good health will enjoy a sound immunological status, thereby resisting/beating pathogenic effects of nematode parasites and other microbial pathogens (Coop and Kyriazakis, 2001). Sufficient nutrients including protein, essential amino acids, vitamins and minerals, will ensure that important biological activities in order of metabolic priorities are attended to (Coop and Kyriazakis, 1999). Biological activities include maintenance, growth, reproduction, and combating of

parasitic organisms and their negative effects. This state in essence, allows for integral biological function that wades off any pathogen establishment and its ensuing pathogenic activity. Animals in this condition will have the capacity to resist nematode parasite infection and/or withstand it (Knox *et al*., 2006), in situations where they are infected. This paints an ideal picture, but what obtains generally in the tropics and subtropics contrast sharply, with characteristic poor quality roughages (Leng, 1990; Jackson and Miller, 2006).

Persistent malnutrition of livetock is common in the tropics and subtropics, and where feeds of good quality are available, the animals are fed insufficient quantity (Waller, 1997a). Either of undernutrition or malnutrition predisposes livestock to parasite infection and disease attack. Some of these factors include favourable temperatures for nematode egg hatch and development, sufficient moisture, degraded and overgrazed pasture, overstocking, stress and poor management, are predisposing factors to nematode parasite infection.

1.1.4 Environmental factors including state of pasture, stocking rate, management and stress

Temperature, moisture, rainfall and humidity are important environmental conditions that have an overbearing influence on development and survival of common ruminant nematode parasites including *H. contorts*, *Teladorsagia circumcinta* and *Trichostrongylus colubriformis* outside the host (Temberly *et al*., 1997; O'Connor *et al*., 2006). Warm temperatures will enhance nematode parasite development, while very low temperatures will have the opposite effect; but moisture is critical to further development to infective stage (Rossanigo and Gruner, 1994). Interaction among environmental factors favourable to nematode egg hatch, larval moulting and survival (Getachew *et al*., 2007), will certainly predetermine the potential level of new infection and reinfection. Closely linked to the environment is the nature and state of pasture.

Good pasture can be maintained by carefully managing stocking rate, to ensure that the requisite number of animals are grazed within the appropriate area in order to retain its integrity (Waller, 2004; Higgins *et al*., 2007). Non respect of these critical parameters by over stocking beyond range capacity to sustainably regenerate pasture, will lead to environmental degradation and greater exposure to parasites. This scenario creates a favourable environment for parasites to thrive and animals to easily encounter them, since they will be grazing very

close to the soil. Degraded pasturelands with over grazed pasture, will therefore make for easy encounter between livestock and nematode parasites, leading to reinfection and new infections (Waller, 2004), besides poor quality of such pasture. Human input by way of management of the state of pasture, stocking rate and incidental environmental conditions becomes a critical input. This important management input either tilts the tide towards creation of favourable conditions for nematode parasite propagation or mitigation/control. The appropriate option will be the later, though with very limited control over climatic conditions such as temperature, rainfall and humidity, since most husbandry activity is outdoor. This leaves us with the singular option of nematode parasite control, since infection and reinfection can only be mitigated and not completely prevented (Thamsborg *et al*., 2010).

1.2 Control of nematode parasites of livestock

Nematode parasite control evolved from the use of plants and plant related products (Waller, 1999; Githiori *et al*., 2006) to development and widespread modern-day reliance on chemical anthelmintics (Waller, 1997b; Coop and Kyriazakis, 2001). This followed remarkable growth of the industry and the quest for more efficient products and easier approaches to mass therapy. Other related approaches in use include biological control (Larsen, 1999; Waller, 2006b) and managerial strategies to evade or mitigate livestock nematode parasite contact and infection, referred to as grazing management control methods or techniques (Barger, 1999; waller, 2006b).The adoption of anthelmintic therapy has not been without major challenges.

1.2.1 Challenges of contemporal nematode parasite control and prospective novel approaches

The adoption of chemical anthelmintic therapy as the most preferred choice of helminth control on the merits of relative clinical efficacy (Besier, 2006) in the livestock industry globally, has been replete with major problems. Selection for chemical anthelmintic resistance has been a major growing challenge (Kaplan, 2004), not long after its introduction in the livestock industry and loomed large in the small ruminant sector (Waller, 1999; Kaplan, 2004). It is imperative that previously adopted control methods that were dropped or no longer commonly used by most stake holders and novel ones be explored for potential refinement and/or further development. These have the advantage of diversifying control methods and conserving their different efficacies, since control will revolve around a number of them.

Other methods of nematode parasite control include phytochemical control using plants and plant related products (Githiori *et al*., 2006), biological control using nematophagous fungi (Larsen, 2006; Ketzis, *et al.*; 2006), use of vaccines to induce and develop immunity against nematode parasites of livestock (Bain, 1999; Hein and Harrison, 2005), selection of animals for genetic resistance against nematode parasites (Stear *et al*., 2001). Parasitic nematodes obtain their nutrients/nourishment from the host animals in various ways; some of which are more harmful than others; leading to various disease conditions. Although a wide range of nematode species affect livestock, *Haemonchus contortus* is economically the most important because of its relative pathogenic nature (Waller and Chandrawathani, 2005).

Fundamentally, these parasites exert a huge impact on the productivity and survival of livestock (Perry *et al*., 2002), and demand appropriate measures to keep them in check or possibly eradicate them.

1.3 Prevailing treatment methods

Several approaches have been adopted to control helminth parasites of livestock (Waller, 1997a), some of which have been dropped in preference for those deemed more effective and manageable. Anthelmintic therapy/prophylaxis originated from plant related products and their extracts (Waller, 1999), but has been overwhelmingly abandoned in preference to chemotherapy (Sargison, 2016b). Strong resurgence of anthelmintic resistance to these methods and products has re-enkindled interest in previously abandoned ones with room for improving anthelmintic efficacy, leading us to set this as a main objective for the project that follows.

1.4 Objectives

The primary objective of this research was to explore how the anthelmintic efficacy of selected plant species can be optimized by combination phytotherapy in small ruminants. Additionally, potent combinations that prove to be more efficacious *in vivo*, will be developed further and placed at the disposal of the livestock farmer to treat livestock.

The secondary objectives included:

(1) Assay selected plant species, in goats and sheep *in vitro*, for their individual anthelmintic efficacy at different doses, as a basis for combination anthelmintic therapy;

(2) Determination of the most effective dose of every selected plant species *in vitro* for both goats and sheep for relative efficacy with regards to concentration, animal species and their interactions;

(3) Quantitatively determine alkaloids, condensed tannins and flavonoids in selected plant species as primary putative anthelmintic macromolecules for their potential association with anthelmintic efficacy;

(4) *In vitro* evaluation of combined anthelmintic efficacy of constituted plant species combinations possessing commonly shared putative anthelmintic macromolecules in sheep;

(5) *In vitro* evaluation of combined anthelmintic efficacy of constituted plant species combinations possessing different putative anthelmintic macromolecules in sheep, and any potential influence of these macromolecules;

(6) Analyse plant species ethanolic crude extracts for their biochemical composition using GC/MS, and profile identified biomolecules for biological activities.

(7) Biochemicals identified are evaluated for their relationship with important parameters of healing traits including expected combined efficacy, observed combined efficacy, simple and Webb's synergies; and

(8) *In vivo* evaluation of selected plant species combinations to determine potential combined doses that will yield least nematode egg shed and, egg hatch and larval recovery over time in Merino sheep.

1.5 Thesis structure

- General introduction (chapter 1).
- Review of literature related to research project (Chapter 2).
- *In vitro* anthelmintic efficacy of ethanolic crude extracts of selected plant species against nematode parasites of goats and sheep (Chapter 3).
- *In vitro* combined anthelmintic efficacy of selected plant species possessing similar putative principles against nematode parasites of sheep (Chapter 4).
- *In vitro* combined anthelmintic efficacy of selected plant species possessing different putative principles in sheep (Chapter 5).
- Identification of major compounds of selected plant species and their concentrations using GC/MS, their biological activities including anthelmintic, antibacterial, antifungal, antioxidant and antiviral activities (Chapter 6).

- Influence of identified biochemical molecules on observed combined efficacy and synergy (chapter 7).
- *In vivo* dose evaluation of selected combinations of plant species in Merino sheep (chapter 8).
- Thesis overview and recommendations for further research (chapter 9).

1.6 Hypothesis

It is hypothesized that combined anthelmintic phytotherapy of small ruminants will not yield better treatment effects relative to that of single plant species and chemotherapy method.

1.6.1 Subhyptheses include:

1. It is hypothesized that plant species exerting anthelmintic activity do not have any effects of concentration, animal species, or interaction of plant species, animal species and concentration on observed efficacy.
2. Combination of crude extracts of plant species exerting anthelmintic activity will produce no synergistic or antagonistic effect and similarly the observed efficacy, synergy or anatagonism is not related to the alkaloid, condensed tannins and flavonoid content of the plant species.
3. Combination of plant species crude extracts containing different anthelmintic macromolecules will interact to produce no synergistic effects and correspondingly, plant species content of any or all of alkaloids, condensed tannins and flavonoids will have no effect on observed efficacy and synergy.
4. Anthelmintic activity exerted by plant species is an attribute of whole plant species, as exhibited by species crude extract and will not relate to a specific compound or a group of phytochemicals.
5. Phytochemicals and related macromolecules exerting anthelmintic activity, will associate with observed efficacy, simple synergy and Webb's synergy. Additionally, phytochemicals exerting anthelmintic activity will interact with other phytochemicals to affect observed efficacy, simple and Webb's synergies.
6. Dosing of sheep with different concentrations of selected plants species combined crude extracts will have no effect on egg count per gram, egg hatch and larval recovery.

The study was carried out in the animal section of the University Research Farm at Ukulinga, using goats and sheep. Laboratory work was done in the post graduate laboratory of Animal

and Poultry Science and related facilities in the School of Agriculture, Earth and Environmental Sciences.

CHAPTER 2

Literature Review

Literature relating to livestock helminths, and specifically gastrointestinal (GIT) nematodes, epidemiology, global prevalence, control schemes, strategies to mitigate deleterious effects on animal health and productivity, and potential approaches to enhance these strategies will be reviewed. This serves as a conceptual basis for possible improvement of one of the pioneer, most strategic, consumer and environmentally friendly but less implemented and complementary scheme, 'Ethnoveterinary control using plants/plant products'. Ethnobotanical control of livestock nematodes is central to this study. Common bioactive principles in plants will be explored, as a basis to identify from an extensive body of knowledge potential and possible anthelmintic principles.

2.1 Global scope and prevalence of helminthiasis in livestock

Gastrointestinal nematode parasite infection represents a major global threat to livestock health and productivity (Perry *et al*., 2002; Vercruysse *et al*., 2006; Waller, 2006a). These parasites occur in tropical/subtropical (Miller, 1996; Stepek *et al*., 2004) and temperate environments (Torres-Acosta and Hoste, 2008), thus affecting stocks across all agro-ecological zones (Githiori *et al*., 2004). The free living developing larval stage have various spans of viability, that is dependent on prevailing climatic conditions such as humidity and temperature (O'connor *et al*., 2006; Chaudary *et al*., 2007).

Infective L_3 larvae have survival span under tropical and subtropical conditions ranging from 1 – 3 months, whereas under temperate conditions the life span ranges from 6 – 12 and sometimes 18 months (Torres-Acosta and Hoste, 2008); when environmental and climatic conditions are favourable. In tropical/subtropical regions hot and wet conditions shorten larval survival, whereas in temperate regions, cool weather prolongs it (Barger, 1999), potentially because of the effect of temperature on metabolic activity. Under drought or frost conditions, survival span is markedly reduced on both sides of the divide because of the susceptibility of L_3 larvae to both temperature extremes (O'Connor *et al*., 2006). Gastrointestinal nematode parasitism has been identified as one of the biggest health challenges of small scale livestock owners in Africa and Asia (Perry *et al*., 2002); whose stock are predominantly goats (*Capra*

hircus L.) and sheep (*Ovis aries* L.), (Krecek and Waller, 2006; Devendra and Solaiman, 2010). Although grazing stocks are generally affected, small ruminants have been acknowledged to be the most affected (Eysker and Ploeger, 2000; Stear *et al*., 2007).

Gastrointestinal nematodes affect efficient production of small ruminants (Sykes, 1994), resulting in major economic losses in the industry at both subclinical and clinical levels (Eysker and Ploeger, 2000). These losses range from reduced weight gain (Ploeger and kloosterman, 1993; Agarwal and Banerjee, 2007), decrease fertility (Ankers *et al*., 1998), decrease milk production (Gross *et al*., 1999), severe weight loss due to depressed appetite (Anthanasiadou and Kyriazakis, 2004) and mortality of young vulnerable stock (Over *et al*., 1992; Eysker and Ploeger, 2000; Sissay *et al*., 2007) as a result of blood or plasma protein losses and damage to the gastrointestinal tract (gastroenteritis). Damage to the gastrointestinal tract (GIT) is usually caused by L_3 and L_4 larval activities, which bore, lodge and form tunnels in the mucosa and submucosa in the process of development to the adult stage. Adult worms in turn exert their own harmful effects on animal host (Eysker and Ploeger, 2000). The challenge of gastrointestinal nematode of livestock also affect large scale resource limited farmers in developing countries (Dhar *et al*., 1982; Lorimer *et al*., 1996; Waller, 1997a; Jackson and Miller, 2006; Sissay *et al*., 2007), a greater bulk of whom rear small ruminants. Even in developed economies with sufficient technical support to nematode parasite control, resources which would have aided further growth and expansion of the industry are directed to the health sector (Waller, 2006b). This results to economic losses globally. The chronic prevalence of livestock parasitic nematodes in grazing livestock sector can be attributed to inadequate knowledge by most stake holders of the epidemiology and poor husbandry practices (Akhtar *et al*., 2000; Bidkar *et al*., 2012), some of which result from shared grazing land and are unavoidable (Papachristou *et al*., 2003). The set-up complicates unilateral management decisions that would effectively disrupt or break the life cycle and stop multiplication and infection.

Nematode larval growth and development entails exsheathment as it grows, moults and progresses from external to internal development and establishment within the animal host. It is explicit that any intervention that interrupts exsheathment, breaks the life cycle and disrupts potential infection (Dakkak *et al*., 1981; DeRosa *et al*., 2005). In this part of the world, grazing livestock husbandry is primarily done extensively on communal lands (Papachristou *et al*., 2003). This common practice renders the implementation of alternative preventive measures, such as grazing management strategies and other improved techniques to control helminthiasis,

impracticable. Another case in point is in areas where land is scarce and animals usually return to previously grazed areas during warm and humid periods, facilitating reinfection (Chaudary *et al*., 2007; Sutherland and Scott, 2010). Additionally, modern veterinary support services are scarce (Wasswa and Olila, 2006; McGaw and Eloff, 2008; Tariq *et al*. 2009), unaffordable or absent (Hammond *et al*., 1979) in most developing economies. The presence and fecundity of nematode parasitism in the warm wet tropical animal milieu is almost always chronic (Tariq *et al*., 2009), considering the prevailing humid environmental climatic conditions and temperature (Miller, 1996; Taylor *et al*., 2007). These conditions allow for recurrent substantial level of egg hatch and survival of free living stages of larval parasite over prolonged periods or incidentally when climatic conditions favour high egg hatch and development. The socioeconomic and nutritional benefits expected from the livestock husbandry, some of which include wealth, essential products such as meat, milk, skin, fibre, vital draft service in some areas and manure (Coop and Kyriazakis, 2001) will be compromised and lost. There is, therefore, the need for efficient control in order to retain these benefits. Sustainable supply of these animal products and related services require that adequate attention be accorded to livestock health, and more especially nematode control. Greater insight will be gained by identifying different nematode parasites of economic importance to grazing livestock.

2.2 Classification of nematode parasites and economic importance to ruminant health

Helminths have two broad phyla, *Platyhelminths* (flatworms) and Nemathelminths (roundworms) (Soulsby, 1982). Central to this study are the round worms, though the flat worms also pose a serious health challenge to ruminants, they fall out of the scope of this study.

Nematodes belong to the phylum Nematoda (Hodda, 2007) and comprise of two major classes, Secernentea and Adenophorea. Secernentea contain major parasites of terrestrial vertebrates, most of which feature among the following orders: Ascaridida, Oxyurida, Rhabditida, Spirurida and Strongylida (Strongylids) (Sissay *et al*., 2007; Sutherland and Scott, 2010). The order Strongylida includes most of the nematode species that cause gastrointestinal diseases to ruminants (Sutherland and Scott, 2010). Strongylida contains several superfamilies, with the superfamily Trichostrongyloidea carrying the bulk of ruminant nematode parasites. They are small and slender, most of which are 2cm long or less. They possess simple, small mouths, which enable them to browse the mucous layer in search of small particles of matter, mucus and dissolve molecules. A few important genera are found in other superfamilies, such as Ancylostomatoidea and Strongyloidea, which are characterized by a stout nature relative to

trichostrongyloids. Ancylostomatoidea contains blood-feeding hook worms, while strongyloidea has a number of plug feeding nematodes, which ingest a plug of host tissue, digest it into liquid form by enzymatic action in the buccal capsule and swallow. Nematodes of the class Adenophorea are aquatic and have two important parasites of vertebrates: *Trichuris* spp and *Trichinella* spp. Of special focus are nematode parasites of livestock.

Nematodes of veterinary importance include *Haemonchus contortus* Rudolphi (1803) (Barbers pole worm), *Ostertagia ostertagi, Ostertagia circumcincta* (small brown stomach worms), *Trichostrongylus colubriformis* Giles (1892), *Trichostrongylus vitrinus* Looss (1905), and *Trichostrongylus axei* Cobbold (1879) (Emery *et al*., 1993). Other species of less potency include, *Nematodirus spathiger* Railliet (1896), *Cooperia curticei* Railliet (1983), *Bunostomum trigonocephalum* Rudolphi (1808) *Oesophagostomum* species. Important insight of potential control strategies will be gained by briefly reviewing the life cycle of most common nematode parasites. The effect of these nematodes on animal health and production is dependent first on the species mode of obtaining nourishment and secondly on the infection load. Review of a typical livestock nematode parasite life-cycle will shed light on the rationale for adopting control strategies or mechanisms and other potential interventions that aid effective control.

2.3 Life cycle of a typical nematode parasite

Typical nematode parasites of small ruminants have a life cycle (Figure 2.1) that does not involve an intermediate host, and sexes are distinctly separate (Sissay, 2007). Worms develop to maturity within host, where breeding occurs, eggs are laid and shaded in faeces. Shade fertilised eggs hatch into first-stage larvae (L_1), which grow under conducive conditions of humidity and temperature within faecal material. L_1 larvae moult into second-stage larvae (L_2). L_2 larvae sustain themselves by feeding on bacteria, and again moult to third-stage larvae (L_3), which are infective. L_3 larvae migrate out of moist faecal material and wriggle up vegetative portions of pasture; moisture playing an important role in the process and are pick up by grazing host. When prevailing temperature and moisture are favourable, development from eggs to infective larvae can take place between 7 and 10 days. This is typical of most tropical or subtropical environments, and summer periods in temperate areas. Within the host (goat and sheep) and under normal conditions, L_3 larvae moult into fourth-stage larvae (L_4), within 3 to 4 days of infecting host and finally moult to young adult parasites between 10 to 14 days (Soulsby, 1982; Coffey *et al*., 2007). Pre-patent period (time from infection of host by L_3 larvae to laying of eggs by adult female worms), differ among various nematode parasite species.

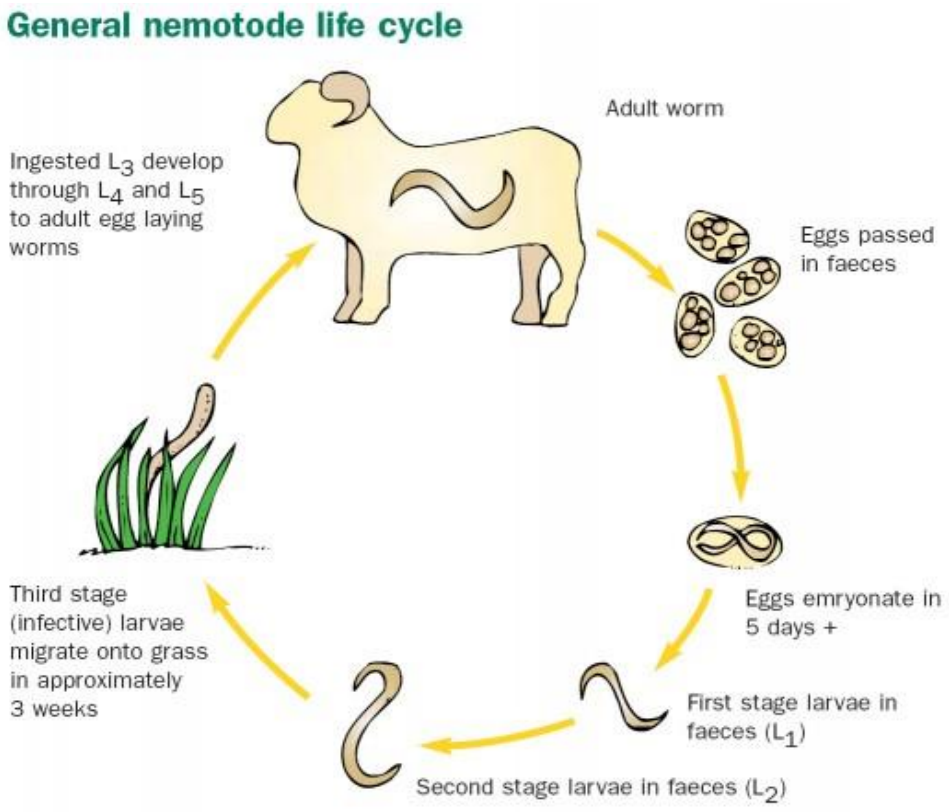


Figure 2. 1: General nematode life cycle: downloaded from
http;//www.farmanimalhealth.co.uk

Considering major nematode parasite species of economic importance, such as *Haemonchus contortus*, the life cycle ranges between 18 and 21 days (Le Jambre *et al*., 1970), *Cooperia* species between 15 and 20 days (Kaufmann, 1996), *Nematodirus* species between 15 and 28 days (Lindahl *et al*., 1970), *Oesophagostomum* species between 6 and 7 days (Talvik *et al*., 1997) and between 20 and 25 days for *Trichostrongylus* species (Kaufmann, 1996). A review of most nematode parasite, genera, some specific morphological details, predilection sites, prepatent periods and major ruminant host are presented in the table below (Table 2.1).

Table 2. 1*:* Common genera of nematodes affecting ruminants and some monogastrics, their predilection sites and pre-patent periods

Family	Genera	Species	Morphology (adult length)	Predilection site	Prepatent period (days)	Ruminant host
Trychostrongyldae	*Haemonchus*	*H. contortus*	♂10-20, ♀ 18-30	abomasum		Goats & sheep
		H. placei, H. similis			18-21	
	Ostertagia	O. *ostertagia, O. lyrata, O. circumcincta*		abomasum		Cattle & sheep
Trichostrongylidae	*Teladorsagia*	*T. circumcincta, T. trifurcate, T. davtiani*			15-21	
	Trichostrongylus	T. axei			15-23	Cattle, sheep, goats, deer, pigs and horses
		T. colubriformis, T. vitrines, T. longispicularis, T. rugatus	Less than 1 cm long	Small intestine	15-23	
Molineidae	*Nematodirus*	*N. battus, N. filicolis, N. spathiger, N. helvetianus*				Sheep, goats & cattle
T**richostrongylidae**	*Coopria*	*C onchophora, C. punctate, C. pectinate, C. surnabada, C. curticei*			14-15	Cattle & sheep
Chabrtiidae	*Oesophagostomum*	*Oe. Radiatum, Oe. Colubianum, Oe. Venulosum*,		Large intestine	40-45	Cattle & sheep
	Charbetia	*C. ovina*			42-50	

Compiled from Soulsby (1982) and Sutherland and Scott (2010)

2.3.1 Specific effects of nematode parasites and gastrointestinal predilection sites in livestock host

Nematode parasite gastroenteritis of livestock is generally manifested by clinical signs such as depressed appetite (Athanasiadou and Kyriazakis, 2004), anaemia, oedema, diarrhoea and anorexia (Eysker and Ploeger, 2000; Roeber *et al*., 2013). Infective nematode larval stage and adults of different nematode species exert different deleterious effect (gastroenteritis) in the

predilection sites of the gastrointestinal tract where they are lodged. They will be examined in the section below following their different predilection sites and their effects on animal health.

2.3.2 Abomasal nematode genera

Haemonchus contortus and Haemonchus placei are the main species of this genus. *Haemonchus contortus*, is a blood sucking nematode that attaches to the abomasal mucosa via its mouth parts, causing anaemia and related submandibular oedema (Eysker and Ploeger, 2000). In severe infection it causes death (Besier, 2007; Roeber *et al*., 2013). *Haemonchus* spp. is the most pathogenic and prolific with females producing in the neighbourhood of 10,000 eggs per day (Sutherland and Scott, 2010). *Haemonchus c*. is mainly a parasite of goats and sheep, but can occur in cattle and some species of deer while *H. placei* is a parasite of cattle. Another species of importance is *H. similis*, which occurs in cattle and deer in North America and Europe. *Haemonchus* spp. generally thrives under warm and moist conditions, and poses a serious challenge in tropical and subtropical regions of the world (Sutherland and Scott, 2010). Common names include twisted stomach worm, Barber's pole worm and wire worm. Adults of these species range between 2 and 3 cm long.

Another abomasal genus of importance is *Ostertagia* spp. The main species include *O. ostertagi* and *O. lyrata*, both of which are considered as the same species (Sutherland and Scott, 2010). *Ostertagia o.* and *Ostertagia circumcincta* larvae penetrate the gastric glands of the abomasum of cattle and sheep, before emerging to lodge on the mucosa. Adult worms are slender, brownish red and about 1cm in length. *Ostertagia spp*. is less prolific relative to *Haemonchus* spp., laying 50 eggs per day. *Ostertagia leptospicularis* is a primary parasite of cervids but has been periodically recovered from cattle and sheep. Other abomasal genera include *Teladorsagia* spp. and *Trichostrongylus* spp.

Teladorsagia, comprises of *T. circumcincta, T. trifurcata and T. davtiani*, all of which occur in goats and sheep. They are brown and similar in colour to *Ostertagia spp*. On the other hand, *Trichostrongylus* spp. is the smallest of abomasal nematodes. *Trichostrongylus axei* is the only species of this genus that has been consistently recovered from the abomasum. *Trichostrongylus axei* occurs in cattle, sheep, goats, deer, pigs and horses.

2.3.3 Small intestinal nematode genera

Trichostrongylus are commonly found in the small intestine and comprises of the following primary species: *T. colubriformis, T. vitrinus, T. longispicularis* and *T. rugatus*. They are less than 1cm in length and relatively small. *Trichostrongylus colubriformis* and *T. vitrinus* are important parasites of sheep and goats, whereas *T. longispicularis* is mainly a parasite of cattle. In subtropical and warmer parts of temperate regions, *Trichostrongylus* spp. contributes significantly to ruminant ill health (Sutherland and Scott, 2010). Animals infected with worms of this genus display very high egg counts due to the prolificacy of the female *Trichostrongylus* spp. Nematode worms of this genus are occasionally referred to as black scour worms because of watery diarrhoea that stains fleece of hind quarters (Roeber *et al*., 2013).

Another small intestinal genus of significance is *Nematodirus,* which has *N. battus, N. filicollis, N. spathiger* and *N. helvetianus* as main species. Morphologically, female nematodes of this genus have narrower anterior relative to posterior, a trait that reflects the prolific egg producing capacity of the parasite. They are described as thin-necked or thread-necked worms (Sutherland and Scott, 2010). *Nematodirus h*. is mainly a parasite of cattle whereas the other species commonly affect small ruminants. *Nematodirus a*. infects calves in addition to small ruminants. *Nematodirus* spp. is different from other *Trichostrongylus* in that larvae develop to the ensheathed L_3 stage within the egg. Their eggs are usually large in size and development is slow.

A third genus of small intestine is *Cooperia,* which has the following main species *C. oncophora, C. punctate, C. pectinata, C. surnabada (C. mcmasteri)* and *C. curticei. Cooperia* spp are parasites of cattle and sheep. *Cooperia c*. is usually recovered from sheep. *Cooperia punctata* and *Cooperia pectinata* have larvae that are more invasive and damaging to animal health, while the other species are of mild pathogenicity (Taylor *et al*., 2007).

2.3.4 Large intestinal nematode genera

There are two main genera of nematode parasites that inhabit the large intestine of livestock, *Oesophagostomum* and *Chabertia ovina. Oesophagostomum* comprises of *Oe. radiatum, Oe. columbianum* and *Oe. venulosum.* They are 2 cm in length and described as nodule worms because of the nodules that develop around larvae that have burrowed into the submucosa of host intestine. *Oesophagostomum venulosum* and *Oe. columbianum* infect sheep, but *Oe. venulosum* is relatively more common. *Oesophagostomum venulosum* is least pathogenic. Nematode parasites of this genus are plug feeding in nature. Adult parasites have shallow

buccal capsules, which mitigate pathogenicity. *Oesophagostomum radiatum* is a worldwide strongyloid parasite of cattle. The larvae burrow and encyst in the submucosa of the distal small and large intestines forming nodules and inflammation. Nodules are subsequently filled with green oesinophilic pus and become more pronounced at repeated infection or exposure. Clinical signs include diarrhoea, which follows the emergence of parasite from submucosal burrows.

Chaberta ovina has a large bell-shape buccal capsule that allows the parasite to take large bites of intestinal mucosa and contrast sharply with *Oesopagostomum*. It is very pathogenic but the parasite load is often low. However, as small as 300 adult parasites can cause clinical signs (Sutherland and Scott, 2010).

Diarrhoea is a common feature of ostertagiosis, trichostrongylosis, nematodirosis (Eysker and Ploeger, 2000). Control of helminth parasitism of livestock will culminate in improved gastrointestinal integrity and feed utilization, productivity and general health (Piedrafita *et al*., 2010). *Haemonchus contortus* is the most pathogenic and endemic species in tropical and subtropical Africa and Asia (Waller, 2003).

Given pathogenic effects of various nematode species that affect grazing livestock and the threat posed to the economy of stake holders and that of nation states, it is but necessary that appropriate and effective control strategies be adopted.

2.4 Control of livestock helminths

Conventionally, orthodox chemical remedies are used overwhelmingly in routine control of livestock helminths (Lorima *et al*., 1996; Waller, 1997c; Githiori *et al*., 2004; Jackson and Miller, 2006; Waller, 2006; Kaminsky *et al*., 2008; López-Aroche *et al*., 2008). From their inception, they have been credited with greater efficacy (Waller *et al*., 2001), easier application at treatment (Mirdeilami *et al*., 2011) and protective cover for a wide variety of helminths and parasitic organisms (Waller, 2006b; Waller, 1997c). Additionally, they have the capacity to provide dual curative and preventive cover (Jackson and Miller, 2006). The merits of chemical anthelmintic control and development of increasingly small dosing having a wider spectre of activities spanning across genera (Waller, 2006b), made it a primary global helminth control method of choice for a long time.

2.4.1 Prevailing methods of livestock nematode parasite control

Control of these parasites is paramount to sustaining animal health and productivity, considering the present and projected global economic losses from their infection (Perry *et al*., 2002; Krecek and Waller, 2006a), if appropriate measures are not taken. Animal gut health (Eysker and Ploeger, 2000) is critical to efficient utilization of feed resources and general productivity. Most control schemes target returning parasite population below the level at which harmful effects will be caused to the host population, rather than complete eradication (Larsen, 2000), which is difficult. Different control schemes have been applied to combat nematodosis in various parts of the world, with mixed outcomes. Control either targets parasite in host or the free living stages in the environment. These methods have various options and include chemotherapeutic control (Waller, 1997c; Stepek *et al*., 2005; Jackson and Miller, 2006), biological control (Grønvold *et al*., 1996), immunological control or vaccination (Knox *et al*., 2010), selection for resistant animal stock to nematode parasites (Wanyangu *et al*., 1997; Stear *et al*., 2007), grazing management (Barger, 1999) and use of ethnoveterinary plants and plant products (Waller, *et al*., 2001). A review of each control scheme or method will shed some light on how it is operated, its benefits to livestock industry and suggested strategies to enhance their different efficiencies.

2.4.2 Chemotherapeutic and chemoprophylactic control

This method of nematode helminths control has been an age-old practice to curb livestock losses (Macedo *et al*., 2010), and involves the use of chemical substances, which exert their activities in various ways. Some of these modes of action include prevention of parasite from establishment or being lodged in host, expelling parasites from gastro-intestinal tract, tissues/organs of livestock or disrupting vital parasite biochemical processes and killing them in the process (McKellar and Jackson, 2004). On the other hand, chemoprophylaxis is the use of a chemical anthelmintic to prevent infection and/or establishment of helminths within the host. The anthelmintic effects of these compounds may be on the adult helminths, larvae and/or eggs. They can also exert anthelmintic activity on a wide variety of helminths; in which case, they are referred to as broad spectrum in activity (Geary *et al.*, 2012). The finest and most efficient forms of current anthelmintic in terms of dose, spectrum and tolerance (Getachew *et al*., 2007), evolved from candidates that had narrow spectre, required huge doses to exert their

anthelmintic activities, had considerable toxicity and were less tolerant (McKellar and Jackson, 2004)

Broad spectrum chemical anthelmintics are ideally recommended for helminth control in livestock because infections are often multi-specific (Waller, 2006b; Ravindra and Anita, 2007). They are grouped into three classes, viz. (1) benzimidazoles and probenzimidazoles, (2) Imidazothiazoles and/or tetrahydropyrimidines and (3) macrocyclic lactones (Stepek *et al*., 2004; Githiori *et al*., 2006; Waller, 2006b; Stear *et al*., 2007). Chemical anthelmintics have been developed from plants and plant material that possess anthelmintic activity, and which were relatively of low efficacy and also perceived to be generally hazardous (Waller *et al*., 2001).

Benefits of chemical control of livestock nematodes have been great, but diverse in efficacy in different parts of the world (Henrioud, 2011), owing to improper use and routine dosing to both highly infected and sub clinically infected animals (Farias *et al*., 1977, Wolstenholme *et al*., 2004; Sargison, 2016b). Generally, the global mode of application has been massive in the livestock industry and more often indiscriminate and abusive (Lorimer *et al*., 1996; Stepek *et al*., 2005; Shaik *et al*., 2006; Kamaraj *et al*., 2011; Bidkar *et al*., 2012). This has led to the crises of selection by livestock nematodes for resistance (Waller, 1997c).

In major parts of the developed world, where chemical anthelmintics are readily available with efficient veterinary support services, deployed resources, modern livestock industrial practices and enforceable legislation, their use in control programs is relatively sound (Waller, 1997b; Henrioud, 2011). However, huge discrepancies exist in the developing world and incidentally in the developed world, depending on know-how of the stockholder, scale of production, available resources, government monitoring/intervention and ability of livestock farmer to properly administer them (Waller, 2006b). On a global scale, most of these anthelmintics and techniques developed for their administration, have been improperly applied (Van Wyk, 2001), further exacerbating the existing crisis of general selection for resistance by parasitic nematodes. Additionally, management of nematode parasite problems in organic livestock regimes, which advocate for non-application of chemical remedies and lack of conventional regulation on drug use (De and Sanyal, 2009), have also contributed immensely to emerging development of resistance against chemical anthelmintics.

2.4.2.1 Global disparities in technical support, availability, affordability and quality of anthelmintic regimes

General lack of sufficient technical knowledge and veterinary support services (Besier, 2006) have been major drivers to improper use of chemical anthelmintics. In countries such as Australia, New Zealand, Brazil, Paraguay, Uruguay and South Africa where the livestock industry is well-developed and chemical anthelmintic remedies regularly available and used, anthelmintic resistance is rived (Prichard, 1990; Van Wyk, 1990; Hennessy, 1997; Jackson and Coop, 2000). Prevalence of resistant parasites in this context renders it absolutely difficult to attribute selection for anthelmintic resistance only to ill-equipped stakeholders in the industry. Where authentic and efficacious anthelmintics are available, most livestock farmers lack of resources to procure them (Githiori *et al*., 2006). Additionally, in developing economies with unregulated markets and absence of quality control, there are anthelmintic products of doubtful quality (Githiori *et al*., 2006), which may largely aid selection for resistant parasites, compromising the purpose for which they were applied. More often in the developed economies, selection for resistant parasites might have arisen from frequent and indiscriminate anthelmintic use (Besier, 2006). This has led to a universal crises of chemical anthelmintic inefficacy and selection for resistant strains of parasites (Newton and Munn, 1999; Henrioud, 2011). Another important means of treatment is the use of copper oxide wire.

2.4.3 Use of copper oxide wire particles

Copper oxide wire particles (COWP) exert anthelmintic activity against *Haemonchus contortus* by reducing infection load in goats (Chartier *et al*., 2000) and sheep (Knox, 2002; Burke *et al*., 2004). Copper oxide particle are able to exert extended anthelmintic activity by lodging in abomasal mucosa and being released gradually to act on susceptible nematode species (Jackson and Miller, 2006). It poses serious threat to the life of multiple born offspring of ewes at late pregnancy and should be set aside at this stage (Burke *et al*., 2005). It will in essence serve as an important alternative, where resistant strains of *Haemonchus contortus* have emerged from long and consistent anthelmintic use. The use of substances or candidates that evoke appropriate immune response and in the process deter anthelmintic use in controlling gastrointestinal nematode parasites is vital.

2.4.4 Immunological control and/or vaccination against livestock helminths

Various immunological control options are being used to reduce or curb dependence on anthelmintics. These include: vaccination, manipulation of the immune system by way of supplemental nutrition (Knox *et al*., 2010) and selection of resistant/resilient animal stocks (Jackson *et al*., 2009). Epidemiology amongst various gastrointestinal nematode (GIN) parasites depends in part on host acquired immunity, while the development and maintenance of immunity hinges on host plane of nutrition (Jackson *et al*., 2009).

Immunization against livestock helminth species stems from resistance of animals to re-infection after a primary parasite infection (Emery *et al*., 1996); in which case parasite antigens evoke some relative immune defence. A low harmless presence of helminths is an important prerequisite to initiate and groom immune response. Two types of antigens have been identified with nematode parasites: (1) Soluble excretory and/or secretory antigens; and (2) somatic antigens that are fixed on the external surface or within the parasite (Newton and Munn, 1999). These antigens ignite immune response in the host at infection and constitute natural antigens. Other antigens exist, which do not ignite any immune response at infection with nematode parasites and are referred to as hidden antigens (Newton and Munn, 1999). These antigens form the basis for the development of vaccines against livestock parasitic helminths. When host is immunized with the right gut membrane from a blood-eating parasite, a high antibody titre is raised, evoking immune related processes. Parasites feed on host and ingest antibodies, which bind on brush borders of intestinal cells, impairing digestive processes leading on-set of starvation, compromising egg production; they become weak, detached from predilection site and are eliminated from the system (Knox *et al*., 1995; Jackson and Miller, 2006). Antigens identified in these processes are proteases that probably take part in digestion of blood meal (Longbottom *et al*., 1997). Contrary to acquired immunity that is developed and transmitted to subsequent generations from primary infection, immunity from vaccines against GIN is referred to as induced and requires subsequent dosing to boost its defence activity.

Acquired and induced immunity restricts parasitism such that the effects of parasitism on host survival and fecundity are markedly reduced to insignificant economic levels potentially because of reduced infection and establishment. Relative to non-resilient livestock to helminth infection and establishment, resilient stock will perform well irrespective of helminth burden (Hoste *et al*., 2006), while non-resilient ones will have their productivity and health greatly impaired or affected. Non-resilient or susceptible livestock to helminth parasitism lack the

ability to function and produce optimally at clinically infective level. Young growing ruminants exhibit a good level of resilience against helminths and eventually establish formidable immunity against gastrointestinal helminth parasites as they grow older (Knox *et al*., 2010), granted there is appropriate nutrition. Given the option to choose between resistant and resilient stock, resistant stock is preferable because it harbours a low harmless helminth burden whereas resilient stock thrives even in the presence of very high helminth burden. Resilient stock will pose a pathological threat to susceptible non-resilient ones. Remarkably, young ruminants progress from resilience to resistance against gastrointestinal helminths. Nutrition exerts a modulating role on the expression of immunity, resilience and resistance of helminth challenged animal host.

2.4.4.1 Effect of nutrition on host immunity, resilience and resistance

Good nutritional status of livestock tends to influence nematode parasite development and establishment. This entails striking a fine balance at the inclusion of sufficient protein (Coop and Kyriazakis, 1999; Ketzis *et al*., 2006; Besier, 2006), macro/micro elements, essential nutrients, vitamins and energy. Energy component is critical to fuel the nutrient pool for growth activities, reproduction, immune response processes and activities. It enhances host ability to accommodate and cope with parasites, improves resistance by limiting establishment, growth, fecundity and persistence, and can well affect parasitism through intake of antiparasitic compounds (Coop and Kyriazakis, 2001; Roeber *et al*., 2013). Different components of feed make various contributions to immune processes and response of animal host.

Fat- rich diets have the potential of suppressing immune response to parasitic nematodes of livestock (Chandra, 1983), rendering them more reliant on treatment. Its inclusion in animal diets must be such that it does not affect immune response to helminth parasites and other pathogens adversely. Supplemental protein tend to improve resilience and resistance in both young and old parasitized ruminants (Holmes, 1993), by ameliorating the expression of immunity to gastrointestinal nematode parasites (Coop and Holmes, 1996; Datta *et al*., 1998). This demands adequate supply of essential amino acids for efficient metabolic function and immune defence. Increased metabolisable protein supplementation resulted in a low nematode egg count in faeces of sheep (Jackson *et al*., 2004; Houdijk *et al*., 2005; Alemayehu *et al*., 2009), indicative of a low nematode burden, establishment and fecundity. Supplementary metabolisable protein enhances immunity to helminth parasitism depending on the need and availability of this essential nutrient to livestock (Kahn *et al*., 2003). Some animals show

exceptional resistance to parasite infection relative to others and will constitute an important base in potential breeding programs for resistance.

2.4.5 Selection of resistant stock

Genetically resistant livestock to helminths is fundamental to sustainable low cost parasite control, in addition to promoting animal health, welfare and productivity (Stear *et al*., 2001). The genetic variation to regulate helminth parasites is heritable in goats (Vagenas *et al*., 2002) and sheep (Morris, 1998), presenting a huge opportunity to use this trait to reduce dependence on anthelmintics in parasite control programs (Tables' 2.2 and 2.3). Different breeds of sheep have in turn expressed various vulnerability to GIT nematode parasite infection (Getachew *et al*., 2007; Zvinorova *et al*., 2016), further highlighting the need to identify useful ones (Table 2.3).

Important phenotypic markers include faecal egg count in highly challenged herds, alongside closely related immunological ones such as antibodies or eosinophils in blood in naturally affected herds (Getachew *et al*., 2007). Use of these breeds to upgrade herd resistance to nematode parasites and other pathogens will be a very critical economic input that will down scale helminth control cost. Resistant stocks have the ability to repel helminth infestation and establishment, while some host animals may harbour traits to interfere with fertility and multiplication (Stear *et al*., 2007). This genetically driven defence mechanism retains infection at subclinical harmless level that will ignite development of appropriate immune defence activity. Animal host immunity, therefore, strongly hinges on genetic factors, in addition to age, production stage, nutrition and history of exposure to parasites (Stear *et al*., 2007).

Naïve or previously uninfected young animals are more susceptible to helminth parasite infection than older stock primarily because of low initial immune response at onset of parasite infection (Colditz *et al*., 1996). Generally, innate immunity to gastrointestinal parasites is related to age and closely linked to physical and chemical differences in gut environment of adult and young animals (Mulcahy *et al*., 2004). Naïve black belly sheep of different ages, were found to have evoked immune response to *H. contortus* infection in age-related pattern (Getachew *et al*., 2007); the older ones being more responsive than the young. The Red Maasai sheep of east Africa (Table 1.3) retains a low faecal egg count (FEC) preceding primary infection with L_3 larvae of *Haemonchus contortus*, following anthelmintic treatment and natural re-infection relative to Dorper ewes (Wanyangu *et al*., 1997). Resistance against

gastrointestinal helminth infection will therefore vary among ruminant species, breeds and within breeds (Gray, 1997; Knox *et al*., 2010); with several parameters factoring in.

Prevailing conditions in the tropics and subtropics subject most indigenous grazing livestock breeds to regular nematode parasite infection and hence the developed genetic capacity to accommodate, tolerate and cope with them through natural selection (Getachew *et al*., 2007). Harsh environmental conditions and inadequate nutrition are additional stressors that have further equipped these breeds to develop formidable resistance relative to their temperate counterparts (Getachew *et al*., 2007). Genetic resources for resistant animal breeds will therefore abound in these regions and require judicious work to identify for further development. Complimenting preceding methods of nematode parasite control, is biological control using nematophagous fungi.

Table 2. 2: Goat breeds with resistant traits against gastrointestinal nematode parasites

Resistant breed	Susceptible breed	Infection	Parasite(s)	References
Sabi	Dorper	N	Hc	Matika *et al*., 2003
Small East African(SEA)	Galla	N	Hc	Baker *et al*., 1994, 1998
Jamunapari	Barbari	N	Hc, *St*, *Oe*	Rout *et al*., 2011
Creole	-	N	Hc, Tc	Mandonnet *et al*., 2001
Creole	-	A	Hc	Bambou *et al*., 2009
Creole	-	N	Hc	de la Chevrotiere *et al* 2012a
West African	-	N	Mixed	Behnke *et al*., 2011

(-) indicates traits which only involve one breed; within breed differences, N- natural infection, Hc- *Haemonchus contortus*, Tc- *Trichostrongylus colubriformis* (Zvinorova *et al*., 2016); St – Strongyloides; Oe – Oesophagostomum spp.

Table 2. 3: Sheep breeds possessing resistant traits against gastrointestinal nematode parasites

Resistant breed	Susceptible breed	Infection	Parasite(s)	References
Gulf Coast Native	-	N	Hc	Pena˜ *et al*., 2004.
F_1 & F_2 SuffolkX Gulf Coast Native	-	N	Hc	Li *et al*., 2001; Miller *et al*., 2006.
INRA 401	-	A	Hc, Tc	Gruner *et al*., 2004.
Merino	-	A	Hc, Tc	Andronicos *et al*., 2010.
Gulf Coast Native	Suffolk	N	Hc, Tc	Miller *et al*., 1998; Shakya *et al*., 2000.
Red Masaai	Blachheaded Somali, Dorper, Romney Marsh	A/N	Hc	Mugambi *et al*., 1997.
Barbados black belly	INRA401	A	*Trichostrongyles*	Gruner *et al*., 2003
Santa Ines	IIe de France, Suffolk	N	Hc, *Oesophagostomum, Columbianum*	Amarante *et al*., 2004.
Texel	Suffolk	N	*Trichostrongyle, Teladorsagia, Nematodirus*	Sayers *et al*., 2005; Good *et al*., 2006.
Florida native, Florida native X Rambouillet	Rambouillet	N	Hc	Amarante *et al*., 1999.
Dorper X Katahdin	Hampshire	A/N	Mixed	Burke & Miller, 2002.
Lohi	Thalli, Kachhi	A/N	Hc	Saddiqi *et al*., 2010.
Caribbean Hair, Katahdin	Crossbred-Dorper	A	Hc	Vanimissetti *et al*., 2004.

(-) indicates traits which only involve one breed; within breed differences, N- natural infection, Hc- *Haemonchus contortus,* Tc- *Trichostrongylus colubriformis* (Zvinorova *et al*., 2016)

2.4.6 Biological control of livestock helminths

This has been an active research area of growing interest on livestock helminth control, considering its biological non-chemotherapeutic nature. Biological control of livestock helminths entails use of a living antagonist by man to reduce helminth burden to within acceptable non-harmful, subclinical level (Grønvold *et al*., 1996). This approach targets the free living stage of helminth in dung pats and on pasture (Waller, 2006a). Nematode-trapping fungus *Duddingtonia flagrans* has shown promising biological control of the free living stages of *Ostertagia ostertagi* and *Cooperia spp* in cattle, whereas in horses, the same fungus is a potential biological control of *Cyasthostomes*, *Strongylus vulgaris* and *Strongylus edentatus* (

Grønvold *et al*., 1996). Additionally, *Duddingtonia flagrans* exhibited natural control over other helminth species such as *Oesophagostomum dentatum* and *Hyostrongylus rubidus* in pigs (Nansen *et al*., 1996).

Duddington flagrans is endowed with the traits and capacity to survive animal gut passage, grow aggressively in freshly deposited dung pat and has prodigious nematophagous capacity (Larsen, 1999). These characteristics render *Duddington flagrans* an excellent biological control agent. *Bacillus thuringiensis* and the fungus *Clonostachys rosea* have also shown individual and combined efficacy in the control of small ruminant helminth burden (Baloyi *et al*., 2011). Similarly, larval development of Merino sheep was significantly reduced using *C. rosae in vitro* (Ahmed, 2013). Grazing management of livestock to avoid or minimize interaction between livestock and nematode parasites is another important control strategy.

2.4.7 Grazing management as control measure of livestock helminths

This concept is aimed at providing grazing livestock clean pasture after anthelmintic treatment (Barger, 1999), to minimize and deter reinfection (Torres-Acosta and Hoste, 2008). Environmental conditions that favour growth and establishment of pasture such as ambient temperature and moisture (Morgan *et al*., 2006), also promote development, survival and transmission of helminth parasites of grazing livestock (Waller, 2006a; Torres-Acosta and Hoste, 2008). Under ambient temperature and moisture, excreted eggs develop and moult into third-stage larvae in the faecal pat, which migrate onto pastures pending being picked up during grazing by livestock. Third-stage larvae are resistant to both physical and chemical factors, with an average life-span in the tropics and subtropics of 1-3months (Torres-Acosta and Hoste, 2008). By the same token, the mean survival rate in the temperate area is potentially longer, ranging from 6-12/18months. Third-stage larvae are susceptible to severe drought and cold conditions (O'Connor *et al*., 2006); probably linking survival span in tropics and subtropics to drought and temperate environments to cold.

The rate of development of shed eggs, migration and mortality of infective larvae outside of the host is dependent on local climatic conditions. This, therefore, implicates adopted production systems in either propagation and/or control of livestock helminths (Piedrafita *et al*., 2010). Grazing management techniques can be implemented such that animals are kept off pastures when climatic conditions favour helminth egg hatch and development to L_3 infective larval stage (Table 2.4). Alternatively, animals can be put to graze when environmental

conditions are hostile to parasite development and survival. Removal of grazing livestock from pasture to previously un-grazed paddocks and reverting to the same paddock after a little while (Barger, 1999) before the onset of high infective L_3 larvae, reduces the frequency of drenching (Jabbar *et al*., 2007; Kahn and Woodgate, 2012). This approach makes available to the animals clean uncontaminated pasture, thus enhancing productivity in addition to retarding selection for resistance by helminths because of reduced host and parasite interaction.

Another option is the use of pastures with a low incidence of worms such as crop stubbles, hay or re-sprouted pasture after bush fire (Jabbar *et al*., 2007). Additionally, alternate grazing of goats and/or sheep with species including cattle and horses, which do not share major nematode parasites species (Barger, 1999; Jabbar *et al*., 2007) can also be adopted. This approach of alternate host grazing is currently being re-examined because small ruminants are progressively becoming more susceptible to cattle helminth species infection (Waller and Thamsborg, 2004).

The relationship between climate and parasite survival constitutes an important tool for biological livestock helminth control via grazing management (Waller and Thamsborg, 2004; Morgan *et al*., 2006). Some helminth species have been identified with particular pastoro-climatic regions of the world. An overview of the distribution indicate that, *Haemonchus* and *Cooperia* are most important in sub-tropical and tropical environments (Waller, 2006a; Torres-Acosta and Hoste, 2008), *Nematodirus* and *Ostertagia* in temperate regions whereas *Trichostrongylus* is common throughout (Waller, 2006a). There exist local peculiarities with various mixes of different species and others that may be of incidental importance. Almost all species of gastro-intestinal nematodes have a common life cycle, allowing for the use of a common grazing management strategy to control them. A sound knowledge of the epidemiology of gastrointestinal nematode parasites is critical to the adoption of grazing management, which have been largely successful in Australia and most of the developed world (Hennessy, 1997). The tropics and subtropics have greater potential of implementing grazing management strategies (Waller and Thamsborg, 2004), but they are rarely implemented. It is of great necessity that this prevention or avoidance strategies be encouraged in Africa and Asia, where a greater bulk of poor livestock farmers who do not have sufficient resources operate. Grazing management strategies, some of which have not been dwelled upon are found in the table below.

Table 2. 4: Different grazing management strategies to minimize contact and infection of livestock

Preventative strategies	Evasive strategies	Diluting strategies
Turning out parasite free animals on clean pasture	Worm challenge is evaded by moving animals from contaminated to clean pastures	Worm challenge is relieved by diluting pasture infectivity
❖ Delayed turnout	❖ Moving to safe pastures within the same season	❖ Avoid stocking rates close to carrying capacity of plant production
❖ Changing pastures between seasons	❖ Alternate grazing of different species	❖ Reduction of the general stocking rate
❖ Moving at weaning	❖ Hay/silage aftermaths	❖ Mixed grazing with other host species
❖ Late lambing	❖ New grass reseeds	❖ Alternate grazing with other host species
❖ Grass reseeds	❖ Cultivation of annual forage crops	
❖ Cultivation of annual forage crops		
❖ Silage/hay aftermaths		
❖ Alternation of different host species		

❖ = *Designates a grazing management strategy;* Adapted from **Younie *et al*., 2004.**

Another historical and essential method of parasite control is the use of plants and plant extracts in livestock helminth control.

2.4.8 Use of plants/plant extracts as anthelmintics in livestock

Plant use in the treatment of various diseases in animals and humans has been a long historical practice (McGaw and Eloff, 2008; Rochfort *et al*., 2008). Modern anthelmintic chemotherapy and chemoprophylaxis and other therapeutic interventions originated from their plant counterparts (Waller *et al*., 2001; Waller and Thamsborg, 2004). Plant material or their crude extracts are being used as athelmintic remedies in several parts of the world (Waller and Thamsborg, 2004; Githiori *et al*., 2006), especially Africa and Asia (Rochfort *et al*., 2008). This is common in the local livestock industry, given their ethno cultural knowledge,

availability and low cost (Torres-Acosta and Hoste, 2008). This practice is more widespread where communication is more developed, and there is intense interaction and flow of indigenous knowledge among various communities, resulting in local plant remedies and exotic ones being commonly used (Waller *et al*., 2001).

Growing interest in both developed and developing world in herbal anthelmintics (Hammond *et al*., 1997; Waller *et al*., 2001) and traditional herbal health remedies (Schillhorn van Veen, 1997; Rochfort *et al*., 2008), is critical to the development of this sector. Moreover, world health organization estimates that 80 % of the population of most developing nations depend on herbal remedies for their health and that of their livestock (Danøe and Bøgh, 1999). Livestock owners in these communities usually draw from the wealth of ethnoveterinary medicine acquired from preceding generations to treat their stock (McGaw and Eloff, 2008). Worldwide switch to chemotherapeutic remedies in most communities, as a result of their high efficacy, relative safety and very broad spectre of activities (Gibson, 1980), seriously dented development of anthelmintic phytotherapy and other ethno botanical therapeutic applications. On the other hand, current pharmacopeia contains 25 % drugs from plant origin and many synthetic analogues based on plant prototype compounds isolated from herbs (Waller *et al*., 2001); again rendering plant remedies very relevant.

The perception of plant remedies as being hazardous is attributable to method of processing than their inherent anthelmintic, antiparasitic and antibiotic traits. The onset of wide scale and ongoing emergence of multi-resistant pest organisms and pathogens, greater appreciation of natural remedies and occurrence of synthetic residues in the food chain and environment are compelling steering factors towards increased research, development and use of herbal remedies (Waller *et al*., 2001). Additionally, greater appeal and world-wide approval for organic livestock production and emphasis on the adoption of organic remedies (Thamsborg *et al*., 1999; Waller and Chandrawathani, 2005), has been another source of motivation for its development. Moreover, the adoption of developed anthelmintic phytotherapy is projected to receive wider approval, since it is commonly practiced and readily available in most parts of the world.

Research in this area from livestock perspective has been based on a few selected plants from a large consortium of identified ones exerting anthelmintic activity (McGaw and Eloff, 2008). Intense research, development and application of phytochemical anthelmintic remedies is expected to markedly reduce residual chemical anthelmintics commonly lodged in meat and

other animal products and environment (Thamsborg *et al*., 1999; Piedrafita *et al*., 2010). Ethnoveterinary plant remedies against helminths present a very important option, given their biological nature. They also have the added advantage of not inducing selection for resistance of human nematodes, cestodes and trematodes to anthelmintic treatment.

Traditional and organic farmers with an extensive knowledge of the use of ethnoveterinary remedies in controlling helminths constitute an important base for this research. Organic farmers are particularly committed to phytochemical or homeopathic remedies, which are authorized in organic livestock industry (Cabaret, 2003). Whereas traditional livestock farmers will adopt phytochemical remedy as primary option based on need and availability, modern veterinary services is secondary in the event of inefficacy. Ultimately, some phytochemicals have been linked to both anthelmintic and antibiotic activity exerted by them. A brief examination of antiparasitic benefits of grazing sheep and goats on some plant species that serve simultaneously as feed and remedy-nutraceuticals, with a greater inclination towards the health benefits, will give some insight to some of the active principles involved.

2.3.8.1 Nutraceuticals

These are forage plants that serve a dual function, both as forage and anthelmintic remedy to livestock (Kumar *et al*., 2012). Primary consideration will be accorded to the health benefits they provide due to their secondary metabolite content (Waller and Thamsborg, 2004). Generally, when most of these forage plant species are grazed or browsed by goats and sheep or fed to them, they reduce gastrointestinal egg count by between 50 – 60 % (Waller and Thamsborg, 2004). For this reason, anthelmintic activity has been closely associated with condensed tannins (Paolini *et al*., 2003a & b) among other secondary plant compounds. Their bio-activities have been linked to various effects, some of which include reduced fecundity, elimination of adult worms and reduced establishment of infective L_3 larvae (Athanasiadou *et al*., 2000a; Athanasiadou *et al*., 2000b; Kahiya *et al*., 2003; Paolini *et al*., 2003a; Paolini *et al*., 2003b).

Some of these plant species exerted their activities in designated sections of the gastrointestinal tract (Athanasiadou *et al*., 2001; Marley *et al*., 2003) of small ruminants. Quebracho that contains condensed tannins reduced small intestinal nematode species load such as *Trichostrongylus colubriformis,* but had no effect on abomasal nematode species of sheep, notably *Haemonchus contortus* and *Teldorsagia circumcincta,* (Athanasiadou *et al*., 2001). The

same results were obtained from dosing goats using Quebracho condensed tannins (Paolini *et al*., 2003a; Paolini *et al*., 2003b), while *Chicory intybus* containing sesquiterpene lactones (Foster *et al*., 2011), on the other hand, reduced abomasal nematode species burdens in sheep (Knight *et al*., 1996; Marley *et al*., 2003). These results suggest that biochemical environment in some sections of the GIT may be more favourable to the activity of some bioactive anthelmintic principles than others. Different biochemical actives have been associated with anthelmintic activity exerted by plants.

2.5 Common bioactive phytochemicals in medicinal plants

Most bioactive plants have a wide array of phytochemicals, many of which have a variety of biological activities; antiparasitic properties among them (Saxena *et al*., 2013). These active biochemical candidates either act directly on the parasite by disrupting its vital processes, or indirectly by affecting host regulatory mechanisms or systems that dislodges parasites from GIT (Jackson and Miller, 2006). Some of these indirect regulatory activities include promotion of host intolerance to nematodes, resilience to adverse effects caused, and resistance to pathogens and their establishment (Jackson and Miller, 2006). Preceding antiparasitic properties of similar bioactive phytochemicals was their antinutritional role when present in high concentration (Dove, 2010), hence the negative connotation or perception towards these biochemicals. However, they have been closely associated with deterring herbivory (Athanasiadou *et al*., 2006), thus playing a crucial role in plant defence and conservation.

These bioactive phytochemicals (Table 2.5) include alkaloids, terpenes, saponins, lactones, glycosides and phenolic compounds (Waller and Thamsborg, 2004; Saxena *et al*., 2013).

Table 2. 5: Common active biochemical compounds in medicinal plants

Classification	Main groups of compounds	Biological functions
Non starch polysaccharides (NSP)	cellulose, hemicellulose, mucilages, pectins, lignins	water holding capacity, delay in nutrient absorption, binding toxins & bile acids
Antibacterial & Antifungal	terpennoids, alkaloids, phenolics	inhibitors of microorganisms, reduce the risk of fungal infections
Antioxidants	polyphenolic compounds, flavonoids, carotinoids, tocopherols, ascorbic acid	oxygen free radical quenching, inhibition of lipid peroxidation
Anticancer	carotenoids, polyphenols, curcumine, flavonoids	Inhibitors of tumours, inhibited development of lung cancer, antimetastatic activity
Detoxifying agents	reductive acids, tocopherols, phenols, Indoles, aromatic isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols	Inhibitors of procarcinogen activation, inhibition of tumourogenesis
Others	alkaloids, terpenoids, volatile flavour compounds, biogenic amines	neuropharmacological agents, anti-oxidants, cancer chemoprevention

Adapted from Saxena et al., 2013

Some plant species, showing sufficient evidence anthelmintic activity (Table 2.6), have related bioactive phytochemicals and/or their analogues. Considering their common occurrence in South Africa, they will play an important role in the current project in attempt to improve their ethnoveterinary capacity in helminth control programs.

Table 2. 6: Some common selected plants species relevant to the study, exerting anthelmintic activity, identified bioactive principles and relevant reference(s)

Plant species	Bioactive principles and reference(s)
Aloe species	Glycoprotein, barbaloin, aloe emodin, mannose-6-phosphate, polysaccharide, acemannan, aloesin, β-sitosterol, diethyltheylphthalate, low molecular weight substances (Choi and Chung, 2003)
Allium cepa	Flavanoids, Proteins, Glycosides, Saponins, Tannnins (cold ethanol extracts) (Bibkar *et al*., 2012); Thiosulphates and volatile sulphur compounds (Marwat *et al*., 2011); Allicin (Vieira *et al*., 1999).
Anana comosus	Cysteine proteinases (Stepek *et al*., 2005); cysteine proteases (bromelain)
Bidens pilosa	Polyacetylenes (Lans, 2007), Polyacetylene, Phenylheptatriyne and Chalcones(Graham *et al*., 1980; Hoffman and Hoelzl, 1988) Phenylpropanoid glucosides, polyacetylenes, diterpenes, flavonoids and flavone glycosides (Chiang *et al*., 2004).
Carica papaya	Cysteine proteinases (Stepek *et al*., 2005) Papain and tannins (Adongo, 2013)
Crinum macowanni	Alkaloids, Coumarins, Glycosides, Triterpenes and Flavanoids; Alkaloids (lycorine type alkaloids and phenolics) (Refaat *et al*., 2012)
Gunnera perpensa	Some bitter principle Celastrine; Alkaloids, Flavonoids, Steroids, Saponins, Tannins and Glycosides (Simelane *et al*., 2010)
Nicotiana tabacum	Nicotine sulphate (British Veterinary Codex, 1953), Nicotine, Nicoteine, Nicotelline and Nicotinine (alkaloids). Also yields Anabasine, Betaine iamylamine, Pyrrolidine and n-methyl pyrroline, resin, Albumen, Gum, extractive matter, and ash containing large amounts of salts (sulphates, nitrates, chlorides, phosphates, malates, and citrates of potassium, ammonium, calcium, etc.). Study isolated a pair of sesquiterpene glucosides – 3 – hydroxysolavetivone-beta-D-glucoside A and B- from the leaves. (Stuart *et al*., 2012)
Ricinus communis	Alkaloids, Flavonoids and Condensed tannins (Rampadarath *et al*., 2014; Wafa *et al.*, 2014), also contains poly unsaturated omega 6,9 fatty acids (linoleic acid, Ricinoleic and 11-eicosenoic acids, Palmitic and Stearic acids), toxic principles present.
Sarcostema viminale	Pregnane derivatives like bregenin [2] and pregnane glycosides like brevine and brevinine [3]. Genin G and H [4], triterpenes like β-amyrin and friedelin [5] and some toxic constituents like sarcovimiside [6], polar compound exert anthelmintic activity (Grime *et al*., 2008)
Trema orientalis	Tannins (Watt and Breyer-Brandwijk, 1962)
Urtica dioica	Flavonoids, Acetylcholine, Phenolic acids, Coumarin, Sterols, high in Calcium, Chromium, Magnesium, Zinc, Cobalt, Manganese, Phosphorus, Potassium, Riboflavin, Selenium, Silicon, Thiamine, vitamin A and C. Seed contains Glycerol, Linoleic acid, Linolenic acid, Oleic and palmitic acids (Haman, 2007)

Vernonia amygdalena	Steroid glucosides, sesquiterpene lactones and flavonoids (Yeap *et al*., 2010)
Zanthozylum capense	Sanshol (Ghisalberti, 2002), Alkaloids and essential oil (cumarins) (Negi *et al*., 2011),
Zingiber officinale	Gingerole and Shogaol (Ghislberti, 2002), Essential oil, Zingiberene, Zingeberole, Sesquiterpene hydrocarbons, including Zigiberene arcurcumene, Sesquiphellandrene, and Bisabolene, Monoterpene aldehydes and Alcohols (Haman, 2007); Alkaloids, Flavonoids, Saponins, Steroids and terpens (Singh *et al*., 2011),
Ziziphus mucronata	Tannins, alkaloids, flavonoids and polar terpenes (sesquiterpene lactones, trip triterpenes, iridoids, flavonoids and alkaloids. (Van Wyk and Wink, 2004); Tannins, other phenolic compounds and alkaloids (Olivier, 2012)

2.6 Challenges arising from chemotherapeutic/chemoprophylactic control of livestock nematode parasites and strategies to mitigate them

General abuse and too frequent use of synthetic anthelmintics has led to widespread emergence of resistant strains of livestock nematodes (Waller, 1986, 1997c; Gill and Lacey, 1998; Van Wyk, 2001; Mortensen *et al*., 2003; Wang *et al*., 2010). Initial identification of single drug anthelmintic resistant parasites (Geerts and Gryseels, 2000), has been closely followed by development of multidrug resistance (MDR) (Table 2.7) in almost all classes of grazing livestock, and largely in the small ruminant industry (Kaplan, 2004). Moreover, selection for anthelmintic resistance has been associated with anthelmintics of all classes (Terrill *et al*., 2001; Jackson *et al*., 2009; Kaplan and Vidyashankar, 2012). Several measures were adopted to deter rapid loss of efficacy that was almost leading to total anthelmintic failure.

Waller (2006b) recommended judicious and sparing use of effective anthemintics as a measure to delay selection for resistant helminth parasites and extensive research efforts for other control options. Frequent and repeated treatment with efficacious anthelmintics and successive ones (Barger, 1995; Stepek *et al*., 2005), was also highlighted as the principal cause of recurrent anthelmintic failure. Diversification of treatment options has the potential to reduce dependence on any one control method, limit frequency of application and curb selection for anthelmintic resistance (Farias *et al*., 1996; Van Wyk *et al*., 1997; Waller, 1997c, 2006; Chandrawathani *et al*., 2003; Kaplan and Vidyashankar, 2012) by nematodes. There is the likelihood of improved efficacy span of most products in use. This has prompted the exploration of alternative control methods in addition to improving existing modes of treatment to enhance efficacy.

Adoption of other non-chemical treatment options have advantages of reducing residual anthelmintic molecules in meat and other animal related products, and also pollutes the environmental (Newton and Munn, 1999; De and Sanyal, 2009; Pediedrafita *et al*., 2010; Kahn and Woodgate, 2012). These deleterious effects are closely linked to persistent use of chemical anthelmintics. The cost of research and development of new anthelmintic candidates is huge and prohibitive to the pharmaceutical industry, besides that of procurement and administration which is a serious constraint to livestock farmers (Jackson *et al*., 2009; Kahn and Vidyashanka, 2012). The general realization of the scale of this crisis, prompted global mobilization of stake-holders from pharmaceutical industries, research institutions and the livestock industry. Successive meetings were held in Armidale, Australia in 1995, Baton Rouge in the USA in 1998; with subsequent ones in in Edinburg, United Kingdom in 2002 and Merida in Mexico in 2005 (Jackson and Miller, 2006) to seek solutions to this crisis. It is imperative to seek other control methods, especially because selection for resistance increases control cost, reduces production efficiency in addition to risk of environmental pollution (Cox, 1999; Coop and Kyriazakis, 2001; Stepek *et al*., 2004; Macedo *et al*., 2010).

Most of rural Africa, Asia (Waller and Thamsborg, 2004) and Latin America use a wide range of herbal ethnoveterinary remedies (Anon, 1994; *Akhtar et al*., 2000; Alawa *et al*., 2003; McGaw *et al*., 2005; Mirdeilami *et al*., 2011), that constitute important control option to mitigating selection for anthelmintic resistance. This is examined from the perspective of deterring selection for anthelmintic resistance from repeated use of anthelmintics, rather than curative or therapeutic as pre-reviewed. Chemical control remains relatively the most widely adopted and efficacious measure globally (Waller, 1997b; Jackson and Miller, 2006; Kaminsky *et al*., 2008), and is predicted to remain the most appropriate for the foreseeable future. The scale of the crises is not relenting following emergence of resistant strains of nematode parasite from the use of all three major classes of chemical anthelmintics viz; benzimidazoles, Imidazothiazoles/tetrahydropyrimdines and macrocyclic lactones (Stepek *et al*., 2004). This development emphasizes adoption of urgent measures to deter near total anthelmintic failure.

Diversification of control methods has the advantage of reducing regular application of chemical anthelmintics and extending their efficacy span (Barger, 1999). The use of various methods interchangeably has allowed for less regular treatment or drenching of livestock using chemical anthelmintics, leading to more sustainable control strategies (Waller, 1997a). Effective control of these livestock parasites will ultimately lead to improved animal health,

improved utilization of feed resources, enhanced productivity and increased economic returns (Piedrafita *et al*., 2010), as primary objectives of the production process.

In the interim, the following strategies have been adopted to deter or mitigate selection for anthelmintic resistance; strategic drenching, quarantine of new stock, reduced frequency of anthelmintic therapy, selective treatment, administration of appropriate dose and anthelmintic combination therapy. A brief review of each of these highlighted strategies in the section that follow, will demonstrate how useful they are to the process.

Table 2. 7: General global state of anthelmintic resistance among livestock hosts

Drug class	**Host with high resistance**	**Host with emerging resistance**	**Major livestock-producing areas where drug is still highly effective in sheep, goats and horses**
Benzimidazoles	Sheep, goats, horses	Cattle	None
Imidothiazoles/tetrahydropyrimidines			
Levamizole (ruminants)	Goats, sheep	Cattle	None
Pyrantel (horses)	Horses (USA only)	Horses	Unknown – few studies out of the United States
Avermectin–milbemycins			
Avermectin	Sheep, goats and cattle	Cattle, horses	Horses – worldwide
Moxidectin	goats	Sheep, goats, cattle	Sheep, goats – Canada/Europe Horses – worldwide Sheep – most regions

*For horses reference is made to cyasthostomin nematodes and trichostrongylid nematodes of small ruminants. Gradation of resistance from emerging to high, refers to resistance that has been identified, prevalence not known and distribution not severe in the former. In the later, level and prevalence of resistance demands general concern and prior test for efficacy before use.*Table adopted from* ***Kaplan, (2004)****.*

2.6.1 Strategic drenching or treatment

Routine treatment of entire herds has generated a lot of challenges, owing to differences in parasite burden among infected animals and others that may not be infected. These differences among animals in a herd have ignited the concept of judicious, appropriate and strategic drenching or treatment of livestock as an important measure to retard selection for resistance by nematodes. Uninfected animals are prevented from being used to currently efficacious anthelmintic and also becoming tolerant. This has a huge potential of conserving the efficacy-status of anthelmintics longer than in routine general periodic treatment (Kahn and Woodgate, 2012) without epidemiological considerations. Strategic use, at the time much required, is aided by a sound knowledge of parasite epidemiology and clinical symptoms (Barger, 1999; Getachew *et al*., 2007). In temperate areas where strategic as opposed to routine anthelmintic treatment is practised, animals at risk and weaned lambs are treated in first grazing season (Getachew *et al.*, 2007). In arid areas where parasite build-up with the onset of rains, two prong treatment is advised; four weeks after beginning of rains and again at the end (Urquhart *et al*., 1996). Animal identified within the herd as heavily infected can be isolated, judiciously treated and reintegrated into the herd. This entail retaining the status of good management in terms of parasite control by carefully handling infected stock to prevent contamination and disruption of set out strategies and programs to avoid too frequent anthelmintic use. Introduction of new stock into the herd has a routine procedure to be implemented as deterrent to contamination referred to as quarantine (Waller, 1997). New animals are isolated, examined, observed and possibly treated if need be, before integration into herd.

2.6.2 Quarantine of new stock

Importation of exotic stock and/or reception of new stock into an existing animal facility, should of necessity be quarantined, faecal egg count and relevant reliable test of non-resistant parasite strains done (Sargison, 2016). Diagnosis in this process is expected to be sensitive, accurate and rapid, given the short quarantine period. This will enable appropriate anthelmintic and other relevant therapy to be administered to rid animals of potentially resistant parasites (Kaplan and Vidyashankar, 2012). Acquisition of new stock without quarantine and treatment has been identified as a major method of introducing resistant helminths parasite into stocks with minor or no incidence of resistance. Other measures of preserving chemical anthelmintic

efficacy by livestock farmers include reduced frequency of treatment, accurate dosing (Sangster and Dobson, 2002), and selective treatment of infected animals.

2.6.3 Reduced frequency of treatment

Frequent dosing tends to promote selection for resistance by helminths against chemical anthelmintics in use (Kettle *et al*., 1983; Hennessy, 1997; Wolstenholme *et al*., 2004; Leathwick and Besier, 2014), as surviving nematode parasites develop considerable tolerance to them. Increased dosing in the phase of waning efficacy, and increased frequency of anthelmintic application (Donald, 1994), also increases the potential of precipitating selection for resistance. This is caused by nematodes that have survived chemotherapy/chemoprophylaxis to become the next generation of worms (Papadopoulos *et al*., 2001), after having been exposed and ineffectively acted upon, in previous dosing. Moreover, protracted use of a particular anthelmintic has been associated with grooming, development and consolidation of resistance (Maingi *et al*., 1996; Hennessy, 1997), because of growing parasite tolerance and genetically aided loss of susceptibility (Jackson and Miller, 2006). In some instances, especially where prevailing abiotic and biotic conditions favour rapid build-up and persistence of free living parasites, frequent treatment is often applied (Waghorn *et al*., 2011). Frequent treatment increases selection pressure (Wolstenholme *et al*., 2004) as the most fit parasites survive, further provoking development of resistance in the long term. Strategic programs are designed to drench livestock when helminth burden is at clinical level in order to conserve anthelmintic efficacy. One of such program is the monitoring of worm egg count (WEC) (Kahn and Woodgate, 2012), which gives a proximate level of helminth parasitism in a stock at different periods of the year. This approach requires skilled manpower, which is scarce in developing countries and insufficient in developed countries (Jackson and Miller, 2006). Alternatively, application of avoidance techniques by moving livestock to cleaner pasture at close to peak periods of L_3 larvae incidence and longer intervals of anthelmintic administration will potentially retain chemical anthelmintic efficacy for longer periods. Papadopoulos *et al*. (2001) recommended complete avoidance of chemical anthelmintic use or occasional administration as strategy to avoid selection for resistant nematodes parasites of livestock. Selective treatment of parasitized stock is as well an important management option.

2.6.4 Selective treatment of infected stock

Targeted treatment of animals, depending on predetermined and standardized techniques of detecting helminthosis of livestock, is required to adopt selective treatment. One of such methods, is FAMACHA© (Malan *et al*., 2001), which is based on anaemic manifestation on the lower eyelid mucous membrane in small ruminants as a morbidity sign for haemonchosis and indicator for treatment (Jackson and Miller, 2006). This approach reduces treatments normally required and minimizes contact or interaction between anthelmintic and prevailing parasites (van Wyk and Bath, 2002) in animal host, which apparently cause no major subclinical or clinical health hazard. It has the advantage of retaining herd parasite populations that have no prior contact with anthelmintics (refugia) (Kaplan *et al*., 2004). This method is thus specific to haemonchosis, and require other disease indicators or markers for clinical nematode infections that manifest otherwise, such as diarrhoea indices, body weight loss, physiopathological or immune related indicators in milk, serum or faeces (Jackson and Miller, 2006). Respect of anthelmintic dose with respect to animal weight is also important to sustaining efficacy.

2.6.5 Administration of appropriate dose

Inappropriate dosing has been a major issue of contention, leading to induced emergence of resistant nematode parasites to most chemical anthemintics in use. Under dosing by inaccurate estimation of animal weight (Leathwick and Besier, 2014) has been one of the causes of selection for resistance by parasitic helminths. It creates anthelmintic tolerant nematode parasites as a result of the administration of insufficient dose, thus reducing action span after administration (Getachew *et al*., 2007; Lawrence *et al*., 2007). It has been recommended that animals be weighed and anthelmintic dosing based on the heaviest weight to maximize anthelmintic efficacy (Hennessy, 1997, Leathwick and Besier, 2014). In this respect, rather than reduced anthelmintic action after administration, it should be increased for those animals that have lower weights from the heavier ones that have been adopted as standard for herd dosing.

2.7 Anthelmintic failure, prospects of recurrent failure of new candidates and recourse to neglected control options

The spectacular development of very efficient anthelmintic therapy/prophylaxis over the years, and lack of relevant education and technical support to enable proper global administration has led to generalized failure (Lanusse *et al*., 2014; Learmount *et al*., 2016; Sargison, 2016). Though selection for anthelmintic resistance certainly sets in with time, inability to properly administer these remedies as prescribed and recommended in the factors above, precipitates recurrent decline of their efficacy. This trend has continued with research and development of new candidates, demanding thorough re-examination of mode of application and exploration of other viable options.

In this state of a global crisis, selection for resistance by nematode species of small ruminants is already filtering in from New Zealand, Uruguay and Holland (Scott *et al*., 2013; Mederos *et al*., 2014; Van de Brom *et al*., 2015) for one of the latest drug candidate 'Zolvix®"; reaffirming apparent absence of any lessons learnt from previous developments or outright irresponsibility. Several flaws in control strategies and practices factored into recurrent failure of different classes of anthelmintics ranging from benzimidazoles and probenzimidazoles, imidazothiazoles/tetrahydropyrimidines and macrocyclic lactones (Stepek *et al*., 2004; Githiori *et al*.,2006; Waller, 2006b; Stear *et al*., 2007; Lanusse *et al*., 2014); most of which have been reviewed in preceding sections. Some of them included ignorance of the implications of general routine administration/dosing; irresponsible use by livestock farmers; dosing by inexperienced non-professionals without relevant consultation or assistance (Farias *et al*., 1977, Wolstenholme *et al*., 2004; Sargison, 2016); adoption of smaller live weight in herds as standard for dosing resulting in under dosing of heavier animals; counterfeit or substandard inefficient anthelmintic products (Githiori *et al*., 2006) that induce parasite tolerance instead of treatment or prevention; insufficient integration of chemotherapy and management schemes (Lanusse *et al*., 2014) as deterrent to frequent anthelmintic use; little knowledge of pharmacological properties and inconclusively, various host related-factors that potentially lead to modification of pharmacokinetic activity and a reduced efficacy of the antiparasitic drug of choice (Lanusse *et al*.,2014).

Pharmacokinetic-based resistant mechanisms include insufficient intracellular drug concentration; improved inactivation of active drug; decreased activation of drug into a more active compound and increased concentration of a metabolic product that inhibits drug action.

Pharmacodynamic resistant mechanisms include modified functional target receptors and remodelled receptor structure that affect drug affinity. Some of the previous practices, leading to anthelmintic failure (Falzon *et al*., 2014), are much more menacing as the choice of efficacious anthelmintic narrows down to a smaller number coupled with declining search for new anthelmintic candidates (Waller, 2006b).

Incidences of resistance to former anthelmintic candidates around the world surpassed those of the latest ones (Falzon *et al*., 2014; Learmont *et al*., 2016), primarily because of their relative longevity or frequency of use, and resulting selection for genetically resistant traits by surviving parasites. Single product use in parasite control programs currently serve as prodigious common risk-factor in selection for resistance (Lanusse *et al*., 2014; Learmont *et al*., 2016), hence the need for combined application as an appropriate measure to deter product failure. Besides, time span of drug concentration and exposure to parasites is key to efficacy, in addition to persistence of activity for most anthelmintics that are applicable to ruminants (Lanusse *et al*., 2014). It is implicit that any strategies that increase anthelmintic drug availability and persistence, will certainly enhance efficacy. Incidences of anthelmintic failure in ruminant species around the world, some of which affected exclusively goats or sheep, and others affected both animal species are presented in Tables' 2.8 and 2.9.

Table 2. 8: Global incidences of anthelmintic resistance in sheep and goats and major nematode species affected

Animal species	Country	Anthelmintic class	Nematode genera	Reference(s)
Goats	Ethiopia	Albendazole, Tetramisole, Ivermectin (BZ, IMID, AVM)	*H. contortus, Trichostrongylus, Teladorsagia spp.*	Sissay *et al*., 2006; Kumsa and Abebe, 2009
	Uganda	Albendazole, Levamisole, Ivermectin (BZ, IMID,AVM)	*H. contortus, Cooperia spp., Oesophagostomum spp*	Byaruhanga and Okwee-Acai, 2013
	Nigeria		*H. contortus*	Chiejina *et al*., 2010
	Pakistan	Oxfendazole, Levamisole (BZ, IMID)	*H. contortus, T. colubriformis*	Saeed *et al*., 2010
Sheep	Zimbabwe	Fenbendazole, Albendazole, Oxfendazole, Levamisole (BZ, IMID)	*H. contortus, Cooperia spp.*	Mukaratirwa *et al*., 1997; Matika *et al*., 2003
	Zimbabwe	Fenbendazole, Albendazole, Levamisole, Rafoxanide (BZ, IMID, SCL)	*H. contortus*	Boersema and Pandey, 1997
	Zambia	Ivermectin, Albendazole (AVM, BZ)	*H. contortus, Trichostrongylus, Oesophagostomum spp.*	Gabriel *et al*., 2001
	Germany	Levamisole, Ivermectin (IMID, AVM)	*Trichostrongylus spp.*	Voigt *et al*., 2012
	Brazil	Ivermectin (AVM)	*H. contortus*	Fortes *et al*., 2013
	Northern Ireland	IvermectinLevamisole (BZ, MLB, AVM, IMID)	*Trichostrongylus, Teladorsagia, Coopria spp.*	McMahon *et al*., 2013

a Benzimidazoles—BZ; Macrocyclic lactones- ML (Avermectins-AVM or Milbemycin−MLB; Nicotinic agonists (Imidothiazoles-IMID or Tetrahydropyrimidines-TETR); Aminoacetonitriles derivatives-AAD; Salicylanilides-SCL. Adapted from: Zvinorova *et al*., 2016

Table 2. 9: Incidences of selection for anthelmintic resistance in sheep and goats; major nematode genera affected

Animal species	Country	Anthelmintic class	Nematode genera	Reference(s)
Goats/Sheep	South Africa	Albendazole, Closantel, Ivermectin, Levamisole (BZ, SCL, AVM, IMID)	*H. contortus*, Trichostrongylus, *Oesophagostomum* spp.	Bakonsi *et al*., 2013, Tsotetsi *et al*., 2013
	Kenya	Ivermectin, Fenbendazole (AVM, BZ)	*H. contortus*, *Trichostrongylus*, *Oesophagostomum spp*.	Nwamachi *et al*., 1995
	Switzerland	Avermectin (AVM)	*H. contortus*, *Trichostrongylus spp.*	Artho *et al*., 2007
	Norway	Albendazole (BZ)	Teladorsagia, *Trichostrongylus spp*.	Domke *et al*., 2012
	India	Fenbendazole, Benzimidazole (BZ)	*H. contortus*, *Trichostrongylus spp.*	Rialch *et al*., 2013
	India	Thiabendazole, Tetramisole (BZ, IMID)	*H. contortus*	Swamkar and Singh, 2011
	Philipppines	Benzimidazole (BZ)	*H. contortus*	Ancheta *et al*., 2004

Benzimidazoles—BZ; Macrocyclic lactones- ML (Avermectins-AVM or Milbemycin−MLB; Nicotinic agonists (Imidothiazoles-IMID or Tetrahydropyrimidines-TETR); Aminoacetonitriles derivatives-AAD; Salicylanilides-SCL.

2.8 Anthelmintic combination(s), pharmacokinetic and pharmacodynamic implications to optimized activity

Single anthelmintic drug use and its related efficacy has been strongly linked to systemic bioavailability of the active principles (Alvarez *et al*., 2012a; Alvarez *et al*., 2012b). Different routes of administration affected this important determinant of efficacy; some of which included intravenous (IV), intraruminal (IR), subcutaneous (SC) and oral routes. Intravenous (IV) administration of albendazole to sheep, resulted in higher plasma availability and improved efficacy relative to intraruminal (IR) administration (Entrocasso *et al*., 2008). Fasting on the other hand, as a pharmacokinetic strategy, did not improve anthelmintic efficacy irrespective of increased systemic bioavailability of albendazole sulphoxide administered to sheep against resistant *Haemonchus contortus* (Alvarez *et al*., 2010), but required a very high dose to attain the desired anthelmintic efficacy. Increases in dose for albendazole and ivermectin as high as 9 and 10 folds the therapeutic dose, respectively, were required to achieve acceptable efficacy against a very resistant strain of *Haemonchus* spp. (Lanusse *et al*., 2014). Expert technical assistance is vital to the method and route of administering either single or combined anthelmintics, in order to achieve the desired efficacy.

Existing anthelmintics that are regarded to have failed can still be employed in resistant nematode control programs by adopting pharmacokinetic strategies to improve parasite exposure thereby extending their efficacy span and that of new anthelmintic candidates by alternate and regulated use (Alvarez *et al*., 2012a). Drug mixtures from different families have been suggested as an important strategy to deter development of anthelmintic resistance (Table 2.10) but improve efficacy (Anderson *et al*., 1988). Several preparations are currently available in Australia, New Zealand and Uruguay (Lanusse *et al*., 2014).

Inherently, combinations resulted to multicomponent active-principle formulations, all exerting different modes of biochemical activity and leaving nematode parasites with insufficient resistance for all of them as opposed to single drug formulations (Lanusse *et al*., 2014). The outcome from such practice is either improved activity, synergy, individual independent activity or mutual destruction. Incidentally, combinations adopted in helminth control programs have exerted very high efficacies (Lanusse *et al*., 2014).

Major sheep producing countries such as Australia, New Zealand and Uruguay have several combined formulations in the market including Triton®, Merail comprising of albendazole, ivermectin and levamisole, Matrix®, Ancare, made of oxfendazole, abamectin and levamisole, multicombinational-drench (Q-Drench, Jurox) consisting of albendazole, levamisole, closantel and abamectin for sheep in Australia (Lanusse *et al*., 2014). The higher the efficacy attained by using combined-formulations, the smaller the resistant strain; which will in turn be diluted by susceptible naïve nematode population. In cases of absolute efficacy from combination therapy, selection for resistance will be lost in the absence of parasite populations from preceding treatment (Dobson *et al*., 2001). This target might appear elusive, but remains a feasible achievement given this novelty.

Table 2. 10: Assessment of pharmacokinetic drug-drug interactions occurring after administration of albendazole (ABZ), ivermectin (IVM), levamisole (LEV) & triclabendazole (TCBZ) administered either alone or under different combined preparations to sheep. The impact of the different routes of administration on the systemic exposure is compared

Athelmintic combination	Assayed moleccule	Route of administration	Systemic exposure expressed as AUC (µgng h d/mL)		Pharmacokinetic drug-drug interaction
			Drug alone treatment	Combined treatment	
ABZ + IVM[1]	ABZ	IV	30.2 ± 5.31	33.9 ± 6.65	No interaction
	IVM	IV	112.3 ± 37.4	210.3 ± 80.6*	Positive interaction
ABZ + IVM[1]	ABZ	IR	19.8 ± 2.55	28.2 ± 3.72*	Positive interaction
	IVM	SC	131.1 ± 70.5	139.7 ± 28.6	No interaction
LEV +ABZ + IVM[2]	LEV	Oral	8.63 ± 5.22	10.5 ± 5.73	No interaction
	ABZ	Oral	30.7 ± 9.01	19.4 ± 7.90*	Negative interaction
	IVM	Oral	30.9 ± 11.6	51.6 ± 16.2*	Positive interaction
TCBZ + IVM[3]	TCBZ	IV	297 ± 74.3	319 ± 70.2	No interaction
	IVM	IV	144 ± 5.83	48.5 ± 46.6*	Positive interaction
	TCBZ	IR	654 ± 141	651 ± 123	No interaction
TCB + IVM[4]	IVM	SC	Not determined	Not determined	-

Data from Alvarez *et al*., 2008; Suarez *et al*. (unpublished); Lifschitz *et al*., 2009; Ceballos *et al*., 2010. IV, intravenous; IR, intraruminal, SC, subcutaneous. AUC: area under the plasma concentration vs time curve. ABZ and TCBZ AUC values are referred to their sulphoxide metabolites, ABZ-sulphoxide and TCBZ-sulphoxide

Chapter 3

In vitro anthelmintic activity of crude extracts of various plant species exerting bioactivity on nematode parasites of small ruminants

Abstract

This study was focused on *in vitro* screening of 15 selected plant species identified to have anthelmintic traits for their individual activity at three concentrations on mixed L3 larvae of goats and sheep grazing contaminated Kikuyu grass (*Pennisetum clandestinum*). It comprised of three sub-studies, based on major bioactive anthelmintic principles from extensive body of research. Sub-study (1) was based on alkaloids and tannins plant species, including *Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Sarcostema viminale, Vernonia amygdalina, Zingiber officinale* and *Zizyphus mucronata.* Sub-study (2) was based on flavonoid plant species and consisted of *Trema orientalis, Urtica dioica* and *Zanthozylum capense.* Ultimately, sub-study (3) was based on proteases and nitrogen compounds containing plant species including *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya,* and *Ricinus communis.* Oven-dried leaf samples (40g; 20g; 10g) of each species were extracted in 70% ethanol, and concentrated to 100ml; constituting 4×, 2× and 1× crude extract concentration. Rectal faecal material from 10 Merino sheep and 25 Nguni goats, pooled within species and hand-mixed. Dung samples (5g) were weighed and cultured for 12 days at 27°C. On day 13, four plates were watered, and others (4) treated with 70% ethanol to correct for solvent effect on mortality. Sub-study (1), had 2 (animal species) x 7 (plant species) x 3 (extract concentrations) factorial design. Sub-study (2), 2 (animal species) x 3 (plant species) x 3 (extract concentration) factorial design. Sub-study (3) had 2 (animal species) x 5 (plant species) x 3 (concentrations) factorial design. In each run, three plates were treated with each crude extract concentration. L3 larvae isolated on day 14, larval counts done, and mortality adopted as indices of percentage dose anthelmintic efficacy. The study was re-run three times. In sub-study (1), animal species (P= 0.0107) and concentration (P= 0.0005) affected efficacy. Increases in crude extract concentration from 1x to 2x and 4x resulted to efficacies of 71.2±2.62%, 88.0±1.88% and 97.9±0.91% for goats, and 93.8±2.62%, 96.0±1.88 % and 98.0±0.91 % for sheep. Interaction between extract concentration and animal species affected efficacy (P= 0.0127). In sub-study (2), concentration affected (P< 0.0001) efficacy. Animal

species affected efficacy (P= 0.0046). Similarly, plant species showed a tendency to affect efficacy (P= 0.0572). There were interactions between concentration and animal species (P= 0.0010), concentration and plant species (P= 0.0123) and among concentration, animal and plant species (P= 0.0435). In sub-study (3), Animal species affected (P= 0.0004) efficacy. Concentration affected (P= 0.0002) efficacy. Interaction between animal species and concentration also affected (P= 0.0015) efficacy. In parasitic helminth control programs, anthelmintic efficacy of plant species can be potentially optimized by according attention to plant species, animal species, crude extract concentration, interaction between concentration and animal species, between plant species and concentration, and among crude extract concentration, plant and animal species.

3.1 Introduction

Given the huge economic set-back to the livestock industry as a result of nematode parasite infection (McLeod, 1995), with a heavier toll on resource-limited livestock farmers (Perry *et al*., 2002; WHO, 2002; Githiori *et al*., 2004) who constitute a bulk of the global industry; there is pressing need for action. Effective and sustainable control of nematode parasites of livestock has become a global challenge that demands prompt attention to curb the growing surge of selection by parasites for chemical anthelmintic resistance (Kaplan, 2004; Waller, 2006b). From the 1950s to the 1990s, during which period benzidazoles, levamisole and avermectins were developed and used to control a wide range of helminth parasites of livestock, the first appearance of resistant strains of nematode parasites to these chemical anthelmintics had already been spotted (Waller, 2006b). Moreover, chemical anthelmintics, notably some macrocyclic lactones such as ivermectins are largely excreted unaltered in faeces and affect dung eating invertebrates, which in turn have the capacity to affect other organisms higher up the food chain (Cox, 1999), among others. This process can potentially induce resistance in other organism by indirectly under-dosing them with the same chemical anthelmintic over time, thus provoking anthelmintic tolerance/resistance by gastro intestinal nematode parasites.

Gastrointestinal nematode parasitism further imposes huge economic constraints on global livestock production at both subclinical and clinical levels of infection; first by depriving their host of essential nutrients or feeding on tissue/blood, and secondly, by causing wasting and death of the host at high levels of infection. *Haemonchus contortus* has been identified in the sheep and goat herds of Ukulinga Research Farm as the primary nematode parasite species 87.27%, *Trichostrongylus* 7.34% and *Oesophagostomum spp*. 5.39% (personal observation).

Haemonchus contortus is highly pathogenic and has the capacity of causing acute disease and high mortality in all classes of grazing stock (Allonby and Urquhart, 1975). The small ruminant industry is strategically very important to most developing and emerging economies.

The global small ruminant industry is dominated by resource poor small farmers (Devendra and Soulaiman, 2010), who depend on their animals for food, clothing, income and manure. At the onset of chemical anthelmintic discovery and application in the 1950s (Waller, 2006b) that was lauded as the most efficient control method. This discovery drew attention and resources from all other control strategies, and nematode parasites of livestock have since been controlled largely using them (Makkar *et al*., 2007). The facility with which chemical anthelmintic therapeutics could be procured and used, the spectrum of activity and remarkable efficacy turned out to be short term gains that militated for rapid development of resistance due to gross misuse (Githiori *et al*., 2004). Overtime, euphoria has diminished with increasing development of resistance to all classes of developed chemical anthelmintics, keeping stakeholders hard at work for solutions. This therefore urges diversification, search for optimization of existing control strategies, development of previously neglected or abandoned approaches, especially ethno veterinary practices and processes of livestock nematode parasite control using phytochemicals/plant material and integrated use of various strategies (Akhtar *et al*., 2000) to mitigate reliance on any one method of control.

Appeals for a more subtle and judicious application to avert/deter development of anthelmintic resistance are facing steep challenge in its implementation, given the wide disparity of technical knowledge possessed by most stakeholders in the industry. Ethno veterinary practice of using plants possessing anthelmintic activity to control helminths of livestock is anticipated to find favour with local livestock farmers considering its cultural base in most communities of Africa (Hammond *et al*., 1997; Githiori *et al*., 2004) and the world. The expectation of this study is that changes in concentration of plant species crude extract will have no effect on anthelmintic efficacy.

The objective of this study was to screen selected plant species crude extracts identified to possess anthelmintic activity for their individual anthelmintic efficacy at three different concentrations on mixed infective L3 larvae of goats and sheep grazing on contaminated kikuyu grass (*Pennisetum clandestinum*). This was part of ongoing studies to explore potential enhancement of the efficacy of these plant species through combination anthelmintic phytotherapy.

It is hypothesized that plant species exerting anthelmintic activity do not have any effects of concentration, animal species, or interaction of plant species, animal species and concentration on observed efficacy.

3.2 Materials and methods

3.2.1 Collection of vegetative plant material, establishment of groups and processing of crude extracts

Fifteen plants that are used traditionally to treat parasitic nematodes of livestock were selected from available literature and allotted to three main groups following reported primary anthelmintic principles. They consisted of: (1) Alkaloids and tannin containing plant species including *Crinum marcowanni* (Refaat *et al*., 2012), *Gunnera perpensa* (Simelane *et al*., 2010), *Nicotiana tabacum* (British Veterinary Codex, 1953; Stuart *et al*., 2012) *Sarcostema viminale* (Grime *et al*., 2008), *Vernonia amygdalina* (Yeap *et al*., 2010), *Zingiber officinales* (Haman, 2007; Singh *et al*., 2011), and *Ziziphus mucronata* (Van Wyk and Wink, 2004; Olivier, 2012). (2) Flavonoids containing plant species comprising of *Trema orientalis* (Watt and Breyer-Brandwijk, 1962), *Urtica dioica* (Haman, 2007) and *Zanthozylum capense*; (3) Proteinases and nitrogen containing plant species namely, *Allium cepa* (Vieira *et al*., 1999; Marwat *et al*., 2011; Bidkar *et al*., 2012), *Ananas comosus* (Stepek *et al*., 2005), *Bidens pilosa* (Graham *et al*., 1980; Hoffman and Hoelzl, 1988), *Carica papaya* (Stepek *et al*., 2005; Adongo, 2013) and *Ricinus communis* (Rampadarath *et al*., 2014; Wafa *et al*., 2014). Plant material was collected from the University of KwaZulu Natal Botanical garden, some from the National Botanical garden, Pietermaritzburg, others from private gardens and some were bought/collected from commercial food and vegetable stores in Pietermaritzburg central business district. Voucher samples were deposited at the UKZN Herbarium, Pietermaritzburg.

Fresh vegetative material was collected, washed, chopped for those with large/long leaves, air dried and subsequently oven dried (Oven mark; LABCON, Model5SOEIB, Maraisburg 1700) to constant weight at 60 °C. Each plant species oven-dried vegetative material was milled using an electric centrifuge mill, mark (RETSCH, GmbH and Co.KG, 5657 HAANI, West Germany), fine enough to pass through a 1 mm sieve. Milled plant samples were then put into air-tight labelled plastic containers and stored in boxes, away from light and moisture at room temperature. Ten grams of each oven-dried plant species material was weighed into labelled thimbles, fitted into distillation columns and extracted with 70 % ethanol as solvent over a

heating unit, mark (Gerhardt Bonn, App. Nr 450893). The extraction process was deemed completed, when the solvent in the thimble carrying unit was apparently free of any coloration. Three other masses of 10 g dry matter were extracted following the same procedure for each of the plant species and later concentrated to 100 ml of plant species crude extract. Half and one quarter of the original crude extract were both made to 100 ml with the same solvent; equivalent to 20 g and 10 g DM crude extracts respectively. The initial crude extract was 4×, the second 2× and the third 1×.

The plant species crude extracts were sealed with parafilm, put into sealed boxes and stored in the fridge for *in vitro* dosing of cultured isolated mixed nematode L3 larvae.

3.2.2 *In vitro* evaluation of plant species extracts

Rectal faecal material was collected from10 Merino sheep and 25 Nguni goats grazing on contaminated Kikuyu grass (*Pennisetum clandestinum*) at Ukulinga Research Farm, pooled within species and thoroughly hand-mixed. Dung samples, each of 5 g were weighed into plates and incubated/cultured (MEMMERT, 854 Schwabach, West-Germany) for 12 days at 27 °C. Samples were watered at 12.00 noon every day during the 12 days of incubation/culturing to keep them moist; considering moisture consistency in the process, in order not to drench and kill hatched developing larvae. On day 13, 4 plates were watered, 4 plates treated with 5 ml of 70% ethanol to correct the solvent effect on mortality and three plates that were allotted to each plant species extract, treated with 5 ml of 1x, 2x and 4x extractions. All the samples were further incubated for 24 hours and nematode isolation done using the Baermann set-up (Hansen and Perry, 1994) in the following 24 hours.

The Baermann technique was used to isolate nematode larvae (Hansen and Perry, 1994). Each faecal culture was placed in a double cheese cloth, a porch loosely formed around and tide with a rubber band. All faecal cultures in the cheese cloth pouches were placed in labelled funnels, big enough to enable complete immersion and supported by appropriate device. Lukewarm water was poured to fill the funnel, care taken to avoid porch from blocking isolated migrating L3 larvae from swimming down the funnel stem. The apparatus was left for 24 hours at room temperature and 15 ml of fluid collected in blood test tubes from the funnel stem. Tubes containing fluid were left to stand for 30 minutes, the supernatant was drawn with a Pasteur pipette, a McMaster slide filled and mounted on a microscope. Samples were examined and larvae viewed and counted using 100 x magnification.

3.2.3 Experimental design and statistical analysis

Sub-experiment (1) had two animal species, seven plant species and three extract concentrations: hence 2 x 7 x 3 factorial design; sub-experiment (2) had two animal species, three plant species and three extract concentrations, resulting to 2 x 3 x 3 factorial design; and sub-experiment (3) had two animal species, five plant species and three extract concentrations, yielding 2 x 5 x 3 factorial design. In each run, three plates were treated with each plant species crude extract in three concentrations (4x, 2x and 1x). Surviving L3 larvae were isolated on day 14, larval counts done, and mortality (based on the mean of three plates) became indices of dosed anthelmintic efficacy. The study was re-run three times. Nematode mortality was calculated using Abbott's formula (Abbott, 1925), as follows:

$$\text{Corrected \% mortality} = \left(1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}}\right) \times 100$$

Where n = number of larvae, T = treated and Co = control.

Data from nematode larval mortality were analysed using the General Linear Model of SAS (2000) to determine the effect of animal species (goats and sheep), plant species, concentration (1×, 2× and 4×) and of various levels of interaction of animal species, plant species, and concentration on mortality. The level of significance was standardized at maximum probability $p \leq 0.05$ for all statistical tests.

$Y_{ijkl} = \mu + A_i + S_j + C_k + (A \times S)_{ij} + (A \times C)_{ik} + (S \times C)_{jk} + (A \times S \times C)_{ijk} + e_{ijkl}$;

Where, Y_{ijkl} = individual observation; μ = overall mean; A_i = effect of animal species; S_j = effect of plant species; C_k = effect of concentration; $(A \times C)_{ik}$ = interaction of animal and concentration effects; $(S \times C)_{ik}$ = interaction of plant species and concentration effects; $(A \times S \times C)_{ijk}$ = interaction of animal effect, plant species effect and concentration; e_{ijkl} = the error term; $_{ijkl}$= number of the respective parametric terms.

3.3 Results

3.3.1 Sub-experiment 1 (alkaloid and condensed tannins containing plant species)

Animal species (P= 0.0107) affected anthelmintic efficacies of plant species possessing alkaloids and tannins. Anthelmintic efficacies for sheep, ranged from a minimum of 89.8 ± 2.62 % for *Nicotiana tabacum* to a maximum 100 ± 0.91 % for both *Nicotiana tabacum* and *Zingiber officinales*; whereas the minimum efficacy for goats was 42.8 ± 2.62 % for *Sarcostema viminale* and 43.7 ± 2.62 % for *Gunnera perpensa* and spanned to a maximum of 100 ± 0.91 % for both *Zingiber officinales* and *Zizyphus mucronata* (Table 3.1). There was a higher initial efficacy for sheep relative to goats, but both animal species efficacies converged at the upper end (Table 3.1; Figures 3.1 & 3.4). At 2× concentration for goats, the efficacies for *Sarcostema viminale* 66.1 ± 1.88 % and *Gunnera perpensa* 74.3 ± 1.88 % were the only ones below the 80 % mark (Table 3.1).

There was interaction between crude extract concentration and animal species (P= 0.0127). The relationship between anthelmintic efficacy of sheep and goats at various concentrations of crude plant extracts given in Figure 3.1 demonstrated that sheep parasite were highly vulnerable to even the lowest concentration of plant crude extracts. Goat parasite succumbed to the activity of these crude extract when subjected to 4x the initial concentration. Thus the spread of efficacy for goats started by being widely dispersed at concentration 1x, closed rank at 2x and at 4x it was between 85 and 100%.

Concentration (P< 0.0001) affected anthelmintic efficacy (Figure 3.1). Wilks' Lambda statistics showed that a change in crude extract concentration from 1×, 2× and 4× resulted to mean efficacy range of between 71.2 ± 2.62 % and 98.0 ± 0.91 % for 1×, 2× and 4× concentrations (Figure 3.2).

Interaction between concentration and animal species affected (P= 0.0127) anthelmintic efficacy (Figure 3.1). Anthelmintic efficacy for goats and sheep at 1x concentration were different (Tables 3.1/Figure 3.3; 71.2 ± 2.62 % vs 93 ± 2.62 %). Additionally, the crude extract efficacy at concentration 2 was also different (P= 0.0446), with mean efficacy of 88.0 ± 1.33 % for goats and 96.0 ± 1.33 % for sheep (Figure 3.2). Efficacy for both animal species converged at concentration 4x, though the trend for both animals was different. There was a more gradual and marginal increase for sheep, whereas that of goats increased more radically from 1x through 2x and 4x concentrations (Figure 3.4).

3.3.2 Sub-experiment 2 (flavonoid containing plant species)

Increase in concentration resulted to an increase in anthelmintic efficacy (P< 0.0001); with mean efficacies of 72.8 ± 7.89 % at 1x concentration, 84.3 ± 5.12 % at 2x concentration and 97. ± 0.78 % at 4x concentration. On the other hand, animal species affected efficacy (p= 0.0046). Goats had lower anthelmintic efficacy relative to sheep (73.9 ± 6.38 % vs 95.8 ± 1.14 %; Figure 3.5). Plant species showed a tendency to affect anthelmintic efficacy (P= 0.0572). There were vast differences in anthelmintic efficacy for both *T. orientalis* and *U. dioica* with progression of efficacy, Whereas *Z. capense* initially had relatively very high efficacy that changed very little with similar progression. Interactions between concentration and animal species, concentration and plant species, and among concentration, animal and plant species affected (P<0.05) anthelmintic efficacy.

Interaction between crude extract concentration and animal species affected (P= 0.0010) anthelmintic efficacy (Figure 3.2). At the lowest concentration anthelmintic efficacy for sheep was much higher than that of goats (91.5 ± 3.86 % vs 54.6 ± 3.86 %); as concentration increased the anthelmintic efficacies increased slowly for sheep but much radically for goats (Figure 3.2).

Similarly, there were effects (P= 0.0123) of interaction between concentration and plant species on efficacy. The differences in anthelmintic efficacy among various plant species at all the different concentrations reduced with increasing concentration, leading to very close efficacies at 4x concentration (Table 3.2). At 1x, 2x and 4x concentrations, *Zanthoxylum capense* had the highest efficacy, which changed marginally as concentration increased.

Ultimately, interaction among concentration, animal and plant species affected (P= 0.0435) anthelmintic efficacy. Efficacy for sheep at 1x, 2x and 4x concentration were generally high for all three plant species, but those for goats varied from low to high. There was an upward shift at 2x concentration, but with more concentration of plant species efficacy in the upper tier and diffused efficacy in the lower tier. Meantime at 4x concentration, there was near absolute concentration of plant species efficacies at the upper tail end (Figure 3.3).

3.3.3 Sub-experiment 3 (proteases and nitrogen containing plant species)

Both animal species had different (P= 0.0004) anthelmintic efficacies, with relatively more radical changes in efficacy for goats than sheep (Figure 3.1) for the same crude extract concentration. At parity, efficacies for goats were generally lower than those of sheep (Table

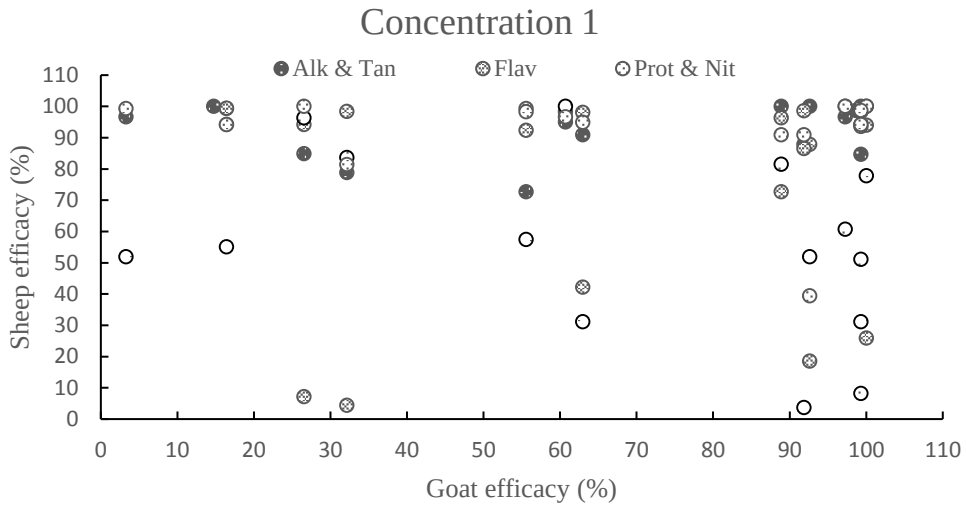
3.1; Figures' 3.1, 3.2 & 3.3). Concentration affected (P< 0.0001) anthelmintic efficacy, with various trends of increases in efficacy following increasing concentration (Figure 3.6). Interaction between animal species and concentration (P= 0.0015) also affected anthelmintic efficacy (Table 3.1; Figure 3.6). At 1x concentration, there was a far lower efficacy for goats relative to that of sheep, which subsequently increased radically at 2x and 4x for the former animal species (goats). On the other hand, efficacy for sheep at 1x concentration was much higher than that of goats but changed marginally with subsequent increases to 2x and 4x concentrations (Figure 3.6). Generally, mean efficacy for both animal species (goats and sheep) were between 66.4 ± 3.13 and 97.5 ± 1.95 % (Figure 3.6).

Table 3. 1: Least square means of anthelmintic efficacy ± standard error of least square means (%) of plant species crude extracts containing alkaloids and/or tannins, flavonoids, proteases and/or nitrogen compounds for both goats and sheep at 1x, 2x and 4x concentration

		Goats			Sheep		
Primary anthelmintic phytochemical(s)	Plant species	Efficacy (%) at 1x Conc.	Efficacy (%) at 2x Conc.	Efficacy (%) at 4x Conc.	Efficacy (%) at 1x Conc	Efficacy (%) at 2x Conc.	Efficacy at (%) 4x Conc.
Alkaloids &	*Crinum m.*	92.4 ± 2.62	95.6 ± 1.88	98.7 ± 0.91	74.7 ± 2.62	96.4 ± 1.88	97.6 ± 0.91
Tannins	*Gunnera p.*	98.1 ± 2.62	100.0 ±1.88	99.4 ± 0.91	43.7 ± 2.62	74.3 ± 1.88	98.3 ± 0.91
	Nicotiana t.	97.8 ± 2.62	100.0 ± 1.88	100.0 ± 0.91	70.1 ± 2.62	91.6 ± 1.88	95.2 ± 0.91
	Sarcostema v.	93.4 ± 2.62	93.4 ± 1.88	97.0 ± 0.91	42.8 ± 2.62	66.1 ± 1.88	95.2 ± 0.91
	Vernonia a.	90.8 ± 2.62	92.9 ± 1.88	96.4 ± 0.91	96.8 ± 2.62	98.6 ± 1.88	99.3 ± 0.91
	Zingiber o.	89.8 ± 2.62	92.9 ± 1.88	100.0 ± 0.91	83.3 ± 2.62	100.0 ± 1.88	100.0 ± 0.91
	Zizyphus m.	94.5 ± 2.62	99.0 ± 1.88	99.0 ± 0.91	87.2 ± 2.62	88.9 ± 1.88	100.0 ± 0.91
Flavonoids	*Trema o.*	22.6 ± 3.86	50.4 ± 3.23	93.0 ± 2.45	93.4 ± 3.86	96.8 ± 3.23	100.0 ± 2.45
	Urtica d.	43.0 ± 3.86	60.8 ± 3.23	80.8 ± 2.45	92.9 ± 3.86	97.2 ± 3.23	98.8 ± 2.45
	Zanthozylum c.	98.3 ± 3.86	100.0 ± 3.23	100.0 ± 2.45	88.1 ± 3.86	96.9 ± 3.23	98.3 ± 2.45

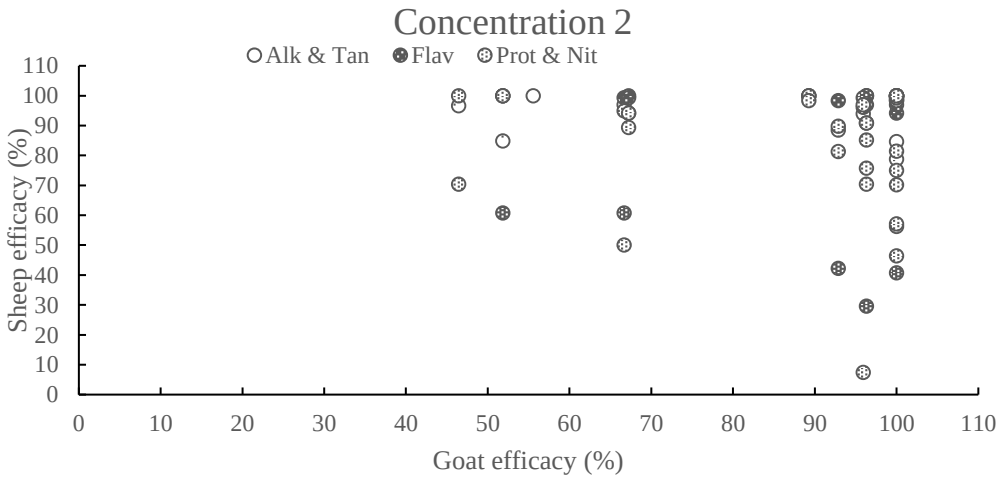
		Goats			Sheep		
Primary anthelmintic phytochemical(s)	Plant species	Efficacy (%) at 1x Conc.	Efficacy (%) at 2x Conc.	Efficacy (%) at 4x Conc.	Efficacy (%) at 1x Conc	Efficacy (%) at 2x Conc.	Efficacy at (%) 4x Conc.
Proteases	*Allium a.*	53.7 ± 3.13	70.3 ± 2.69	77.7 ± 1.95	79.0 ± 3.13	91.4 ± 2.69	93.4 ± 1.95
&/or	*Ananas c.*	46.7 ± 3.13	62.6 ± 2.69	69.5 ± 1.95	96.2 ± 3.13	98.1 ± 2.69	99.4 ± 1.95
Nitrogen	*Bidens p.*	75.3 ± 3.13	83.3 ± 2.69	94.8 ± 1.95	90.8 ± 3.13	90.8 ± 2.69	97.2 ± 1.95
compounds	*Carica p*	67.5 ± 3.13	82.2 ± 2.69	96.2 ± 1.95	96.1 ± 3.13	97.9 ± 2.69	98.7 ± 1.95
	Ricinus c.	37.3 ± 3.13	51.3 ± 2.69	83.2 ± 1.95	95.4 ± 3.13	98.4 ± 2.69	99.0 ± 1.95

Crinum m.= Crinum macowanni, Gunnera p.= Gunnera perpensa, Nicotiana t.= Nicotiana tabacum ,Sarcostema v.=Sarcostema viminale, Vernonia a.= Vernonia amygdalina, Zingiber o.= Zingiber officinales and Zizyphus m.= Zizyphus mucronata, Trema o.= Trema orientalis, Urtica d.= Urtica dioica, Zanthozylum c.= Zanthozylum capense,. Allium c= Allium cepa, Ananas c.= Ananas comosus, Bidens p.= Bidens pilosa, Carica p.= Carica papya, Ricinus c. = Ricinus communis, SELSM= standard error of least square mean, Conc= Concentration



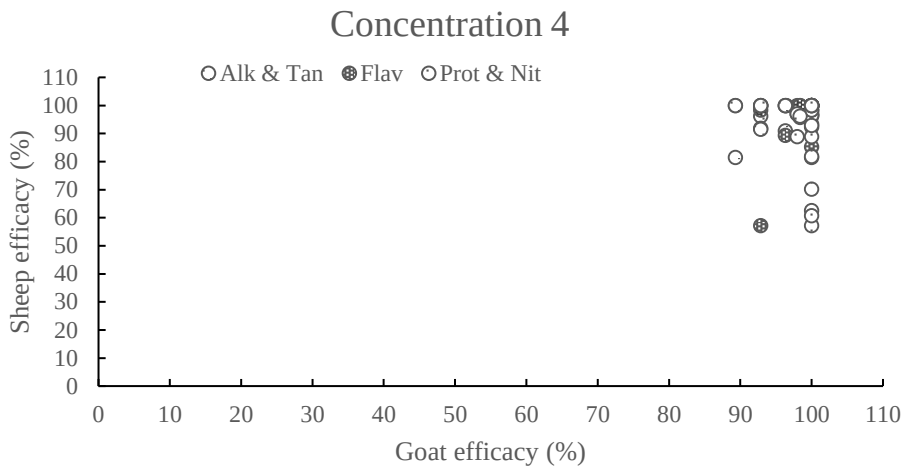
Alk & Tan = Alkaloid and tannin containing species; Flav = Flavonoid containing species; Prot & Nit = Proteases and nitrogen compound containing species

Figure 3. 1 : General distribution of *in vitro* phytotherapeutic efficacy (%) for goats and sheep at 1x concentration for selected plants possessing alkaloids/Tannins, Flavonoids and Proteases/Nitrogen compounds



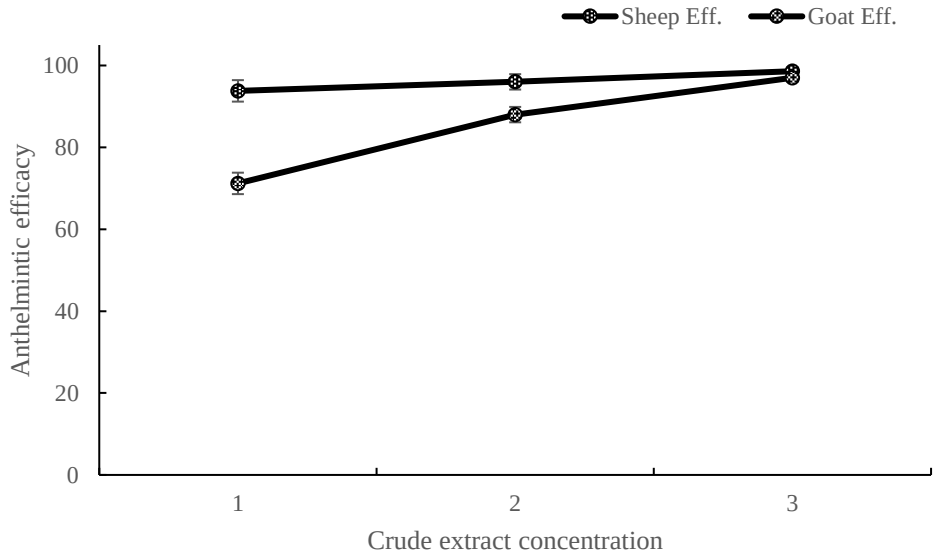
Alk & Tan = Alkaloids and tannin containing species; Flav = Flavonoid containing plant species; Prot & Nit = Proteases and nitrogen containing plant species

Figure 3. 2: General distribution of *in vitro* phytotherapeutic efficacy (%) for goats and sheep at 2x concentration for selected plants possessing alkaloids/Tannins, Flavonoids and Proteases/Nitrogen compounds



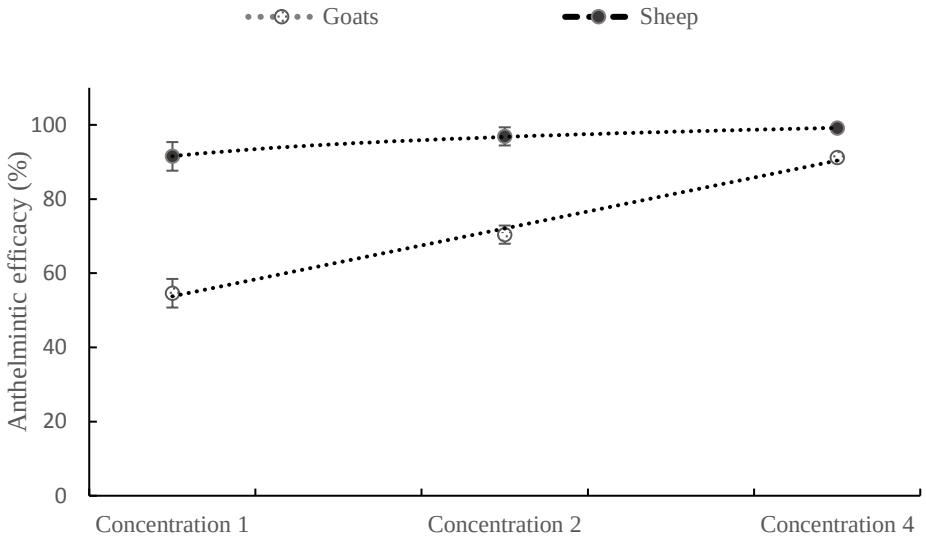
Alk & Tan = Alkaloids and tannin containing species; Flav = Flavonoid containing plant species; Prot & Nit = Proteases and nitrogen containing

Figure 3. 3: General distribution of *in vitro* phytotherapeutic efficacy (%) for goats and sheep at 2x concentration for selected plants possessing alkaloids/Tannins, Flavonoids and Proteases/Nitrogen compounds



Sheep Eff = Mean anthelmintic efficacy for sheep, and Goat Eff. = mean anthelmintic efficacy for goats, SELSM = standard error of least square mean, 1, 2 & 3 = 1x, 2x and 4x concentration

Figure 3. 4: Trend of anthelmintic efficacy (%) of plant species possessing tannins and alkaloids for both goats and sheep at 1x, 2x and 4x concentration



SELSM = standard error of least square means, concentrations 1, 2 and 4 are equivalent to 1x, 2x and 4x concentration

Figure 3. 5: Progression of anthelmintic efficacy (%) of plant species containing flavonoids for both goats and sheep (Least square means (LSM) ± standard error of least square meansmeans (SELSM)) at 1x, 2x and 4x concentration

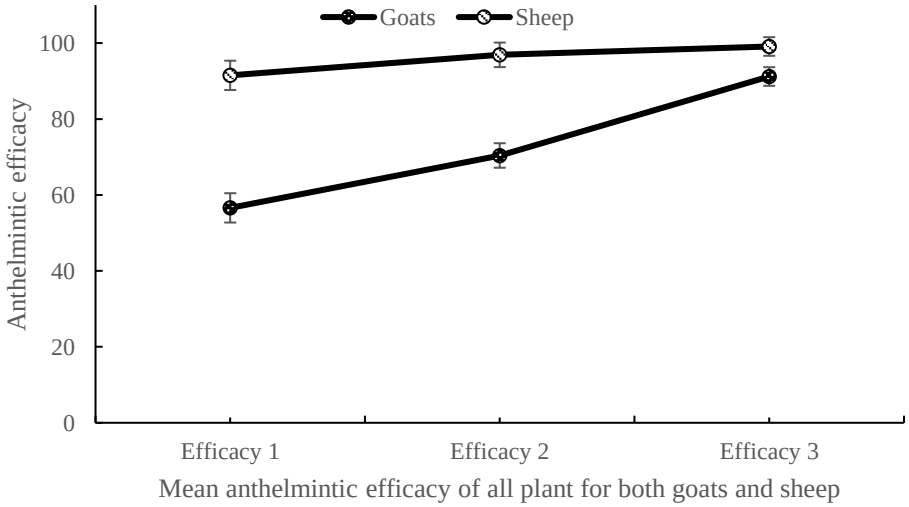


Figure 3. 6: Progression of anthelmintic efficacy (%) of plant species containing proteases and nitrogen compounds for both goats and sheep (Least square means (LSM) ± standard error of least square means (SELSM)) at 1x, 2x and 4x concentration

3.4 Discussion

3.4.1 Sub-experiment one (alkaloids and tannins)

Anthelmintic efficacy of the same plant species crude extract possessing condensed tannins and alkaloids at the same concentration was different for both goats and sheep (Figures' 3.1 and 3.4), suggesting that animal species related traits might have affected efficacy as concentration increased (Paolini *et al*., 2003; Hoste *et al*., 2006). This is consistent with the suggestion that specific requirements of animal species be considered in order to maintain or enhance anthelmintic efficacy of relevant plant species because of inherent disparities (Vercruysse *et al*., 2001). At higher concentration the anthelmintic efficacies for both goats and sheep were either similar or within close range of each other (Figure 3.4), showing how close efficacies of both animal species become at higher concentration. Contrary to our expectation increase in crude extract concentration resulted to increase in efficacy. Other possible causes may be responsible for the differences in efficacy as a result of animal species.

Differences in anthelmintic efficacy for the same plant species between goats and sheep (Figures' 3.1 and 3.4) at lower concentrations may be attributed to their feeding behaviour and forages encountered; goats browse/graze (Skarpe *et al*., 2007; Dove, 2010) whereas sheep mainly graze naturally (Gordon, 2003). Plant secondary metabolites including alkaloids, flavonoids, tannins and saponins abound in browse (Nguyen *et al*., 2005; Athanasiadou *et al*., 2007) and/or forbs, consequently, goats might too frequently have been pre-exposed to these compounds relative to sheep. Their foraging behaviour might have aided to develop the biochemical capacity to counteract tanniferous and alkaloidal anthelmintic activities at lower concentration (Hoste *et al*., 2006) and/or having worms with better adaptation strategies. At higher concentration, the threshold of animal species difference is potentially exceeded/overwhelmed and counteracted, thus concentration exerts its full effect on anthelmintic efficacy giving rise to the highly close efficacy for both species (Figure 3.4). Secondary chemical metabolites of forage consumed by both animal species may well have served as another contributing factor for the differences observed in anthelmintic efficacy.

Fundamentally, browse and forbs, and grass differ in their chemistry (Gordon, 2003); browse plants contain higher levels of secondary compounds (alkaloids, flavonoids, saponins and tannins) and other nitrogenous compounds (Gordon, 1989) relative to grasses. This trait might have increased the level of tolerance by goats due to frequent pre-exposure to alkaloids and

tannins, since browse constitutes a major proportion of their diet. Sheep consumed a lower level of tannin rich *Lespedeza sericea* (Terrill *et al*., 1989) relative to goats grazing the same forage species (Terrill *et al*., 1989; Min and Hart, 2003), reaffirming goats' tolerance to higher level of tannins and alkaloids. That is potentially why lower concentrations of plant species crude extract containing alkaloids and tannins yielded a higher efficacy for sheep than goats, but exhibited similar anthelmintic efficacy at higher concentration (Figure 3.4). On the other hand, a change in concentration entails a higher concentration of the active anthelmintic principle(s) (alkaloids and/or tannins) and increased efficacy (Figure 3.4). This concurs with *in vivo* studies in sheep, goats and deer suggesting that a minimum range of 3-4 % condensed tannins has to be attained for any discernible anthelmintic activity and further increases in concentration resulted to increases in efficacy (Hoste *et al*., 2006). This threshold marks the concentration below which administration of plant crude extracts containing alkaloids and/or tannins to livestock in helminth control programs will be regarded as under dosing and thus ineffective.

There was similar interaction of anthelmintic efficacy and condensed tannin concentration administered *in vivo,* potentially linking increased activity to increased concentration (Min and Hart, 2003). Increased tannin extract concentration from legumes also resulted to a decrease in egg hatch and larval development of *Trichostrongylus colubriformis* (Molan *et al.*, 2000). *In vivo* administration of condensed tannins to nematode infected sheep has to be carefully managed because beyond some threshold, adverse effects will set in, negating the positive anthelmintic effect that they possess. Administration of 8 % of food intake to sheep infected with a single dose of *Teladorsagia colubriformis* of Quebracho extract rich in tannins, resulted to reduction in faecal egg count (FEC) and worm burden lasting for a week (Athanasiadou *et al*., 2000), but with consistent follow-up administration, FEC remained low. Similar results were obtained from *in vivo* studies wherein sheep were dosed with crude plant extracts of *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata* and *Warburgia salutaris* weekly for forty two days (Ahmed *et al*., 2014). Athanasiadou *et al*., (2001) subsequently found that dosing the same sheep infected with *Teladorsagia colubriformis* once with 8 % of Quebracho extracts relative to daily food intake reduced FEC and could potentially be retained at that level for a month. Sheep receiving an initial dose of 16 % Quebracho extract compared to daily food intake had a more reduced FEC, but their food intake fell significantly from day 28 and they manifested severe signs of diarrhoea after the second dose of Quebracho extract administration. Reed (1995) discouraged administration of high levels of some classes of

condensed tannins to ruminants because of their toxic effects. Condensed tannins differ widely in chemical structure/activity (Alonso-Díaz, *et al*., 2009; Rochfort *et al*., 2008) and different types occur in different plant species (Barry, 1985), additionally, plants contain a wide array of secondary metabolites (Makkar, 2006; Hoste *et al*., 2008a) which may collectively influence the plant species level of anthelmintic activity among others, thus contrasting sharply with the concept of a primary putative bioactive anthelmintic principle. The biochemical mode of action of most plant secondary metabolites (PSM) on adult nematode fecundity and egg hatch is not clearly understood.

Plant secondary metabolites, especially condensed tannins and other polyphenolic compounds form complexes with organic macromolecules, some of which are proteins and carbohydrates (Ademola *et al*., 2005; Hoste *et al*., 2006; Mueller-Harvey, 2006; Alonso-Diaz *et al*., 2011). The formation of these complexes is suggestive of deprivative effect on proteins, essential amino acids and carbohydrates that will drive vital biochemical activities within the developing/developed nematode egg, growing larva and adult nematode. Tannins-protein/amino acid complexes formed, culminate to potential loss of egg capacity to develop or developed mature eggs from hatching, hence decreasing egg hatch and count (Athanasiadou *et al*., 2000; Molan *et al*., 2002). The same interaction with other macromolecules (Mueller-Harvey, 2006) of developing nematode larvae, possibly will impair biochemical activity that promote development and growth, decreasing or arresting larval development (Molan *et al*., 2002). Concentration of some tannins and other polyphenolic compounds as low as 75 μgml^{-1} interfered and related negatively with some biological processes of *Haemonchus contortus* (Alonso-Diaz *et al*., 2011; Azando *et al*., 2011). In the nematode parasite life cycle, any biochemical activities/intervention that either lead to decrease in egg hatch or impede larval development will potentially reduce infective L3 larvae and adult nematode parasite burden (Athanasiadou *et al*., 2000). Alkaloids in their own group are naturally occurring chemical compounds that contain mostly basic nitrogen atoms in the heterocycle. It is claimed the same reaction may be plausible with alkaloids (Alonso-Diaz *et al*., 2011; Azando *et al*., 2011) because they possibly exert the same effect and some of them are alleged to be polyphenolic in nature (Ademola *et al*., 2005). There is vast empirical evidence implicating alkaloids of exhibiting anthelmintic activity (Ademola *et al*., 2005; Hoste et *al*., 2006, Alonso-Díaz *et al*., 2012), but their biochemical mode of action is not clearly understood and has been likened to that of tannins.

The trend of efficacy at the lowest concentration for goats was *Vernonia amygdalina>Zizyphus mucronata> Zingiber officinale>Crinum macowanii>Nicotiana tabacum>Gunnera perpensa>Sarcostema viminale* (table 1) relative to sheep that were within close range of *Vernonia* (table 1) which exhibited the highest efficacy for goats. This trend reaffirms suggested differences in response of different animal species (Paolini *et al*., 2003; Hoste *et al*., 2008a) to the use of plant species possessing anthelmintic activity in parasite control programs. Additionally, the trend of efficacy at the lowest concentration for all plant species is suggestive of differences in the concentration and number of bioactive anthelmintic principle(s) which may synergise each other, given the increase in concentration that brings about increased efficacy to various extents for all plant species (Figures 3.1 and 3.4). This is analogous to more than just a dose dependent anthelmintic activity that is exhibited by most plant species possessing antiparasitic activity (Shrivka and Kuma, 2003; Iqbal *et al*., 2006; Jabbar *et al*., 2007), thus suggesting there could be interacting multiple principles in each plant. For both animal species, all plant species exhibited mean anthelmintic efficacy above 60% (Figure 3.4) concurring with previous findings (British Veterinary codex, 1953, 1965; Grime *et al*., 2008; Haman, 2007; Refaat *et al*., 2012; Simelane *et al*., 2010; Van Wyk and Wink, 2004; Yeap *et al*., 2010).

3.4.2 Sub-experiment two (flavonoids)

Goats and sheep had various levels of efficacy for same plant species crude extract (Table 3.1), but the differences in efficacy for both animal species decreased as efficacy increased until they were statistically very close (Figure 3.5). The wide differences in efficacy between goats and sheep potentially have been borne from animal species related trait influences (Vercruysse *et al*., 2001; Chaudry *et al*., 2007) on bioactive anthelmintic principles involved and resulting anthelmintic efficacy. Anthelmintic efficacies of sheep for *Trema orientalis* and *Urtica dioica* were higher than those of goats (Table 3.1). Exceptionally, this trend was different for the third species *Zanthozylum capense* that had very high values for both goats and sheep; but with numerically higher efficacy for goats than sheep. The above observations reaffirm suggestions that animal species trait (Vercruysse *et al*., 2001; Makkar *et al*., 2007) might have caused the different trends in efficacy for *Trema orientalis* and *Urtica dioica*; *Zanthozylum capense* broke rank from consistency and exhibited its own unique anthelmintic efficacy trend. The anthelmintic activity of *Zanthozylum capense* is probably linked either to very powerful potent bioactive anthelmintic principle(s) or the absence of any prior encounter by both animal

species. Animal species feeding behaviour and diet composition also appear to play a major role in the observed differences in anthelmintic efficacy between goats and sheep. Goats browse and graze (Alonso-Diaz *et al*., 2008; Skarpe *et al*., 2007; Dove, 2010), while sheep naturally graze (Gordon, 2003; Alonso-Diaz *et al*., 2009). Goats are pre-exposed to some of the bioactive classes of compounds such as tannins, terpenes, alkaloids beside flavonoids that possess anthelmintic activity and abound in browse and Forbs (Papanastasis *et al*., 2008; McDonald *et al*., 2011). Pre-exposure by goats most likely has created some level of tolerance to flavonoids at low concentration in the present context, leading to marginal differences in efficacy for both animal species at higher concentration relative to wider differences initially observed at lower concentrations. Additionally, variation in anthelmintic efficacy between goats and sheep might have arisen from genetic differences in resistance between them; consistent with observed significant differences in resistance of gastrointestinal nematode parasites of goat and sheep breeds of the same species (Chaudary *et al*., 2007). This is suggestive of huge variation for different animal species such as goats and sheep, making goats a good animal model for potential anthelmintic studies.

There were various effects of initial concentration and subsequent changes to 2x and 4x concentration (Figures' 3.1, 3.2 & 3.3). Plant speciation (Hammond *et al*. 1997; Aherne and O'Brien, 2002; Makkar *et al*. 2007; Cala *et al*., 2012) is suggested to be a primary driver of the various effects of concentration on efficacy; as biochemical composition (Hammond *et al*., 1997; Wenk, 2003; Shaik *et al*., 2006) is unique to any of *T. orientalis, U. dioica* and *Z. capense* among other factors. The nature, chemical structure, molecular size and variety of the anthelmintic principle(s) and related biochemical candidates (Harborne, 1986) that support and enhance anthelmintic/antibiotic activity of any of the plant species are potential factors that might have influenced the effect of concentration on efficacy. This is essentially because most plant species possessing/exhibiting anthelmintic activity have a wide range of antibiotic principles and also exercise broad spectrum anthelmintic activity (Satrija *et al*., 2001). Additionally, a low dose of anthelmintics expelled helminths, while a sufficiently high dose was potent (Sollmann, 1918), reaffirming the differences in anthelmintic activity/efficacy as a result of increased concentration. Interaction of concentration and animal species exerted different effects on efficacy.

An evaluation of interaction between animal species and concentration in the current study showed that progression in concentration brought about elaborate changes in anthelmintic efficacy for both goats and sheep. These results concur with previous studies, wherein lower

levels of inclusion of *Sericea lespedeza* (25% and 50%) in goat diet resulted in reduced faecal egg count (FEC), whereas 80% inclusion resulted to reduction in abomasal worms (Terrill *et al.,* 2009). Similar results were obtained for both goats and sheep at 75-80% of *Sericea lespedeza* inclusion (Lange *et al*., 2006; Shaik *et al*., 2006). Interaction between concentration and plant species also exerted various effects on efficacy. Though *T. orientalis, U. dioica* and *Z. capense* all putatively possess a common class of bioactive anthelmintic principle (flavonoids), they retain their specific differences in phytochemical nature, variety (Hammond *et al*., 1997; Makkar *et al*., 2007) and concentration (Cala *et al*., 2012) within the same and different concentrations hence different levels of efficacy. Similar observations whereby plant species from the same family (Bazh and El-Bahy, 2013) exhibited different anthelmintic efficacies at the same crude extract concentration concur with that of the present study; possibly reaffirming the natural variation of plant species (Wenk, 2003; Cala *et al*., 2012). After all, interaction of concentration, animal and plant species had diverse effects on efficacy.

In the present study, efficacies obtained at various concentrations were different in accordance with Hördegen *et al*., (2006), while animal species also affected efficacy (Vercruysse *et al*., 2001; Makkar *et al*., 2007) at all three concentrations, and plant species in turn exerted their specific differences, suggestive of their biochemical variation (Hammond *et al*., 1997; Gordon 2003; Makkar *et al*., 2007) by exhibiting various effects on efficacies (Hördegen *et al*., 2006) at the same and at different concentrations in the current study. These differences in anthelmintic efficacy resulting from the effects of concentration, animal and plant species potentially point to significant interaction as statistically tenable in the current study. However, how flavonoids exercise their biochemical activity in control of parasitic nematodes of livestock is enigmatic.

Empirical evidence is suggestive of sufficiently acceptable levels of anthelmintic activity by plant possessing flavonoids (Perry and Metzger, 1980; Duke, 1992; Barrau *et al*., 2005; Haman, 2007; Kerboeuf *et al*., 2008; Eguale and Giday, 2009; Azando *et al*., 2011; Refaat *et al*., 2012: Bibkar *et al*., 2012). Flavonoids inhibited larval migration of *H. contortus* as much as condensed tannins did. Sequential fractional precipitation of flavonoids that occur in plant vegetative material alongside condensed tannins using polyvinyl pyrrolidone (PVPP) eliminated inhibitory activity of larval migration caused (Barrau *et al*., 2005). In a similar study, but that which dealt with L3 larval exsheathment inhibitory assay (LEIA), flavonoids and tannins inhibited the process (Azando *et al*., 2011); which was restored to near negative control levels with addition of Polyethylene glycol (PEG). Tannins bind and form insoluble

complexes with organic macromolecules such as proteins and carbohydrates (Ademola *et al*., 2005; Hoste *et al*., 2006; Mueller-Harvey, 2006; Alonso-Diaz *et al*., 2011). These macromolecules support major vital biochemical activities and growth of nematode parasites, and their developmental intermediate stages, hence disruption of larval motility and larval exsheathment (Barrau *et al*., 2005; Azando *et al*., 2011). This reaction is evocative of a similar biochemical mode of action of flavonoids and tannins. There is fundamentally very close relationship between flavonoids and tannins, both of which are polyphenols, with flavan-3-ols as basic phenolic monomers (Barrau *et al*., 2005); potentially supporting closely similar biochemical mode of action against livestock parasitic helminths between the two.

3.4.3 Sub-experiment three (proteases and nitrogen compounds)

Animal species had different *in vitro* anthelmintic efficacies (Figure 3.6) regardless of the plant species (Table 3.1). The trend in anthelmintic activity in relation to animal species is in agreement with (Vercruysse *et al*., 2001), who suggested consideration of animal species differences at dosing with anthelmintics in order to ensure high and consistent efficacy. Similarly, differences in anthelmintic efficacy of plant remedies, emanating from differences in animal species (Makkar *et al*., 2007) were observed and highlighted to be taken care of at dosing, in accordance with the observation of the current study in order to obtain optimum anthelmintic efficacy in helminth control programs. In the process of developing anthelmintic, it is perhaps better to use the animal (goats) with a lower efficacy than sheep, given that whichever plant species exerts anthelmintic activity for the former, does exceedingly well for the later (Figures 3.1, 3.2, 3.3 & 3.6). Cysteine proteinases from pawpaw that were found to be very effective against parasitic helminths showed variable efficacy *in vivo* with different strains of rat (Luoga, 2012). Although, the observation was from an *in vivo* trial, the present *in vitro* study highlights subject variety differences with strong implications to greater species differences in anthelmintic efficacy between goats and sheep.

Given the same concentration of plant species crude extract, the anthelmintic efficacy for larvae drawn from sheep was far higher than that from goats (Figure 3). This trend was suggestive of prior exposure to the same or similar active anthelmintic phytochemical by goats relative to sheep, in effect, creating some level of tolerance at low concentration. The most probable route of prior encounter is likely to have been through animal species diet (Gordon, 1989), in which plant species or plant varieties possessing similar phytochemicals constituted some portion of their diet. Goats browse and graze, whereas untrained sheep graze (Solaiman, 2010), rendering

tree parts such as shoots, leaves, growing parts of shrubs and forbs main constituents of goats' diet. These forages contain plenty of plant secondary metabolites (McAllistair *et al*., 2005; Rogosic *et al*., 2008), some of which are implicated in control of parasitic nematodes and other microbial organisms. Sheep on their part seldom encounter these plant secondary metabolites because their diet is predominantly grass in nature (Gordon, 1989). Differences in anthelmintic efficacy were also observed in the treatment of infected lambs and mice with extracts of *Albizia anthelmintica Brong* (Githiori *et al*., 2003), wherein, there was no anthelmintic activity observed against mice parasites. Though the outcome contrasted with that of the current study in which plant species possessing proteases and protein compounds exhibited anthelmintic activity against both goats and sheep *in vitro*, it highlights animal species differences vis a vis anthelmintic efficacy of the same plant species

Concentration effect of different plant species crude extract was also observed to influence differences in anthelmintic efficacy in this study. Increase in concentration of plant species crude extract from 1x through 2x to 4x concentration for both animal species resulted to various changes in anthelmintic efficacy in conformation with dose-dependent activity of *Ficus racemose* (Linn.) bark crude extract (Chandrashekhar *et al*., 2008) on adult earth worms. The primary anthelmintic bioactive phytochemical in the above crude extract is proteases in nature (Chandrashekhar *et al*., 2008), and similar to that of some plant species in the current study. Cysteine proteinases from papaya latex administered to sheep infected with *Haemonchus contortus* and *Trichostrongylus colubriformis* (Buttle *et al*., 2011), also exhibited dose dependent anthelmintic activity. Additionally, aqueous extracts of pineapple skin and bromelain exhibited dose-dependent *in vitro* activity on *Haemonchus contortus* egg hatch development inhibition and larval development inhibition in sheep (Domigues *et al*., 2013). All of the former examples are corroborative of dose dependent anthelmintic activity of the same plant species and others in the current study. Variable anthelmintic efficacy emanating from different plant species possessing proteases such as pineapple, Kiwi fruits and pawpaw at the same and different concentration (Luoga, 2012) are in accord with results of the current study. Concentration or dose effect may also vary between *in vitro* and *in vivo* activity in consonance with (Luoga, 2012), as a result of animal host internal environment. There were differences between animal species and concentration. How proteinases and related phytochemicals exercise their activity in helminth parasite control is worth brief exploration.

Proteinases have a wide range of characteristics, some of which include tissue dissolution and remodelling (Knox, 2007). The same biochemical mode of action is used by parasites to

penetrate host tissues in order to obtain nutrients, be it plug tissue feeding *Strongyloides* (Sutherland and Scott, 2010) or other parasitic species that burrow and lodge in the subcutaneous walls of the gastrointestinal tract among others. These parasites protect themselves from host protease analogues which aid protein digestion and other biochemical processes by secreting proteases inhibitors (Knox, 2007). Proteases exert their activity in context by binding to protein or peptide substrate in their active site, in the process cleaving them (Sajid and Mckerrow, 2002), and retaining site specificity by amino acids on both sides of the cleavage. It is implicit that several active sites are therefore involved in dissolution of parasite protein or peptide, for them to exert anthelmintic activity. Extensive *in vitro* and *in vivo* cuticular damage was done to rodent intestinal nematode *Heligmosomoid bak*eri by cysteine proteases from Kiwi fruit (*Actinidia deliciosa*), latex of *Carrica papaya,* and, stem and fruit bromelain of *Ananas comosus* (Luoga *et al*.,2015), reaffirming tissue digesting trait of proteases. So a combination of proteases could even become more devastating on helminths.

3.5 Conclusion

Animal species effect has to be accorded adequate attention in the use of crude plant extracts containing bioactive anthelmintic principles, be they tannins, alkaloids, flavonoids, proteases or nitrogen compounds. This is essential to ensure that there is accurate dosing to obtain the desired high efficacy. In the use of condensed tannins, *in vitro* treatment with high concentrations may yield higher efficacy, but they have proven to be a huge limitation to animal health and productivity due to their negative interaction with proteins and other biological macromolecules essential to biochemical function and metabolic activities. Different plant species exerted different levels of anthelmintic activity, regardless of the anthelmintic principle(s) involved. Concentration of the various plant species crude extracts was also central to the level of athelmintic efficacy. In nature, tannins, alkaloids, flavonoids, proteases and nitrogenous bioactive macromolecules are a wide variety of different plant compounds with the potential of exercising different levels of anthelmintic activity that culminate to the observed plant species activity. Combinations of the various plant species extract, will have the capacity of increasing the range of bioactive compound classes, macromolecular variety pool and potential of optimizing anthelmintic activity at a lower concentration or dose. Combination therapy has the potential of enhanced anthelmintic activity at lower concentrations thus reducing the dose and adverse effects that may arise from excessively high concentration, especially condensed tannins.

Important insight has been gained from key vehicles of anthelmintic efficacy of plants possessing different bioactive anthelmintic principles. These included animal species, plant species, dose/concentration of crude extract and their interactions. This therefore provide some bearing or guide to subsequent studies. Moreover, the potential to enhance anthelmintic efficacy by exploration of other modes of dosing will be recommended for further research in the following studies.

Chapter 4

In vitro combination anthelmintic phytotherapy for sheep using plants possessing similar bioactives

Abstract

This study evaluated *in vitro* anthelmintic efficacy of combined plant species on mixed nematode of sheep in three sub-experiments, comprising of plant species containing (1) alkaloids and/or condensed tannins, (2) flavonoids and (3) proteases and/or nitrogen. Sub-experiment (SEP) one had twenty one (21) combinations; SEP two, three (3) and SEP three, ten (10). Each experiment was run three times. Crude extract of each plant species was obtained by extracting 4 g dry matter (DM) in 70 % ethanol and made up to 100 ml. Sheep dung, collected from the rectum, was pooled and mixed to constitute test samples for incubation. Five (5 g) of dung was weighed into Petri dishes and cultured for 12 days at 27ºC. On day 13, treatments were dosed with combined extract at 2.5 ml x 2 (doubled concentration of each constituting pair), and controls moistened. Surviving L3 larvae were isolated and counted using McMaster slide. Expected combined efficacy computed as (a + b)/2, and simple synergy (differences between combined and expected efficacies) were also computed. Webb's synergy was computed using Webb's fractional product method. Alkaloids, condensed tannins and flavonoids contents were quantified and, simple and multiple regression analyses were run to determine their contribution to anthelmintic efficacy. High efficacies for combined plant species of SEP 1 (93.8 ± 0.67 %), SEP 2 (94.3 ± 5.88 %), and SEP 3 (97.9 ± 0.30 %.) were observed but within sub-experiments efficacies were not different (P>0.05). Simple synergies were mostly positive, with means of 2.5 ± 0.67 % (SEP 1), 1.8 ± 1.19 % (SEP 2), and 2.8 ± 0.30 % (SEP 3). However, Webb's synergy were largely negative for SEP 1 (-5.4 ± 0.67 %), SEP 2 (-5.2 ± 1.19 %), and SEP 3 (-1.9 ± 0.30 %), each being lower than zero. Among plant combinations, in SEP 1, condensed tannin and flavonoid contents were different (P< 0.0001) but not in alkaloid contents (P= 0.3037). In SEP 2 condensed tannin (P< 0.009) and flavonoid (p= 0.0211) contents were different but not in alkaloid contents (P= 0.07). In SEP 3, condensed tannin contents were not different (P> 0.4312), while the alkaloid (P= 0.0135) and flavonoid contents (P< 0.0001) were different. For all these macro-molecules, there was no discernible association with anthelmintic efficacy. In conclusion, there was potent activity arising from combinations as exemplified by high efficacy, which in the absence of any correlation could be potentially attributed to activity of all macromolecules including alkaloids, condensed

tannins and flavonoids, and bioactivity of other related phytochemicals. This is suggestive of a more complex and intricate macromolecular and biochemical interaction in combinations.

4.1 Introduction

In the phase of waning chemo-anthelmintic efficacy (Terrill *et al*., 2001; Jackson *et al*., 2009; Kaplan and Vidyashankar, 2012) and general selection by nematode parasites and other pathogenic micro-organisms for single (Geerts and Gryseels, 2000) or multiple drug resistance (Kaplan, 2004), it is imperative to explore how efficacy of plants possessing anthelmintic activity among other biological activities can be optimized. High plant species anthelmintic efficacy, though often lower than that of their orthodox counterparts, has advantage of retaining nematode load below that which will affect production negatively; referred to as "economic threshold" (Ketzis *et al*., 2006).

Non-chemical helminth control methods of this nature will serve as viable alternatives (options), which will reduce recurrent use of relevant chemical anthelmintics or reliance on a few. This, in effect, deters selection for resistant parasites, as has been strongly implicated in the current crisis (Besier, 2007). Plants, by their very nature, contain a wide variety of bioactive macromolecules belonging to the same and/or different classes of compounds (Klongsiriwet *et al*., 2015) and can be likened to combined remedies (Sajid and McKerrow, 2002; Choi and Chung, 2003; Makkar, 2006; Hoste *et al*., 2008a), setting a precedence to anthelmintic therapy.

Combination anthelmintic therapy or prophylaxis has currently been adopted to retain high drug efficacy (Entrocasso *et al*., 2008; Bartram *et al*., 2012) and simultaneously recycled those anthelmintics that have failed or currently exert relatively low efficacy (Bartram *et al*., 2012; Lanusse *et al*, 2014; Lanusse *et al*., 2015). Additionally, combination therapy widens spectrum of nematode parasite control within particular and different sites of infection in the gastrointestinal tract hosting them (Athanasiadou *et al*., 2001; Marley *et al*., 2003). Gastrointestinal nematode infection of livestock poses a huge economic challenge to health and productivity of grazing-livestock globally (Vercruysse *et al*., 2006; Waller, 2006a; Besier, 2007; Athanasiadou *et al*., 2008; Sutherland and leathwick, 2011), relative to those in confinement. This problem has attained unprecedented level in small ruminants (goats and sheep), in addition to challenges of current control strategies (Jackson and Coop, 2000). Given

the role of small ruminants as an important source of wealth and animal protein to low resource households of Africa, Asia and most developing nations (Perry *et al*., 2002), any perturbation to their productivity or mortality resulting from infection by these parasites will have a huge setback on their income and livelihood. Consistent and progressive failure of chemical anthelmintics (Waller, 1997c; Jackson and Miller, 2006; Kaminsky *et al*., 2008; López-Aroche *et al*., 2008), which has been the primary method of nematode parasite control from their inception, demand a review of previous and current modes of application and possibly a fundamental change to re-establish high efficacy. Additionally, potential methods of improving ethnobotanical anthelmintic efficacy will prove to be a very useful and crucial tool in the control process.

These changes are critical, because they will potentially arrest rapid development of resistant parasites and improve the efficacy span of anthelmintics in use, including subsequent candidates that will be developed. Additionally, research and development of new and more effective chemical candidates had similar high efficacy with preceding ones (Coop and Kyriazakis, 2001) turned ineffective, as resistant nematode strains still emerge sooner or later (Várady *et al*., 2011). Extensive research and development of new anthelmintic candidates could well be out of favour with the economic interest of major pharmaceutical industries (Besier, 2007), because of the sheer size of small ruminant industry that is mostly affected relative to other domestic ruminant species (Várady *et al*., 2011). These challenges raise a lot of concerns, reaffirming dire need to conserve the efficacy of current anthelmintics and concurrently seek avenues of optimization. Existing control strategies not precluded, other viable and sustainable options should be explored, with the former ones serving as important platform for future research, innovation and development. Two principal methods of gastrointestinal parasite control of livestock have been implemented; external animal environmental control strategy that is prophylactic in nature, and internal gastrointestinal control using anthelmintic remedies that are either therapeutic or prophylactic. External strategies of control, some of which include grazing management (Waller, 2006a), use of treated or conserved forages (Fleming *et al*., 2006) among others, seek to deter build-up of intermediate developing stages of parasites in the animal host environment. Internal anthelmintic remedies are either chemical or bioactive principles that combat parasites within the host animal.

The later phase of gastrointestinal parasite control is critical to improve animal health and productivity. Wide-spread application of chemical anthelmintics (Lorimer *et al*., 1996; Stepek

et al., 2004; Shaik *et al*., 2006; Kamaraj *et al*., 2011; Bidkar, 2012) has been fraught with wide ranging challenges, some of which include emergence of resistant strains to all classes of chemical anthelmintics (Terrill *et al*., 2001; Jackson *et al*., 2009; Kaplan and Vidyashankar, 2012), lodging of residues in animal products (Kaemmerer and Butenkotter, 1973; Athanasiadou *et al*., 2008; Tariq *et al*., 2009), environmental pollution by excreted un-metabolised chemical anthelmintics (Hammond *et al*., 1997; Yeap *et al*., 2010) and induced resistance by rendering them unduly available to untargeted organisms. Innovation of this method of control by adopting combination therapy has been another option of enhancing anthelmintic efficacy. It involves combined administration of two or more chemical anthelmintics, following waning efficacy of any one of them (Hennessy, 1997; Kabasa *et al*., 2000; Savioli *et al*., 2004; Hotez *et al*., 2007), thus altering and improving pharmacokinetic and pharmacodynamic activities of one or both (Entrocasso *et al*., 2008). These practices are commendable though resistant strains of gastrointestinal nematodes still emerge with time, when consistently used without alternating with other effective options. It is therefore critical to further explore and diversify other options, in view of attaining this goal. Besides, emergence of resistant gastrointestinal nematode parasites strain because of treatment with particular anthelmintics or anthelmintic combination(s), is irreversible (Prichard *et al*., 1980). Naturally, animals forage on various plants and may employ the activity of various bio-compounds in self-cure prevention. Similar practice can be employed using plants possessing anthelmintic activity.

Combination anthelmintic phytotherapy has been used for a long time, without any sound scientific basis of the bioactive principles involved, and interactions leading to improved efficacy. It is hypothesized that combination of plant species crude extract exerting anthelmintic activity will produce no synergistic or antagonistic effects, and correspondingly observed efficacy, synergy and antagonism will not relate to plant secondary metabolites including alkaloids, condensed tannins and flavonoid content of plant species. The specific objective was to evaluate and identify plant extracts with potential of being used in combination to develop a more effective livestock nematode control remedy. This study also elucidated the quantitative contribution of alkaloids, condensed tannins and flavonoids of component plant species to anthelmintic efficacy of plant combinations.

4.2 Materials and Methods

4.2.1 Collection of vegetative plant material and processing of crude extracts

Sixteen plants that are used traditionally to treat infectious helminths of livestock were selected from available literature and allotted to three main groups (Table 3.1) following their putative primary anthelmintic principles. It is worthy of note that there may be overlap of phytochemical composition, but emphasis is laid on suggested primary bioactive principle(s) or macromolecule(s). They consisted of:

(1) Alkaloids and condensed tannins containing plant species which included *Crinum macowanii* (Refaat *et al*., 2012), *Gunnera perpensa* (Simelane *et al*., 2010), *Nicotiana tabacum* (British Veterinary Codex, 1953; Stuart *et al*., 2012) *Sarcostema viminale* (Grime *et al*., 2008), *Vernonia amygdalina* (Yeap *et al*., 2010), *Zingiber officinale* (Haman, 2007; Singh *et al*., 2011), *Zizyphus mucronata* (Van Wyk and Wink, 2004; Olivier, 2012) and *Aloe vanbalenii* (McGaw *et al.*, 2000*;* Ahmed *et al.*, 2013).

(2) Flavonoids containing plant species comprised of *Trema orientalis* (Watt and Breyer-Brandwijk, 1962), *Urtica dioica* (Haman, 2007) and *Zanthozylum capense* (McGaw *et al*., 2000*;* Maphosa and Masika, 2010); and

(3) Proteinases and nitrogenous compound containing plant species were made of, *Allium cepa* (Vieira *et al*., 1999; Marwat *et al*., 2011; Bibkar *et al*., 2012), *Ananas comosus* (Stepek *et al*., 2005), *Bidens pilosa* (Graham *et al*., 1980; Hoffman and Hoelzl, 1988), *Carica papaya* (Stepek *et al*., 2005; Adongo, 2013) and *Ricinus communis* (Rampadarath *et al*., 2014; Wafa *et al*., 2014).

Table 4. 1: Plant species sharing similar primary anthelmintic bioactives principles of (a) tannins and alkaloids, (b) flavonoids and (c) protease and nitrogen compounds

Tannins and Alkaloids (Experiment one)	Flavonoids (Experiment two)	Nitrogen compounds (Experiment three)
Aloe vanbalenii	*Trema orientalis*	*Allium cepa*
Crinum macowanii	*Urtica dioica*	*Ananas comosus*
Gunnera perpensa	*Zanthozylum capense*	*Bidens pilosa*
Nicotiana tabacum	-	*Carica papaya*
Sarcostema viminale	-	*Ricinus communis*
Vernonia amygdalina	-	-
Zingiber officinales	-	-
Zizyphus mucronata	-	-

These plants species were categorized (Table 4.1) and their anthelmintic properties evaluated in three experiments, dubbed sub experiments 1, 2 and 3. Samples of plant material were collected from the University of KwaZulu-Natal Botanical garden, some from the National Botanical garden, Pietermaritzburg, others from private gardens, while some were bought from commercial food and vegetable stores in Pietermaritzburg central business district. Voucher samples were deposited at the UKZN Herbarium, Pietermaritzburg.

For each plant, fresh vegetative material was collected, washed, chopped and processed as described in the previous section. Those with large and long leaves, were first air dried to reduce moisture content and subsequently oven dried (Oven mark; LABCON, Model 5SOEIB, Maraisburg 1700) to constant weight at 60°C. Oven-dried material of each plant species was milled using an electric centrifuge mill (RETSCH, GmbH and Co.KG, 5657 HAANI, West Germany), fine enough to pass through a 1-mm sieve. Milled plant samples were then put into air-tight labelled plastic containers and stored in boxes, away from light and moisture at room temperature.

A milled sample (4 g) of each plant species was weighed into labelled thimbles, fitted into distillation columns and extracted in 70% ethanol over a heating unit (Gerhardt Bonn, App. Nr

450893). The extraction process was completed when the solvent in the thimble carrying unit was apparently free of any coloration. At that point, plant crude extracts were obtained from bottles into which they were drained and concentrated. Bottles, which fell below the 100 ml mark, were made up to standardized volume by adding solvent (70% ethanol) and sealed with parafilm® (Parafilm® American National Can$_{TM}$, Neenah, W154956). They were packaged into boxes and stored in a fridge for *in vitro* dosing of mixed cultured isolated nematode L3 larvae.

4.2.2 Analysis of alkaloids, flavonoids and condensed tannins in plant samples

Alkaloids were determined following Harborne (1973). Five grams ground sample of each plant species vegetative material was weighed into a 250 ml beaker, into which 200 ml of 10 % acetic acid in ethanol was added and covered to extract for 4 hours. The solution was filtered using a fine sieve into a beaker of similar capacity to the first and the extract concentrated in a water bath to ¼ its original volume at 100ºC. Concentrated ammonium hydroxide was added drop wise to completely precipitate extract and solution allowed to settle. A precipitate was collected and washed with dilute ammonium hydroxide 50:50 volume for volume. The residue was filtered using Whatman^{TM} 42 filter paper (GE Healthcare UK Limited, Amersham Place Little Chalfont, Buckinghamshire HP7 9NA, UK, Made in China) oven dried under low heat and weighed.

Flavonoids content of selected plant vegetative material was determined following Boham and Kocipal-Abyazan (1974). Ten grams (10 g) of oven-dried plant material was milled to pass through a 1-mm sieve, weighed into a 250 ml sterile beaker, and 100 ml of 80 % aqueous methanol added to it. The content was allowed to stand for 10 hours at room temperature, while being stirred intermittently with a magnetic stirring bar over a magnetic rotor without heat. Each solution was filtered individually through Whatman^{TM} No 42 filter paper. The filtrate of each sample was transferred into pre-weighed 250 ml conical flask and evaporated to dryness in a water bath at constant temperature (80ºC). Flasks and their contents were allowed to cool and subsequently placed in the desiccator for one hour to rid them of any moisture. Each of them was weighed, and the weight of the sterilized conical flask deducted from that of flask and flavonoid. The difference was computed as a percentage of flavonoid content of different species.

Condensed tannins were analysed following HCl-Butanol proanthocyanidin assay (Porter *et al*., 1986) as leucocyanidin equivalent (Makkar, 1995). By which, one and a half grams (1.5 g)

of this material was weighed into pre-weighed filter paper (Whatman^{TM} , number 1, diameter 110 mm, Cat number 1001 – 110, GE Healthcare UK limited, Amersham Place, Little Chalfont, Buckinghamshire. HP7, 9NA, UK, and Made in China) and Sohxlet extracted in 1% glacial in petroleum ether to rid them of pigments and fats that could interfere with quantitative determination of condensed tannins. It should be noted that the addition of glacial to petroleum ether serves as antioxidant, and prevents condensed tannins from being oxidized and bound to vegetative material. Weights of maximum 0.2001 g samples of the different plant species were measured into 100 ml plastic centrifuge tubes, and 10 ml of 70% aqueous acetone added to extract condensed tannins. Centrifuge tubes and their contents were Vortex mixed and placed in an ice bath. Samples were subjected to ultrasonic treatment for 3 minutes in ice cold water and vortex mixed intermittently for 12 minutes, resulting to 4 ultrasonic treatments in all. The content was centrifuged at 5000 rotations per minute (rpm) for 20 minutes at 4 ºC, and supernatant carefully collected in a glass test tube and stored on ice. Appropriate dilutions of tannin extracts with 70% aqueous acetone were made. Butanol reagent of volume 6 ml (950 ml of butanol and 50 ml of HCl 37%) and 0.2 ml of ferric reagent (16.6 ml of concentrated HCl 37 % diluted to 100 ml with water to make 2 M HCl and 2 g of ammonium ferric sulphate dissolved in it) was added to the tubes and vortex mixed. Tubes were covered and placed in a heating bath adjusted to between 96 – 100 ºC for 60 minutes. At the end of the incubation, they were cooled and absorbances measured using Beckman DU®640 Spectrophotometer at visible wavelength of light 550 nm. From each of these absorbances red, was deducted that of an unheated mixture (blank). The method allows for appropriate absorbances between 0.30 and less than or equal to 0.60 to be considered stable and most appropriate. Percentage condensed tannins in each of the plant samples was computed following the formula below:

Percentage condensed tannins in dry matter= A_{550nm} x 78.26 x D/% dry matter,

where: A_{550nm}= Absorbance at 550 nm; 78.26= Accumulative factor taking into account: extinction coefficient of leucocyanidin, mass of sample (200 g) and other factors except dilution; D= Dilution factor

4.2.3 Extraction and *in vitro* dosing of sheep dung

Dung was collected from 18 sheep as per rectum, pooled together and thoroughly mixed. Five grams of faecal material was weighed into a Petri dish and incubated at 27°C for 12 days. On day 13, each cultured faecal sample was dosed with 5 ml of combined plant extract, or 2.5 ml

each of the designated combined pair of crude plant species extracts (double concentration; 2 x 2.5 ml). Dosed samples were further incubated for one day (combined extract was 0.4x that of the concentration in the previous trial; at 50: 50 for biochemical contribution of the component species). The control was watered and left untreated. Larvae (L3) that survived combined dosing were isolated on day 14, including controls following the Baermann method. Fluid of volume 10 ml was drawn from the stem of each funnel into a labelled test tube of capacity 15 ml and allowed to settle for 15 minutes. Fluid was further drawn from the supernatant using a Pasteur pipette, filled into a McMaster slide and surviving L3 larvae counted. Corrected mortality was computed using Abott's formula (Abbott, 1963), and used as indices of *in vitro* combined anthelmintic efficacy.

4.2.4 Experimental design, computation of additive and synergistic effects and statistical analysis

Plant species were grouped according to the primary putative anthelmintic principle, and combinations established by permutation. Following these groups, plant species combinations possessing similar classes of bioactive macromolecules were retained as separate sub-experiments to test their efficacies. Sub-experiment one comprised of combinations involving alkaloids and tannins; sub-experiment two was based on combinations of plant species possessing flavonoids; and sub-experiment three was based on plant species containing proteinases and nitrogen compounds. In all three experiments, only sheep faeces was used. Each combination of plant species was evaluated in three replications, each of which was run on faeces collected on the same day. Sub-experiment one had 21 combinations, sub-experiment two 3 and sub-experiment three 10 combinations.

Two approaches were used to compute additive and synergistic anthelmintic effects resulting from *in vitro* combination therapy. In the first method, mean efficacies of component plant species were computed (a + b)/2 and deducted from their observed combined efficacy. Positive differences measured synergistic effects, whereas negative differences measured antagonistic effects. In the second method, expected efficacy of plant species combination were estimated following Webb's fractional product method (Webb, 1963). Following this method, if the efficacies of two plant species "A" and "B" represented by "a" and "b" proportion of worms killed, then expected efficacy of combinations assuming additive effect is computed thus:

Efficacy (A + B) = 1 – ((1 – a) x (1 – b)). Synergistic effect is considered to have occurred when the response of combined administration is greater than additive.

Data collected in each of these three experiments was analysed following general linear model (GLM) of SAS (2000). Pearson correlation was used to seek possible relationship between anthelmintic efficacy on the one hand and each of alkaloids, flavonoids and condensed tannins as primary putative anthelmintic macromolecules. Additionally, multiple regression analysis was run to seek explanations of the role of various variables including alkaloids, flavonoids and condensed tannins to observed trends of anthelmintic efficacy. Means separation was done using Student Neuman Keul's statistic, aided by SAS (2000).

Table 4. 2: Alkaloids, condensed tannins and flavonoid content (± standard error of means) g/Kg DM of selected plant species possessing anthelmintic activity

	Plant species	n	Alkaloids (gDM/Kg)	n	Cond. Tannins (gDM/Kg)	n	Flavonoids (gDM/Kg)
Alkaloids ad condensed tannins							
	Crinum m.	2	20.9 ± 1.10^{A}	6	5.5 ± 1.28^{A}	2	117.9 ± 1.75^{B}
	Gunnera p.	2	44.4 ± 15.20^{A}	5	7.6 ± 1.30^{B}	2	26.0 ± 2.60^{A}
	Nicotiana t.	2	37.1 ± 3.20^{A}	5	6.4 ± 1.42^{B}	2	202.6 ± 0.75^{A}
	Sarcostema v.	2	46.7 ± 8.50^{A}	2	2.8 ± 0.01^{B}	2	117.0 ± 2.76^{B}
	Vernonia a.	2	42.4 ± 8.20^{A}	6	3.4 ± 0.63^{B}	2	125.0 ± 13.57^{B}
	Zingiber o.	2	48.3 ± 4.50^{A}	6	3.4 ± 0.55^{B}	2	172.1 ± 17.60^{A}
	Zizyphus m.	2	30.6 ± 0.68^{A}	6	13.7 ± 1.99^{B}	2	124.3 ± 10.67^{B}
Flavonoids							
	Trema o.	2	72.5 ± 13.80^{A}	3	11.5 ± 2.14^{A}	2	207.5 ± 1.66^{A}
	Urtica d.	2	23.6 ± 17.90^{A}	6	11.2 ± 1.61^{A}	2	138.6 ± 6.63^{B}
	Zanthozylum c.	2	16.3 ± 1.22^{A}	6	3.9 ± 1.47^{B}	2	129.1 ± 16.01^{B}
Proteases and or nitrogen compounds							
	Allium c.	2	5.7 ± 0.30^{A}	6	4.7 ± 0.97^{A}	2	550.4 ± 25.42^{A}
	Ananas c.	2	47.5 ± 6.70^{A}	6	4.4 ± 0.75^{A}	2	133.5 ± 5.15^{B}
	Bidens p.	2	39.5 ± 6.10^{A}	6	5.9 ± 1.09^{A}	2	163.5 ± 1.92^{B}
	Carica p.	2	40.5 ± 6.10^{A}	4	2.6 ± 0.76^{A}	2	167.7 ± 12.38^{B}
	Ricinus c.	2	43.0 ± 4.80^{A}	6	4.4 ± 1.56^{A}	2	149.6 ± 10.27^{B}

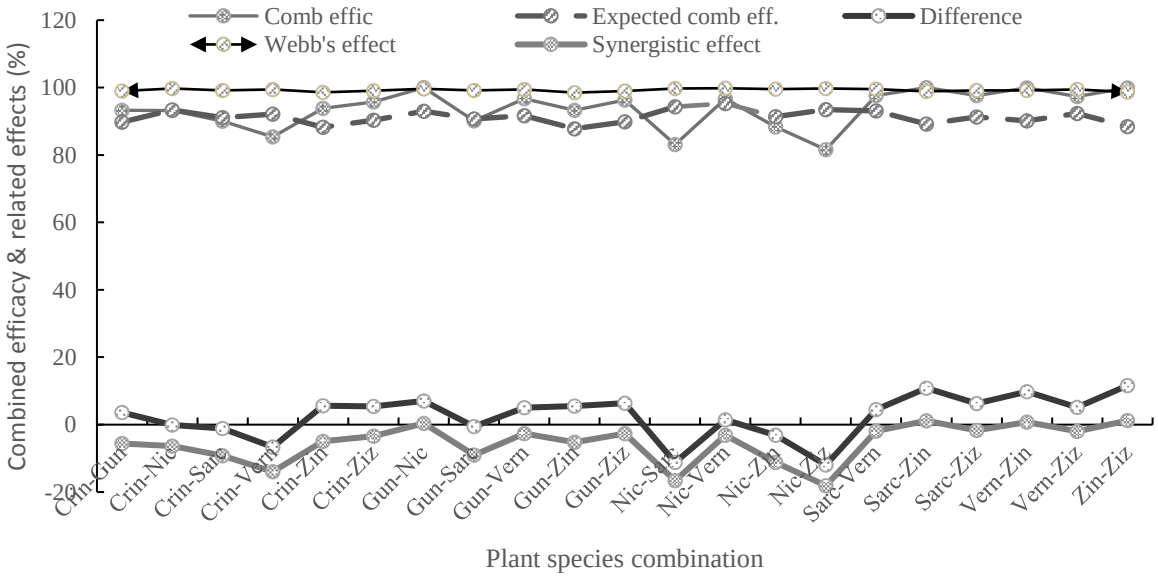
gKg^{-1}= grams per kilogram; DM= dry matter; cond. Tannins= condensed tannins; crinum m.= *Crinum macowanni*; *Gunnera p.= Gunnera perpensa*; *Nicotiana t.= Nicotiana tabacum*; *Sarcostema v.= Sarcostema viminale*; *Vernonia a.= Vernonia amygdalina*; *Zingiber o.= Zingiber officinale*; *Zizyphus m.= Zizypus mucronata*; *Trema o= Trema orientalis*; *Urtica d.= Urtica dioica*; *Zanthozylum c.= Zanthozylum capense*; *Allium c.= Allium cepa*; *Ananas c.= Ananas comosus*; *Bidens p.= Bidens pilosa*; *Carica p.= Carica papaya*; *Ricinus c.= Ricinus communis*

4.3 Results

Results are presented in Table 4.2 bearing phytochemical properties, and in Table 4.3 and 4.4 bearing the effects of various combination of members of each group on efficacy.

4.3.1 Sub-experiment one (combined efficacies of plant species containing alkaloids and tannins)

Observed efficacies of plant species containing alkaloids and tannins (Figure 4.1) were high but not different (P= 0.5595), with mean combined efficacy of 93.8 ± 0.67 % for all plant species combinations. Expected combined (A+B/2) efficacies were different (P< 0.0001), with mean 91.3 ± 0.67 %. Simple synergy (differences between observed and expected efficacies) ranged from 11.6 to -12.0 % (Figure 4.1) but they were similar (P= 0.2477); with mean of 2.5 ± 0.67 %, which is greater than zero. Webb's additive effects for combinations were high with mean 99.3 ± 0.67 %. Webb's synergistic effect arising from combinations were not different from each other (P= 0.5114), and had a mean of -5.4 ± 0.67 %, which is lower than zero.



Crin = Crinum macowanni, Gun = Gunnera perpensa, Nic = Nicotiana tabacum, Sarc = Sarcostema viminale, Vern = Vernonia amygdalina, Zin = Zingiber officinales, Ziz = Zizyphus mucronata, Trem= Trema orientalis, Urt=Urtica dioica, Zan= Zanthozylum capense; Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webb's effect= Webb's additive effect

Figure 4. 1: Combination of plant species possessing alkaloids and condensed tannins: their efficacies (%), expected efficacies (%), Webb's or additive effect (%), simple and Webb's synergistic effect (%)

Alkaloids, condensed tannins and flavonoids were identified and their concentration evaluated in these plant species. Alkaloid contents were similar (P= 0.304), with mean concentration of 38.6 ± 0.68 g/KgDM (Table 4.2). Concentrations of condensed tannins of the different plant species were different (P< 0.0001), with mean content of 6.0 ± 0.13 g/KgDM. The trend of tannin content was: *Z. mucronata*> *G. perpensa*> *N. tabacum*> *C. macowanii*> *V. amygdalina*> *Z. officinale*> *S. viminale* (Table 4.2). Correspondingly, flavonoid contents were different (P= 0.0006), and had mean content 152.1 ± 0.74 g/KgDM. The trend of flavonoid content was thus*: N. tabacum*> *Z. officinale*> *V. amygdalina*> *Z. mucronata*> *C. macowanii*> *S. viminale*> *G. perpensa* (Table 4.2). The order of macro biochemical content for all plant species in this group was, flavonoids> alkaloids>condensed tannins. There was no correlation between combined efficacy and any of alkaloids (r= 0.1458; P= 0.2543), condensed tannins (r= 0.0059; P= 0.9637) or flavonoids (r= -0.0293; P= 0.8199). Alkaloids, condensed tannins and flavonoids were all poor predictors of combined efficacy for plant species possessing alkaloids and tannins in a multi-regression analysis as no variables met the criterion of P= 0.15 level of significance.

4.3.2 Sub-experiment two (combined efficacies of plant species containing flavonoids)

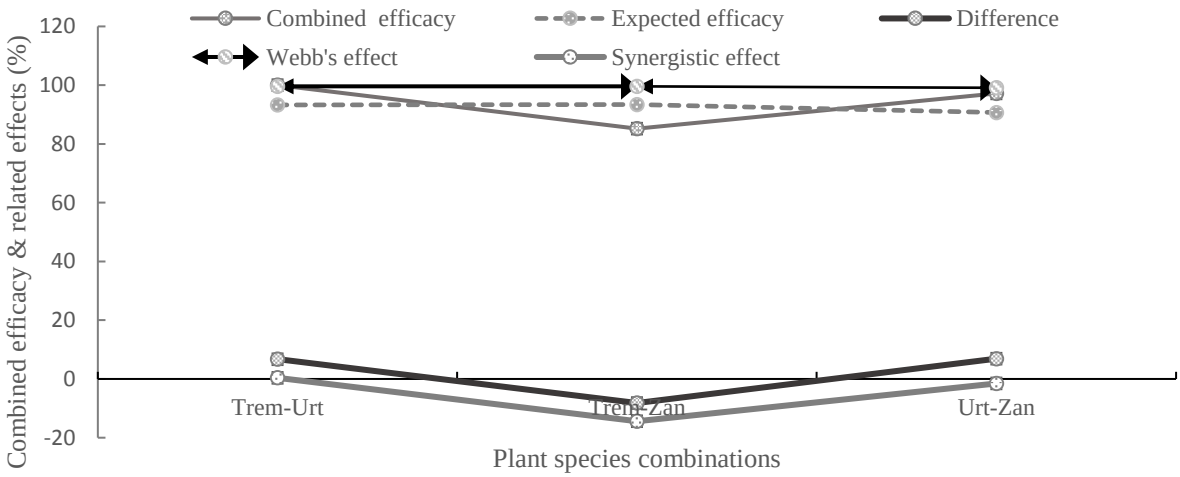
Combined or observed efficacy (Table 4.3) of these plant species were high but not different (P= 0.3183), with a mean of 94.3 ± 5.88 %. Contrary, expected efficacies for these plant combinations were different P< 0.0001), and had mean 92.5 ± 1.19 %. Simple synergies (differences between combined and expected efficacies) of this group were not different (P= 0.2671), they ranged from – 8.3 ± 1.97 % to 6.9 ± 1.97 % with mean of 1.8 ± 1.19 % (Figure 4.2). Webb's additive effect for combinations of these plant species were high but different (P< 0.0001) and had mean 99.5 ± 1.19 %. Webb's synergistic effects were not different (P= 0.3099), and had a mean of -5.2 ± 1.19 %, which differed from zero (Figure 4.2).

Quantitatively, content of condensed tannins, alkaloids and flavonoids (Table 4.2) of these plant species had various relationships. Condensed tannin content were different (P= 0.0089), with mean 8.3 ± 0.91 g/KgDM, whereas alkaloid content were not different (P= 0.07), and had mean 37.5 ± 1.93 g/KgDM. Additionally, flavonoid content of these plants were different (P= 0.0211), with mean 156.4 ± 1.78 g/KgDM. There was no discernible association between anthelmintic efficacy and any of alkaloids (r= -0.1411; P= 0.7173), condensed tannins (r= 0.3361; P= 0.3765) or flavonoid (r= -0.1457; P= 0.7084) content of these plant species. Multiple regression analysis of alkaloids, condensed tannins and flavonoid content as predictors of combined efficacy did not prove useful, as none entered the model at P= 0.15.

Table 4. 3: Combined anthelmintic efficacy (%) of plant species possessing alkaloids and/or tannins (sub-experiment 1), and of those containing flavonoids (sub experiment 2)

Putative anthelmintic principle	Plant species combination	Combined efficacy (%)	Plant species combination	Combined efficacy (%)
Alkaloid and condensed tannins containing combinations				
	Crin-Gun	93.3± 0.67	*Nic-Sarc*	83.1 ± 0.67
	Crin-Nic	93.2 ± 0.67	*Nic-Vern*	96.7 ± 0.67
	Crin-Sarc	90.0 ± 0.67	*Nic-Zin*	88.3 ± 0.67
	Crin-Vern	85.4 ± 0.67	*Nic-Ziz*	81.6 ± 0.67
	Crin-Zin	93.8 ± 0.67	*Sarc-Vern*	97.6 ± 0.67
	Crin-Ziz	95.7 ± 0.67	*Sarc-Zin*	100 ± 0.67
	Gun-Nic	100 ± 0.67	*Sarc-Ziz*	97.6 ± 0.67
	Gun-Sarc	90.1 ± 0.67	*Vern-Zin*	99.9 ± 0.67
	Gun-Vern	96.7 ± 0.67	*Vern-Ziz*	97.4 ± 0.67
	Gun-Zin	93.3 ± 0.67	*Zin-Ziz*	99.9 ± 0.67
	Gun-Ziz	96.3 ± 0.67		
Flavonoids containing species combinations				
	Trem-Urt	100.0 ± 1.97	*Trem-Zan*	85.2 ± 1.97
	Urt-Zan	97.6 ± 1.97	-	-

Crin = Crinum macowanni, Gun = Gunnera perpensa, Nic = Nicotiana tabacum, Sarc = Sarcostema viminale, Vern = Vernonia amygdalina, Zin = Zingiber officinales, Ziz = Zizyphus mucronata; *Trem= Trema orientalis, Urt=Urtica dioica, Zan= Zanthozylum capense* SELSM = standard error of least square means expected efficacy; Webb's effect= Webb's additive effect



Trem= Tremia orientalis; *Urt= Urtica dioica*; *Zan= Zanthozylum capense*; Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webb's effect= Webs additive effect

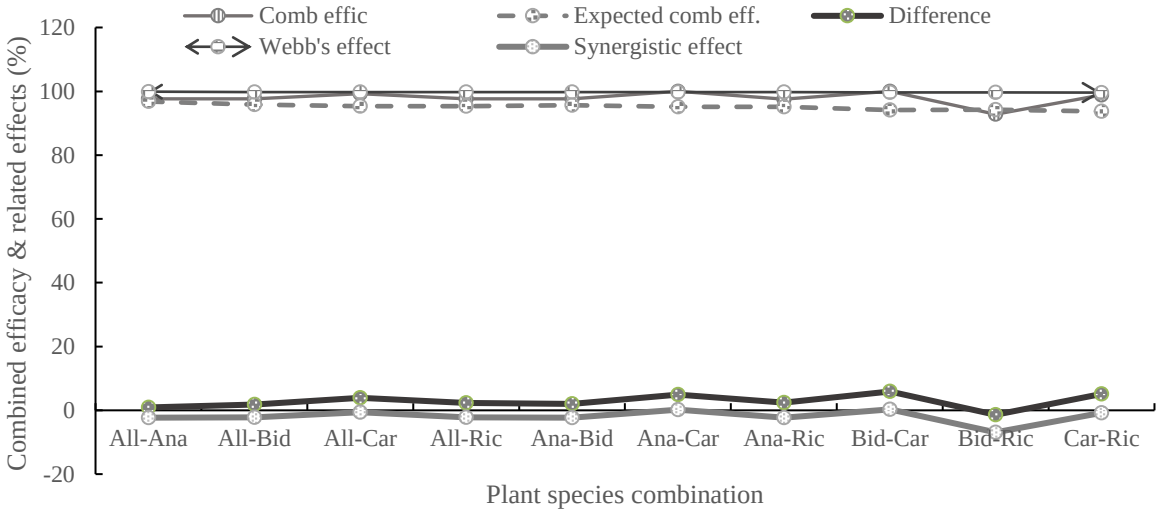
Figure 4. 2: Combination of plant species possessing flavonoids: their efficacies (%), expected efficacies (%), Webb's or additive effect (%), difference (%) and synergistic effect (%)

4.3.3 Sub-experiment three (combined efficacies of plant species containing proteases and nitrogen compounds)

Observed efficacies of these plant species were high, but similar (P= 0.785), with mean 97.9 ± 0.30 % (table 4.4). Expected efficacies for combinations were different (P< 0.0001). Simple synergies (differences between observed and expected efficacies) of theses plant species were not different (P= 0.7162), with a mean of 2.8 ± 0.30 %, which is higher than zero. Webb's additive effect for combinations of plant species were high and different from each other (P< 0.0001). Webb's synergistic effects were similar (P= 0.7882); with a mean of -1.9 ± 0.30 %, which is lower than zero (Figure 4.3).

Alkaloid contents were different (P= 0.0135) and had mean 35.2 ± 0.78 g/KgDM (Table 4.2), whereas condensed tannin contents were similar (P= 0.4312), with mean 4.5 ± 0.46 g/KgDM. Lastly, flavonoid contents were also different (P< 0.0001) with mean 232.9 ± 1.24 g/kgDM. There was no correlation between any of alkaloids (r= -0.02774; P= 0.8843), condensed tannins (r= -0.3071; P= 0.0987) or flavonoids (r= 0.0359; P= 0.8505) and observed efficacy. A multi-

regressions of alkaloids, condensed tannins and flavonoids as predictors of combined anthelmintic efficacy also gave no relationship. Overall trend of concentration for all three biochemical compounds was flavonoids> alkaloids> condensed tannins (Table 4.2).



All= Allium cepa, *Ana= Ananas comossus*; *Bid= Bidens pilosa*; *Car= Carica papaya*; *Ric= Ricinus communis;* Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webs effect= Webs additive effect

Figure 4. 3: Combination of plant species possessing proteases/nitrogen compounds: their efficacies (%), expected efficacies (%), Webs or additive effects (%), differences (%) and synergistic effects (%)

Table 4. 4: Combined anthelmintic efficacy (%) of plant species possessing proteases and/or nitrogen compounds (sub-experiment 3)

Plant species combinations	Combined efficacy (%)	Plant species combinations	Combined efficacy (%)
All-Ana	97.6 ± 0.30	*Ana-Car*	100.0 ± 0.30
All-Bid	97.6 ± 0.30	*Ana-Ric*	97.6 ± 0.30
All-Car	99.2± 0.30	*Bid-Car*	100.0 ± 0.30
All-Ric	97.6 ± 0.30	*Car-Ric*	92.8 ± 0.30
Ana-Bid	97.6 ± 0.30	*Car-Ric*	98.8 ± 0.30

All = Allium cepa, Ana = Ananas comosus, Bid = Bidens pilosa, Car = Carica papaya, Ric = Ricinus communis

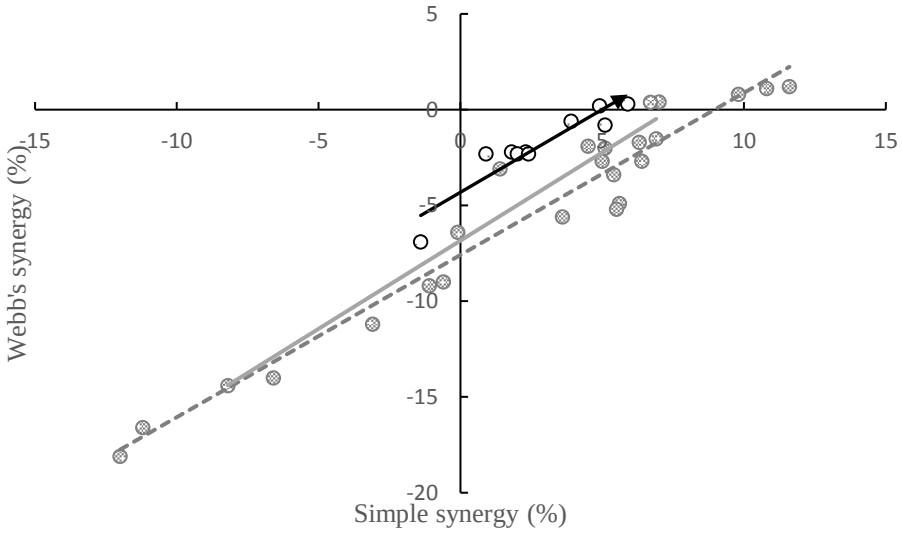


Figure 4. 4: Scattered plot of relationship between simple and Webb's synergies for combinations involving tannin/tannin, flavonoid/flavonoid and proteases/proteases containing plant species

4.4 Discussion

Combination of plant species in phytoanthelmintic therapy is expected to improve efficacy and spectrum of cover to that which is higher than that of the component plant species constituting a pair (Bartram *et al*., 2012; lanusse *et al*., 2014). Two approaches were adopted to evaluate synergism and improved anthelmintic activity arising from combination of these plant species. The first was that of differences between observed and expected efficacies (simple synergy), and the second was computed using Webb's fractional method (Webb, 1963). Contrary to our expectation, there was synergistic activity from both simple synergy (Figure 4.4) and Webb's synergy. For the former, the differences were mostly positive, alluding to improved anthelmintic activity in all three sub experiments as a result of constituted combinations. From the later, synergistic activities of most combinations were overwhelmingly negative in all three sub-experiments suggesting antagonistic activity. Remarkably, graphical trends of activities were closely similar, suggesting some common pattern of activities, though in opposite directions (Figures 4.4). While simple synergy was largely positive, the later was oppositely negative, but both related linearly (Figure 4.4). Though combined concentration of crude extract was reduced in the current trial to 40%, computed additive effect was virtually at 100 %. It is most likely in the current study that reduction in dose might not have accorded adequate room to sufficiently evaluate synergistic effect, as shown in the general trend of additive activities in all three sub experiments. There is, therefore, need to further reduce the dose in order to target less additive effect than that obtained from the current study.

In all three sub experiments, alkaloids, condensed tannins and flavonoids were quantitatively evaluated for all plant species. Given that empirical evidence closely links alkaloids (Ademola *et al*., 2005; Hoste *et al*., 2006), condensed tannins (Athanasiadou *et al*., 2000; Molan *et al*., 2000; Min and Hart, 2003; Hoste *et al*., 2006) and flavonoids (Perry and Metzger, 1980; Duke, 1992; Barrau *et al*., 2005; Haman, 2007; Kerboeuf *et al*., 2008; Eguale and Giday, 2009; Azando *et al*., 2011; Refaat *et al*., 2012; Bibkar *et al*., 2012) to exerting anthelmintic activity, a natural expectation would be some level of association of these macromolecules with anthelmintic efficacy in plant species in which they occur. Contrary to this expectation, there was no discernible correlation of all three macromolecular compounds with anthelmintic efficacy. In combinations, the collective activity of these macromolecules and others that might not have been identified, may be responsible for this activity. Additionally, anthelmintic potency of these principles put together, may far exceed or shield that of individual

macromolecules including alkaloids, condensed tannins and flavonoids. In attempt to formulate some predictive measure of combined anthelmintic efficacy or interchangeably observed efficacy based on the contribution of alkaloids, condensed tannins and flavonoids in the current study, a multi-regression analysis did not prove to be useful. This reaffirms collective rather than strong individual contribution of various anthelmintic principles to efficacy.

Plant species containing alkaloids generally have a wide variety of these macromolecules and/or other related biochemicals with different chemical structures, some of which are isomers and others have different molecular weights (Nair *et al*., 2000; Jin, 2003; Madala *et al*., 2016). A similar pool of various types of tannins occurred in plant species possessing condensed tannins (Makkar *et al*., 2007; Rochfort *et al*., 2008; Alonso-Díaz *et al*., 2009). Flavonoid, containing plant species likewise have a wide variety of different flavonoids and other biochemicals (Kubola and Siriamornpun, 2008; Madala *et al*., 2016), and sometimes different isomers of the same flavonoid occur in the same and also in different plant species (Madala *et al*., 2016). Similarly, the biochemical content of proteases and nitrogen compounds in plant species containing this macromolecular class would have been structurally diverse (Butts *et al*., 2016), in addition to other related anthelmintics.

This structural biochemical diversity of alkaloids and tannins, flavonoids, and, proteases and nitrogen compounds (Makkar *et al*., 2007; Kubola and Siriamornpun, 2008; Butts *et al*., 2016; Madala *et al*., 2016) in different plant species confers on them different properties and biochemical activities (Alonso-Díaz *et al*., 2009; Madala *et al*., 2016; Butts *et al*., 2016). Therefore, a wider pool of biochemical compounds in combinations, different interactions among them or pharmacodynamic activities, and by inference various pharmacokinetic activities (kundu *et al*., 2014). This would occur first, within a similar class of anthelmintic bioactive pool (Barry, 1985) such as alkaloids, condensed tannins and flavonoids in plant species and cumulatively in different plant species (Quijada *et al*., 2015) constituting various combinations (combined biochemical pool), yielding improved efficacy as targeted and observed in the current study. This further suggest collective than individual anthelmintic activity.

Based on the current study, a much more reduced combined dose is required to properly address synergistic activity. While the other parameters show that there is potentially greater potency in combined anthelmintic phytotherapy, synergy is more negative than positive. Improved observed efficacy in the current study is generally in accord with its primary objective.

Antagonistic and synergistic activities as observe from the both correlation matrix and multiregression occur in plant species exerting anthelmintic activities and other relevant antiparasitic activities. The biological activity identified in plant species suggest that various modulating chemical activities occur within each plant species or combinations to enable them exercise their activity without causing harm to livestock that is treated. Additionally, different types of active compounds may occur in the same plant species (Table 4.2) or in different plant species (Barry, 1985), conferring on them broad spectrum nematode parasite control capacity (Adamu *et al*., 2006). This biochemical nature, inherently accords advantage to anthelmintic activity. Combination therapy, therefore, yields multicomponent active principle, which exert different modes of biochemical activity, leaving nematode parasites insufficient capacity to develop resistance against all of them relative to single plant species treatment (Lanusse *et al*., 2014).

Though combination brings together a variety of bioactive anthelmintic principles, in nature, individual plant species harbour different primary biochemicals including alkaloids, condensed tannins and flavonoids (Table 3.2) and other compounds not determined in this study in different concentrations. Naturally, anthelmintic biochemical variety exist at the level of individual plant species. Some of the bioactive anthelmintic principles including alkaloids, tannins, flavonoids and terpenes among others, are plant secondary metabolites (Villalba and Provenza, 2009; Dove, 2010), which when present in diets beyond certain thresholds negate their beneficial effects (Dove, 2010). A variety of these macromolecules in diets have been suggested to mitigate or modulate the negative effects of individual plant species, implicitly by dilution or counteraction of deleterious effects of each other. These interactive and modulatory effects enhance the nutritional and curative benefits to animal production and health (Villalba and Provenza, 2009). In the same vein, combinations of plant species exerting anthelmintic activity that result to enhanced activity greater than that of individual component species, is an expression of the merits of a much broader positive interaction. Measurable and relatively enhanced anthelmintic efficacy emanating from combination of different plant species has been adopted from its application in chemical or orthodox anthelmintic combination (Webb, 1963).

Improved activity from combination anthelmintic phytotherapy will result to more potent activity relative to that of individual component species, and similar to what obtains in combination chemical anthelmintic therapy (Lanusse *et al*., 2014). This heightened activity has the likelihood of complete parasite elimination at contact or parasite/biochemical interaction. Remnants of surviving parasites that would not have had any contact or interaction with

combined extract will retain their integral vulnerability to subsequent dosing. This is typical of the concept of refugia (Sutherland and Leathwick, 2011) where in, the surviving portion of parasites in a herd of animals is made to have absolutely no contact or interaction with anthelmintic drug in use, while those with any contact are killed. Efficacious drug disposition of this nature is critical in livestock nematode control programs because of prevention of selection for resistant parasites and build-up of resistant alleles that usually lead to drug failure.

Among different plant species possessing similar biochemical class but with different structural formulae, some of them may exert anthelmintic activity and others not (Werne *et al*., 2013), suggesting that all plant species possessing these tagged bioactive principle classes may not be necessarily anthelmintic in nature, or may not exert this activity to the same extent when it exists. Additionally, plant species content of any of alkaloids, condensed tannins and flavonoids does not conclusively translate to anthelmintic biological activity, because not all macromolecules exert this activity (Werne *et al*., 2013). This raises the need for a much finer biochemical profiling, leading to extensive identification of all potential contributors to this anthelmintic trait. There is need to ascertain advantages of combination anthelmintic phytotherapy or chemotherapy offered over either of them individually.

The genetic basis of combination anthelmintic phytotherapy or chemotherapy is crucial in nematode parasite control programs. Chemical anthelmintic therapy presents peculiar cases of anthelmintic resistance, wherein selection for resistance against an anthelmintic does not necessarily affect others in the same or different groups (Mottier and Prichard, 2008) because of different mechanisms of action. Independent selection for resistance tie with the genetic control of this trait, which is in turn controlled by different alleles (Bartram *et al*., 2012). Combination anthelmintic therapy renders genetic selection for resistance difficult, though not impossible, as many alleles will have to be involved in the process. Combination therapy, therefore, affords the opportunity to recycle anthelmintics, which otherwise would have been ineffective or exerting low efficacy individually, the opportunity to exert acceptable levels of efficacy because of the genetic disposition towards this trait. Additionally, component anthelmintics in combination can influence and radically improve pharmacokinetic and pharmacodynamic interactions of the duo (Lanusse *et al*., 2014) resulting to additive and synergistic effects on efficacy. Similarly, plant bioactive anthelmintic principles constitute a pool of various biochemical classes and types of macromolecules, rendering genetic control much more intricate and complicated. Plants by their biological nature require huge genetic alterations in parasites to take place in order for them to select for resistance; which is

farfetched and inherently advantageous in livestock nematode parasite control programs. Ethno veterinary phytochemical combination anthelmintic therapy therefore constitute an important option in parasite control that should be accorded adequate attention in research, development and treatment.

4.5 Conclusion

Combination anthelmintic phytotherapy generally increases the concentration of similar principles in the component species and potential activity. Though, there was some antagonism as shown by negative synergistic activity, additive anthelmintic effects occurred. Additionally, combination anthelmintic therapy diversifies anthelmintic bioactive options, enhances efficacy and potentially other activities that promote animal health and productivity. By extension, the likelihood of selecting for resistance by nematode and related parasites is remote, because of involvement of various anthelmintic/antibiotic principles at macro biochemical level in plant combinations. This in turn demands a much more extensive work to further test many more plant species in combination in order to validate and provide more benign consumer/ecological friendly anthelmintic options**.** The next question is, "Will plant combinations possessing different biochemical anthelmintic principles yield similar results like those of the current study?"

Chapter 5

In vitro anthelmintic combination phytotherapy of sheep with selected plants possessing different bioactives

Abstract

Plant species in groups containing identical putative macromoleules including alkaloids/condensed tannins, flavonoids and proteases/nitrogen compounds were reconstituted into combinations across groups to evaluate efficacy and synergistic effects *in vitro* in sheep, in search of more efficacious remedies. Intergroup combinations were thirty two (32) for condensed tannins/alkaloids and proteases/nitrogen compounds in sub experiment one (SEP 1); 13 combinations for flavonoids and alkaloids/tannin plant species (SEP 2); and 15 combinations for proteases/nitrogen compound and flavonoid containing plant species (SEP 3). Each experiment was run thrice. Four grams of plant species vegetative material was extracted in 70 % ethanol and made up to 100 ml. Sheep dung collected from rectum and pooled to constitute test samples. Five grams (5 g) samples weighed into Petri dishes and cultured for 12 days at 27º C. On day 13, treatments dosed with combined plant species crude extract at 2.5 ml with double dose concentration of each constituting pair, while some controls were moistened and others treated with 70 % ethanol to eliminate solvent killing effect. Surviving mixed L_3 larvae were counted on day fourteenth. Corrected mortalities were adopted as indices of observed combined efficacies. Synergistic effects computed following Webb's method and alternatively simple synergy from differences between observed and expected efficacies (a + b)/2. Combined efficacies of SEP 1 related species were not different (P= 0.2760), but high, mean (95.5 ± 0.12 %). Synergistic activities were not different (P= 0.3217), with mean (-4.0 ± 0.12 %). No association occurred between any of alkaloids, condensed tannins or flavonoids with observed efficacy. Multiple regression analysis to establish relationships among quantified macromolecules with efficacy was not useful. Efficacy of combinations SEP 2 were not different (P= 0.4318), with mean (95.2 ± 0.34 %). Synergistic means were not different (P= 0.2685), with mean (-5.4 ± 0.34 %). Multiple regression analysis of macromolecules as predictors of observed efficacy was not useful. Observed efficacy of combinations relating to SEP 3 were similar (P= 0.5968) and high, mean (95.8 ± 0.04 %). Webb's synergy for SEP 3 combinations were not different (P= 0.6264) and had mean (-3.8 ± 0.04 %). There was no correlation between any of the macromolecules and observed efficacy. Multiple regression analysis of different macromolecules as predictors of observed efficacy was not useful. All synergistic means were negative. Crude extracts of all combinations exhibited anthelmintic activity, but could not be attributed to any specific macromlecule(s). This is suggestive that there are wide ranging biochemical interactions that retain anthelmintic and other relevant traits, but may be potentially different from those exerted by individual plant species. Evidently, there is more to the active principles involved than has been examined in the current study, warranting a more detailed study in the succeeding chapter.

5.1 Introduction

Plant species exerting anthelmintic activity are widely reported to have a variety of active phytochemicals (Sajid and McKerrow, 2002; Choi and Chung, 2003; Makkar, 2006; Hoste *et al*., 2008a), which collectively give them their anthelmintic attribute (Efferth and Koch, 2011). Moreover, very closely related plant species or cultivars from same geographic location share some similar principles and others that belong to different classes of biochemicals; more often occurring in various concentrations (Rees and Harborne, 1985; Foster *et al*., 2011). This anthelmintic or antibiotic biochemical disposition among others is suggestive of different efficacies for each of them, and the potential of some interaction if such plant cultivars were to be used in combination to treat livestock helminths. Constituting plant species combinations with similar or different anthelmintic principles, most likely increases the pool of phytochemicals, concentration of those compounds shared by both plant species, and the potential scope/efficacy of anthelmintic activity. These biochemical interactions in combination therapy have plenty of possible outcomes, some of which may result to improved activity (synergistic effect), parallel or independent activity, antagonistic or mutually inhibitory interaction and bioactive domination of some principles by others.

The failure of most chemical anthelmintics in contemporary control programs has ignited research, development and implementation of other options that will potentially broaden and increase control strategies. Many more control strategies will provide the opportunity to employ various options interchangeably in order to sustain efficacy at economically beneficial levels (Shalaby, 2013). One of such novel practices is the combined use of narrow and broad spectrum anthelmintics of different classes (Shalaby, 2013) at carefully targeted and specific times to maximize efficacy (Dobson *et al*., 2001; Leathwick *et al*., 2001), with attendant effectiveness of 99.9 % or more (Krecek and Waller, 2006). Adoption of such treatment regime has to be judiciously implemented to ensure that animals with unselected sub populations of parasites on the farm are retained untreated (van Wyk *et al*., 2006, Shalaby, 2013), in order to avoid development and propagation of resistant alleles. This strategy has been projected to conserve efficacy span of combinations for as long as 20 years, if adopted from inception of anthelmintic discovery that makes up the combination (Leathwick, 2012). Similar combinations have been inadvertently applied in ethnoveterinary and ethnobotanical medicines, in the treatment of helminths and other microbial infections without profound understanding of pharmacological and biochemical bases of their function. Relative to efficacy

of component members of a wide combination of plant species exerting anthelmintic activity, the combined anthelmintic efficacy was largely more potent (Dwivedi *et al*., 2009) at treatment of livestock nematodes. Similarly, a combined ethnobotanical formulation for treatment of round worms in native chicken proved to be very efficient and was comparable to existing anthelmintics of choice (Ozaraga *et al*., 2015). Socio-economic implications of ethnobotanical treatment of livestock helminth are important considerations (Ozaraga *et al*., 2015) in the promotion and establishment of efficient and effective treatment regimes, beside pharmacological, consumer health and environmental benefits.

Efficacious plant combinations as treatment against helminths, will tremendously reduce the frequency of anthelmintic use (Shalaby, 2013), and in the process conserve efficacy span. Economically, cost of production will be reduced and enormous production gains realized (Ketzis *et al*., 2006; Ozaraga *et al*., 2015) as a result of better gut integrity, better feed conversion efficiency and reduced host morbidity. Selection for anthelmintic resistance by helminth parasites and correspondingly selection for antibiotic resistance by microbes, has been intimately linked to frequency of treatment (Shalaby, 2013) among others. Evolution of resistant alleles from recurrent use of anthelmintics will be briefly examined to explore some of the advantages plant combinations potentially confer on nematode control.

Constituting anthelmintic combinations from diverse anthelmintic classes usually renders the evolution of multi-alleles for resistance intricate and long drawn (Bartram *et al*., 2012; Leathwick *et al*., 2012). Single plant species exerting anthelmintic activity on their part, naturally harbour a variety of bioactive compounds (Makkar, 2006; Hoste *et al*., 2008a), at which level, evolution of resistant anthelmintic alleles is complicated. With the constitution of plant combinations, phytochemical anthelmintic principles increase tremendously, further complicating and rendering evolution of phytochemical anthelmintic resistant alleles far more remote or untenable, as huge and fundamental genetic changes will be required. This pharmacological and genetic interaction strongly motivates the use of plant-plant combinations in nematode control programs of livestock. Plants by their biological nature are supposedly non-resistible and better suited for sustainable helminth and other parasite control programs because of their inherent diverse biochemical disposition.

It is hypothesized that combinations of plant species possessing different anthelmintic macromolecular principles will interact to produce no synergistic effect and correspondingly, plant species content of any or all of alkaloids, condensed tannins and flavonoids will not have

any effect on observed efficacy and synergy. The objective of this study was to identify which of plant combinations possessing different macromolecules can be developed as a more effective remedy for livestock nematode control.

5.2 Materials and methods

5.2.1 Collection of vegetative plant material, establishment of groups and processing of crude extracts

Sixteen plant species used in the preceding work were collected and processed as described in section 3.2.1, chapter 3.

Plant species combinations were constituted by: (1) those containing alkaloids/condensed tannins and others containing proteases/nitrogen compounds; (2) those containing flavonoids and others containing alkaloids/condensed tannins; and (3) those containing proteases/nitrogen compounds and their counterparts containing flavonoids, to evaluate their anthelmintic efficacy on mixed nematode parasites of sheep *in vitro*.

Primarily in the current study, intermacromolecular class combinations were constituted to explore how phytochemical interaction will affect anthelmintic efficacy and synergy. Correspondingly, quantitative content of alkaloids, condensed tannins and flavonoids were also examined for their implication on observed efficacy, simple and Webb's synergies.

5.2.2 Extraction and dosing of sheep dung

Four grams of each milled plant sample was weighed and extracted in 70% ethanol. When extraction was completed, the volume of crude extract was made up to 100 ml. Dung was collected from 18 sheep as per rectum, pooled and thoroughly mixed. Five gram sample of faecal material was weighed into Petri dishes and incubated at 27°C for 12 days. On day 13, each of the cultured sample was dosed with 5 ml of combined plant extract, or 2.5 ml each of the designated combined pair of crude extracts (double concentration; 2 x 2.5 ml). Negative or untreated controls were processed thus: some control samples were watered at time of dosing treated samples, while others were treated with 5 ml of 70 % ethanol. Mean parasite count from ethanol treated controls was deducted from that of negative controls to eliminate any influence on killing by the solvent. All samples were further incubated for one day. L3 larvae that survived combined dosing were isolated on day 14, including those of controls following the Baermann method. Fluid of volume 10 g was drawn from the stem of each funnel into a labelled

test tube and allowed to settle for 15 minutes. Fluid was again drawn from the supernatant of the test tube using a Pasteur pipette, filled into a McMaster slide and isolated L3 larvae counted. Corrected mortality for different samples was computed using Abott's formula (Abott, 1925) and served as indices of *in vitr*o combined anthelmintic efficacy. Synergistic activity from combination therapy was evaluated following two different methods. One of them was simple synergy, computed as difference between observed and expected efficacies, the other, Webb's synergy was computed following Webb's fractional method.

5.2.3 Experimental design and statistical analysis

Designated macromolecular classes including alkaloids/condensed tannins, flavonoids and proteases/nitrogen. These groups consisted of: (1) plant species containing alkaloids/tannins and those containing proteases/nitrogen compounds (sub-experiment one (SEP 1)); (2) plant species containing flavonoids and others containing alkaloids/condensed tannins (SEP 2); and (3) plant species containing proteases/nitrogen compounds and others designated as containing flavonoids (SEP 3). All combinations of plant species were run three times, each run being replicated thrice. Sub experiment one had 32 combinations, experiment two 13 and sub-experiment three, 15. Data was analysed using the general linear model (GLM), Pearson correlation and multiple regression of SAS (2000).

Table 5. 1: Anthelmintic efficacies (%) of combined plant species possessing alkaloids/tannins and those containing proteases/nitrogen compounds

Plant species combinations	Combined efficacy (%)	Plant species combinations	Combined efficacy (%)
All-Crin	95.2 ± 4.72	*Bid-Nic*	97.2 ± 2.78
All-Gun	97.5 ± 2.33	*Bid-Sarc*	95.1 ± 2.98
All-Nic	97.6 ± 2.38	*Bid-Vern*	97.1 ± 2.72
All-Sarc	99.9 ± 0.12	*Bid-Zin*	83.6 ± 4.07
All-Vern	100.0 ± 0.00	*Bid-Ziz*	96.3 ± 3.18
All-Zin	97.6 ± 2.38	*Car-Crin*	87.7 ± 8.51
All-Ziz	100.0 ± 0.00	*Car-Gun*	97.0 ± 2.67
Ana-Crin	100.0 ± 0.00	*Car-Nic*	99.8 ± 0.22
Ana-Gun	99.8 ± 0.22	*Car-Sarc*	95.1 ± 4.89
Ana-Nic	99.4 ± 0.52	*Car-Vern*	94.0 ± 0.12
Ana-Sarc	95.2 ± 4.76	*Car-Zin*	92.3 ± 7.67
Ana-Vern	96.0 ± 2.11	*Car-Ziz*	93.2 ± 2.44
Ana-Zin	97.6 ± 2.38	*Ric-Sarc*	80.8 ± 10.88
Ana-Ziz	95.2 ± 4.72	*Ric-Vern*	96.6 ± 3.28
Bid-Crin	92.9 ± 6.46	*Ric-Zin*	100.0 ± 0.00
Bid-Gun	93.6 ± 3.23	*Ric-Ziz*	95.2 ± 4.76

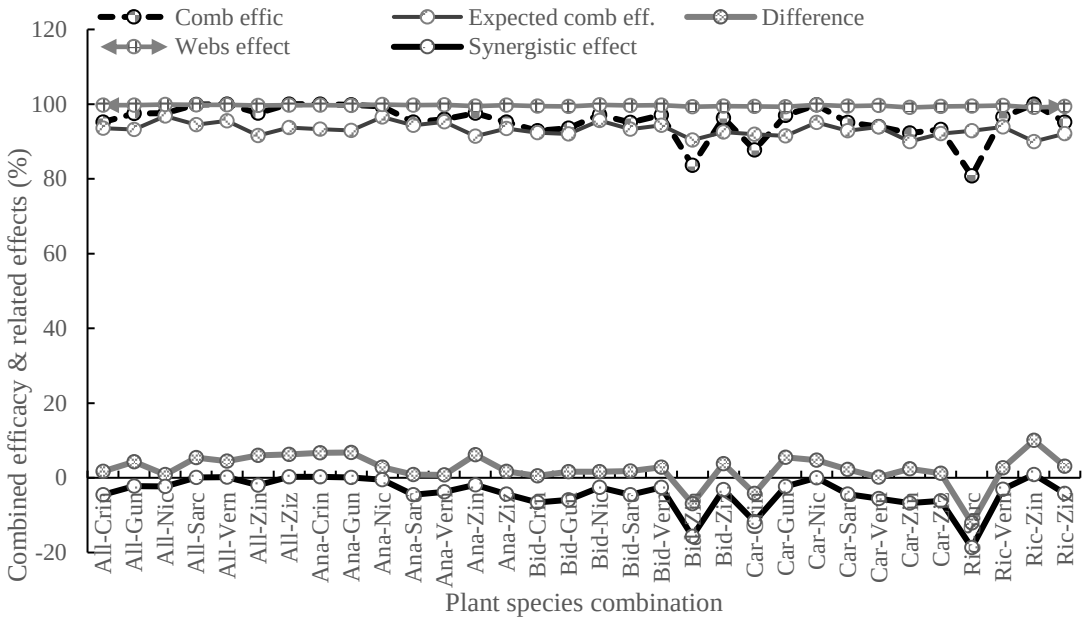
All = *Allium cepa*, Ana = *Ananas comosus*, Bid = *Bidens pilosa*, Car = *Carrica papaya*, Crin = *Crinum macowanii*, Gun = *Gunnera perpensa*, Nic = *Nicotiana tabacum*, Ric = *Ricinus communis*, Sarc = *Sarcostema viminale*, Vern = *Vernonia amygdalina*, Zin = *Zingiber officinale*, Ziz = *Zizyphus mucronata*

5.3 Results

5.3.1 Sub experiment one (alkaloids/tannins and proteases/nitrogen compounds)

Observed efficacies of plant species containing alkaloids/tannins and of those containing proteases/nitrogen compounds were not different (P= 0.276) but high, with mean 95.6 ± 0.12 %. Efficacies of combined plant species ranged from 80.8 ± 0.12 % for *Ricinus communis/Sarcostema viminale,* to 100 ± 0.12 % for *Allium cepa/Vernonia amygdalina*, *Allium cepa/Zizyphus mucronata*, *Ananas comosus/Crinum macowanii*, and *Ricinus communis/Zingiber officinale* (Table 5.1). The differences between observed and expected efficacies (simple synergy) were not different (P= 0.47350) with a mean 2.4 ± 0.12 % (Figure

5.1). Webb's synergy of these combinations were similar (P= 0.322), with mean -4.0 ± 0.12 %, and ranged from -18 ± 0.12 % for *Ricinus communis*/*Sarcostema viminale* to 0.9 ± 0.12 % for *Ricinus communis*/*Zingiber officinale* (Figure 5.1). There was no correlation of either alkaloids or condensed tannins to combined anthelmintic efficacy, but for that of flavonoids (r= 0.204; P= 0.0459). Multiple-regression analysis of alkaloids, condensed tannins and flavonoids as predictors of combined anthelmintic efficacy yielded no positive results as none of them entered the model at (P= 0.15) significant level.



All = *Allium cepa;* Ana= *Ananas comosus;* Bid =*Bidens pilosa;* Car = *Carica papaya;* Crin= *Crinum macowanii*; Gun= *Gunnera perpensa*; Nic= *Nicotiana tabacum*; Ric= *Ricinus communis;* Sarc= *Sarcostema viminale*; Vern= *Vernonia amygdalina*; Zin= *Zingiber officinale*; Ziz= *Zizyphus mucronata*; Comb effic= Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webb's effect= Webb's additive effect

Figure 5. 1: Combinations of plant species possessing alkaloids/condensed tannins and those of proteases/nitrogen compounds, and their projected additive and synergistic effects (%)

5.3.2 Sub experiment two (flavonoids and alkaloids/tannins)

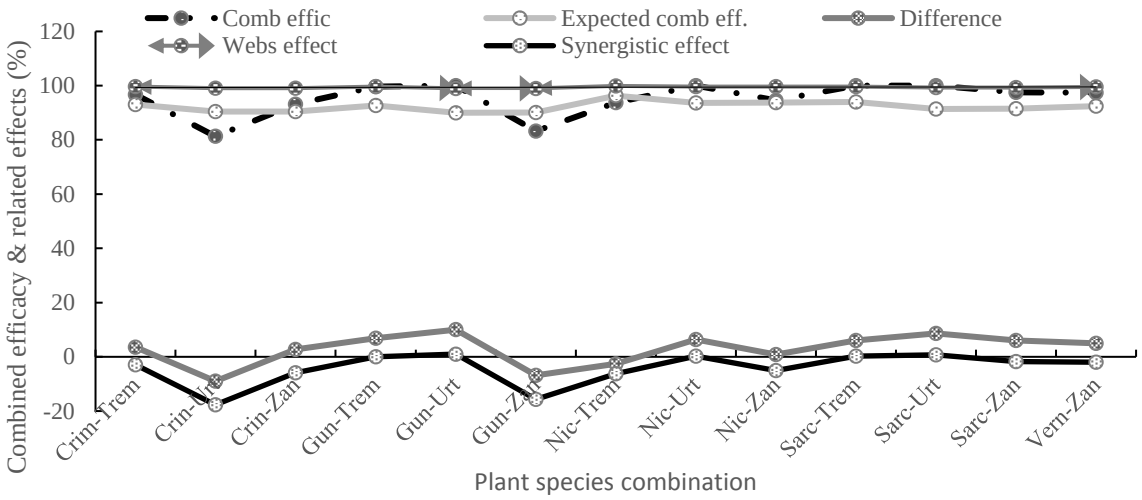
Observed anthelmintic efficacies of these combined plant species were similar (P= 0.432) and but had high mean 95.2 ± 0.34 %. Observed efficacies ranged from 81.3 ± 0.34 % for *Crinum marcownii*/*Urtica dioica* to 100.0 ± 0.34 % for *Gunnera perpensa*/*Urtica dioica*, *Nicotiana*

tabacum/*Urtica dioica*, *Sarcostema viminale*/*Trema orientalis* and *Sarcostema viminale*/*Urtica dioica* (Table 5.2). Simple synergies were not different (P= 0.3814) among combined plant species and had a mean of 2.9 ± 0.36 % (Figure 5.2). Webb's synergy of anthelmintic effects resulting from combinations were not different (P= 0.2685) among pairs, with a mean -5.4 ± 0.34 % and ranged from -17.7 ± 0.34 % for *Crinum marcowanii*/*Urtica dioica* to 1.0 ± 0.34 % *for Gunnera perpensa*/*Urtica dioica* (Figure 5.2). There was no association of any of alkaloids, condensed tannins or flavonoids with combined anthelmintic efficacy of these plant species. Additionally, multi-regression analysis of alkaloids, condensed tannins and flavonoids as predictors or explanatory variables of combined anthelmintic efficacy did not qualify any to enter at (P = 0.15) significance level.

Table 5. 2: Anthelmintic efficacies (%) of combined plant species possessing flavonoids and those containing alkaloids/tannins

Plant species combination	Combined efficacy (%)	Plant species combination	Combined efficacy (%)
Crin-Trem	96.7 ± 3.33	*Trem-Vern*	97.5 ± 2.33
Crin-Urt	81.3 ± 5.62	*Trem-Zin*	99.4 ± 2.41
Crin-Zan	93.2 ± 6.61	*Trem-Ziz*	99.9 ± 0.07
Gun-Trem	99.6 ± 0.44	*Urt-Vern*	96.5 ± 2.06
Gun-Urt	100.0 ± 0.00	*Urt-Zin*	100.0 ± 0.00
Gun-Zan	83.3 ± 16.67	*Urt-Ziz*	99.9 ± 0.07
Nic-Trem	93.7 ± 2.87	*Vern-Zan*	97.4 ± 2.29
Nic-Urt	100.0 ± 0.00	*Zan-Zin*	100.0 ± 0.00
Nic-Zan	94.6 ± 2.72	*Zan-Ziz*	97.6 ± 2.35
Sarc-Trem	100.0 ± 0.00		
Sarc-Urt	100.0 ± 0.00		
Sarc-Zan	97.5 ± 2.33		

Crin = *Crinum macowanii*, Gun = *Gunnera perpensa*, Nic = *Nicotiana tabacum*, Sarc = *Sarcostema viminale*, Trem = *Trema orientalis*, Urt = *Urtica dioica*, Vern = *Vernonia amygdalina*, Zan = *Zanthozylum capense*, Zin = *Zingiber officinale*, Ziz = *Zizyphus mucronata*



Crin= *Crinum macowanii*; Gun= *Gunnera perpensa;* Nic = *Nicotiana tabacum;* Sarc = *Sarcostema viminale; Trema orientalis*; Urt= *Urtica dioica*; Vern= *Vernonia amygdalina;* Zan= *Zanthozylum capense*; Zin= *Zingiber officinale*; Ziz= *Zizyphus mucronata* Comb effic= Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webs effect= Webs additive effect

Figure 5. 2: Combinations of plant species possessing flavonoids and those of alkaloids/condensed tannins, and their projected additive and synergistic effects (%)

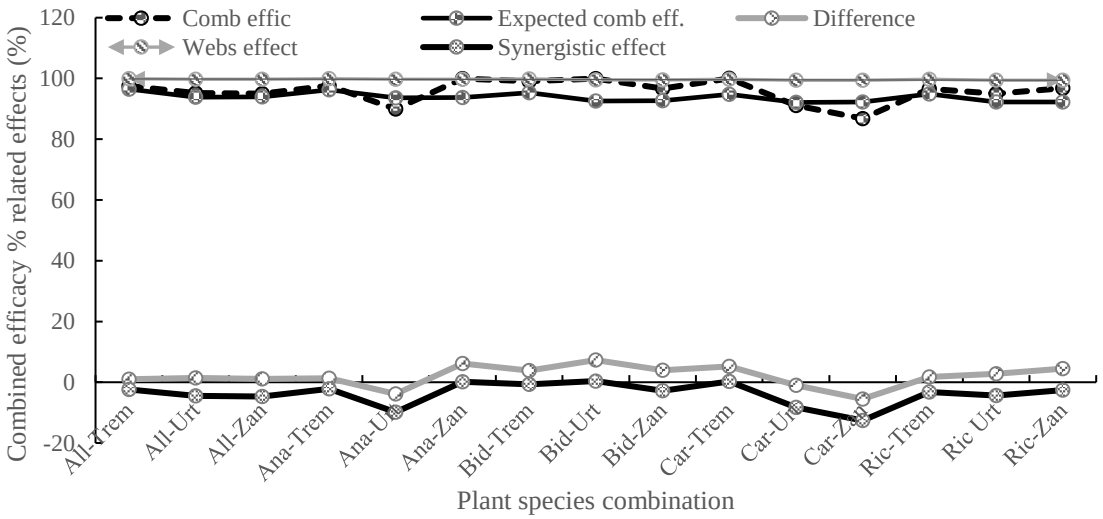
5.3.3 Sub experiment three (proteases/nitrogen compounds and flavonoids)

Observed combined efficacies of plant species containing proteases/nitrogen compounds and flavonoids were not different (P= 0.597) but relatively high, with a mean of 95.8 ± 0.04 %. Observed efficacies ranged from 86.8 ± 0.04 % for *Carica papaya/Zanthozylum capense* to 100.0 ± 0.04 % for *Carica papaya/Trema orientalis* (Table 5.3). Simple synergies were similar (P= 0.756), with mean of 2.0 ± 0.27 %. Webb's synergy resulting from these combinations were not different (P= 0.626), with a mean -3.8 ± 0.04 % and ranged from -12 ± 0.04 % for *Carica papaya*/*Zanthozylum capense* to 0.4 ± 0.04 % for *Biden pilosa*/*Urtica dioica* (Figure 5.3). There was no correlation between any of alkaloids, condensed tannins or flavonoids content of plant species and anthelmintic efficacy. Correspondingly, multiple regression analysis of alkaloids, condensed tannins and flavonoid content as predictors of combined anthelmintic efficacy was so poor that none entered the model at P=0.15 level of significance.

Table 5. 3: Anthelmintic efficacies (%) of combined plant species possessing proteases/nitrogen compounds and those containing flavonoids

Plant species combination	Combined efficacy (%)	Plant species combination	Combined efficacy (%)
All-Trem	97.5 ± 2.33	*Bid-Zan*	96.7 ± 2.33
All-Urt	95.2 ± 5.83	*Car-Trem*	100.0 ± 0.00
All-Zan	95.0 ± 4.66	*Car-Urt*	91.1 ± 8.88
Ana-Trem	97.6 ± 2.38	*Car-Zan*	86.8 ± 6.61
Ana-Urt	89.8 ± 6.86	*Ric-Trem*	96.6 ± 3.38
Ana-Zan	99.9 ± 0.13	*Ric-Urt*	95.0 ± 2.55
Bid-Trem	99.1 ± 0.89	*Ric-Zan*	96.8 ± 2.58
Bid-Urt	99.9 ± 0.11	-	-

All = *Allium cepa*, Ana = *Ananas comosus*, Bid = *Bidens pilosas*, Car = *Carrica papya*, Ric = *Ricinus communis*, Trem = *Trema orientalis*, Urt = *Urtica dioica*, Zan = *Zanthozylum capense*



All = *Allium cepa*; Ana = *Ananas comosus;* Bid = *Bidens pilosa;* Car = *Carica papaya;* Urt= *Urtica dioica*; Ric = *Ricinus communis* Zan= *Zanthozylum capense*, Comb effic= Combined efficacy; expected comb eff= expected combined efficacy; Difference= Difference between combined efficacy and expected efficacy; Webs effect= Webs additive effect

Figure 5. 3: Combined efficacies of plant species possessing proteases/nitrogen compounds and those of flavonoids, and their projected additive and synergistic effects %

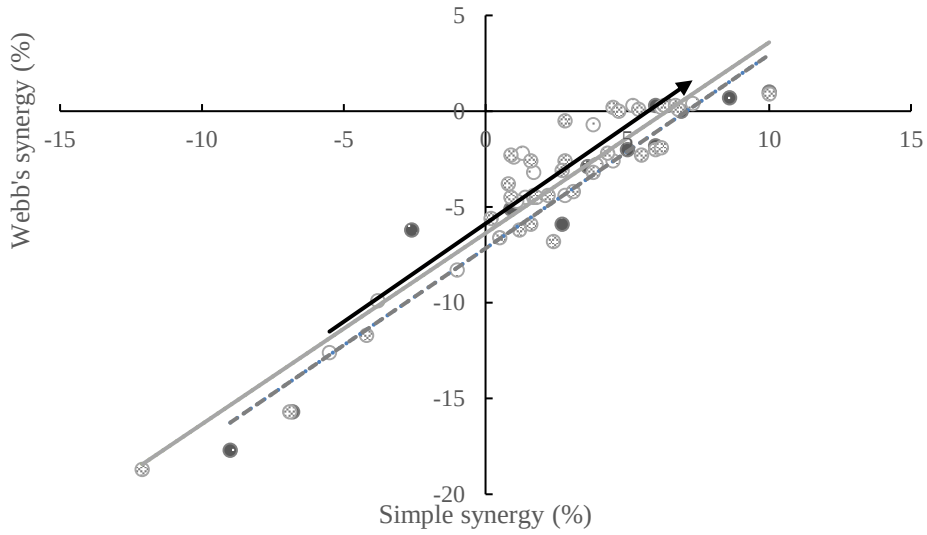


Figure 5. 4: Scattered plot showing relationship of simple and Webb's synergy in sub experiments 1, 2 and 3

5.4 Discussion

Observed combined anthelmintic efficacies of plant combinations carrying different classes of bioactive compounds in all three sub experiments were high, but had various trends. These trends are suggestive of various interactions among different classes of anthelmintic principles involved (Kubola and Siriamornpun, 2008; Rochfort *et al*., 2008; Madalaa *et al*., 2016), and others that might have aided the process of combined anthelmintic efficacy. Nonetheless, improved anthelmintic activity from plant combinations as exemplified by the trend of simple synergies is a potential indication of some positive biochemical interaction, which was not discernible at the level of putative macro-biochemical interaction including alkaloids, condensed tannins and flavonoids. Fundamentally, plant species in their nature and true to their species trait (Hammond *et al*., 1997; Aherne and O'Brien, 2002; Makkar *et al*., 2007; Cala *et al*., 2012) are suggested to be responsible for primary differences in combined anthelmintic activity (Hammond *et al*., 1997; Wenk, 2003; Shaik *et al*., 2006); these same differences are pooled in combinations resulting to various interactions. These species differences may therefore lead to many more interactions and further differences in anthelmintic activity exerted by various combinations. Basically, the nature, chemical structure, molecular size and variety of anthelmintic principle(s), and other related biochemical candidates (Harborne, 1986) in the pool of each pair of combination, most likely would contribute to explain trends of observed combined efficacies.

Mean combined anthelmintic efficacy of combinations involving alkaloids/tannins and proteases/nitrogenous compounds bearing plant species (95.6 ± 0.12 %), and that of proteases/nitrogenous compound and flavonoids bearing plant species (95.8 ± 0.04 %) were proportionally different as opposed to their statistical similarity, while that of combinations involving plant species carrying flavonoids and alkaloids/tannin containing plant species was relatively higher (98.9 ± 0.34 %) in accordance with unique biochemical nature of component plant species (Makkar *et al*., 2007; Cala *et al*., 2012) constituting combinations. These observed differences most likely would have arisen from phytochemical interactions within various combinations. Combinations of different plant species would have produced a rich pool of different biochemical compounds, some of which potentially interacted and improved on anthelmintic effect, while others produced some antagonistic effects (Efferth and Koch, 2011; Che *et al*., 2013). The net effect from these interactions being the largely positive simple synergies, though Webb's synergies were largely negative.

Improved anthelmintic activity in combination phytotherapy was measured first, by simple synergy and alternatively, by any further response above additive effect qualified as synergistic following Webb's fractional product method (Webb, 1963). However, both approaches interacted linearly though simple synergy was largely positive, whereas Webb's synergy was oppositely negative (Figure 5.4). Combined dose adopted in the current study yielded high observed efficacies and were mostly close to 100%, leaving very little room to adequately evaluate synergistic effects for most combinations in the process. Lower combined doses, relative to the current one, will potentially better address determination of synergistic effect and any extensive improvement that will arise from combined phytoanthelmintic therapy. As hypothesized, there is neither rejection nor acceptance following the dose adopted in the current study.

In sub experiment one (SE 1) flavonoids associated with observed efficacy, whereas alkaloids and condensed tannins did not. Additionally, in sub experiments two (SE 2) and three (SE 3) there was no association of any of flavonoids, condensed tannins and alkaloids with observed efficacy and other related parameters including simple and Webb's synergies. Plant species containing or carrying some of these bioactive molecular classes, usually have a mixture of different molecular types, some of which may lack bioactive attributes for the relevant trait (Klongsiriwet *et al*., 2015). In this context, if the molecular constitution of the biochemical class were more of the inactive portion (Klongsiriwet *et al*., 2015), then, there is likelihood that

the activity will be obscured and rendered insignificant. It is likely to occur when molecular classes of alkaloids, condensed tannins and flavonoids are considered holistically. On the other hand, if combinations containing different biochemical principles that exert anthelmintic and other related activities are overwhelmingly of the active type, additive and/or synergistic effects will occur. Improved observed efficacy of some combinations in the current study is in accord with the objective of adopting combination therapy in helminth treatment and other therapeutic processes. Anthelmintic efficacy of combinations involving alkaloids/tannins and proteases/nitrogen, flavonoids and alkaloids/tannin), and proteases/nitrogen compounds and flavonoids, all produced positive simple synergic effects. This is suggestive of a collective anthelmintic activity in combinations that might have arisen from a variety of active principles including alkaloids, condensed tannins and flavonoids, with no outstanding strong evidence of individual contributions.

Following Webb's formula for computing additive and synergistic effects from anthelmintic drug combinations (Webb, 1963), additive effect of two acting independent drugs, is the product of the surviving or unaffected fractions after treatment with either agents alone. The basis of adoption of combined anthelmintic therapy has been because, generally, the resulting combined efficacy is usually higher than that of any of the component anthelmintics (Leathwick and Besier, 2014). An analogous expectation is that plant species combinations possessing different anthelmintic principles will yield similar results to those of drug combinations. Though some combinations yielded antagonistic effects, it is important to select combinations with favourable responses to evade any negative effect. Phytochemical interactions in phytotherapeutic remedies is usually complex with synergistic and antagonistic processes taking place concurrently to enable healing (Efferth and Koch, 2011). Alternative evaluation focussing on simple synergy yielded overall positive mean in contrast to largely negative values following Webb's fractional method. While results of simple synergy paint a picture of positive synergism, Webb's method yielded antagonistic interaction. Our general observation showed that there was a tendency for some marginal increase in anthelmintic potency as a result of plant species combinations possessing different principles. Anthelmintic and other biological activities that are identified with these selected plant species most likely resulted from net synergism as opposed to antagonism by relevant phytochemicals

This tool has been useful in regulating and mitigating selection for resistance, given that higher anthelmintic efficacy from combination therapy and synergistic interaction will eliminate

almost all nematode parasites at drug/parasite interphase (Bartram *et al*., 2012). Anthelmintic efficacy that is close to 100% leaves virtually only parasites and their intermediate developmental stages that have no contact or interaction with anthelmintic remedy (Geary *et al*., 2012) and so, conserve their susceptibility for subsequent treatment or dosing. In this scenario, non-interaction of sub parasite populations with potent combination can be likened to selective treatment of affected animals (van Wyke *et al*., 2006; Shalaby, 2013) that leaves untreated ones with parasites that are highly vulnerable to killing effect. Drug combination apart, augmentation of protein content of livestock diets resulted to much more resilient animals (Burke *et al*., 2007). Proteins of nutritional benefit administered as supplement concurrently with copper wire particles to lambs and goats, reduced nematode egg count significantly and was suggested to have acted synergistically (Burke *et al*., 2004; Burk *et al*., 2007).

Better control of nematodes and other economically important parasites of grazing livestock is therefore not limited to application of combination anthelmintic therapy and those of plants possessing different bioactive principles. This opens ample space for multifaceted research on combination therapy and a huge potential of diverse approaches to this novel method of control. Protein supplementation and its interaction with plant secondary metabolites, especially condensed tannins (Athanasiadou *et al*., 2008) is suggested to be another method of combination therapy and can also serve a prophylactic role. It exerts an indirect role similar to that of supplementation in diets of infected livestock by being temporally bound to tannins and evading ruminal microbial degradation, thus enabling their supply to the abomasum and small intestine for efficient enzymatic digestion, absorption and assimilation (Barry and McNabb, 1999). This reinforces protein availability for vital metabolic processes and immune defence activities against nematode parasites and other pathogens. However, this among other methods, may reduce nematode burden considerably. Anthelmintic combination therapy and prophylaxis represent a more effective and efficient method, while anthelmintic bioactive plant species combinations have budding potentials to be implemented and improved upon.

5.5 Conclusion

Combinations of plant species exerting anthelmintic activity, and also possessing different anthelmintic bioactive principle classes are an important method of livestock nematode parasite control, in addition to other pathogens. Intergroup combinations apparently showed some marginal positive synergistic effects, while others were antagonistic, because of the wide range of bio-chemicals involved. Phytotherapy naturally involves both processes, but with a net positive or synergistic effect that engenders healing. Antagonism may potentially modulate some phytochemicals whose interaction may impact negatively on killing and healing if left unchecked. While those combinations that have proven useful can be adopted for subsequent studies, it is also important to identify those that produce negative effects. The dose used in the current study has to be reduced to sufficiently address the element of synergy. There is need for a more consistent method of evaluating synergism given the differences in outcome from the current study. A much more critical or finer evaluation of these plant species is also required to identify other principles that aid and facilitate anthelmintic activity.

Chapter 6

Comprehensive biochemical analysis of selected plant species exerting anthelmintic activity

Abstract

Sixteen selected plant species including *Allium cepa, Aloe van balenii*, *Ananas comosus, Bidens pilosa, Carica papaya, Crinum marcowanii, Gunnera perpensa, Nicotiana tabacum, Ricinus communis, Sarcostema viminale, Urtica dioica, Vernonia amygdalina, Zanthozylum capense, Zingiber officinale* and *Zizyphus mucronata* were analysed for phytochemical composition using GC/MS, in search of phytochemicals/ bioactive compounds related to anthelmintic activity. Four grams (4 g) dry matter (DM) of each species vegetative material was extracted in 70 % ethanol, from which 2 µl was injected into a chromatoprobe trap and analysed for biochemical composition. Compound identification carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, where available, and also comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps run in GC/MS as controls to identify background contamination. Compounds present at higher or similar percentages in controls were contaminants and excluded from analysis. For quantification, each peak area in each sample was quantified and converted to percentage of emission, and emitted mass in Nano grams. Phytochemicals identified belonged to aldehydes, amines, sulphur compounds, nitrogen compounds, Ketones, aliphatic acids, benzenoids, alcohols, lactones, amides, alkaloids, furans and esters. Reference to previous screening and related bodies of work identified and profiled phytochemicals with anthelmintic and other related biological activities. Fourty six phytochemicals had antibacterial activity, 42 antioxidant activity, 38 antifungal activity, 24 antiviral activity, and 13 anthelmintic activity. Allotment of thirteen anthelmintic related phytochemicals according to occurrence in selected plant species indicated that 2 plants had one, 6 plants had two, four plants had three phytochemicals, and 4 plants four phytochemicals. It is most plausible that anthelmintic and other related biological activities exerted by plant species are closely linked to some phytochemical(s).

6.1 Introduction

Anthelmintic activity of plant species possessing this natural trait has been closely associated with several biochemical principles. Some of these exert very little or no metabolic function to vital activities of relevant plant species, and are tagged plant secondary metabolites (PSM) (Bennett and Wallsgrove, 1994; Zhao *et al*., 2005). Their primary role is in plant defense, including protection from herbivory, pest and other pathogenic organisms (Bennett and Wallsgrove, 1994; Athanasiadou *et al*., 2006; Solaiman and Owens, 2010). True to the nature of plants, each plant may have several classes of plant secondary metabolites and related principles (Ghisalberti, 2002; Olivier, 2012; Refaat *et al*., 2012; Rampadarath *et al*., 2014), exercising the same function, but having different biochemical modes of action and in different gastrointestinal sites (Terrill *et al*., 1994; Desrues *et al*., 2017) on livestock nematode parasite and/or related developing stages.

Some relevant secondary metabolites include cyanogenic glucosides, glucosinolates, non-protein amino acids, alkaloids, plant phenolics, plant terpenes, sesquiterpenes and sterols, phytoalexins, salicylic acid and methyl jasmonate (Bennett and Wallsgrove, 1994). A vast body of research work has identified some key plant secondary metabolites involved in livestock helminth control. These include alkaloids (Refaat *et al*., 2012), condensed tannins (Min and Hart, 2003; Ademola *et al*., 2005; Alonso-Diaz *et al*., 2011), flavonoids (Azando *et al*., 2011; Iqbal *et al*., 2011: Singh *et al*., 2011), proteases and nitrogen compounds (Luoga *et al*., 2012; Domingues *et al*., 2013) among others. In addition to the wide variety of plant bioactive anthelmintic principles, there are differences in their content and concentration even in the same plant species from the same or different regions due to a number of factors (Sampaio *et al*., 2016).

Generally, the biochemical content of most plant species has been found to vary with seasons (Scogings *et al*., 2003), climatic regions (Waghorn, 2008), soil type and fertility (Jat and Gajbhiye, 2017). Climatic stress and threat or outright herbivory are critical to their production and accumulation in most plant species (Akula and Ravishankar, 2011). Arid accessions of the same plant species are richer in plant secondary metabolites than their analogues from humid and more benign climatic regions. If biochemical anthelmintic activity were dependent solely on these principles, they will potentially influence the trend of activity when collected from different sites or climatic regions and at different seasons.

It is hypothesized that anthelmintic activity exerted by plant species is an attribute of whole plant species, as exhibited by species crude extract. Therefore, it would be necessary to carry out a finer biochemical analysis of these plant species to proof the contrary and provide a profile of phytochemicals responsible for anthelmintic and other related activities. The objective of this study was to analyze selected plant species for phytochemical content, relate them to known primary anthelmintic and other biological activities including antibacterial, antifungal, antioxidant and antiviral activities as plausible proof of their involvement.

6.2 Materials and methods

6.2.1 Selection of plant species and extraction of vegetative material

The following plant species were selected and identified based on previous knowledge of their ethno veterinary use in control of parasitic helminth in various communities around the world. *Allium cepa* (Vieira *et al*., 1999; Marwat *et al*., 2011; Bidkar *et al*., 2012), *Aloe van balenii, Trema orientalis* (Watt and Breyer-Brandwijk, 1962), *Ananas comosus* (Stepek *et al*., 2005), *Bidens pilosa* (Graham *et al*., 1980; Hoffman and Hoelzl, 1988), *Carrica papaya* (Stepek *et al*., 2005; Adongo, 2013), *Crinum macowanii* (Refaat *et al*., 2012), *Gunnera perpensa* (Semelane *et al*., 2010), *Nicotiana tabacum* (British Veterinary Codex, 1953; Stuart *et al*., 2012), *Ricinus communis* (Rampadarath *et al*., 2014; Wafa *et al.*, 2014), *Sarcostema viminale* (Grime *et al*., 2008), *Urtica dioica* (Haman, 2007), *Vernonia amygdalina* (Yeap *et al*., 2010), *Zanthozylum capense* (Negi *et al*., 2011) and *Zingiber officinale* (Haman, 2007; Singh *et al*., 2011), and *Zizyphus mucronata* (Van Wyk and Wink, 2004; Olivier, 2012).

Four grams dry matter (4 g DM) of each plant species vegetative material was weighed into labelled thimbles and closed with a ball of cotton wool wrapped in piece of cheese cloth. Thimbles containing plant material were mounted into distillation units and extracted using 70 % ethanol as solvent. Crude extracts were refluxed into extraction bottles of volume 100 ml. Extraction process was completed when solvent in thimble carrying section was free of any coloration. Extraction bottles containing crude extracts were withdrawn, allowed to cool and Volume of crude extract was made up to 100 ml. volumes of 4 ml each were poured into glass vials for GC/MS analysis.

6.2.2 Biochemical analysis of crude extracts of plant species using GC/MS

Volatile samples were analysed using a coupled Varian 3800 gas chromatography (Varian Palo Alto, California, and USA) and Varian 1200 mass spectrometer (GC-MS). The gas chromatography (GC) was equipped with an Alltech EC - WAX column of 30m x 0.25 µm internal diameter x 0.25 µm film thickness (Alltech Associates Inc., Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. From each plant species extract, 2 µl was injected into a chromatoprobe trap prepared by cutting glass tubes of similar size to that of chromatoprobe quartz microvials (length: 15 mm; inner diameter: 2 mm) and filled with 2 mg of a 50:50 mixture of Tenax TA (Alltech Associates, USA) and graphitized carbon (Carbotrap™, Supelco, USA). These tubes were closed on both ends with glass wool. Chromatoprobe traps were placed in a Varian 1079 injector by means of a Chromatoprobe fitting and thermally desorbed. The temperature of the injector was 40 °C, and was held for 2 minutes with a 20:1 split ratio and then increased to 200 °C. It was held at 200 °C min^{-1} in splitless mode for thermal desorption.

Compound detection was delayed for 6 minutes. After a 3 minutes hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C min^{-1} and retained for 12 minutes. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, as well as comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps were run in GC-MS as controls to identify background contamination. Compounds present at higher or similar percentages in controls were considered as contaminants and excluded from the analysis. For quantification of compounds, known amounts of standards of dominant compounds were injected into cartridges and thermally desorbed under identical conditions to samples. The peak area of compounds in samples were compared with those of standards and used to calculate the total amount of compound per gram of substrate.

6.2.3 Evaluation of biochemical composition and statistical analysis

Phytochemical content of selected plant species was determined in Nano grams (Ng) per gram dry matter. Phytochemicals identified in these plants were subsequently browsed for their anthelmintic and other biological activities including antibacterial, antifungal, antioxidant and antiviral activities. Simple statistical evaluation for minimum and maximum content, including sum, mean content and standard deviation was done using means procedure of SAS (2000).

6.3 Results

Simple statistics of biochemicals of selected plant species are given in Table 6.1

An array of 51 biochemical compounds were identified and included candidates belonging to aldehydes, alcohols, amines and amides, sulphur compounds, nitrogen compounds, ketones, aliphatic acids, benzenoids, lactones, alkaloids, furans and esters.

Aldehyde: Furfural occurred in *Allium cepa, Aloe van balenii, Gunnera perpensa,* and *Ricinus communis,* while 5-hydroxymethylfurfural was identified in *Aloe van balenii*, *Crinum macowanii*, *Gunnera perpensa* and *Sarcostema viminale*. The other member molecules occurred in only one species each and included, hexanal in *Zingiber officinale* and 2-furancarboxaldehyde, 5- methyl in *Ricinus communis*.

Alcohols: Alcohols included, furanmethanol which occurred in all sixteen crude extracts, 2,3-butanediol in *Allium cepa*, *Nicotiana tabacum* and *Zizyphus mucronata,* while phytol occurred in *Trema orientalis*, *Urtica dioica* and *Zizyphus mucronata*. The other members of alcohols which featured in two and one plant species included glycerin in *Zanthoxylum capense* and *Zingiber officinale;* 2,7-Octadiene-1,6-diol in *Aloe vanbalenii*;1,2,3-propanetriol in *Zizyphus mucronata* and 2,6-dimethyl in *Allium cepa*.

Amines, amide and nitrogenous compound: These compounds included acetamide in *Ananas comosus, Biden pilosa, Carica papaya,* and *crinum macowanii*, whereas 2-propenamide featured in *Allium cepa* and *Ananas comosus.* Methylamine, N,N- dimethyl- featured only in *Sarcostema viminale*. Nitrogen compounds included 2,5-dimethylpyrazine, which was identified in *Ananas comosus, Trema orientalis* and *Urtica dioica*. Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Nicotine) and 2,5-dihydro-1H-pyrrole in two plant species each; the former in *Nicotiana tabacum* and *Sarcostema viminale,* and the later in *Bidens pilosa* and *Carica papaya*. Other nitrogen compounds occurred in only one plant species each, including 2,6-dimethylpyrazine in *Zingiber officinale,* 2-ethyl-5-methylpyrazine and 8 azabicyclo[3.2.1]octane-3-carbonitrile,8 methyl- in *Zizyphus mucronata,* and Benzyl nitrile in *Carica papaya.*

Sulphur compound: Sulphur compounds occurred in 10 plant species, except *Aloe van balenii, Ananas comosus, Sarcostema viminale, Zanthozylum capense, Zingiber officinale* and *Zizyphus mucronata*. Other sulphur compounds that occurred in much smaller concentrations in selected

plant species included, 3,4-dimethylthiophene, 1-propenyl methyl disulphide and dimethyl trisulphide in *Allium cepa*.

Ketones: Ketones featured strongly in most of the selected plant species and included 1-hydroxy-2-propanone which occurred in crude extract of all sixteen plant species; 2,5-dimethyl-4-hydroxy-3(2H)-furanone occurred in half of the selected plant species inclusive of *Allium cepa, Aloe vanbalenii, Ananas comosus, Bidens pilosa, Carica papaya, Ricinus communis, Trema orientalis* and *Zanthozylum capense.* 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one was identified in *Allium cepa, Aloe van balenii, Ananas comosus, Bidens pilosa, Trema orientalis, Urtica dioica, Zanthozylum capense* and *Zingiber officinale*. Other ketones were: (1) 1-(1H-pyrrol-2-yl)-ethanone occurring in *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya, Crinum macowanii, Ricinus communis* and *Zingiber officinale;* and (2) dihydro-4-hydroxy-2-(3H)-furanone occurring in *Allium cepa, Ananas comosus, Crinum macowanii, Ricinus communis* and *Sarcostema viminale*. Least occurring ketones included 4-cyclopentene-1,3-dione in *Zanthozylum capense* and *Zizyphus mucronata;* 4-methyl-5H-furan-2-one in *Sarcostema viminale*; 2(3H)-furanone in *Allium cepa* and 2(3H)-furanone, dihydro-3-hydroxy-4,4-dimethyl- in *Urtica dioica*.

Aliphatic acids: Aliphatic acids were also identified among the most common biochemical molecular class, with acetic acid featuring in all 16 plant species, propanoic acid in three including *Zanthozylum capense, Zingiber officinale* and *Zizyphhus mucronata*. On the other hand, 4-hydroxy-butanoic acid occurred only in *Carica papaya* and *Zanthozylum capense*, whereas, sorbic acid featured principally in *Sarcostema viminale*.

Benzenoid: They featured in twelve plant species, inclusive of *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya, Crinum macowanii, Gunnera perpensa, Ricinus communis, Sarcostema viminale, Vernonia amygdalina, Zanthozylum capense, Zingiber officinale* and *Zizyphus mucronata*. The order of distribution of different molecules included, 2-methoxy-4-vinylphenol in ten plant species, excluding *Aloe vanbalenii, Gunnera perpensa, Nicotiana tabacum, Trema orientalis, Urtica dioica* and *Vernonia amygdalina*. Benzene acetaldehyde occurred in *Allium cepa, Carica papaya, Bidens pilosa* and *Ricinus communis*. Other molecules of benzenoids occurred in smaller concentrations including benzaldehyde in *Vernonia amygdalina,* vanillin and 2-butanone, 4-(4-hydroxy-3-methoxyphenyl)- in *Zingiber officinale*.

Lactones: Lactones included 2-pyrrolidone that occurred in 12 plant species, excluding *Allium cepa, Aloe van balenii, Sarcostema viminale* and *Urtica dioica*. It was closely followed by

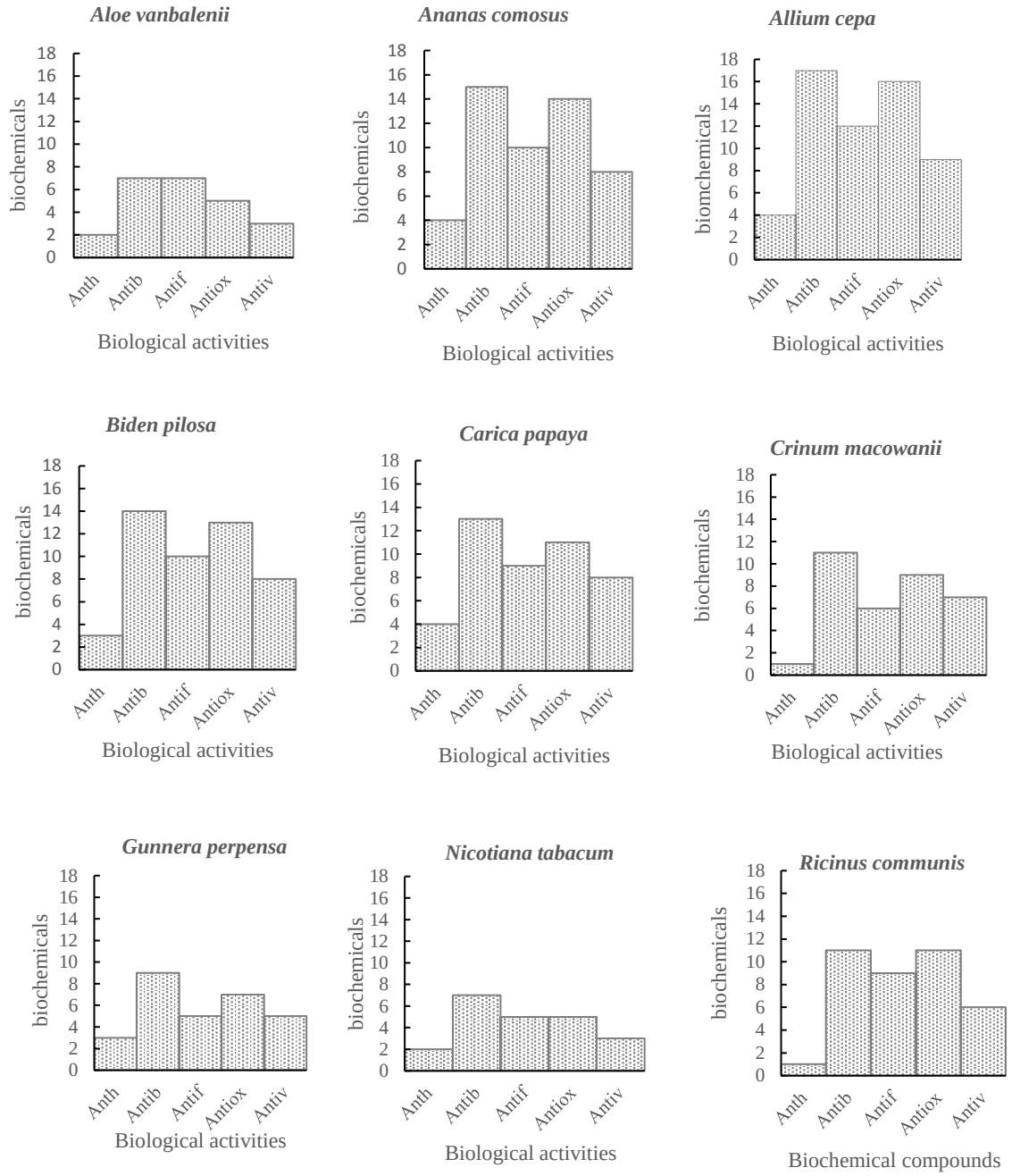
butyrolactone in nine species excluding *Allium cepa*, *Aloe vanbalenii*, *Crinum macowanii*, *Ricinus communis*, *Sarcostema viminale*, *Zanthozylum capense*, and *Zizyphus mucronata*. 2-hydroxy-gamma-butyrolactone least featured and occurred in six species, including *Allium cepa*, *Ananas comosus*, *Ricinus communis*, *Sarcostema viminale*, *Zanthozylum capense* and *Zizyphus mucronata*.

Alkaloids, were also identified in some of the plant species, and included glutarimide which occurred in *Trema orientalis* and *Urtica dioica*, while its other collateral, Z-1-(1-butenyl) aziridine was identified in *Trema orientalis*. Ultimately, there was furan related biomolecule, primarily benzofuran, 2,3-dihydro-, which occurred in *Allium cepa*, *Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Trema orientalis*, *Vernonia amygdalina*, *Zanthozylum capense*, *Zingiber officinale and Zizyphus mucronata.*

Table 6. 1: Major biochemical molecules in selected plant species possessing anthelmintic activity, their mean, standard deviation (st dev) minimum (min) and maximum (max) concentrations

Biochemical	n	Mean (Ng/g)	st dev	Min (Ng/g)	Max (Ng/g)
Hexanal	16	0.22	2.57	0	10.31
Methylamine,N,N-dimethyl	16	0.02	0.20	0	0.27
3,4-Dimethylthiophene	16	0.05	0.73	0	1.18
1-propenyl methyl disulphide	16	0.06	0.86	0	1.40
Dimethyl trisulphide	16	0.03	0.45	0	0.73
2,6-dimthylpyrazine	16	0.11	1.30	0	1.73
2,5-Dimethylpyrazine	16	0.10	0.66	0	4.64
1-hydroxy-2-propanone	16	1.98	5.25	0.41	8.32
Furfural	16	0.96	10.37	0	16.78
Acetic acid	16	32.21	92.39	5.77	124.70
Benzaldehyde	16	0.06	0.73	0	0.97
Propanoic acid	16	0.05	0.33	0	0.37
2-Furancarboxaldehyde, 5-methyl-	16	0.12	0.86	0	1.02
2,3-butanediol	16	5.24	70.10	0	113.59
Sorbic acid	16	0.09	1.03	0	1.38
4-Cyclopentene-1,3-dione	16	0.06	0.50	0	0.62
Dimethyl Sulfoxide	16	0.38	1.51	0	1.75
Benzene acetaldehyde	16	0.46	2.58	0	2.34
Butyrolactone	16	0.62	2.41	0	2.35
4-hydroxy-butanoic acid	16	0.05	0.43	0	0.48
2-Furanmethanol	16	1.16	7.11	0.01	12.02
5-methyl-2-Furanmethanol	16	0.13	1.23	0	1.94
4-Methyl-5H-furan-2-one	16	0.04	0.50	0	0.67
2(3H)-Furanone	16	0.05	0.67	0	1.08
Acetamide	16	0.21	1.05	0	1.22
2,5-dihydro-1H-Pyrrole	16	0.07	0.56	0	0.62
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	16	3.38	35.87	0	48.09
Phenylethyl Alcohol	16	0.09	0.55	0	0.49

Biochemical	n	Mean (Ng/g)	st dev	Min (Ng/g)	Max (Ng/g)
Benzyl nitrile	16	0.22	2.48	0	3.31
2-Propenamide	16	0.32	2.07	0	2.62
1-(1H-pyrrol-2-yl)-ethanone	16	0.28	0.39	0	2.18
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	16	0.59	2.17	0	2.98
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-	16	0.05	0.53	0	0.71
2-Pyrrolidinone	16	0.29	0.79	0	0.92
2-Ethyl-5-methylpyrazine	16	0.07	0.53	0	0.61
8-Azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl	16	0.14	1.66	0	2.22
2-Hydroxy-gamma-butyrolactone	16	0.17	0.88	0	1.18
2-Methoxy-4-vinylphenol	16	0.42	1.41	0	1.30
Z-1-(1-butenyl) aziridine	16	0.12	1.38	0	1.84
2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4	16	12.09	85.45	2.46	143.34
2,7-Octadiene-1,6-diol, 2,6-dimethyl-	16	0.03	0.47	0	0.75
Glycerin	16	0.14	2.37	0	2.77
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a	16	0.15	0.99	0	1.34
Glutarimide	16	0.13	1.38	0	1.85
Benzofuran, 2,3-dihydro-	16	0.87	4.11	0	5.11
5-Hydroxymethylfurfural	16	5.64	62.34	0	101.35
Vanillin	16	0.02	0.19	0	0.26
1-Butanol, 3-methyl-, acetate	16	0.81	9.33	0	12.46
Phytol	16	0.15	11.28	0	1.64
Dihydro-4-hydroxy-2-(3H)-furanone	16	0.17	1.09	0	1.57
2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-	16	0.13	1.44	0	1.92
Cumulative (All)	16	288.2	553.43	50.4	5878.0
Efficacy	495	95.6	7.72	39.4	100



Anth= anthelmintic activity; Antib= antibacterial activity; Antif= antifungal activity; Antiox= antioxidant activity; Antiv= antiviral activity

Table 6. 2: Histograms of biological activities and phytochemical distribution in relation to selected plant species

6.3.1 Profiles of plant species

Allium cepa had nineteen major biomolecules, and also the highest number phytochemicals identified. These included one aldehyde (furfural), three alcohols (2-furanmethanol, 2,3-butanediol and 2,6-dimethyl), one amide/amine (2-propenamide), three sulphur compounds (3,4-dimethylthiophene, 1-propenyl methyl disulphide and dimethyl trisulphide), six ketones (1-hydroxy-2-propanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one, 2(3H)-furanone and dihydro-4-hydroxy-2-(3H)-furanone), one aliphatic acid (acetic acid), two benzenoids (benzene acetaldehyde and, 2-methoxy-4-vinylphenol), one lactone (2-hydroxy-gamma-butyrolactone) and one furan (benzofuran, 2,3-dihydro-). *Allium cepa* is rich in different biochemical molecules with an upper tier ranging from 430.40 to 229.69 Ng/g, consisting of 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one; 2,3 butanediol,5-Hydroxymethylfurfural and acetic acid, a second tier of 50.35 to 24.96 Ng/g, comprising of furfural, 2-furanmethanol and 1-hydroxy-2-propanone, and third tier of range 8.93 Ng/g to 0.54 Ng/g that carried a bulk of other biochemicals.

Aloe vanbalenni had nine major biochemical molecules belonging to four biochemical classes including two aldehydes (furfural and 5-hydroxymethylfurfural), two alcohols (2-furanmethanol and 2,7-Octadiene-1,6-diol, 2,6-dimethyl-), three ketones (1-hydroxy-2-propanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one), one aliphatic acid (acetic acid) and one lactone (2-Hydroxy-gamma-butyrolactone). *Aloe van balenii* had two main tiers of biochemical concentration; the upper one of range 24.95 Ng/g and 14.47 Ng/g, comprising of acetic acid, 5-Hydroxymethylfurfural and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one. The bottom tier carried the bulk of biochemicals and ranged from 4.73 Ng/g to 0.4 Ng/g.

Ananas comosus in turn had sixteen major biochemical molecules which belonged to eight biochemical classes, including alcohols with one biochemical molecular type (2-furanmethanol), amine/amides with two (acetamide and 2-propenamide), nitrogen compound with one (2,5-dimethylpyrazine), ketones with five candidates (1-hydroxy-2-propanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one and dihydro-4-hydroxy-2-(3H)-furanone), aliphatic acids with one (acetic acid), benzenoids with two (benzene acetaldehyde and 2-methoxy-4-vinylphenol,), lactones with three (butyrolactone, 2-pyrrolidone and 2-hydroxy-gamma-

butyrolactone) and furans with one candidate (benzofuran, 2,3-dihydro-). The biochemical concentrations were 374.09 Ng/g for acetic acid, 36.71 Ng/g for 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one, and 15.32 to 2.02 Ng/g for a bulk of other biochemical molecules.

B*idens pilosa* had fifteen molecules belonging to nine biochemical classes, viz: alcohols with one member (2-furanmethanol), amines/amides two (acetamide and 2-propenamide), sulphur compounds had one (dimethyl sulfoxide), nitrogen compounds one (2,5-dihydro-1H-pyrrole) and furans with one candidate (Benzofuran, 2,3-dihydro), aliphatic acids two (acetic acid and 4-hydroxy-butanoic acid), benzenoids two candidates (benzene acetaldehyde and 2-methoxy-4-vinylphenol) and lactones with two members (butyrolactone and 2-pyrrolidone), and ketones with four (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one). The concentration of these biochemical molecules was such that, the highest was acetic acid with 63.88 Ng/g, followed by 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one at 19.53 Ng/g and a bulk of other biomolecules lying within the range 6.74 to 0.39 Ng/g (table 5.2).

Carica papaya had thirteen prominent compounds including one each of alcohols (2-furanmethanol), amines/amides (acetamide), sulphur compounds (dimethyl Sulfoxide), aliphatic acids (acetic acid), benzenoids (2-methoxy-4-vinylphenol) and furans (benzofuran, 2,3-dihydro-); two each of nitrogen compounds (2,5-dihydro-1H-pyrrole and benzyl nitrile), and lactones (butyrolactone and 2-pyrrolidone), and ketones with three compounds (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone and 2,5-dimethyl-4-hydroxy-3(2H)-furanone). These biochemical molecules were partition into three different tiers; the upper most ranging from 24.58 to 35.78 Ng/g, comprised of 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one and acetic acid, the middle tier of concentration ranged between 9.94 to 3.17 Ng/g consisting of benzyl nitril, butyrolactone, Benzofuran , 2,3-dihydro-, 2-methoxy-4-vinylphenol and Dimethyl Sulfoxide, and the third tier that carried the bulk of biochemicals ranged from concentration 2.79 to 0.42 Ng/g.

Crinum macowanii was the next plant species in the queue and had fourteen major biochemical candidates. The above biomolecules belonged to eight main biochemical classes, and included two aldehydes (2-Furancarboxaldehyde, 5-methyl- and 5-Hydroxymethylfurfural), one alcohol (2-furanmethanol), one amine/amide (acetamide), one sulphur compound (dimethyl sulfoxide), one aliphatic acid (acetic acid), one lactone (2-pyrrolidone), five ketone (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,5-Dimethyl-4-hydroxy-3(2H)-furanone, 2,3-

dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one and dihydro-4-hydroxy-2-(3H)-furanone) and two benzenoids (phenylethyl alcohol and 2-methoxy-4-vinylphenol). These molecules ranged 32.71 to 17.34 that comprised of 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one and acetic acid, and the lower level ranging from 3.26 to 0.40 Ng/g (table 5.2).

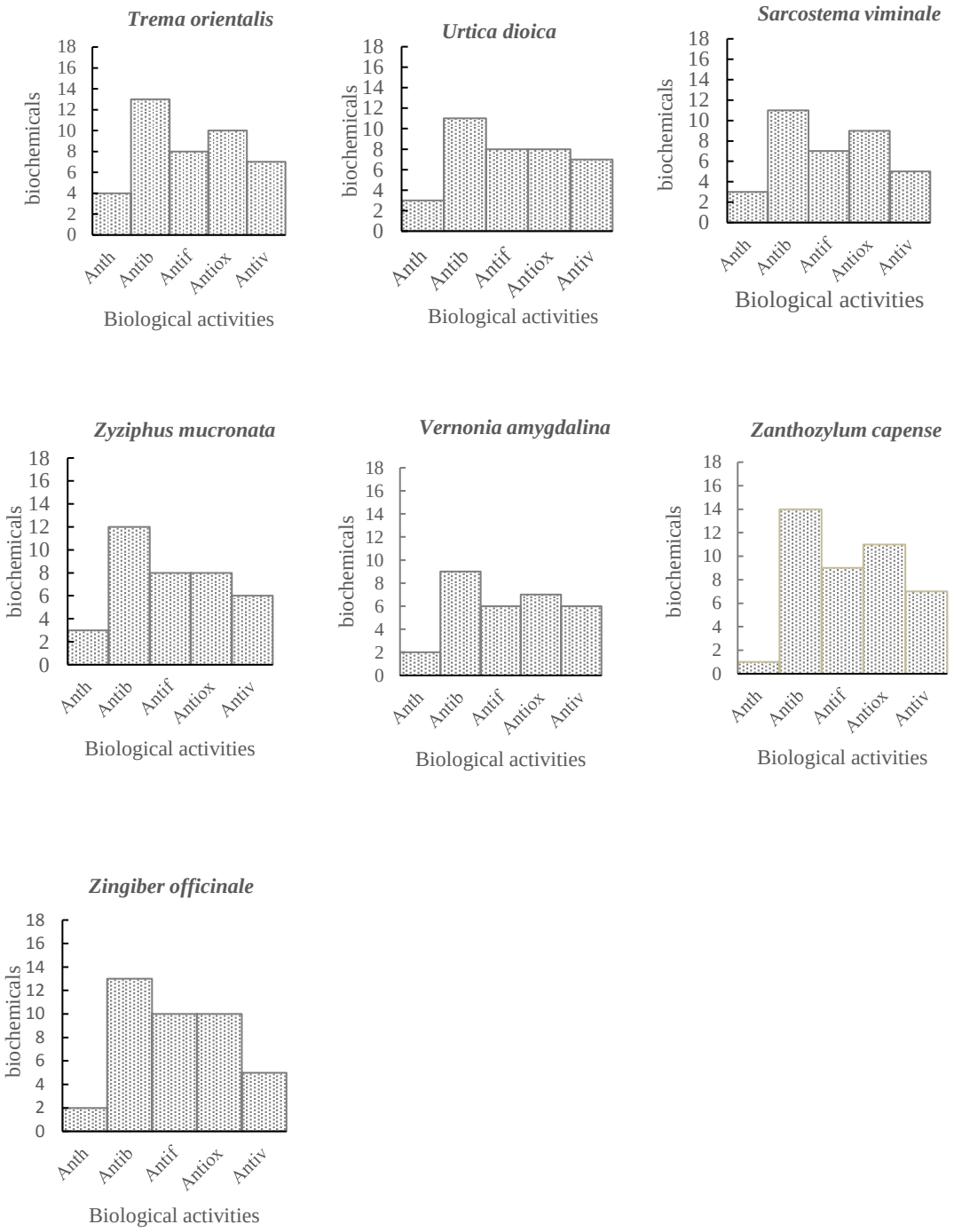
Gunnera perpensa had twelve biomolecules belonging to seven biochemical classes including one alcohol (2-furanmethanol), one sulphur compound (dimethyl Sulfoxide), two ketones (1-hydroxy-2-propanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one), one aliphatic acid (acetic acid) and one benzenoid (phenyl alcohol), three aldehydes (furfurals, 2-Furancarboxaldehyde, 5-methyl- and 5-hydroxymethylfurfural), two lactones (butyrolactone and 2-pyrrolidone) and unclassified molecule one (2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a). The concentration of biomolecules in the high echelon ranged from 25.59 to 14.12 Ng/g and comprised of 5-Hydroxymethylfurfural, acetic acid and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one. The second and lower echelon ranged from 4.76 to 0.45 Ng/g in concentration.

Nicotiana tabacum in turn had eleven biochemical molecules belonging to seven classes: one sulphur compound (dimethyl sulfoxide), one nitrogen compound (pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-), two ketones (1-hydroxy-2-propanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one) and one aliphatic acid (acetic acid), four alcohols (2,3-butanediol, 2-furanmethanol, 5-methyl-2-Furanmethanol and Glycerin) and two lactones (butyrolactone and 2-pyrrolidone). Relating to their different concentrations, there were those in very high concentration between 159.24 to 144.27 Ng/g and comprised of acetic acid and Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- and the other bulk ranging in concentration from 6.42 to 0.42 Ng/g constituted the rest of biomolecules (table 5.1).

Ricinus communis was one the selected plant species, and comprised of eighteen major biomolecules. The distribution of biomolecules relative to the different biomolecular classes was, alcohols three members (2-furanmethanol, 5-methyl-2-Furanmethanol and glycerin), sulphur compound, one (dimethyl sulphuroxide), nitrogen compound, one (Pyridine, 3-(1-methyl-2-pyrrolidinyl)-,(s)-) and aliphatic acid, one (acetic acid), aldehydes, three (furfurals, 2-furancarboxaldehyde, 5-methyl and 5-Hydroxymethylfurfural), benzenoids, two (benzene acetaldehyde and 2-methoxy-4-vinylphenol), lactones, two (2-pyrrolidone and 2-hydroxy-gamma-butyrolactone), and ketones, five (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-

pyran-4-one and dihydro-4-hydroxy-2-(3H)-furanone) (table 5.3). Identified biomolecules had various concentrations. There was 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and acetic acid that occurred in relatively very high concentration at 35.14 and 30.89 Ng/g, followed by 5-Hydroxymethylfurfural at 15.25 Ng/g, and the other bulk of biomolecules within the range 7.01 to 0.44 Ng/g (table 5.2).

Sarcostema viminale had various compound classes: one aldehyde (5-hydroxymethylfurfural), one alcohol (2-furanmethanol); one amine/amide (methylamine, N,N- dimethyl-), one nitrogen compound (pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-), one benzenoid (2-methoxy-4-vinylphenol); one lactone (2-Hydroxy-gamma-butyrolactone), two aliphatic acids (acetic acid and sorbic acid); and five ketones (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,5-Dimethyl-4-hydroxy-3(2H)-furanone, 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4 and Dihydro-4-hydroxy-2-(3H)-furanone). Concentration of the various biomolecules was such that acetic occurred in relatively very high concentration 67.25 Ng/g, followed by 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one at 12.31 Ng/g and the rest of biomolecules within 6.43 to 0.40 Ng/g (table 5.1).



Anth= anthelmintic activity; Antib= antibacterial activity; Antif= antifungal activity; Antiox= antioxidant activity; Antiv= antiviral activity

Figure 6. 1: Histograms of biological activities and phytochemical distribution in relation to selected plant species

Trema orientalis had fifteen biomolecules comprising of one sulphur compound (dimethyl sulphuroxide); one nitrogen compound (2,5-dimethylpyrazine), one aliphatic acid (acetic acid), one furan (benzofuran, 2,3-dihydro-), two alcohol (2-furanmethanol and phytol), two lactones

(Butyrolactone and 2-pyrrolidone), two alkaloids (Z-1-(1-butenyl) aziridine and glutarimide) and three ketones (1-hydroxy-2-propanone, 4-Methyl-5H-furan-2-one, 2,5-dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one). The concentrations of these biomolecules ranged 99.78 Ng/g for acetic acid, through 13.03 to 7.86 Ng/g for both 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one and 2-propenamide, to 5.56 to 0.27 Ng/g for a bulk of biomolecules .

Urtica dioica had ten compounds including one of sulphur (Dimethylsulphuroxide), nitrogen compound (2,5-dimethylpyrazine), aliphatic acid (acetic acid), lactone (butyrolactone) and alkaloid (glutaimide), and three each of both alcohols (2-Furanmethanol, phytol and glycerin) and ketones (1-hydroxy-2-propanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one). Biomolecular concentration was such that acetic acid was relatively very high at 38.03 Ng/g, followed by 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one (9.13 Ng/g) and 1-hydroxy-2-propanone (5.62Ng/g), and the rest ranged from 1.35 to 0.01 Ng/g (5.2).

Vernonia amygdalina had thirteen compounds including: two alcohols (2-furanmethanol and glycerin); one sulphur compounds (dimethyl sulphuroxide); three ketones (1-hydroxy-2-propanone, 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4 and 2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-); one aliphatic acid (acetic acid); one furan (benzofuran, 2,3-dihydro-); two benzenoids (benzaldehyde and phenylethyl alcohol); two lactones (butyrolactone and 2-pyrrolidone), and unclassified compound (2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a). Biomolecular concentrations ranged from high (acetic acid of 30.56 Ng/g), followed by 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one (14.90 Ng/g), glycerin (8.32 Ng/g) and benzofuran, 2,3-dihydro- (7.99 Ng/g). The others occurred in very low concentration ranging from 4.04 to 0.01 Ng/g.

Zanthozylum capense had sixteen biomolecules which included: one benzenoids (2-methoxy-4-vinylphenol); one furans (benzofuran, 2,3-dihydro-); two alcohols (2,3-butanediol and glycerin); two lactones (2-pyrrolidone and 2-hydroxy-gamma-butyrolactone); three aliphatic acids (acetic acid, propanoic acid and 4-hydroxy-butanoic acid); four ketones (1-hydroxy-2-propanone, 4-cyclopentene-1,3-dione, 2,5-dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one); and one unclassified molecule (2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a). The concentrations ranged from acetic acid, 84.05 Ng/g, followed by 1-butanol, 3-methyl-, acetate (37.37 Ng/g), 2,3-dihydro-3,5-dihydroxy--6-

methyl-4H-pyran-4-one (13.82 Ng/g), 1-hydroxy-2-propanone (15.49 Ng/g), and the rest in low concentration ranging from 4.19 to 0.23 Ng/g .

Zingiber officinale had seventeen biomolecules in nine classes such as: one aldehyde (Hexanal); two amines/amides (Acetamide and 2-Propenamide); one nitrogen compound (2,6-dimethylpyrazine); one furan (benzofuran, 2,3-dihydro-); one alcohol (2-furanmethanol); two aliphatic acids (acetic acid and propanoic acid); two lactones (butyrolactone and 2-pyrrolidone); four ketones (1-hydroxy-2-propanone, 1-(1H-pyrrol-2-yl)-ethanone, 2,5-Dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one) and three benzenoids (2-methoxy-4-vinylphenol, 2-butanone, 4-(4-hydroxy-3-methoxyphenyl)- and vanillin). Acetic acid was the highest (66.34 Ng/g), followed by hexanal (10.37 Ng/g) and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one (6.64 Ng/g). The bulk of compounds was within concentration range 5.75 to 0.3 Ng/g (table 5.1).

Zizyphus mucronata had fourteen compound in eight classes including: one amine/amide (2-Propenamide); one benzenoid (2-methoxy-4-vinylphenol); one lactone (2-pyrrolidone); one furan (benzofuran, 2,3-dihydro-); two aliphatic acids (acetic acid and propanoic acid); two nitrogen compounds (2-ethyl-5-methylpyrazine and 8 azabicyclo[3.2.1]octane-3-carbonitrile,8 methyl-); three alcohols (2,3-butanediol, 2-furanmethanol and phytol); and three ketones (1-hydroxy-2-propanone, 2,5-Dimethyl-4-hydroxy-3(2H)-furanone and 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one). These concentrations can be partitioned into four tiers; 133.46 Ng/g for acetic acid; 23.16 Ng/g for 2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one; and the third of range between 8.82 (7.70) and 6.65 Ng/g for 1-hydroy-2-propanone, 2,3-butanediol and 8 azabicyclo[3.2.1]octane-3-carbonitrile,8 methyl-. The fourth tier ranged between 4.92 and 0.44 Ng/g, and constituted the greater bulk of biochemical molecules.

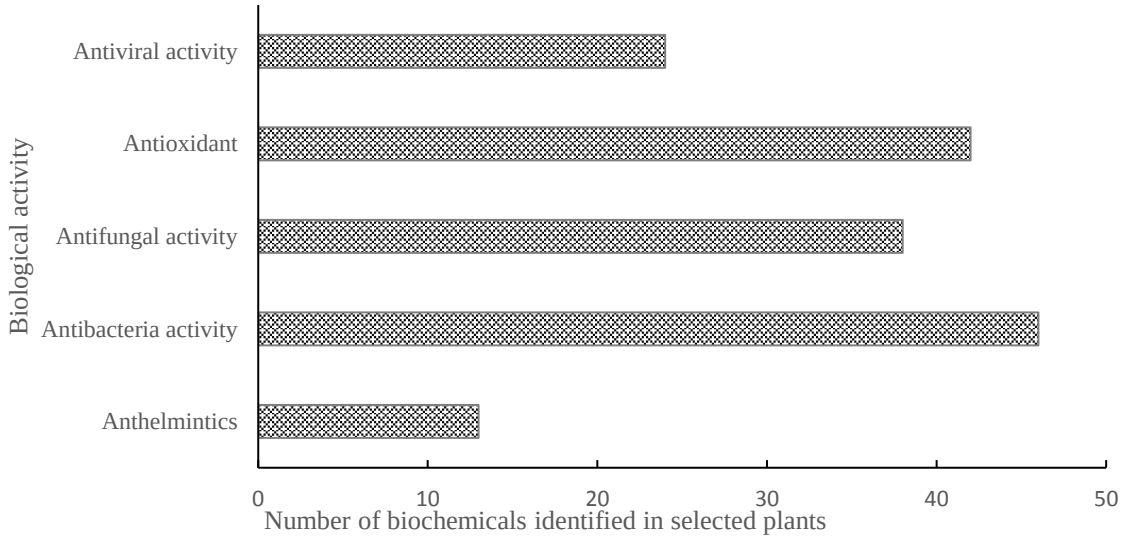


Figure 6. 2: Prevailing bioactivities and number of related phytochemicals in selected plant species

6.3.2 Bioactivity of phytochemicals

Table 5.2 shows phytochemicals in various plants, and their biological activities, and table 5.3 gives sources of literature demonstrating these activities. Bioactivities examined were anthelmintic, anti-bacteria, anti-fungal, antioxidant and antiviral activities. Following these biological activities, thirteen phytochemicals exerted anthelmintic activity, forty seven had antibacterial activity, thirty eight had antifungal activity, forty two had antioxidant activity and twenty four had antiviral activity (Figure 5.2).

Table 6. 3: Selected plant species, major phytochemical classes, related phytochemicals and their biological activities

	Plant speceies																Biological activity				
Compound class & related phytochemicals	*Allium c.*	*Aloe vb.*	*Ananas c.*	*Biden p.*	*Carica p.*	*Crinum m.*	*Gunnera p.*	*Nicotiana t.*	*Ricinus t.*	*Sarcostema V.*	*Trema o.*	*Urtica d.*	*Vernonia a.*	*Zanthozylum c.*	*Zingiber o.*	*Zizyphus m.*	Athelmintic	Antibacteria	Antifungal	Antioxidant	Antiviral
Aldehydes																					
Furfural	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+
Hexanal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-
5-Hydroxymethylfurfural	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-
Alcohol																					
2-Furanmethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+
2,3-butanediol	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
2,7-Octadiene-1,6-diol	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phytol	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+
1,2,3-Propanetriol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
2,6-dimethyl	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Glycerin	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	+
Amine/Amide																					
Acetamide	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
2-Propenamide	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Methylamine, N,N- dimethyl	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+
Sulphur compounds																					
Dimethyl Sulfoxide	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	+	+
3,4-Dimethylthiophene	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
1-propenyl methyl disulphide	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-

	Plant speceies																Biological activity				
Compound class & related phytochemicals	*Allium c.*	*Aloe vb.*	*Ananas c.*	*Biden p.*	*Carica p.*	*Crinum m.*	*Gunnera p.*	*Nicotiana t.*	*Ricinus t.*	*Sarcostema V.*	*Trema o.*	*Urtica d.*	*Vernonia a.*	*Zanthozylum c.*	*Zingiber o.*	*Zizyphus m.*	Athelmintic	Antibacteria	Antifungal	Antioxidant	Antiviral
Dimethyl trisulphide	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
N-compound																					
Nicotine (Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-)	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	+	+	+	-
2,6-dimethylpyrazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-
2-Ethyl-5-methylpyrazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
8 Azabicyclo[3.2.1]octane-3-carbonitrile,8 methyl-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-
2,5-Dimethylpyrazine	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+
2,5-dihydro-1H-Pyrrole	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Benzyl nitrile	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Ketones																					
1-hydroxy-2-propanone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	+	+	+	+	+	-	-	-	+	-	+	-	-	+	-	-	-	+	-	+	-
1-(1H-pyrrol-2-yl)-ethanone	+	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	+	+	+
2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one	+	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+	-
4-Cyclopentene-1,3-dione	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	+
4-Methyl-5H-furan-2-one	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+
2(3H)-Furanone	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Dihydro-4-hydroxy-2-(3H)-furanone	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-
Aliphatic acids																					
Acetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+

	Plant speceies																Biological activity				
Compound class & related phytochemicals	*Allium c.*	*Aloe vb.*	*Ananas c.*	*Biden p.*	*Carica p.*	*Crinum m.*	*Gumnera p.*	*Nicotiana t.*	*Ricinus t.*	*Sarcostema V.*	*Trema o.*	*Urtica d.*	*Vernonia a.*	*Zanthozylum c.*	*Zingiber o.*	*Zizyphus m.*	Athelmintic	Antibacteria	Antifungal	Antioxidant	Antiviral
Propanoic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+
Sorbic acid	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-
4-hydroxy-butanoic acid	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+
Benzenoids																					
Benzaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+
Benzene acetaldehyde	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
Phenylethyl alcohol	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	+	-	+	+
2-methoxy-4-vinylphenol	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	-	+	-	+	-
Vanillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-
2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-
Lactones																					
Butyrolactone	-	-	+	+	+	-	+	+	-	-	+	+	+	-	+	-	+	+	+	+	-
2-pyrrolidone	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+	-
2-Hydroxy-gamma-butyrolactone	+	-	+	-	-	-	-	-	+	+	-	-	-	+	-	+	-	+	+	+	-
Alkaloids																					
Z-1-(1-butenyl) aziridine	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-
Glutarimide	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+
Furan																					
Benzofuran, 2,3-dihydro-	+	-	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
Esther																					
1-Butanol, 3-methyl-, acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-

-= not available; += available or inherent trait

Table 6. 4: References to various biological activities exerted by identified biomolecules reported in table 6.2; 1= anthelmintic activity; 2= antibacterial activity; 3= antifungal activity; 4= antioxidant activity and 5= antiviral activity, NA= not available.

Biological activities and related references					
Compound class & related biochemicals	**Anthelmintic activity**	**Antibacterial activity**	**Antifungal activity**	**Antioxidant activity**	**Antiviral activity**
Aldehydes					
Fufural	Tharachand *et al*., 2015; Ortu, 2015	Tharachand et al., 2015.	Jung et al., 2007	Vignoli et al., 2014	Kalt and Cock, 2014
Hexanal	NA	Trombetta *et al*., 2000	Gardini *et al*., 1997.	NA	NA
5-Hydroxymethylfurfural	Ntalli *et al*., 2010	Diris *et al*., 2017	NA	Ghaidaa *et al*., 2016	NA
2-Furancarboxaldehyde, 5-methyl	NA	Ghaidaa *et al*., 2016	Ghaidaa *et al*., 2016.	Kim *et al*., 2014.	NA
Alcohol					
2-Furanmethanol	NA	Kalt and Cock, 2014.	NA	Wei *et al*., 2001.	Udaweediye and Ginigandarage, 2016.
2,3-butanediol	NA	NA	NA	NA	NA
2,7-Octadiene-1,6-diol	NA	NA	NA	NA	NA
Phytol	de Moraes *et al*., 2014.	Ghaneian *et al*., 2015.	Omoruyi *et al*., 2014.	Pasricha *et al*., 2014.	Santoyo et al., 2010.
1,2,3-Propanetriol	NA	NA	NA	NA	NA
2,6-dimethyl	NA	Kanazawa *et al*., 1994.	Hameed *et al*., 2016.	Sanmartín-Suárez *et al*., 2011.	Sendl *et al*., 1996.
Glycerin	NA	Nalawade *et al*., 2015.	Lind *et al*., 2010.	Lind *et al*., 2010.	Zandi *et al*., 2009.
Amine & amides					
Acetamide	Sawant and Kawade, 2011.	Kumar and Mishra, 2015.	Ugwu and Okoro, 2014.	Kadhum *et al*., 2011.	Stamatiou *et al*., 2003.
2-Propenamide	Meenakshisundaram *et al*., 2017.	Kaur and Wakode, 2016.	Mares *et al*., 1994.	Devi *et al*., 2016.	Katen *et al*., 2010.
Methylamine, N, N-dimethyl	Patani and LaVoie, 1996.	Abd El-Wahab., 2012.	Rami *et al*., 2013.	Al-Huqail *et al*., 2013.	Ivashchenko *et al*., 2014.
Sulphur compounds					
Dimethyl sulpuroxide	NA	Hassan, 2014.	Hazen, 2013.	Sanmartín-Suárez *et al*., 2011.	Aguilar, 2002.
3,4-Dimethylthiophene	NA	Mohammad Asif Iqbal *et al*., 2012.	Fokialakis *et al*., 2006.	Madhavi and Sree Ramya., 2017.	NA
1-propenyl methyl disulphide	NA	Mnayer *et al*., 2014.	Kasaian *et al*., 2016.	Liguori *et al*., 2017.	NA
Dimethyl trisulphide	NA	Mnayer *et al*., 2014.	Fernando *et al*., 2005.	Morales-López *et al*., 2017.	NA
N-compounds					

Biological activities and related references

Compound class & related biochemicals	Anthelmintic activity	Antibacterial activity	Antifungal activity	Antioxidant activity	Antiviral activity
Nicotine (pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (s)-)	Behnke *et al*., 2008.	Pavia *et al*., 2000.	Pavia *et al*., 2000.	Newman *et al*., 2002.	NA
2,6-dimethylpyrazine	NA	Miyazawa and Usami, 2014.	Miyazawa and Usami, 2014.	NA	NA
2-Ethyl-5-methylpyrazine	Shrestha *et al*., 2016.	Saikachi and Matsuo, 1966.	NA	NA	NA
8 Azabicyclo[3.2.1]octane-3-carbonitrile,8 methyl-	NA	Pala *et al*., 2016.	Marson, 2011.	Sishu *et al*., 2005.	NA
2,5-Dimethylpyrazine	NA	Abu-Youssef *et al*., 2006.	Wang and Tao, 2009.	Peng *et al*., 2015.	Alcaide *et al*., 2014.
2,5-dihydro-1H-pyrrole	NA	Mahboobi *et al*., 2008.	Dabur *et al*., 2005,	Volkovoy *et al*., 2017.	Bhanushali and Zhao, 2012
Benzyl nitrile	Surikova *et al*., 2017.	Aires *et al*., 2009.	Fleming *et al*., 2010.	Asif, 2014	Singh and Lakshman, 2009.
Ketones					
1-hydroxy-2-propanone	NA	Yang *et al*., 2016.	NA	NA	NA
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	NA	Schwab, 2013.	NA	Koga *et al*., 1998.	NA
1-(1H-pyrrol-2-yl)-ethanone	NA	Shehab *et al*., 2016.	Shehab *et al*., 2016.	Kim *et al*., 2014.	Avan, 2011.
2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4-one	NA	Ahmed Subeh., 2011	NA	echovska´ *et al*., 2011.	NA
4-Cyclopentene-1,3-dione	NA	Revathi *et al*., 2014.	Babu *et al*., 2006.	NA	Lapshina *et al*., 2010.
4-Methyl-5H-furan-2-one	NA	Mrityunjay Banerjee *et al*., 2012.	Pour *et al*., 2000.	Montazeri *et al*., 2013.	Mrityunjay Banerjee *et al*., 2012.
2(3H)-Furanone	Asif Husain *et al*., 2015.	Alam *et al*., 2010.	Husain *et al*., 2009.	Yanagimoto *et al*., 2002.	Husain *et al*., 2009.
Dihydro-4-hydroxy-2-(3H)-furanone	NA	Ndagijimana *et al*., 2006.	Paulitz *et al*., 2000.	Yu *et al*., 2012.	Flefel *et al*., 2012
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-	NA	Gurnani *et al*., 2016.	Just, 2007.	Gurnani *et al*., 2016.	NA
Aliphatic acids					
Acetic acid	NA	Ryssel *et al*., 2009.	Cabo *et al*., 2002.	NA	Shirey *et al*., 2011
Propanoic acid	NA	Thompson and Hinton, 1997.	Fernandez *et al*., 2017.	Drachevaa *et al*., 2009.	Stahla-Beek *et al*., 2012.
Sorbic acid	NA	Eklund, 1983.	Razavi-Rohani and Griffiths, 1999.	Winkler *et al*., 2006.	NA
4-hydroxy-butanoic acid	NA	Gołezbiowski *et al*., 2013.	Hassan *et al*., 2015.	Politeo *et al*., 2007.	Rajendiran *et al*., 2017.
Benzenoids					
Benzaldehyde	NA	Ullah *et al*., 2015.	Kim *et al*., 2011.	Ullah *et al*., 2015.	Tolstorozhev *et al*., 2012.
Benzene acetaldehyde	NA	NA	NA	Yang *et al*., 2015.	NA

Biological activities and related references

Compound class & related biochemicals	Anthelmintic activity	Antibacterial activity	Antifungal activity	Antioxidant activity	Antiviral activity
Phenylethyl alcohol	NA	Corre et al., 1990.	NA	Wang *et al*., 2015.	Kishimoto *et al*., 2005.
2-methoxy-4-vinylphenol	NA	Feng *et al*., 2010.	NA	Fukai *et al*., 2009.	NA
Vanillin	NA	Ngarmsak *et al*., 2006.	Boonchird and Flegel, 1982.	Tai *et al*., 2011.	NA
2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-	NA	Ahmad *et al*., 2017.	Svetaz *et al*., 2014.	Ahmad *et al*., 2016.	NA
Lactones					
Butyrolactone	Maaz *et al*., 2015.	Cazar *et al*., 2005.	Wu *et al*., 2016.	Salat *et al*., 2012.	NA
2-pyrrolidone	NA	Phaechamud *et al*., 2012.	Al-Amiery *et al*., 2012.	Thangam *et al*., 2013.	NA
2-Hydroxy-gamma-butyrolactone	NA	Verma and Bansal, 2015.	Rana *et al*., 2016.	Min *et al*., 2004.	NA
Alkaloids					
Z-1-(1-butenyl) aziridine	NA	Al-Majeed and Al-Ghizawi, 2016.	NA	Owokotomo *et al*., 2012.	NA
Glutarimide	Lee *et al*., 2003.	Cheng *et al*., 2006.	Kim *et al*., 1999.	Dhivare, 2016.	Saito *et al*., 1976.
Furan					
Benzofuran, 2,3-dihydro-	Swargiary *et al*., 2016.	Mohamed *et al*., 2013.	Abu-Hashem *et al*., 2014.	Rangaswamy *et al*., 2017.	Yar *et al*., 2009.
Esther					
1-Butanol, 3-methyl-, acetate	NA	Strobel *et al*., 2001.	Sánchez-Oritz *et al*., 2016.	Palani *et al*., 2011.	NA

NA= Not available; += existing biological activity; -= absence of biological activity

Nine biomolecules including furfural, phytol, acetamide, 2-propenamide, methylamine, N, N-dimethyl, benzyle nitrile, 2(3H)-furanone, glutarimide and benzofuran, 2,3-dihydro were found from previous works to exert all highlighted bioactivities. In all, thirty nine biomolecules, were identified to exert three or more of the highlighted bioactivities. Most of the these and others in the list, have been identified as intermediate molecules in the syntheses of more active and potent compounds of anthelmintic, antibacterial, antifungal, antioxidant and antiviral importance. In all, fifty one major biomolecules were identified, one of which has no name. Every plant species had biochemical compounds that exerted all five biological activities.

Thirteen phytochemicals with anthelmintic activity are given in Figure 6.3, indicating that two plants had one phytochemical (*Ricinus communis* and *Zanthozylum capense*), five plant had two phytochemicals each (*Aloe vanbalenii*, *Crinum macowanii*, *Nicotiana tabacum, Vernonia amygdalina* and *Zingiber officinale*), four plants had three phytochemicals each (*Biden pilosa, Gunnera perpensa Sarcostema viminale, Urtica dioica* and *Zizyphus mucronata*) and four plants had four phytochemicals each (*Allium cepa*, *Ananas comosus*, *Carica papaya* and *Trema orientalis*). Mean concentration of phytochemicals can be allotted to four tiers, the first of which ranged from 0.02 ± 0.20 Ng to 0.13 ± 1.38 Ng. These included methylamine, N, N-dimethyl (0.02 ± 0.20 Ng), 2(3H) furanone (0.05 ± 0.67 Ng), 2-ethyl-5-methylpyrazine (0.07 ± 0.53 Ng) and glutarimide (0.13 ± 1.38 Ng). The second tier comprised of phytol (0.15 ± 11.28 Ng), acetamide (0.21 ± 1.05 Ng), benzyl nitrile (0.22 ± 2.48 Ng), and 2-propenamide (0.32 ± 2.07 Ng). Tier number three included butyrolactone (0.62 ± 2.41 Ng), benzofuran, 2,3-dihydro (0.87 ± 4.11 Ng) and furfural (0.96 ± 10.37 Ng). The fourth and last tier with the highest concentration comprised of pyridine, 3-(1-methyl-2-pyrrolidinyl)- (3.38 ± 35.87 Ng) and 5-Hydroxymethylfurfural (5.64 ± 63.34 Ng).

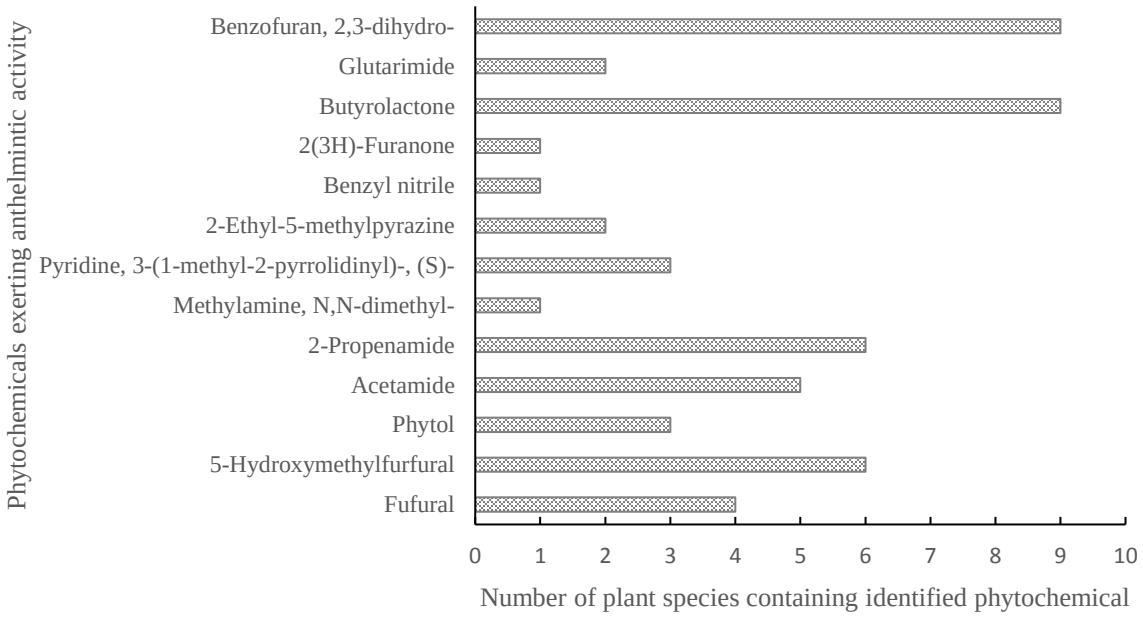
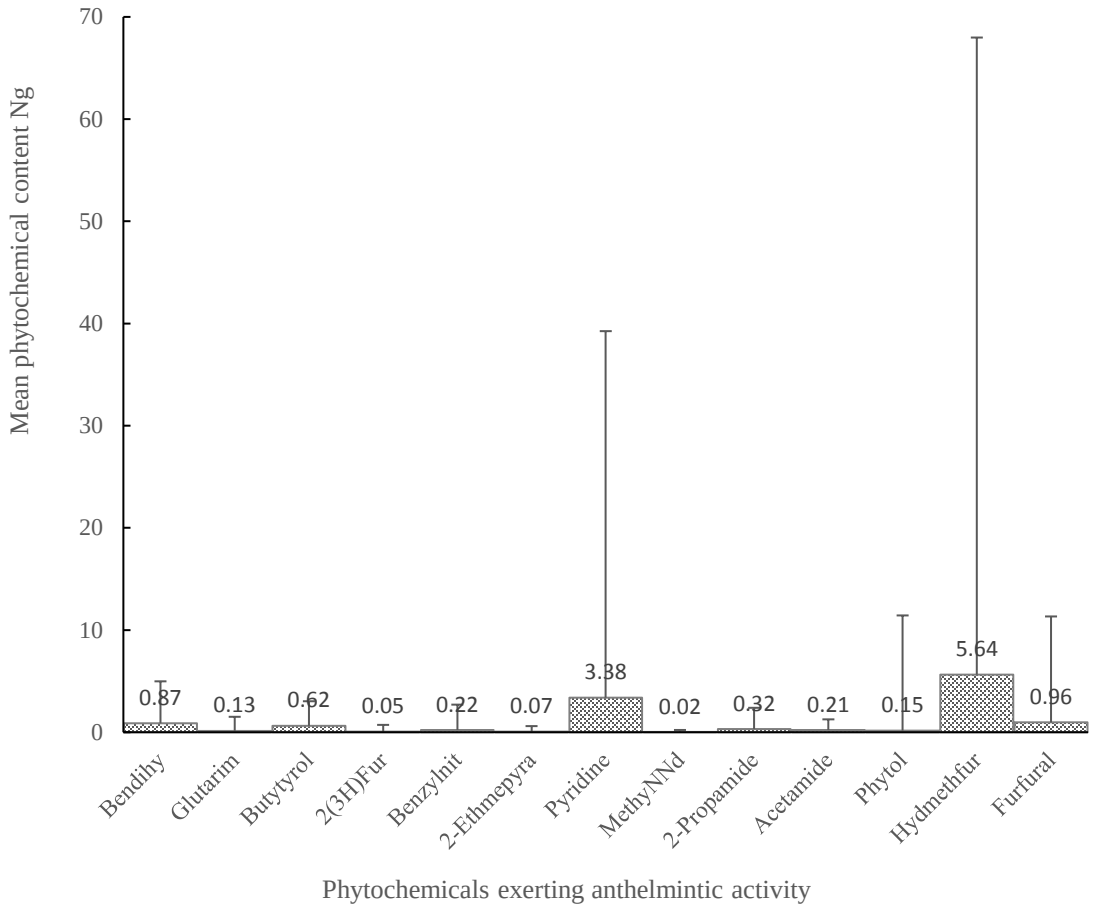


Figure 6. 3: Number of selected plant species in which particular anthelmintic phytochemicals were observed.



Bendihy= Benzofuran, 2,3-dihydro-; Glutarim= Glutarimide; Butyrol= Butyrolactone; 2(3H) Fur= 2(3H) Furanone; Benzylnit= Benzyl nitril; 2-Ethmepyra= 2-Ethyl-5-methylpyrazine; Pyridine= pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (s)-); MethylNNd= Methylamine, N, N-dimethyl; Hydmethfur= 5-Hydroxymethylfurfural; Ng= nanogram

Figure 6. 4: Mean content of phytochemicals exerting anthelmintic activity in selected plant species

6.4 Discussion

Aldehydes, amides, sulphur compounds, most nitrogen compounds, ketones, lactones, alkaloids and furans are biomolecular classes found to carry a number of anthelmintic candidates and others

exerting various important activities that promote health. *Allium cepa* and *Trema orientalis* were the richest plant species in relation to anthelmintic phytochemicals with five each. This was closely followed by *Ananas comosus*, *Biden pilosa*, *Carica papaya* and *Gunnera perpensa.* Three plant species including *Vernonia amygdalina*, *Zingiber officinale* and *Zizyphus mucronata* carried three phytochemicals. The greater bulk comprised of *Aloe van balenii*, *Crinum macowanii*, *Ricinus communis*, *Sarcostema viminale*, *Urtica dioica* and *Zanthozylum capense* contained two. *Nicotiana tabacum* was the lone plant species with one anthelmintic phytochemical (plate 5.1). These bioactive molecular dispositions of the different plant species auger well for both single and combination phytoanthelmintic therapy (Che *et al*., 2013), as it broadens the scope and spectre of anthelmintic and other related therapeutic activities (Zhou *et al*., 2016). Therapeutic efficacy of phytotherapy is generally dependent on combined action/interaction of mixtures of bioactive phytochemicals, as most relevant plant species contain two or more candidates (Efferth and Koch, 2011). Modern veterinary practice has adopted combination anthelmintic therapy (Bartram *et al*., 2012) from bioactive disposition that prevails in plant species (plate 5.1). Additionally, numerous anthelmintic bioactive candidates make it very difficult for nematodes to genetically select for resistance and other related parasites to act correspondingly . Single or multi-herb anthelmintic phytotherapy therefore present a sterling example of multicomponent treatment, given a number of bioactive phytochemicals in single plant species and more so in two or more in combinations.

Apart from anthelmintic activity, most of the compounds identified have been found to exert other activities that are also critical to animal health, welfare and productivity. Cognizant of the nature of plants in relation to their rich biochemical content, these activities cannot be separated from that in focus. They included antibacterial, antifungal, antioxidant and antiviral activities. Fundamentally, the cause of most diseases in nature is multifactorial than single (Efferth and Koch, 2011), rendering plant bioactive disposition our first line of choice. A breakdown of various compound classes identified, the plant species in which they occured and their relevant biological activities will be examined below.

Aldehyde containing compounds, most of which were identified in *Allium cepa*., *Crinum macowanii*, *Gunnera perpensa*, *Ricinus communis*, *Sarcostema viminale* and *Zingiber officinale* in the current study have been found to exercise anthelmintic activity (Davyt *et al*., 2001; Ntalli *et*

al., 2010). This is in accordance with the initial selection of these species as adopted and used by various communities in ethnoveterinary medicine as phyto-anthelmintic therapeutics. Additionally, plants containing this class of compounds were found to exert antibacterial activity (Dorman and Deans, 2000), antifungal activity (kurita *et al*., 1981; Kim *et al*., 1999; Dorman and Deans, 2000) and antiviral activity (Ji, Xing-yue *et al*., 2010). Suggestive of the diverse role of their constituent bioactive molecules or some particular ones exercising these crucial role of improving and sustaining animal health among other functions. Importantly, a good number have been screened and found to exercise antioxidant or free radical scavenging activity (Gulcin *et al*., 2004; Wang *et al*., 2008), by either serving as reducing agents in donating hydrogen or oxygen donors which oxidize. Both processes stabilize marauding debilitating free radicals that risk disrupting essential biochemical processes that promote efficient metabolic function by initiating counter reactions. This role is critical to maintaining sound integral animal function by ensuring that its essential biochemicals are directed to drive those metabolic processes which ensure effective organ system function.

Amides are an important biochemical class of compounds identified in some of the selected plant species possessing anthelmintic activity. These included *Ananas comosus, Bidens pilosa, Carica papaya,* and *Crinum macowanii,* all of which contained sufficiently high concentrations of amide related compound, acetamide. *Ananas comosus* and *Carica papaya,* are some of the plant species noted for their proteolytic anthelmintic activity (Stepek *et al*., 2005), which were not measured. Acetamide has been closely linked to a number of essential therapeutic processes, some of which included antibiotic (Göker *et al*., 1998; Özden *et al.*,2005; Capleton *et al*., 2006), antifungal (Göker *et al*., 1998) and anthelmintic (Özden *et al.*,2005) activities. Acetamides are therefore very important to the current study and contribute to protection of livestock against helminths. Additionally, they promote other biological processes that enhance health and productivity.

Sulphur containing compounds were also identified as key role players in livestock helminth control activity (Watt and Breyer-Brandwijk, 1962; Lyantagaye, 2011) of some of the selected plant species. *Allium cepa*, *Carica papaya*, *Crinum macowanii*, *Gunnera perpensa*, *Nicotiana tabacum*, *Ricinus communis, Trema orientalis*, *Urtica dioica* and *Vernonia amygdalina* were those that contained sulphur compounds. Among them, *Allium cepa* was the most endowed, with three different bioactive molecules (3,4-dimethylthiophene and 1-propenyl methyl disulphide

exclusively in *A. cepa*) and dimethyl sulfoxide. Other important biological roles exerted by sulphur containing compounds include antibacterial (Ross, 2003; El-Wakil *et al*., 2015), antifungal (Ross, 2003; Pyun and Shin, 2006) and antioxidant (Lyantagaye, 2011; El-Wakil *et al*., 2015) activities.

Nitrogen containing compounds were additionally important biochemical constituents of some of the selected plant species. *Ananas comosus, Carica papaya*, *Bidens pilosa*, *Nicotiana tabacum*, *Sarcostema viminale*, *Trema orientalis*, *Ricinus communis*, *Urtica dioica*, *Zingiber officinale* and *Zizyphus mucronata* all fell in this group. Different nitrogen compounds occurred in various plant species: 2,6-diemthylpyrazine occurred in *Z. officinale*; 2,5-dimethylpyrazine in *A. comosus*, *T. orientalis* and *U. dioica*; 2,5-dihydro-1H-Pyrrole in *B. pilosa and C. papaya*; *p*yridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (nicotine) in *N. tabacum, R. communis* and *S. viminale,* and ultimately 8-azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl- in *Z. mucronata*. Besides being closely associated with anthelmintic activity (Bondock *et al*., 2013), they also have antibacterial (Silici and Kutluca, 2005; Abdalla *et al*., 2009; Singh and Tabane, 2015), antifungal (Dabur *et al*., 2005, Singh and Tabane, 2015) and antioxidant properties (Singh and Tabane, 2015). Among some of these nitrogen compounds that exerted anthelmintic activity, was nicotine (Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-), which is commonly attributed to *Nicotiana tabacum* (Hammond *et al*., 1997), and was also identified in *Sarcostema viminale*.

Ketones were another common group of organic compounds that occurred in all selected species, though represented by a wide array of different compounds including 1- hydroxy-2-propanone; 4-cyclopentene-1,3-dione; 4-Methyl-5H-furan-2-one; and 2,5-Dimethyl-4-hydroxy-3(2H)-furanone. These ketones have been closely linked to anthelmintic properties exerted by some of the selected plant species (Kumar and Joshi, 2008; Ahmad *et al*., 2011; Ortu *et al*., 2016), in addition to antibacterial (Inayama *et al*., 1976), antifungal (Inayama *et al*., 1976) and antioxidant activities (G. Su *et al*., 2011). A wide variety of organic compounds in different plant species in which ketones occur, potentially creates a huge possibility of different interactions in combination therapy (Quijada *et al*., 2015), and much more leverage of enhanced activities.

Benzenoids were among a wide range of biochemicals identified to exert anthelmintic activity (Patel *et al*., 2010; Olounladé *et al*., 2012). Though bezenoids do not have sufficient evidence of anthelmintic activity, they were more or less intermediate molecules in the synthetic process of

more potent compounds (Salahuddin *et al*., 2012). Additionally, they exhibited antibacterial (Al-Snafi, 2016; Ahmadi *et al*., 2015), antifungal (Ahmadi *et al*., 2015) and antioxidant properties (Al-Snafi, 2016). In accord with multifactorial cause of most diseases (Efferth and Koch, 2011), these bioactive phytochemicals play an important role in healing. Lactones also exerted anthelmintic activity (Foster *et al*., 2011; Kumar and Tyagi, 2013).

Lactone related bioactive molecules including butyrolactone and 2-pyrrolidone were identified in most selected species and the former exerted anthelmintic activity in accordance with our primary focus (Wang *et al*., 2010). This bioactivity adds to the pool of others in the same plant species exerting anthelmintic activity, in addition to their antibacterial (Barbour *et al*., 2004; Du *et al*., 2017), antioxidant (GüIçin, 2011) and antifungal (Xinhua *et al*., 2014) properties.

Alkaloids are another biochemical class of great importance in nematode parasite control. Various studies have closely associated alkaloids to anthelmintic traits and their use in livestock nematode control (Negi *et al*., 2011; Refaat *et al*., 2012). Two molecules, Z-1-(1-butenyl) aziridine and glutarimide were identified. The first occurred in *Trema orientalis*, and the later in both *Trema orientalis* and *Urtica dioica.* The occurrence of Z-1-(1-butenyl) aziridine and glutarimide in *Trema orientalis* is in accord with the observation that different types of molecules from the same biochemical class occur in the same plant species (Makkar *et al*., 2007). Alkaloids also possess antibacterial (Grosvenor *et al*., 1995; Daciana and Băra, 2007), antifungal (Singh *et al*., 1990; Singh *et al.*, 2000), antioxidant (Harborne, 1973; Velioglu *et al*., 1998; Suresh Kumar *et al*., 2008), and antifungal traits, according livestock better protection from a wide range of pathogens and oxidation of essential biomolecules that disequilibrate various metabolic processes. Additionally, it is worthy to highlight the initial misconception of tagging or ascribing single bioactive molecular class to certain plant species. Biomolecular profiling renders attribution of a single biochemical class to any or most plant species conflationary and untrue in nature (figure 5.2). This is exemplified in two plant species identified to have significant alkaloids: *Trema orientalis* and *Urtica dioica. Trema orientalis* has three different bioactive molecular classes, and *Urtica dioica* three; all of them coincidentally of a similar molecular class. It can be a tenable and accepted tribute, if reference were made based on assayed profile of biochemicals that exert the activity in perspective and its concentration status, such that the bioactive molecular class in highest concentration is taken for the species biochemical class.

Alcohols, phytols and aliphatic acids were not directly linked to bioactive anthelmintic activity, but the outcome from combination can be diametrically different as a result of their different interactions with other chemical analogues and bioactive molecules. Combinations of bioactive molecules, just like that of nutrients in diets, has the potential of bringing about effects which are not related to those of the component constituents (Diplock *et al*., 1998); some of which may be anthelmintic. Acetic acid for example induces writhing by earthworms (Nirmale *et al.*, 2007), and will potentially aid the activity of a more potent bioactive anthelmintic phytochemical in livestock nematode control. Additionally, structural biochemical differences of bioactive molecules of the same chemical class have brought about differences in their activity and efficacy (Quijada *et al*., 2015). All of these molecular classes have been closely linked to antibacterial (Thorp *et al*., 1998; Togashi *et al*., 2007), antifungal (kurita *et al*., 1981; Makovitzki *et al*., 2006), antioxidant (Rice-Evans *et al*., 1997; Budak and Guzel-Seydim, 2010) and antiviral (De Clercq and Holy, 1979) activities (figure 5.2). Therefore, interactions of these biomolecules resulting to anthelmintic activity is not remote. There is thus a likelihood that these molecules could well be part of a larger and more elaborate set of biochemicals exerting anthelmintic activity. Contrary to our hypothesis, some biochemical compounds exerted anthelmintic activity individually, but there was much more potent consortia activity arising from combined activity.

Furans were uniquely represented by benzofuran, 2,3-dihydro-, that was identified in *Allium cepa, Ananas comosus*, *Bidens pilosa*, *Carica papaya*, *Vernonia amygdalina*, *Zanthozylum capense*, *Zingiber officinale* and *Zizyphus mucronata.* This bioactive molecule has been linked to anthelmintic (Kumaraswamy *et al*., 2008), antibacterial (Kamal *et al*., 2011), antifungal (Kamal *et al*., 2011) antioxidant (Hosseiny Davarani *et al*., 2006) and antiviral (Ugarkar *et al*., 1984) activities. This wide spectrum of activities makes furans important bioactive candidates to control and treat livestock of parasites and related diseases.

6.5 Conclusion

Phytochemical analyses and bioactivity profiling in the current study have shown that, plant species more often have multiple candidates exerting anthelmintic and other related activities. Bioactivity therefore emanates from individual phytochemicals and builds up to more potent

activity from their interaction. How these multiple bioactive phytochemicals relate and interact with others will be examined in the next chapter.

Chapter 7

Using correlations and multiple regression relationships of phytochemicals with anthelmintic efficacy to confirm activity of biochemicals

Abstract

This study analyzed and identified phytochemical composition of plant species including *Allium cepa, Aloe van balenii, Ananas comosus, Bidens pilosa, Carica papaya, Crinum marcowanii, Gunnera perpensa, Nicotiana tabacum, Ricinus communis, Sarcostema viminale, Urtica dioica, Vernonia amygdalina, Zanthozylum capense, Zingiber officinale* and *Zizyphus mucronata,* in search of their relationship with observed anthelmintic efficacy, simple and Webb's synergies. Phyto-active compounds were analyzed using GC/MS. Four grams (4 g) dry matter (DM) of each plant species material was extracted in 70 % ethanol, from which 2 µl was injected into a chromatoprobe trap and analysed for biochemical composition. Compound identification carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, where available, and also comparisons between calculated Kovats retention indices and those published in the literature. Pearson correlation coefficient was run to explore association of phytochemical candidates with observed efficacy, simple and Webb's synergies. Multiple regressions were also run to explore influence of various phytochemicals on efficacy, simple and Webb's synergies, by conducting 10 searches to identify any of such influence(s). Some phytochemicals had positive (benzofuran, 2,3-dihydro; pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-; 2 propenamide; phytol; 5-hydroxymethylfurfural; furfural) and others negative influences. Furfural and 5-hydroxymethylfural that were selected to exerted positive influence, did so on both observed efficacy and simple synergy. Both correlation matrix and multiple regression relationships pointed to more consortia of phytochemical action. It is most likely that many other phytochemical molecules must have been involved in anthelmintic activities observed from plant-plant combinations, but might not have been picked up within slated time of the analysis.

7.1 Introduction

Plant species crude extract contain a wide range of phytochemicals, some of which may influence particular biological activities. Combination phytotherapy will pool a greater variety of phytochemicals relative to single plant species. Biological activities exerted by plant species combinations are expected to result from their combined or collective biochemical interaction that are relevant to that trait, given that in many of these plants more than one phytochemical exert similar activity. Additionally, phytochemical interaction in combinations has the likelihood of bringing about bioactivities that are not associated with some of these candidates individually (Diplock *et al*., 1998). In combinations, there are various phytochemical interactions, some of which may be positive, negative, neutral or antagonistic (kyaw *et al*., 2012) to each other. Crude plant extract analyses using GC/MS have identified a wide range of phytochemicals in different concentrations (Chapter 5), and a number of them influence particular biological activities. Some of these phytocemicals were found to exert more than one biological activity as exemplified by glycerin that exerted both antifungal and antioxidant activities (Lind *et al*., 2010), and 2-furancarboxaldehyde, 5-methyl that exerted both antibacterial and antifungal activities (Ghaidaa *et al*., 2016). Additionally, increased concentration of plant species extract resulted to greater biological activity (Menghini *et al*., 2011). Combination of *Trema orientalis* and *Urtica dioica*, both of which contain anthelmintic phytochemical glutarimide (Lee *et al*., 2003), will result to increased concentration. Implicitly, plant-plant combination with similar active phytochemicals will result to greater activity as its concentration will increase in the process.

Plant species exerting some healing or biological activity have been closely associated with several biochemical principles, some of which make very little or no contribution to metabolic function and are tagged plant secondary metabolites (PSM) (Bennett and Wallsgrove, 1994; Zhao *et al*., 2005). They primarily serve in plant defense, including protection from herbivory, pest and other disease causing organisms (Bennett and Wallsgrove, 1994; Athanasiadou *et al*., 2006; Solaiman and Owens, 2010). Naturally, each plant may have several classes of plant secondary metabolites and related principles (Ghisalberti, 2002; Olivier, 2012; Refaat *et al*., 2012; Rampadarath *et al*., 2014), exercising the same function, but having different biochemical modes of action. Some sites of gastrointestinal tract may favour bioactive activity of some PSM than others.

Generally, some PSM including alkaloids (Refaat *et al*., 2012), condensed tannins (Min and Hart, 2003; Ademola *et al*., 2005; Alonso-Diaz *et al*., 2011), flavonoids (Azando *et al*., 2011; Iqbal *et al*., 2011; Singh *et al*., 2011), proteases and nitrogen compounds (Luoga *et al*., 2012; Domingues *et al*., 2013) among others, have been closely associated with livestock nematode control, though a finer resolution has not been done in Africa. It is hypothesized that phytochemicals and related plant macromolecules exerting anthelmintic activity, will associate with observed efficacy, simple and Webb's synergies. Additionally, phytochemicals exerting anthelmintic activity will interact with other phytochemicals to affect observed efficacy, simple and Webb's synergies.

The main objective of this section is to explore how different biochemicals relate to affect plant species anthelmintic efficacy, and potentially similar activities in combinations and new candidates that may aid the process.

7.2 Materials and methods

7.2.1 Plant species and extraction of vegetative material

Allium cepa (Vieira *et al*., 1999; Marwat *et al*., 2011; Bidkar *et al*., 2012), *Aloe van balenii*, *Trema orientalis* (Watt and Breyer-Brandwijk, 1962), *Ananas comosus* (Stepek *et al*., 2005), *Bidens pilosa* (Graham *et al*., 1980; Hoffman and Hoelzl, 1988), *Carrica papaya* (Stepek *et al*., 2005; Adongo, 2013), *Crinum macowanii* (Refaat *et al*., 2012), *Gunnera perpensa* (Semelane *et al*., 2010), *Nicotiana tabacum* (British Veterinary Codex, 1953; Stuart *et al*., 2012), *Ricinus communis* (Rampadarath *et al*., 2014; Wafa *et al*., 2014), *Sarcostema viminale* (Grime *et al*., 2008), *Urtica dioica* (Haman, 2007), *Vernonia amygdalina* (Yeap *et al*., 2010), *Zanthozylum capense* (Negi *et al*., 2011) and *Zingiber officinale* (Haman, 2007; Singh *et al*., 2011), and *Zizyphus mucronata* (Van Wyk and Wink, 2004; Olivier, 2012) were identified based on previous knowledge of their ethno veterinary use in livestock nematode control.

Four grams dry matter (4 g DM) of each plant species vegetative material was weighed into labelled thimbles and closed with a ball of cotton wool wrapped in a piece of cheese cloth. Thimbles containing plant material were mounted into distillation units and extracted using 70 % ethanol as solvent. Crude extracts were refluxed into extraction bottles of volume 100 ml. The extraction process was completed when the solvent in thimble bearing section was free of any

coloration. Extraction bottles containing crude extracts were withdrawn, allowed to cool and Volume of crude extract was made up to 100 ml. volumes of 4 ml each were poured into glass vials for GC/MS analysis.

7.2.2 Biochemical analysis of crude extracts of plant species using GC/MS

Volatile samples were analysed using a coupled Varian 3800 gas chromatography (Varian Palo Alto, California, and USA) and Varian 1200 mass spectrometer (GC-MS). The gas chromatography (GC) was equipped with an Alltech EC - WAX column of 30m x 0.25 µm internal diameter x 0.25 µm film thickness (Alltech Associates Inc., Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. From each plant species extract, 2 µl was injected into a chromatoprobe trap prepared by cutting glass tubes of similar size to that of chromatoprobe quartz microvials (length: 15 mm; inner diameter: 2 mm) and filled with 2 mg of a 50:50 mixture of Tenax TA (Alltech Associates, USA) and graphitized carbon (Carbotrap™, Supelco, USA). These tubes were closed on both ends with glass wool. Chromatoprobe traps were placed in a Varian 1079 injector by means of a Chromatoprobe fitting and thermally desorbed. The temperature of the injector was 40 °C, and was held for 2 minutes with a 20:1 split ratio and then increased to 200 °C. It was held at 200 °C min^{-1} in splitless mode for thermal desorption.

Compound detection was delayed for 6 minutes. After a 3 minutes hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C min^{-1} and retained for 12 minutes. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, as well as comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps were run in GC-MS as controls to identify background contamination. Compounds present at higher or similar percentages in controls were considered as contaminants and excluded from the analysis. For quantification of compounds, known amounts of standards of dominant compounds were injected into cartridges and thermally desorbed under identical conditions to samples. The peak area of compounds in samples were compared with those of standards and used to calculate the total amount of compound per gram of substrate. These constituent compounds helped in grouping plant species as either made of similar or different classes of compounds.

7.2.3 Evaluation of biochemical composition and statistical analysis

Phytochemical composition of individual plant species were determined. Pearson correlation was run to explore association of different phytochemicals with observed efficacy, simple synergy (differences between observed and expected efficacy), and Web's synergy (Webb, 1963). Multiple regression relationships between phytochemicals and anthelmintic related variables (observed efficacy, simple synergy and Webb's synergy) were run invoking a stepwise option. These searches were made ten times to determine the contribution of phytochemicals. However, during succeeding searches the first most influential phytochemical in the preceding step was eliminated so that new ones could be identified. However, in some cases some phytochemicals were recycled. The criterion for selection of a biochemical during every search was $p \leq 0.15$. All data were analysed using SAS (2000).

7.3 Results

A number of phytochemicals exerted considerable influence on observed efficacy, simple and Webb's synergies as indicated on correlations (Table 6.1). Some phytochemicals associated with observed efficacy and included 2,5-dimethylpyrazine (r= 0.11; P= 0.038), acetic acid (r= 0.11; P= 0.029), dimethylsulphuroxide (r= -0.10; P= 0.048), propenamide (r= 0.11; P= 0.037), 2,5-dimethyl-4-hydroxy-3(2H)- (r= 0.12; P= 0.028) and all compounds identified (r= 0.11; P= 0.035). Those that tended to influence observed efficacy positively included 1-hydroxy-2-propanone (r= 0.10; P= 0.052), 3,4-dimethylthiophene (r= 0.10; P= 0.065), 1-propenyl methyl disulsulphide (r= 0.10; P= 0.065), dimethyl trisulphide (r= 0.01; P= 0.065), furfural (r= 0.10; P= 0.060), 2,3-butanediol (r= 0.10; P= 0.065), 2-furanmethanol (r= 0.09; P= 0.078), 5-methyl-2-furanmethanol (r= 0.09; P= 0.104), 2(3H)-furanone (r= 0.10; P= 0.065), phenylethyl alcohol (r= -0.08; P= 0.149), 2-hydroxy-gama-butyrolactone (r= 0.09; P= 0.106), 2,3-dihydro-3,5-dihydroxy-6-methyl- (r= 0.10; P= 0.069), benzofuran 2,3-dihydro (r= 0.08; P= 0.109), 5-hydroxymethylfurfural (r= 0.10; P= 0.064) and dihydro-4-hydroxy-2-(3H)-furanone (r= 0.10; P= 0.071).

Some biochemicals positively correlated with simple synergy including hexanal (r= 0.17; P= 0.001), 2,6-dimethylpyrazine (r= 0.17; P= 0.001), vanillin (r= 0.17; P= 0.001) and 2-butanone, 4-(4-hydroxy-3- (r= 0.17; P= 0.05); while others did so negatively including dimethylsulphuroxide

(r= -0.17; P= 0.001), butyrolactone (r= -0.12; P= 0.029), 2,5-dihydro-1H-pyrrole (r= -0.11; P= 0.038), and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (r= -0.13; P= 0.014). Biochemicals that tended to associate with simple synergy included propanoic acid (r= 0.08; P= 0.134), 8-azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl (r= 0.08; P= 0.139) and phytol (r= 0.09; P= 0.097).

Phytochemicals that tended to associate positively with Webb's synergy included 3,4-dimethylthiophene (r= 0.08; P= 0.114), 1-propenyl methyl disulsulphide (r= 0.08; P= 0.114), dimethyl trisulphide (r= 0.08; P= 0.114), 2,5-dimethylpyrazine1- (r= 0.10; P= 0.063), 1-hydroxy-2-propanone (r= 0.09; P= 0.083), furfural (r= 0.09; P= 0.104), acetic acid (r= 0.10; P= 0.074), 2,3-butanediol (r= 0.083; P= 0.114), 2-furanmethanol (r= 0.08; P= 0.134), propenamide (r= 0.10; P= 0.064), 2,5-dimethyl-4-hydroxy-3(2H)- furanone (r= 0.10; P= 0.061), 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (r= 0.08; P= 0.124), 5-hydroxymethylfurfural (r= 0.08; P= 0.11), dihydro-4-hydroxy-2-(3H)-furanone (r= 0.08; P= 0.139) and all phytochemicals (All; r= 0.09; P= 0.081). Dimethylsulphuroxide associated negatively with Webb's synergy (r= -0.11; P= 0.031), just as it did with both observed efficacy and simple synergy.

Based on multiple regression relationship, phytochemicals that had positive influence on observed efficacy included 2,5-dimethyl-4-hydroxy-3(2H)-furanone, propenamide, acetic acid, all phytochemicals (All), furfural, 2,5-dimethylpyrazine, 2-butanone, 4-(4-hydroxy-3-, 2(3H)-furanone, dihydro-3-hydroxy-4,4-dimethyl, 1-hydroxy-2-propanone, benzofuran, 2,3-dihydro, 2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-, 5-hydroxymethylfurfural, dihydro-4-hydroxy-2-(3H)-furanone and 2,3-butanediol. Phytochemicals with a negative influences on observed efficacy included methylamine,N,N-dimethyl, dimethylsulphuroxide, 4-hydroxy-butanoic acid, 1-(1H-pyrrol-2-yl)-ethanone, Z-1-(-butenyl) aziridine, 1-(1H-pyrrol-2-yl)-ethanone, phenylethyl alcohol and 1-butanol, 3-methyl- and acetate.

Multiple regression relationship with simple synergy found that some phytochemicals exerted positive influences, and included 2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-, 2,6-dimethylpyrazine, hexanal, Vanillin, furfural, 5-hydroxymethylfurfural, phytol and propanoic acid. Phytochemicals with negative influences on simple synergy included methylamine,N,N-dimethyl, dimethylsulphuroxide, 4-hydroxy-butanoic acid, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2,5-dihydro-1H-Pyrrole, pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-, 2-

furancarboxaldehyde, 5-methyl, butyrolactone, 5-methyl-2-Furanmethanol, glutarimide, azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl and 1-butanol, 3-methyl-, acetate.

Based on multiple regression relationships with Webb's synergy, phytochemicals with positive influence included 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2,5-dimethylpyrazine, furfural, vanillin, propenamide, acetic acid, all phytochemicals (All), pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-, dihydro-4-hydroxy-2-(3H)-furanone, 5-hydroxymethylfurfural and 1-propenyl methyl disulsulphide. Phytochemicals with negative influences included 2,5-dihydro-1H-Pyrrole, 1-(1H-pyrrol-2-yl)-ethanone, Z-1-(-butenyl) aziridine, acetamide, pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-, 4-hydroxy-butanoic acid, phenylethyl alcohol and 5-methyl-2-furanmethanol.

Table 7 1: Correlation matrix of phytochemicals with observed efficacy, differences between observed and expected efficacies (simple synergy), and Webb's synergy

Biochemical compound	Observed efficacy		Simple synergy		Webb's synergy	
	r	P	r	P	r	P
Hexanal	0.03	0.58	0.17	0.001*	0.05	0.359
Methylamine,N,N-dimethyl	-.07	0.205	-.05	0.328	-.06	0.221
3,4-Dimethylthiophene	0.10	0.065+	-.00	0.971	0.08	0.114+
1-Propenyl methyl disulsulphide	0.10	0.065+	-.00	0.971	0.08	0.114+
Dimethyl trisulphide	0.10	0.065+	-.00	0.971	0.08	0.114+
2,6-dimethylpyrazine	0.03	0.584	0.17	0.001*	0.05	0.359
2,5-dimethylpyrazine	0.11	0.038*	0.03	0.550	0.10	0.063+
1-Hydroxy-2-propanone	0.10	0.052+	0.02	0.662	0.09	0.083+
Furfural	0.10	0.060+	0.00	0.933	0.09	0.104+
Acetic acid	0.11	0.029*	-.03	0.642	0.10	0.074+
Benzaldehyde	0.03	0.556	0.00	0.949	0.03	0.606
Propanoic acid	-.02	0.757	0.08	0.134+	-.00	0.963
2-Furancarboxaldehyde, 5-methyl	-0.06	0.256	-0.03	0.526	-0.06	0.290
2,3-Butanediol	0.10	0.064+	-0.002	0.964	0.083	0.114+
Sorbic acid	-0.07	0.205	-0.05	0.328	-0.06	0.221
4-Cyclopentene-1,3-dione	0.01	0.9062	0.07	0.185	0.01	0.766
Dimethylsulphuroxide	-0.10	0.048*	-0.17	0.001*	-0.11	0.031*
Benzene acetaldehyde	0.06	0.289	-0.06	0.251	0.04	0.459
Butyrolactone	-0.02	0.676	-0.12	0.029*	-0.04	0.495
4-Hydroxy-butanoic acid	-0.06	0.229	-0.07	0.190	-0.06	0.222
2-Furanmethanol	0.09	0.078+	-0.01	0.927	0.08	0.134+
Trisulphide, di-2-propenyl						
5-methyl-2-Furanmethanol	0.09	0.104+	-0.03	0.517	0.07	0.194
4-Methyl-5H-furan-2-one	0.07	0.180	0.001	0.987	0.06	0.251
2(3H)-Furanone	0.10	0.065+	-0.002	0.971	0.08	0.114+

Biochemical compound	Observed efficacy		Simple synergy		Webb's synergy	
	r	P	r	P	r	P
Acetamide	-0.01	0.892	-0.03	0.599	-0.01	0.841
2,5-dihydro-1H-Pyrrole	0.07	0.190	-0.11	0.038*	-0.08	0.155
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	-0.05	0.354	-0.13	0.014*	-0.06	0.246
Phenylethyl alcohol	-0.08	0.149+	-0.03	0.672	-0.07	0.197
Benzyl nitrile	-0.05	0.320	-0.01	0.170	-0.06	0.295
Propenamide	0.11	0.037*	0.03	0.580	0.10	0.064+
1-(1H-pyrrol-2-yl)-ethanone	0.05	0.322	-0.02	0.655	0.04	0.431
2,5-dimethyl-4-hydroxy-3(2H)-furanone	0.12	0.028*	-0.004	0.939	0.10	0.061+
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl	0.03	0.556	0.003	0.949	0.03	0.606
2-Pyrrolidinone	-0.03	0.568	-0.04	0.427	-0.03	0.546
2-Ethyl-5-methylpyrazine	-0.001	0.984	0.06	0.221	0.01	0.869
Azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl	0.03	0.579	0.08	0.139+	0.04	0.488
2-Hydroxy-gama-butyrolactone	0.09	0.106+	0.0001	0.999	0.07	0.164
2-Methoxy-4-vinylphenol	0.06	0.275	0.01	0.923	0.05	0.342
Z-1-(-butenyl) aziridine	0.07	0.180	0.001	0.987	0.06	0.251
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	0.10	0.069+	-0.01	0.882	0.08	0.124+
2,7-Octadiene-1,6-dimethyl-	0.02	0.686	0.05	0.394	0.03	0.635
Glycerin	-0.003	0.962	-0.04	0.447	-0.01	0.880
2(4)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a	0.02	0.725	0.01	0.917	0.02	0.749
Glutarimide	0.07	0.166	0.01	0.910	0.06	0.231
Benzofuran, 2,3-dihydro	0.08	0.109+	-0.01	0.838	0.07	0.181
5-Hydroxymethylfurfural	0.10	0.064+	0.004	0.938	0.08	0.110+
Vanillin	0.03	0.584	0.17	0.001*	0.05	0.359
1-Butanol, 3-methyl-, acetate	-0.05	0.374	-0.01	0.921	-0.04	0.439
Phytol	0.06	0.271	0.09	0.097+	0.06	0.236
Dihydro-4-hydroxy-2-(3H)-furanone	0.10	0.071+	-0.02	0.642	0.08	0.139+
2-Butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-	0.03	0.584	0.17	0.001*	0.05	0.359
All	0.11	0.035*	-0.02	0.669	0.09	0.081+

+= tending towards association; *= outright association

Table 7 2: Multiple regression relationships of phytochemicals in plant species with combined or observed efficacy

Search round	Selected Biochemical	Direction	r^2 (%) Max	P
	2,5-dimethyl-4-hydroxy-3(2H)-furanone	+ve		0.028
1	Propenamide	+ve	2.13	0.092
2	methylamine,N,N-dimethyl	-ve		0.134
	acetic acid	+ve		0.029
	dimethylsulphuroxide	-ve		0.101
	4-hydroxy-butanoic acid	-ve	3.52	0.081
3	All	+ve		0.035
	propenamide	+ve	2.33	0.047
4	furfural	+ve		0.039
	Propenamide	+ve		0.037
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.090
	Z-1-(-butenyl) aziridine	-ve	4.11	0.062
5	2,5-dimethylpyrazine	+ve		0.038
	furfural	+ve		0.027
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.081
	2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-	+ve	4.21	0.079
6	methylamine,N,N-dimethyl	-ve		0.050
	dimethylsulphuroxide	-ve		0.048
	4-Hydroxy-butanoic acid	-ve		0.090
6	phenylethyl alcohol	-ve		0.095
	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl	+ve	4.35	0.123
7	1-hydroxy-2-propanone	+ve		0.052
	1-(1H-pyrrol-2-yl)-ethanone	-ve	2.87	0.049
	1-butanol, 3-methyl-, acetate	-ve		0.100
8	furfural	+ve		0.060
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.087
	benzofuran, 2,3-dihydro	+ve		0.062
	2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-	+ve	3.54	0.091
9	5-hydroxymethylfurfural	+ve		0.064
	benzofuran, 2,3-dihydro	+ve		0.090
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.075
	2-Butanone, 4-(4-hydroxy-3-methoxymthoxyphenyl)-	+ve		0.102
	dihydro-4-hydroxy-2-(3H)-furanone	+ve	4.05	0.109
10	2,3-Butanediol	+ve		0.064
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.093
	dihydro-4-hydroxy-2-(3H)-furanone	+ve		0.082
	2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-	+ve	3.97	0.023

+ve= positive; -ve= negative

Table 7 3: Multiple regression relationships of phytochemicals in plant species with simple synergy of combined anthelmintic phytotherapy

Search round	Biochemical selected	Direction	r^2 (%) Max.	p
1	Methylamine,N,N-dimethyl	-ve		0.050
	Dimethylsulphuroxide	-ve		0.0003
	4-hydroxy-butanoic acid	-ve		0.025
	2,5-dimethyl-4-hydroxy-3(2H)-furanone	-ve	6.91	0.126
	2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-	+ve		0.057
2	Methylamine,N,N-dimethyl	-ve	6.29	0.050
	2,6-dimethylpyrazine	+ve		0.057
	Dimethylsulphuroxide	-ve		0.0003
	4-hydroxy-butanoic acid	-ve		0.025
	2,5-dimethyl-4-hydroxy-3(2H)-furanone	-ve	6.91	0.126
3	Hexanal	+ve		0.057
	Methylamine,N,N-dimethyl	-ve		0.050
	Dimethylsulphuroxide	-ve		0.0003
	4-hydroxy-butanoic acid	-ve		0.025
4	Methylamine,N,N-dimethyl	-ve		0.050
	Dimethylsulphuroxide	-ve		0.0003
	4-hydroxy-butanoic acid	-ve		0.025
	2,5-dimethyl-4-hydrox-3(2H)-furanone	-ve	6.91	0.126
	Vanillin	+ve		0.056
5	Methylamine,N,N-dimethyl	-ve		0.022
	Dimethylsulphuroxide	-ve		< 0.0001
	4-hydroxy-butanoic acid	-ve		0.008
	2,5-dimethyl-4-hydroxy-3(2H)-furanone	-ve	5.95	0.065
6	2,5-dihydro-1H-Pyrrole	-ve	3.22	0.018
	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	-ve		0.007
7	Methylamine,N,N-dimethyl	-ve		0.038
	Furfural	+ve		0.016
	2-furancarboxaldehyde, 5-methyl	-ve		0.002
	Butyrolactone	-ve		0.003
	4-hydroxy-butanoic acid	-ve		0.003
	5-methyl-2-Furanmethanol	-ve		0.039
	Glutarimide	-ve	6.72	0.144
	5-hydroxymethylfurfural	+ve		0.031
8	2,5-dihydro-1H-Pyrrole	-ve	1.20	0.038
9	Phytol	+ve	0.77	0.097
10	Propanoic acid	+ve		0.001
	Azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl	-ve	3.66	0.011
	1-butanol, 3-methyl-, acetate	-ve		0.001

+ve= positive; -ve= negative

Table 7 4: Multiple regression relationships of phytochemicals in plant species with Webb's synergy of combined anthelmintic phytotherapy

Search round	Biochemical selected	Direction	r^2 (%) Max	p
1	2,5-dihydro-1H-Pyrrole	-ve		0.116
	2,5-dimethyl-4-hydroxy-3(2H)-furanone	+ve	1.66	0.047
2	2,5-dimethylpyrazine	+ve		0.016
	Furfural	+ve		0.004
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.022
	Vanillin	+ve	3.92	0.038
3	Furfural	+ve		0.006
	Propenamide	+ve		0.012
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.027
	Z-1-(-butenyl) aziridine	-ve	3.48	0.068
4	Acetic acid	+ve		0.003
	Acetamide	-ve		0.025
	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	-ve		0.040
	Vanillin	+ve	3.16	0.102
5	All	+ve	0.85	0.081
6	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	+ve		0.044
	4-Hydroxy-butanoic acid	-ve	1.55	0.110
7	Furfural	+ve		0.035
	Phenylethyl alcohol	-ve		0.0031
	Vanillin	+ve		0.016
	Dihydro-4-hydroxy-2-(3H)-furanone	+ve	3.47	0.041
8	Phenylethyl alcohol	-ve		0.002
	5-hydroxymethylfurfural	+ve		0.028
	Vanillin	+ve		0.013
	Dihydro-4-hydroxy-2-(3H)-furanone	+ve	3.58	0.024
9	1-propenyl methyl disulsulphide	+ve		0.013
	5-methyl-2-furanmethanol	-ve	4.31	0.145
	1-(1H-pyrrol-2-yl)-ethanone	-ve		0.001
	Vanillin	+ve		0.011
	Dihydro-4-hydroxy-2-(3H)-furanone	+ve		0.010
10	-	-	-	-

+ve= positive; -ve= negative

7.4 Discussion

Plant species in the current study had one to four phytochemicals that have been screened for anthelmintic activity among others. Correlations of phytochemicals with efficacy, simple and Webb's synergies were explored. Secondly, how these phytochemicals, including those that do not have any known relationship with relevant bioactivity interacted to effect anthelmintic and other related highlighted biological activities, were also examined in multiple regression relationships with the same variables. Different phytochemicals correlated either positively or negatively with observed efficacy, simple and Webb's synergies. In multiple regression relationships, some phytochemicals related positively whereas others related negatively with observed efficacy, simple and Webb's synergies. Bioactive compounds exhibit their healing pharmacological effects by either synergistic or antagonistic interaction, or both synergistic and antagonistic interactions concurrently of their many phytochemicals (Efferth and Koch, 2011).

Table 7 5: Phytochemicals selected by multiple regression matrix to exert positive and negative influences from among those exerting anthelmintic activity and those with non-available anthelmintic activity

	Expected	**Unexpected**
Positive influence	benzofuran, 2,3-dihydro; pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-; 2 propenamide; phytol; 5-hydroxymethylfurfural; furfural	2,5-dimethyl-4-hydroxy-3(2H)-furanone; acetic acid; all; 2,5-dimethylpyrazine; 2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-; 2(3H)-furanone dihydro-3-hydroxy-4,4-dimethyl; 1-hydroxy-2-propanone; dihydro-4-hydroxy-2-(3H)-furanone; 2,3-butanediol; 2,6-dimethylpyrazine; hexanal; Vanillin; 5-hydroxymethylfurfural; and propanoic acid.
Negative influences	Glutarimide; butyrolactone; pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-; methylamine,N,N-dimethyl; acetamide	Dimethylsulphuroxide; 4-Hydroxy-butanoic acid; 1-(1H-pyrrol-2-yl)-ethanone; Z-1-(-butenyl) aziridine; phenylethyl alcohol; 1-butanol, 3-methyl-, acetate; 2,5-dihydro-1H-pyrrole; 2-furancarboxaldehyde 5-methyl; 5-methyl-2-furanmethanol; 5-hydroxymethylfurfural; azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl; 1-butanol, 3-methyl-, acetate

Phytochemicals that tended to associate positively (table 6.1) with observed efficacy included 3,4-dimethylthiophene, 1-propenyl methyl disulsulphide, dimethyl trisulphide, furfural, 2,3-butanediol, 2-furanmethanol, 5-methyl-2-furanmethanol, 2(3H)-furanone, 2-hydroxy-gama-butyrolactone, 2,3-dihydro-3,5-dihydroxy-6-methyl-, benzofuran 2,3-dihydro, 5-

hydroxymethylfurfural and dihydro-4-hydroxy-2-(3H)-furanone. Only phenylethyl alcohol tended to associate negatively with observed efficacy (table 6.1). Other biomolecules associated out rightly with anthelmintic efficacy and included 2,5-dimethylpyrazine, 1-hydroxy-2-propanone, acetic acid, propenamide, 2,5-dimethyl-4-hydroxy-3(2H)-furanone and interaction of all pooled phytochemicals (all). The overall positive correlation of all phytochemicals identified (table 6.1) is suggestive of the characteristic anthelmintic activity exerted. Dimethylsulphuroxide associated negatively with observed efficacy.

Synergistic activities arising from plant species inherently possessing multiple bioactives or plant species combinations were optionally evaluated using two different methods. The first was the difference between expected and observed efficacy (simple synergy), whereas the second was Webb's efficacy (Webb, 1963). Phytochemicals that tended to associate with simple synergy included propanoic acid, 8-azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl and phytol. The other set of phytochemicals associated with simple synergy. Those with positive association included hexanal, 2,6-dimethylpyrazine, vanillin and 2-butanone, 4-(4-hydroxy-3-dimethylsulphuroxide. Three phytochemicals including phytol (de Moraes *et al*., 2014), butyrolactone (Maaz *et al*., 2015) and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Behnke *et al*., 2008) are known to exert anthelmintic activity. Butyrolactone and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- correlated negatively, whereas phytol correlated positively. Lastly, biomolecules which correlated with Webb's synergy included 3,4-dimethylthiophene, 1-propenyl methyl disulphide, dimethyl trisulphide, 2,5-dimethylpyrazine1-, 1-hydroxy-2-propanone, furfural, acetic acid, 2,3-butanediol, 2-furanmethanol, 2(3H)-furanone, propenamide, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, 5-hydroxymethylfurfural, dihydro-4-hydroxy-2-(3H)-furanone and 'all' biochemicals combined. In this set, dimethylsulphuroxide associated negatively with Webb's synergy, just as it did with both observed efficacy and simple synergy. Among these phyto-compounds are eight candidates that exerted anthelmintic activity and included benzofuran 2,3-dihydro (Swargiary *et al*., 2016), butyrolactone (Maaz *et al*., 2015), furfural (Tharachand *et al*., 2015), phytol (de Moraes *et al*., 2014), propenamide (Meenakshisundaram *et al*., 2017), pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Behnke *et al*., 2008), 2(3H)-furanone (Asif Husain *et al*., 2015) and 5-hydroxymethylfurfural (Ntalli *et al*., 2010). Correlation matrix has the potential of identifying phytochemicals with related anthelmintic activity as corroborated by the current study. Other phytochemicals correlated to anthelmintic

activity for which there is no available evidence, and have to be screened for this important trait. It is also possible that a phytochemical may lack anthelmintic activity individually but exert this in combination as a result of its interaction with other phytochemicals. That is probably why some individual biochemichemicals were associated with anthelmintic efficacy, while others which had no direct effect, associated with either simple or Webb's synergy that played the role of enhancing anthelmintic activity. Anthelmintic activity in combination phytotherapy is possibly the outcome of various biochemical interaction, some of which may play indirect roles. Additionally, some phytochemicals that individually lack anthelmintic activity could become anthelmintically active in combination as exemplified by acetic acid and vanillin.

Multiple regression relationships were used to identify possible phytochemicals that had any bearing on observed efficacy, simple and Webb's synergies; whether positive or negative. Several phytochemicals exerted different influences. Phytochemicals that had positive influence on anthelmintic activity included benzofuran, 2,3-dihydro (Swargiary *et al*., 2016), 2-propenamide (Meenakshisundaram *et al*., 2017), phytol (de Moraes *et al*., 2014), pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Behnke *et al*., 2008), 5-hydroxymethylfurfural (Ntalli *et al*., 2010) and furfural (Tharachand *et al*., 2015; Ortu, 2015). Phytochemicals identified to have exerted anthelmintic activity with oppositely negative contributions included acetamide (Sawant and Kawade, 2011), butyrolactones (Maaz *et al*., 2015), glutarimide (Lee *et al*., 2003), methylamine, N,N-dimethyl (Patani and LaVoie, 1996) and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Behnke *et al*., 2008). Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- was at one stage selected to have exerted a positive role, while in another it exercised a negative role. Its role is therefore confounding and in context possibly dependent on other phytochemicals with which it interacted. Phytochemicals with known anthelmintic bioactivity that were selected to have contributed negatively, most likely would have done so as a result of their interaction with other phytochemicals. Methylamine, N, N-dimethyl among others (table 6.5) that exerted anthelmintic activity (Patani and LaVoie, 1996) had a negative influence on observed anthelmintic efficacy, suggestive of a confounding factor. Known anthelmintically active biomolecules including 2(3H)-furanone (Asif Husain *et al*., 2015), benzyl nitrile (Surikova *et al*., 2017) and 2-ethyl-5-methylpyrazine (Shrestha *et al*., 2016) were not selected in 10 searches; probably more searches are needed. It is also tenable that as some anthelmintically active phytochemicals were selected to exert a positive role and others negative

role, these three phytochemicals would have had their activity suppressed as a result of their interaction with other molecules.

Other phytochemicals exerted influences on any of observed efficacy, simple and Webb's synergies, but can be regarded as outsiders because they have not been linked to anthelmintic activity from available literature and were not expected (table 6.5). Phytochemicals that had positive influences included 2,5-dimethyl-4-hydroxy-3(2H)-furanone, acetic acid, interaction of all phytochemicals (all), 2,5-dimethylpyrazine, 2-butanone 4-(4-hydroxy-3-methoxymethoxyphenyl)-, 2(3H)-furanone dihydro-3-hydroxy-4,4-dimethyl, 1-hydroxy-2-propanone, dihydro-4-hydroxy-2-(3H)-furanone, 2,3-butanediol, 2,6-dimethylpyrazine, hexanal, Vanillin, 5-hydroxymethylfurfural and propanoic acid (table 6.5). Acetic acid, which has not been identified to have anthelmintic activity, was selected to positively influence efficacy and synergy. Acetic acid induced writhing by earthworms (Nirmale *et al.*, 2007), and can possibly enhance or exert anthelmintic activity in its interaction with other phytochemicals. Additionally, structural biochemical differences of bioactive molecules of the same chemical class have brought about differences in their activity and efficacy (Quijada *et al.*, 2015). Therapeutic efficacy of phytotherapy is dependent on a mixture of constituents (Efferth and Koch, 2011) whose interaction (especially in combination) may diametrically change bioactivities for which they have been previously screened. In combination anthelmintic phytotherapy, it is plausible that the biological outcome of individual phytochemicals emanating from their interaction may not necessarily tie with their known contribution to certain biological activities. While some candidates will retain their bioactivity profiles, others may produce astoundingly different outcomes. Vanillin and acetic acid require close evaluation to ascertain their contribution to anthelmintic activity because they were consistently selected owing to their positive contribution to observed efficacy and related variables.

Phytochemicals without any available anthelmintic activity, but with a negative influence included dimethylsulphuroxide, 4-hydroxy-butanoic acid, 1-(1H-pyrrol-2-yl)-ethanone, Z-1-(-butenyl) aziridine, phenylethyl alcohol, 1-butanol, 3-methyl-, acetate, 2,5-dihydro-1H-pyrrole, 2-furancarboxaldehyde 5-methyl, 5-methyl-2-furanmethanol, 5-hydroxymethylfurfural, azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl and 1-butanol, 3-methyl-, acetate. Some biomolecules notably acetic acid, furfural, 2,5-dimethylpyrazine, propenamide, 2,5-dimethyl-4-

hydrox-3(2H)-furanone, 5-Hydroxymethylfurfural and a collection of all selected biochemicals positively affected both observed efficacy and synergistic effects. Furfural and 5-hydroxymethylfurfural that were identified by models to be supportive to observed efficacy also contributed positively to simple and Webb's synergies. Some biomolecules, with no available anthelmintic activity were selected to be supportive of simple synergy (table 6.1). Combinations of bioactive molecules, just like that of nutrients in diets, have the potential of bringing about biological activities which are not related to those of the component constituents (Diplock *et al*., 1998); some of which may be anthelmintic. In combination anthelmintic therapy and other therapeutic activities involving plants, the wide variety of biochemical compounds appear to play mixed roles to treatment or healing (Efferth and Koch, 2011). This collective modulating role of both negative and positive effects culminates to healing as exemplified in the positive role of holistic phytochemical interaction on both observed efficacy and Webb's synergy. It is most likely that both negative and positive influences of phytochemicals in plants exerting important healing biological activity may help to alley any negative attributes of some of these candidates. Additionally, the absence of strong positive individual correlations of identified phytochemicals, seemingly reaffirms the collective rather than individual strength or contribution to healing. Beside some phytochemicals being identified with anthelmintic activity and others doing so as a result of their interaction in combination, they also exercise other biological activities. All of these phytochemicals have been closely linked with antibacterial (Thorp *et al*., 1998; Togashi *et al*., 2007), antifungal (kurita *et al*., 1981; Makovitzki *et al*., 2006), antioxidant (Rice-Evans *et al*., 1997; Budak and Guzel-Seydim, 2010) and antiviral (De Clercq and Holy, 1979) activities. Multi-causative effects of most diseases (Efferth and Koch, 2011), can be better managed by phytotherapeutic application of related plant species. Therefore, interactions of these biomolecules resulting to anthelmintic activity is not remote. There is thus a likelihood that these molecules could well be part of a larger and more elaborate set of biochemicals exerting anthelmintic activity. Contrary to our hypothesis, some biochemical compounds exerted anthelmintic activity individually, but there could well be much more potent consortia action arising from combined activity.

7.5 Conclusion

Plant species possessing anthelmintic activity in isolation or combination have shown that this trait is the combined activity of diverse phytochemicals. Combinations will potentially play a pivotal role in nematode parasite control of livestock by providing numerous sites for anthelmintic activity. This will militate against selection for resistance by parasites that is of common occurrence in orthodox anthelmintics with relatively fewer principles and potentially less sites of activity. Multiple phytochemical relationships have shown how some biochemical compounds may exert mild biological activity and evade attention as a result of our search for stronger and more potent activity. While individual phytochemicals lacked strong activity as corroborated by correlation matrix, they collectively exerted strong activity as shown by their holistic interaction. *In vivo* assay of some of these selected combinations that have exhibited potent *in vitro* anthelmintic activity in the next section will presents an opportunity to test the same results.

Chapter 8

In vivo combination anthelmintic phytotherapy and dose determination of some selected plant species in sheep

Abstract

The study was designed to evaluate and identify the most effective combined dose of *Allium cepa* and *Vernonia amygdalina* (COMBP1)*;* and *Ananas comosus* and *Carica papaya* (COMBP2) at 50:50 weight for weight relative to positive control, "Zolvix" on natural nematode infected sheep. Sheep were fed 1.2 kg each of 4 % urea treated hay, dry matter (DM) 923 gKg^{-1}, NDF 746 gKg^{-1}, ADF 417 gKg^{-1} and CP 91 gKg^{-1}; Lucerne of DM 911 gKg^{-1}, NDF 697 gKg^{-1}, ADF 485 gKg^{-1} and CP 71 gKg^{-1}; and yellow maize, DM 900 gKg^{-1}, NDF 155 gKg^{-1}, ADF 32 gKg^{-1} and CP 80 gKg^{-1}. Inclusion ratio was 1:1:1, adjusted at 10 more of intake for heavy feeders. Treatment doses included 5 g, 10 g, 15 g, and 20 g dry matter equivalent extract in 100 ml of 70 % ethanol. Two experiments; the first, evaluated egg per gram (epg) change post treatment and the second, egg hatch and larval recovery post treatment. Fifty six Merino ewes averaging 45.0 ± 0.09 kg were weighed and initial epg done. Both parameters served as covariates at allotting sheep to treatment doses and control of experiment one. Effects of treatment, time, interaction between time and treatment on epg were evaluated weekly for 4 weeks. In the second trial, faecal grabs from treatments in the former were pooled, mixed and three sub-samples of 4 g incubated and cultured for egg hatch and larval recovery post treatment on days 1, 14 and 28, relative to negative control from untreated sheep. Effects of treatment, time and, interaction between treatment and time evaluated relative to egg hatch and larval recovery. In trial one, for COMBP1, initial sheep weight was not different, whereas final weight was relatively high ($P< 0.05$). Epg predosing and epg post treatment including those at the end of weeks' 1, 2 and 3 were low, whereas Epg at end of week 4 were high ($P< 0.05$). For COMBP2, initial sheep weight preceding treatment were not different, whereas weight post treatment were mostly similar, except weight of treatment 1, (5 g DM equivalent crude extract), ($P< 0.05$). Initial epg pre-treatment for combination *A. comosus* and *C. papaya* (COMP2) were similar ($P> 0.05$). Mean epgs' post treatment for end of weeks' 1, 2 and 3 were low, while those at end of week 4 were high ($P< 0.05$). In trial two, *e*gg hatch and larval recovery for COMP1 were low for all treatments on day 1 and 14, but oppositely high for day 28 ($P< 0.05$). Mean egg hatch and larval recovery of COMBP2 for days 1 and 14 were low, whereas that of day 28 post treatment were high ($P< 0.05$). Egg hatch and larval recovery increased with time ($P< 0.0219$). Similarly interaction of treatments and time resulted to high egg hatch and larval recovery ($P= 0.0496$).

Treatment trends for both combinations COMBP1 and COMBP2 were seemingly consistent for the first two weeks post treatment, suggesting that a follow-up second dose would have been appropriate.

8.1 Introduction

Prior to the advent of commercial anthelmintics, livestock were treated against gastrointestinal nematodes and other parasites primarily with plant based remedies and related products (Waller *et al*., 2001). These plant species are widely distributed in the tropics (Hammond *et al*., 1997; Waller *et al*., 2001), temperate and colder regions of the world (Waller *et al*., 2001; Hrckova and Velebny, 2013). Though, cocktail or mixtures of plant extracts have been used over the years to treat various livestock and human diseases (Smidt, 1997), the dose that yields the best results is seldom known. Evaluation of different combined doses *in vivo* of selected plant species is expected to identify which of them will yield the most desired efficacy. This process is critical to improved phyto-anthelmintic therapy and related healing processes, as potential results will be known.

This is projected to translate the results obtained in the previous sections into a nematode control package, with a palpable effect in the industry, given the near total dependence on conventional anthelmintics. Plants naturally contain a wide variety of phytochemical molecules and compounds (Sajid and Mckerrow, 2002; Choi and Chung, 2003; Hoste *et al*., 2008a), and those associated with anthelmintic activity are similarly many and variable (Makkar *et al*., 2007), even when they belong to the same class of compounds (Hammond *et al*., 1997; Makkar *et al*., 2007).

Inherently, combination anthelmintic phytotherapy increases the pool of anthelmintic principles and may broaden spectrum of activity, first by increasing the concentration of similar bioactives that occur in component plant species. Worldwide availability of this vital resources has resolved one of the most important challenges faced by livestock farmers, especially resource limited farmers in remote areas with poor or no commuting facilities and infrastructure. On this merit, they are invaluable resources to develop and improve their anthelmintic potential. The existence and practice of both ethnoveterinary botanical and contemporary anthelmintic controls, presents important health, environmental and economic advantages, when meticulously adopted in the prevailing context of helminth or more specifically nematode parasite burden challenge of livestock.

While ethnobotanical nematode control can be adopted to retain parasite load significantly low and harmless to health and productivity of livestock host (Ketzis *et al*., 2006), anthelmintic interventions are carried out incidentally when infection levels are critically high and life threatening (Shalaby, 2013). Integrated use of both methods is strongly encouraged to promote those advantages inherent in use of the former and those of the later, while on the other hand deterring loss of efficacy of the later from too frequent and unnecessary use (Wolstenholme *et al*., 2004). In both circumstances and importantly, with application of ethnobotanical control, appropriate *in vivo* dose should be determined to enable attainment of the desired outcome. Various treatment effects have been obtained from the use of these plant remedies, most of which may be attributed to differences in dose/concentration of bioactive principles responsible for anthelmintic activity, modulated by climate, soil type, growth stage and period of collection among other factors (Athanasiadou *et al*., 2005; Estell, 2010, Scogings *et al*., 2015).

Variations in treatment effects obtained from different regions using similar animal and plant species, demand development of standards of treatment that are elastic and capable of accommodating, even to different animal species. This necessitates determination of effective concentration locally within seasons, leading to the establishment of a system that can enable prediction and effective use of these plant remedies. Such will serve as an important milestone towards validation of global application of these species in nematode and other parasite control programs. Like is the role in anthelmintic and other chemotherapeutic practices with definitive and standardized drug concentrations/doses (Geary *et al*., 1999, Lanusse *et al*., 2014) for effective treatment *in vivo*, ethnobotanical veterinary therapy requires *in vitro* pre-screening a priori to ascertain anthelmintic activity. This is followed by *in vivo* dose determination to attain the primary objective of definitive treatment or healing effect.

It is expected that dosing of sheep with different treatment concentrations of combined plant species extract will have no effect on egg count per gram and, egg hatch and larval recovery. This study was aimed at determining *in vivo*, the most efficacious dose treatment of selected combined plant species including *Allium cepa* and *Vernonia amygdalina, and Ananas comosus* and *Carica papaya* extract on both egg count and, egg hatch and larval recovery of naturally infected sheep, to effectively control these parasites. Two experiments were carried out in the current study; one

involved monitoring the egg count per gram after intra-ruminal drenching with combined plant species crude extract at four (4) different doses, and a positive control, and the second was based on evaluation of egg hatch and larval development (egg hatch and larval development/recovery assay) following treatments in the preceding experiment.

8.2 Materials and methods

8.2.1 Study site

The study was carried out in the livestock section of the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg. It is situated at 700 m above sea level with subtropical climatic conditions. Annual rainfall is 735 mm, most of which falls between October and April. The mean maximum and minimum temperatures are 25.7º C and 8.9º C, respectively. There is occasional light frost in winter.

8.2.2 Extraction and constitution of plant species combined crude extract

Four plant species, comprising of *Allium cepa*, *Ananas comosus*, *Carica papaya* and *Vernonia amygdalina* were collected and processed as described in chapter 2. Milled vegetative material of 10g weighed into thimbles and extracted in 70 % ethanol using a Sohxlet system. Each plant material was again weighed into thimbles after completion of the first and extracted similarly. The process was repeatedly carried out to ensure that adequate stock of crude extract was obtained to meet experimental requirements. Plant species crude extract from the first and second extractions were concentrated by draining solvent from the distillation column to a final volume of 100ml. The resulting crude extract for each plant species was equivalent to 20g dry matter (DM). Volumes equivalent to 5g, 10g and 15g DM were measured and made up to 100ml with 70 % ethanol. Combinations of *Allium cepa* and *Vernonia amygdalina* (COMBP1), and *Ananas comosus* and *Carica papaya* (COMBP2) were constituted at 50:50 volume for volume and weight for weight, yielding 5g, 10g, 15g and 20g of crude extract as treatment doses.

8.2.3 Diet

Ingredients were analysed for moisture content using procedures prescribed by Association of Official Analytical Chemists (AOAC, 1999). Crude protein was evaluated from nitrogen content using LECO Truspec nitrogen analyser (LECO FP2000, LECO Pretoria, South Africa), and quantified by multiplying nitrogen content by a factor of 6.25 (crude protein = nitrogen content x 6.25). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed using ANKOM A220 fibre analyser (ANKOM Technology, New York, USA). Hemicellulose determined as the difference between neutral detergent fibre and acid detergent fibre (hemicellulose content = neutral detergent fibre – acid detergent fibre). Crude fat (CF) content was evaluated using the Soxhlet method on Soxhet Buchi 810 fat analyser (Soxhlet Buchi Switzerland). Urea treated hay had dry matter (DM) 923 gKg^{-1}, NDF 746 gKg^{-1}, ADF 417 gKg^{-1}, hemicellulose 329 gKg^{-1}CP 91 $gKg^{-}1$, and CF 12 gKg^{-1}. Lucerne on the other hand had DM 911 gKg^{-1}, NDF 697 gKg^{-1}, ADF 485 gKg^{-1}, hemicellulose 212 gKg^{-1}, CP 71 gKg^{-1} and CF 12 gKg^{-1}. Correspondingly, yellow maize had DM 900 gKg^{-1}, NDF 155 gKg^{-1}, ADF 32 gKg^{-1}, and hemicellulose 123 gKg^{-1}, CP 80 gKg^{-1}, and CF 42 gKg^{-1}. Sheep were each served a mixture of 400 g of urea treated milled hay, 400g of crushed maize and 400g of milled Lucerne hay, with the target of supplying 1.5 times their maintenance nutrient requirements. Heavy feeders were served 10 % more feed (0.12 kg), to prevent them from being underfed. Every morning, feeders were emptied of left over feed and fresh feed served. Animals were served fresh water ad libitum from automated drinkers, but where dysfunctional, clean buckets held in place by wire rings tied to the pens were used.

8.2.4 Experimental design, allotment of experimental sheep to treatments and data collection

There were 56 Merino sheep ranging from 3 to 5 years of age, with mean weight 45.0 ± 0.09 kg. Sheep were housed and confined in individual pens (1.5 x 0.69 m) for the duration of trial. Prior to commencement of the trial, animals were first adapted for 10 days, after which they were weighed and pre-treatment egg count taken. First, floatation fluid comprising of salt and sugar solution was constituted. One litter of water was measured into a 2000 ml beaker and 400 g of common salt added. The solution was stirred on a rotor using a stirring bar at very low heat until completely dissolved. Sugar of 500 g was added and again stirred until completely dissolved. The specific gravity of this salt and sugar solution was 1.280. Faecal mass of 4 g was weighed into a

250 ml beaker and 56 ml of floatation fluid added. Faecal material in solution was blended using a hand blender, and sieved into a clean beaker of capacity 100 ml. Few drops of amyl alcohol were added to each sample to disperse gas bubbles and foams. A Pasteur pipette was used to draw fluid potentially containing nematode eggs from a beaker, McMaster slide filled and mounted on a light microscope. Both chambers of the slide were viewed at 100x magnification after 5 minutes and eggs counted. Total number of eggs from both slide chambers were multiplied by 50 to obtain egg count per gram (epg). Egg count per gram was log transformed for analyses and presented in tables. Both initial weight and pre-trial egg count served as bases to sort, randomize and allot animals to different treatments.

Within the facility, and in the adjoining open space to pens, sheep were restrained and all the pens opened. Sheep were driven into the pens, and where more animals entered into one pen, the first was retained and the others driven out to own pen(s). The entire process ensured good level of randomization of sheep to pen spaces, with almost all neighbouring animals belonging to different treatments.

There were two primary combinations to study. In Trial one, *Allium cepa* and *Vernonia amygdalina* (COMP1) and Trial two, *Ananas comosus* and *Carica papaya* (COMBP2), each of which had 5 g, 10 g, 15 g and 20 g equivalent combined crude extract in 100 ml of 70 % ethanol as dose treatments. Each combination was allotted 24 sheep, and each treatment dose 6 sheep. All sheep belonging to different treatments were drenched once intra-ruminally using stomach tube at the beginning of trial. Positive control was allotted 8 sheep and treated with anthelmintic of choice "Zolvix®". Rectal faecal samples were collected at the end of each of four weeks spanning first trial to take egg count post-treatment.

Relating to the second experiment that was based on egg hatch and larval recovery, rectal faecal samples were collected 24 hours after treatment. These samples were pooled together for each treatment, mixed and three subsamples of 4g each constituted for incubation (Memmert,Schwabach, Germany®) at 27ºC. Incubation and culturing lasted for 12 days, during which they were moistened and stirred daily. Isolation of larvae was done following the Baermann funnel technique (Hansen and Perry, 1994) on day 13. Following this method, cultured faecal material was loosely tied in a double piece of cheese cloth using a rubber. Funnels with flexible silica rubber tubing of about 12 cm fitted to stems, folded and tied with rubber band to retain fluid

containing isolated L3 larvae. Prepared labelled funnels were fitted onto a rack and platform carrying holes to hold them in place. Ball of cultured faecal samples in cheese cloth were placed in labelled funnels across the neck to allow free passage of isolated L3 larvae. Funnels were filled with lurk warm water to completely immerse ball of cultured faecal sample. The whole device was left in place for larvae to migrate into fluid and swim down the funnel stem by gravity for one day (24 hours). Fitted tubing was untied and fluid containing larvae released into 10 ml glass tube. Glass tubes containing fluid and larvae were allowed to stand for 15 minutes and supernatant drawn using a Pasteur pipette. McMaster slides were filled and L3 larvae counted following McMaster technique (Hansen and Perry, 1924) on day 14 using light microscope at 100x magnification. Larvae count from both slide chambers were multiplied by 50 to obtain larval recovery per gram of faecal matter. Adjusted count was log transformed and presented in tables. Control adopted, was dung/rectal faecal grabs from ten randomly untreated sheep served urea treated hay supplemented with lucern. Faecal material was rectally collected, pooled, mixed and six sub samples of 4g each constituted for incubation, isolation and counting similarly done as were treatment samples. Samples were subsequently collected on days 14 and 28, and processed similarly for data collection. Log transformed larval count was back-transformed and presented in histogram for interpretation.

8.2.5 Statistical analysis

Mixed nematode egg counts were analysed using model below:

$$Y_{ijkln} = \mu + T_i + G_j + L_k + t_l + (T_i * t_l) + e_{ijk1n;}$$

Where: Y_{ijk} = individual observation, μ = overall mean; T_i = effect of treatment; G_j = co-variate effect of initial egg count per gram; L_k = co-variate effect of initial live weight; t_l = effect of time in days; $T_i * t_l$ = interaction between treatment and time; e_{ijkl} = the error term, time was introduced as a repeated measure.

Egg count per gram (epg) were transformed using natural log and presented in the tables, while larval counts of faecal cultures were analysed similarly using General Linear Model of SAS (2000) in accordance with statistical model below:

$$Y_{ijk} = \mu + T_i + C_j + t_k + (C_j * t_k) + e_{1jk;}$$

Where: Y_{ijk} = individual observation; μ = overall mean; T_i = effect of treatment; C = effect of larval count; t_k= effect of time in days; $C_j * t_k$ = interaction between larval count and time; e_{ijk}= error term; time was introduced as a repeated measure. In both statistical analyses, mean separation was done following student Newman-Keuls Test.

8.3 Results

8.3.1 Trial one

Initial weight of sheep for treatments relating to combination *Allium c*. and *Vernonia a*. (COMBP1; Table 8.1) were not different (P>0.05) while the final weight were different (P< 0.05) among treatments. Sheep that were dosed with 5 and 10 g DM equivalent crude extract of the combination *A. cepa*. and *V. amyddalina* had higher final weight than sheep on the other treatment doses. However, weight changes for these sheep were not different among the treatments (Table 8.1). Mean egg count per gram (epg) prior to commencement of the trial were not different among all the treatments. Similarly egg count per gram for all treatment doses after the 1st, 2nd and 3rd week post-treatment didn't differ (p>.005). Egg count per gram of sheep allotted to all treatment doses for week four post treatment were high (P< 0.05). There were no high increases in epg with time, and also interaction between treatment and time did not result to high epg. The other combined remedy was *Ananas comosus* and *Carica papaya*, tagged COMBP2. Initial weight of all sheep allotted to all treatment doses were not different (P> 0.05). Final weight of sheep allotted to dose treatments 2, 3 and 4 didn't differ (P>0.05), but were higher than for dose one (Table 8.2). Weight changes for different treatment doses were not different (P>0.05). Sheep place on control treatment lost weight (Table 8.1), while those on dosages 1 and 4 gained weight. Egg count per gram (epg) for sheep allotted to all treatment doses prior to drenching were not different (Table 8.2). Those of weeks' one and two post-drench for all treatment doses were similarly not different. Oppositely, epg for sheep of all treatment doses were different for weeks' 3 and 4 post drench (P< 0.05).

Table 8. 1: Mean weight of sheep (± SEM) Kg, pre and post treatment, natural log of egg count per gram (± SEM) pre and post treatment, and natural log of egg hatch and larval recovery per gram of dung for combinations of *Allium cepa* and *Vernonia amygdalina* (COMBP1) for four weeks

Allium cepa* and *Vernonia amygdalina						
Changes in weight (Kg)						
Dose treatments	Control	1 (5g)	2 (10g)	3 (15g)	4 (20g)	RMSE
n	8	6	6	6	6	
Initial weight (Kg)	45.5 ± 1.02^{A}	48.1 ± 1.36^{A}	48.7 ± 1.36^{A}	43.5 ± 1.36^{A}	45.8 ± 1.36^{A}	33.23
Final weight	44.5 ± 0.43^{B}	48.3 ± 0.43^{A}	48.8 ± 0.43^{A}	44.4 ± 0.43^{B}	45.8 ± 0.43^{B}	3.30
Change in weight	-1.0 ± 0.10^{A}	0.2 ± 0.30^{A}	0.2 ± 0.30^{A}	0.9 ± 0.30^{A}	-0.1 ± 0.30^{A}	3.30
Egg count per gram (log epg)						
Epg for week 0	6.4 ± 0.56^{A}	5.4 ± 0.78^{A}	5.0 ± 0.74^{A}	5.5 ± 0.74^{A}	5.4 ± 0.74^{A}	9.96
Epg for week 1	1.8 ± 0.39^{A}	3.1 ± 0.52^{A}	4.9 ± 0.52^{A}	4.7 ± 0.52^{A}	3.9 ± 0.52^{A}	4.92
Epg for week 2	2.2 ± 0.46^{A}	3.3 ± 0.61^{A}	6.1 ± 0.61^{A}	3.9 ± 0.61^{A}	4.0 ± 0.61^{A}	6.76
Epg for week 3	1.3 ± 0.43^{A}	2.5 ± 0.58^{A}	4.6 ± 0.58^{A}	3.7 ± 0.58^{A}	4.3 ± 0.58^{A}	6.00
Epg for week 4	2.5 ± 0.37^{B}	2.4 ± 0.50^{B}	4.5 ± 0.50^{BA}	5.9 ± 0.50^{A}	6.0 ± 0.50^{A}	4.48
Egg hatch & larval recovery (log larvae per gram)						
n	6	3	3	6	3	
Day 1	3.7 ± 0.84^{A}	9.0 ± 1.68^{A}	8.8 ± 1.68^{A}	4.4 ± 0.84^{A}	9.8 ± 1.68^{A}	12.66
Day 14	7.3 ± 0.66^{A}	2.6 ± 1.32^{A}	4.7 ± 1.32^{A}	4.9 ± 0.66^{A}	7.7 ± 1.32^{A}	7.82
Day 28	9.6 ± 0.15^{A}	7.1 ± 0.30^{C}	7.9 ± 0.30^{BC}	9.1 ± 0.15^{A}	8.8 ± 0.30^{BA}	0.40

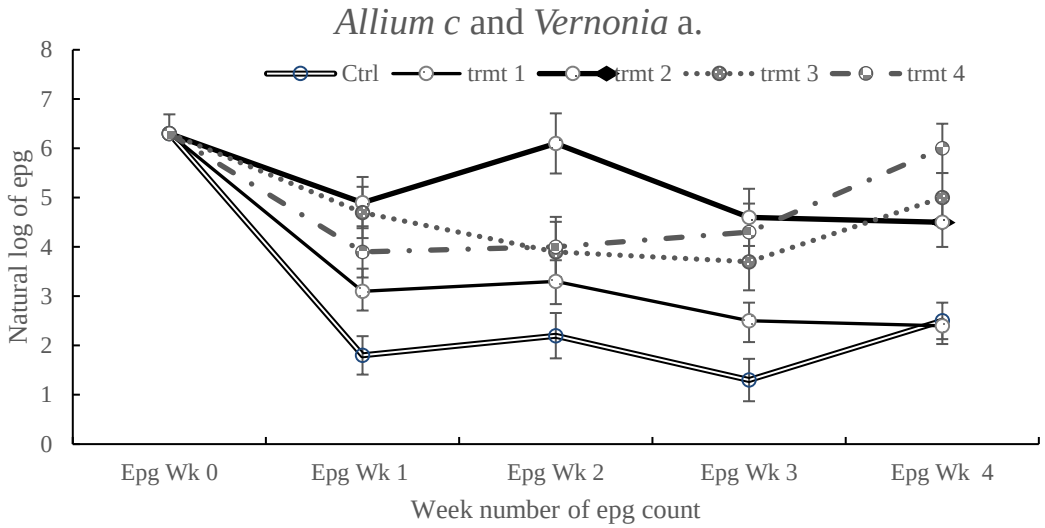
8.3.2 Trial two

Egg hatch and larval recovery for treatment doses of combination *Vernonia amygdalina* and *Allium cepa* (COMBP1; Figures 8.1, 8.3) for all treatment doses on days' 1 and 14 after drenching were not different ($P > 0.05$). Egg hatch and larval recovery on day 28 post-treatment (Figure 8.3) for all treatment doses were high ($P < 0.05$). Egg hatch and larval recovery did not increase highly over time. Interaction between treatment doses and time did not result to high egg hatch and larval recovery. Egg hatch and larval recovery for combination *A. comosus* and *C. papaya* (COMBP2) on days' 1 and 14 post drench for sheep belonging to all treatment doses were not different (Figure 8.2). Ultimately, egg hatch and larval recovery for day 28 post drench (Figure 8.4) for treatment doses were high ($P < 0.05$). Egg hatch and larval recovery increased with time ($P = 0.0219$). Interaction between treatments and time resulted to increased egg hatch and larval recovery for COMP2 ($p = 0.0166$).

Table 8. 2: Mean weight of sheep (± SEM) Kg, pre and post treatment, natural log of egg count per gram (± SEM) pre and post treatment, and natural log of egg hatch and larval recovery per gram of dung for combinations of *Ananas comosus* and *Carica papaya* (COMBP2) for four weeks

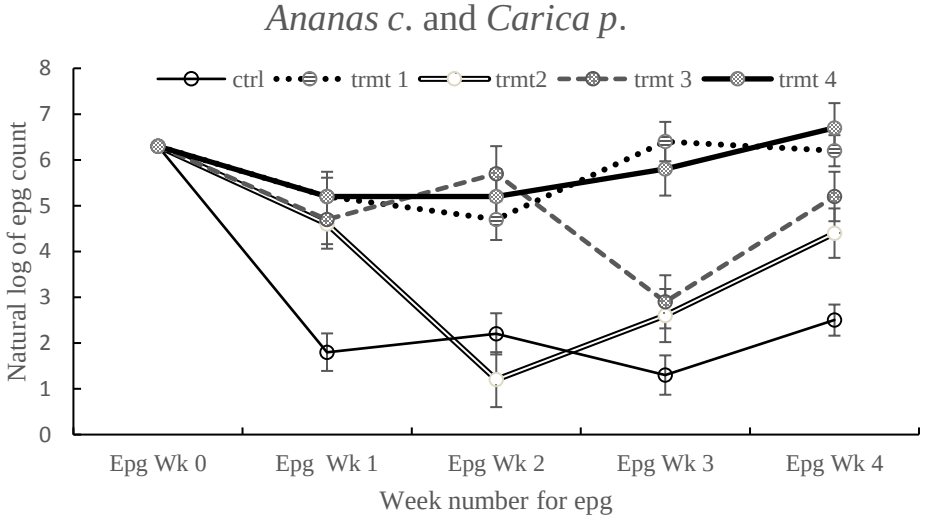
Ananas comosus* and *Carica papaya						
Weight change (kg)						
Treatments	Control	1 (5g)	2 (10g)	3 (15g)	4 (20g)	RMSE
n	8	6	6	6	6	
Initial weight (Kg)	45.5 ± 0.93^{A}	39.8 ± 1.24^{A}	44.2 ± 1.24^{A}	46.3 ± 1.24^{A}	42.4 ± 1.24^{A}	27.78
Final weight	44.5 ± 0.38^{A}	40.8 ± 0.50^{B}	43.9 ± 0.50^{A}	45.8 ± 0.50^{A}	43.7 ± 0.50^{A}	4.53
Change in weight	-1.0 ± 0.38^{A}	1.0 ± 0.50^{A}	-0.3 ± 0.50^{A}	-0.5 ± 0.50^{A}	1.3 ± 0.50^{A}	4.53
Egg count per gram for various treatments (log epg)						
Epg week 0	6.4 ± 0.56^{A}	5.5 ± 0.74^{A}	5.3 ± 0.74^{A}	5.2 ± 0.74^{A}	6.1 ± 0.74^{A}	9.90
Epg week 1	1.8 ± 0.41^{A}	5.2 ± 0.54^{A}	4.6 ± 0.54^{A}	4.7 ± 0.54^{A}	5.2 ± 0.54^{A}	5.26
Epg week 2	2.2 ± 0.44^{A}	4.9 ± 0.60^{A}	2.0 ± 0.60^{A}	5.7 ± 0.60^{A}	5.2 ± 0.60^{A}	6.41
Epg week 3	1.3 ± 0.43^{B}	6.4 ± 0.58^{A}	2.6 ± 0.58^{BA}	2.9 ± 0.58^{BA}	5.8 ± 0.58^{A}	6.02
Epg week 4	2.5 ± 0.34^{A}	6.2 ± 0.45^{A}	4.4 ± 0.45^{BA}	5.2 ± 0.45^{A}	6.7 ± 0.45^{A}	3.60
Egg hatch and larval recovery (log larvae per gram)						
n	6	3	3	3	3	
Day 1	3.7 ± 0.83^{A}	9.8 ± 1.63^{A}	9.0 ± 1.63^{A}	7.8 ± 1.63^{A}	6.2 ± 1.63^{A}	12.00
Day 14	7.3 ± 0.57^{A}	9.0 ± 1.14^{A}	4.9 ± 1.14^{A}	6.2 ± 1.14^{A}	8.8 ± 1.14^{A}	5.89
Day 28	9.6 ± 0.14^{A}	9.6 ± 0.27^{A}	7.8 ± 0.27^{B}	8.7 ± 0.72^{BA}	9.4 ± 0.27^{A}	0.34

RMSE= root mean square



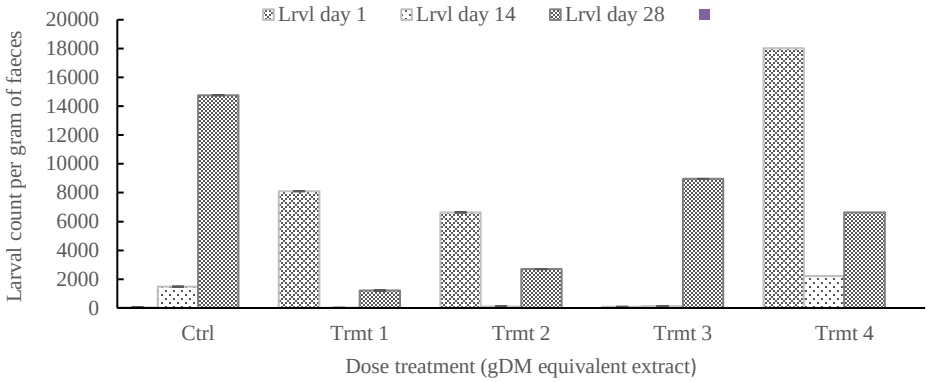
Ctrl = Positive control; trmt 1 = treatment at 5 g equivalent combined crude extract, trmt 2 = treatment at 10 g equivalent crude extract; trmt 3 = treatment at 15 g equivalent combined crude extract; trmt = treatment at 20 g equivalent combined crude extract; Epg Wk = weekly nematode egg count per gram post treatment

Figure 8. 1: Egg count per gram (Epg) post treatment with plant species combination Allium cepa/Vernonia amygdalina (COMBP1) for the different treatment doses



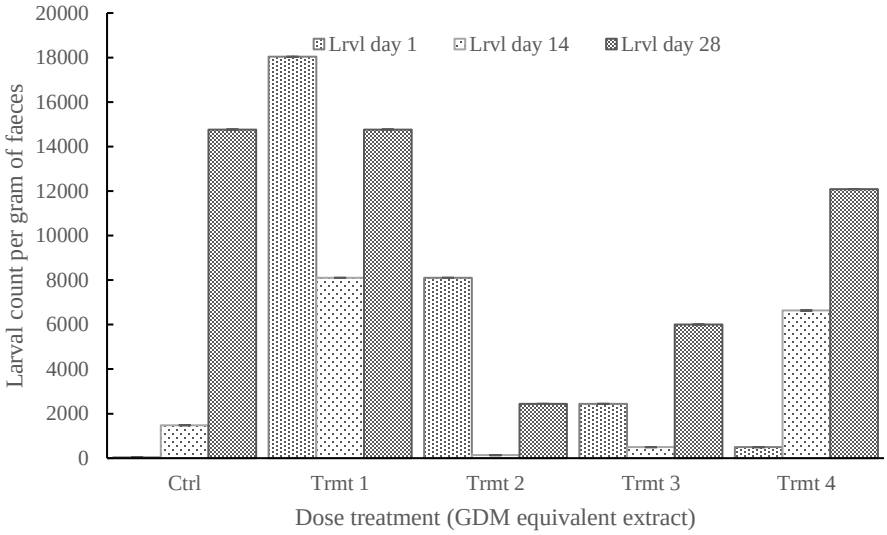
Ctrl = Positive control; trmt 1 = treatment at 5 g equivalent combined crude extract, trmt 2 = treatment at 10 g equivalent crude extract; trmt 3 = treatment at 15 g equivalent combined crude extract; trmt = treatment at 20 g equivalent combined crude extract; Epg Wk = weekly nematode egg count per gram post treatment

Figure 8. 2: Egg count per gram (Epg) post treatment with plant species combination *Ananas comosus*/*Carica papaya* (COMBP2) for the different treatment doses



Lrvl Day 1 = Larval count for day 1; Lrvl Day 14 = Larval count day 14; Lrvl Day 28 = Larval count day 28; trmt 1 = treatment at 5 g equivalent combined crude extract, trmt 2 = treatment at 10 g equivalent crude extract; trmt 3 = treatment at 15 g equivalent combined crude extract; trmt = treatment at 20 g equivalent combined crude extract

Figure 8. 3: Larval count recovery post treatment of sheep for days 1, 14 and 28 for plant species combination *Allium cepa* and *Vernonia amygdalina* relative to negative control.



Lrvl Day 1 = Larval count for day 1; Lrvl Day 14 = Larval count for day 14; Lrvl Day 28 = Larval count for day 28; trmt 1 = treatment at 5 g equivalent combined crude extract, trmt 2 = treatment at 10 g equivalent crude extract; trmt 3 = treatment at 15 g equivalent combined crude extract; trmt 4 = treatment at 20 g equivalent combined crude extract

Figure 8. 4: Larval count recovery post treatment of sheep for days 1, 14 and 28 for plant species combination *Ananas comosus* and *Carica papaya* (COMBP2) relative to negative control

8.4 Discussion and conclusion

Results from preceding *in vitro* and *in vivo* studies showed that *A. cepa* (Fajimi *et al*., 2001), *A. comosus*, *C. papaya* (Fajimi *et al*., 2001; Stepek *et al*., 2005) and *V. amygdalina*. (Alawa *et al*., 2000; Chiezey *et al*., 2000) individually exerted anthelmintic activity on nematode parasites of small ruminants. In another *in vivo* study with lambs, *A. comosus* exerted excellent anthelmintc activity against *Haemonchus c*. (Ahmed *et al*., 2013). Additionally, *V. amydalina* showed antiparasitic traits (Hussain *et al*., 2008). From the trend of mean egg count per gram (epg) for combinations of *A. cepa* and *V. amygdalina* (COMBP1)*;* and *A. comosus*. and *C. papaya*. (COMBP2), it is suggestive of similar activity in combination in accordance with expectations of the current project. Though the desired efficacy might not have been achieved, potential changes in mode of dosing may yield desired results. It is plausible that individually and in designated combinations, these plants could exert anthelmintic activity to various extents. The short span in combined anthelmintic activity of plant species may well be attributed to multifaceted factors.

The span of exposure of parasites to combined bioactives is influenced by interaction of these active candidates from component plant species (Lanusse *et al*., 2015). Some of them may increase or decrease concentration of other molecules or again influence activity negatively or positively (chapter 7). Biochemical analyses showed that *A. cepa* and *V. amygdalina* both have alcohol, aliphatic acids, benzenoid, ketones, lactones and sulphur related compounds. Combining crude extracts of these two plant species is bound to alter bilateral concentration(s) of other molecules and potentially increase their biochemical activities, if they do not counteract or destroy each other in the process. Similarly, combination of *A. comosus* and *C. papaya* has contributions of various alcohols, amides, ketones, lactones and nitrogen compounds (chapters 6 and 7) from both plant species. Additionally, based on previous studies (Stepek *et al*., 2005; Adongo, 2013), these two plant species have been closely linked to harbouring proteases. Proteases exert their biochemical activities by breaking down cuticular and structural proteins, and could well interfere with enzymatic, hormonal and other metabolic activities of nematode parasites, to the extent of destroying their inherent enzymatic activity.

Trends in epg count for both combinations, *Allium cepa* and *Vernonia amygdalina*; and *Ananas comosus* and *Carica papaya* and that of egg hatch and larval recovery point to a decrease followed

by a recovery two-to-three weeks after treatment. This suggests a need for prolonged period of interacting with parasites, which could be achieved following repeated dosing; otherwise using a dosage with capacity to sustained activity and prolong anthelmintic effect. Observations showed egg hatch and larval recovery for treatments of *Ananas comosus* and *Carica papaya* decreased initially and increased over time post-dosing to a high. Similarly, interaction between treatment and time resulted to increased egg hatch and larval recovery. Both observations reaffirm loss of activity as time post-treatment elapsed, and the need to add a second dose. Further dosing, following the preceding one, will potentially revive waning anthelmintic activity and promote further decrease of epg. Adjusting the dosing of nematode infected sheep from 8% of their feed intake (Athanasiadou *et al*., 2000) to 16 % (Athanasiadou *et al*., 2001) with Quebracho condensed tannins yielded a reduced and harmless parasite load for a longer period. Though further reduction of epg occurred, caution has to be exercised to avoid adverse effects of excessive condensed tannins binding proteins and other nutrients irreversibly (Solaiman 2010; Muir, 2011). Binding of these nutrients denies host animal access and metabolic use. Similar results of low sub-therapeutic nematode burden for forty days was obtained by weekly drenching of sheep allotted to different treatments with various plant species crude extract (Ahmed *et al*., 2014). Both of these research groups reaffirm the need to test varied dosing strategies.

Another possible reason for the short drop in egg count and subsequent rise, might be from break down (metabolism) of bioactive compounds by GIT and microbial enzymes (Lanusse *et al*., 2014). Breakdown results to fast waning activity necessitating another dosage to sustain activity for adequate killing of nematode parasite. Wide differences in efficacy observed between in vitro and *in vivo* dosing (Githiori *et al*., 2006) are potentially linked to biochemical interaction of microbial enzymes and phytochemical bioactives in the rumen, and other relevant sections of the GIT. Reduced epg shedding is likely to result from biochemical activity on adult nematode parasites and/or a killing effect, while reduced egg hatch and larval recovery assay points to possible and/or residual effects on the viability of eggs. Coincidentally, epg drops to very low on day seven for most treatments of combinations *Allium cepa-Vernonia amygdalina* and day fourteen for *Ananas comosus-Carica papaya*. A similar trend was observed for egg hatch and larval recovery (Figures' 8.3 and 8.4). There was a relatively more prolonged activity on egg hatch and larval recovery for combination, *Ananas comosus-Carica papaya*, relative to combination *Allium cepa-Vernonia amygdalina*. This is in accord with observed increase in egg hatch and larval recovery for the

former relative to the later with time. The biochemical mode of action might have been a killing-effect on the more prolific and dominant egg-producing *Haemonchus conturtus*. species in the herd. It could also be as a result of impaired egg production, or again, destruction of egg viability. Individual plant species may have many active anthelmintic phytochemicals, and implicitly more in combinations. Multiple interactions may potentially result to greater activity vis a vis vital metabolic activities of parasite/eggs. There was a drop in epg for most treatments doses relating to both combinations post treatment (figures 8.1 and 8.2), possibly linking to the killing-effect of combined extracts (Stepek *et al*., 2005) on adult parasites.

Growth or gain by sheep was marginal (tables 8.1 and 8.2), and may be attributed to differences in feed conversion efficiency among sheep on various treatments (Arthur *et al*., 2004). Age of Merino sheep used in the current trial ranged from 3 to 5 years, most of which must have exceeded the phase of accelerated growth and featured within phase of decelerated growth (Owens *et al*., 1993). Age (Malhado *et al*., 2009) is a possible factor affecting gain in sheep on various treatments; as growth rate reduces with aging (Halloran *et al*., 2002).

Empirically, anthelmintic chemotherapy currently remains the most efficacious strategy to livestock gastrointestinal nematode and other pathogenic parasite control (Fatima *et al*., 2014), but has shortfalls that must be addressed. Regular and too frequent use has resulted to drug toxicity and depressed immune system (Chang, 2011), rendering infected and treated animal host more vulnerable to other infectious diseases. Additionally, environmental pollution by non-metabolised, non-degradable drug residues and those that rest in animal product are other present-day concerns that are compelling to search for options that will mitigate these effects. One of such options is enhanced anthelmintic phytotherapy using plant species combinations. This option has shown some potential in the current study, but requires repeated dosing to retain sufficient bioactive concentration at parasite/combined crude extract interphase to enable sustained and increased efficacy.

It is recommended that repeated in vivo dosing be done, as follow up to the current study, among other pharmacokinetic studies that can increase combined crude extract availability and activity. For both combinations in the current study, there was apparently no negative effect on sheep productivity and health, giving leeway for further research and validation.

Chapter 9

General discussion and conclusions

9.1 Summary of aims and results

Generally, none of intensive or extensive grazing livestock husbandry systems is free from the debilitating effects of nematode on their host. Infection, treatment, reinfection and repeated treatment becomes a perennial cyclical process to retain these parasites at below sub clinical level, in order to promote/sustain animal health and productivity. The frequency of dosing in poorly managed grazing set-ups in the tropics and subtropics is pre-occupying, especially because favourable climatic conditions for nematode survival and propagation occur during a greater portion of the year. This approach is generally adopted because infection of livestock is recurrent, with parasite load surging each time environmental conditions favour hatching, molting, and reinfection. That is why selection for resistant nematode parasites as a result of frequent use of major anthelmintic classes has attained global proportion (Waller, 1999; Van Wyk, 2001), and is currently a major crises. The scale of this problem has attained unprecedented magnitude in the small ruminant industry (Waller, 1999).

Remarkably, this industry makes up a huge portion of the rural economy to which it contributes to nutrition as protein source, wealth/savings and social status (Devendra, 2001). It is strongly recommended that other control methods be embraced in order to reduce frequent dosing, mitigate selection for resistance by nematodes and avoid other harmful effects resulting from this practice. Nematode species of livestock cause different effects to their host.

Recapitulation of some of the most poignant effects will be pertinent to substantiate control, control processes and ultimate need for diverse options. Different nematode parasite species of livestock derive nourishment from their host in different ways and so exert various deleterious effects on their health and productivity. *Haemonchus species* is the most potent nematode species (Roeber *et al*., 2013) and females are very prolific (Sutherland and Scott, 2010). It feeds on host blood by attaching to abomasal mucosa with its mouth parts, thereby provoking anaemia, submandibular oedema (Eysker and Ploeger, 2000) and mortality at high burden. Incidentally, it is a common parasite of goats and sheep (small ruminants) that thrives in tropical and subtropical

regions (Sutherland and Scott, 2010). The other lot of nematode species provoke gastroenteritis, interfering with gastric enzyme production/secretion, nutrient digestion and integral function of GIT where they lodge (Sutherland and Scott, 2010) and derive nourishment. It is, therefore, compelling to control incidences and prevalence, in order to avoid harmful effects on health of relevant host livestock and productivity.

Chemical anthelmintic remedies are widely used to control livestock nematode parasites (Jackson and Miller, 2006; Kaminsky *et al*., 2008). They are credited with very high efficacy (Waller *et al*., 2001), ease of administration at treatment (Mirdeilami *et al*., 2011), broad spectrum protection (Waller, 2006b) and can both prevent infection and provide treatment. Irregular and too frequent treatment with these classes of anthelmintics (Kaplan, 2004) has led to general selection for resistant parasites resulting to progressive treatment failure (Stepek *et al*., 2004). Selection for resistant nematodes and anthelmintic failure occur when the prescribed/recommended dose ceases to yield desired efficacy as was from inception, and increasing proportion of parasites survive treatment (Jabbar *et al*., 2006; Coffey *et al*., 2007). Additionally, growing consumer awareness of possible residual synthetic drug material in animal products (Kaemmerer and Butenkotter, 1973; Athanasiadou *et al*., 2008; Tariq *et al*., 2009) and environmental pollution from unmetabolised products (Hammond *et al*., 1997; Yeap *et al*., 2008) are also major comtemporal concerns. Economic consequences on low resource livestock farmers who dominate the small ruminant industry is huge.

Scarce resources of relevant livestock farmers are directed to procurement of efficacious anthelmintic for treatment of livestock. This and other challenges posed by expensive efficacious anthelmintics further complicate and exacerbate nematode control processes, reducing economic gains. Development of combination anthelmintic therapy, with abundant relevant plant species globally, will be of enormous benefit to livestock farmers. Other options including biological control using nematophagous fungi, selection and breeding for nematode resistant animal species and management strategies to minimize host/parasite contact. Enhanced application of plant and plant related products in combination anthelmintic therapy was our choice for innovation.

The importance of adopting the use of plant species exerting anthelmintic activity in livestock nematode parasite control has been highlighted in literature (Waller, 1999; Githiori *et al*., 2006) and further use of plant species combinations has the potential of enhancing efficacy and widening

spectrum of parasites under control (Dobson *et al*., 2001; Leathwick *et al*., 2001). Plant species in their nature have a wide range of biochemical molecules (Sajid and Mckerrow, 2002; Choi and Chung, 2003; Makkar, 2006), many of which exert anthelmintic (Davyt *et al*., 2001; Ntalli *et al*., 2010), antibacterial (Grosvenor *et al*., 1995; Dorman and Deans, 2000), antifungal (Singh *et al*., 2000; Pyun and Shin, 2006), antiviral (Kishimoto *et al*., 2003; Alcaide *et al*., 2014) and antioxidant activities. Combination anthelmintic phytotherapy accords several advantages, one of which is a large pool of anthelmintic biochemicals. Interaction among some of these biochemicals may mitigate deleterious effects by either reduced concentration in combination of plant species or interactions among biochemical compounds, some of which may potentially neutralise harmful effects of others.

The project was executed primarily to explore potential enhancement of livestock nematode parasite control by adopting combination phytoanthelmintic therapy. Additionally, plant combinations found to exert potent anthelmintic activity will be further developed, patented and placed at the disposal of livestock farmers to treat their animals. Sixteen plant species were identified from preceding works as possessing and exerting anthelmintic activity. They included *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya, Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Sarcostema viminale, Trema orientalis, Urtica dioica, Vernonia amygdalina, Zanthozylum capense, Zingiber officinale, Zizyphus mucronata* and *Aloe van balenii*. These plant species were subsequently allotted to three groups based on their primary putative anthelmintic principles and treated as sub experiments (SEP). Sub experiment one comprised of alkaloid and tannin containing species, and included *Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Sarcostema viminale, Vernonia amygdalina, Zingiber officinale* and *Zizyphus mucronata*. Sub experiment 2 was based on flavonoid containing plant species and consisted of *Trema orientalis, Urtica dioica* and *Zanthozylum capense*; and Sub-study 3 was of proteases and nitrogen compounds, and consisted of *Allium cepa, Ananas comosus, Bidens pilosa, Carica papaya,* and *Ricinus communis.* Two animal species goats and sheep were used in this initial study and the others that followed were based on sheep

In all three sub experiment for both animal species, mean efficacies were lower for goats relative to sheep at 1x and 2x concentrations. At 4x concentration, mean efficacies of alkaloid and tannin containing plant species were virtually at parity, whereas those of flavonoids and

proteases/nitrogen containing compounds were very close for goats and sheep. Differences observed were suggested to be attributable to type and biochemical composition of plant species foraged upon by goats and sheep, and their various interactions. This served as a basis for *in vitro* combination phytoanthelmintic therapy with sheep as a model experimental animal.

Combinations were constituted first within groups consisting of plant species sharing similar primary anthelmintic principle(s), retaining SEP 1, SEP 2 and SEP 3 in former experiment. Combinations in groups constituted were evaluated for their expected efficacy, observed efficacy, simple and Webb's synergies. Alkaloids, condensed tannins and flavonoids as putative anthelmintic chemical contents were quantified, simple and multiple regression analyses were run to determine their contribution to anthelmintic efficacy. Observed efficacies for combined plant species of SEP 1, SEP 2, and SEP 3 were high but within sub-experiments were not different ($P>0.05$). Simple synergies were mostly positive. However, Webb's synergy were largely negative for SEP 1 and SEP 3, each being lower than zero. Among plant combinations, in SEP 1, condensed tannin and flavonoid contents were different ($P< 0.0001$), while alkaloid contents was not different ($P= 0.3037$); in SEP 2 condensed tannin ($P< 0.009$) and flavonoid ($P= 0.0211$) contents were different but alkaloid contents were similar ($P= 0.07$); and in SEP 3, condensed tannin contents were similar ($P= 0.4312$), while the alkaloid ($P= 0.0135$) and flavonoid contents ($P< 0.0001$) were different. For all these macro-molecules, there was no discernible association with anthelmintic efficacy. Potent anthelmintic activity arising from combinations as exemplified by high efficacy credits combination anthelmintic phytotherapy. Absence of any correlation of macromolecular compounds including alkaloids, condensed tannins and flavonoids, and bioactivity of other related phytochemicals, is suggestive of more complex and intricate macromolecular and biochemical interaction in combinations.

In chapter 5, plant species in groups containing identical putative macromolecules including alkaloids/condensed tannins, flavonoids and proteases/nitrogen compounds were reconstituted into combinations across groups to evaluate efficacy, simple and Webb's synergies in vitro in sheep, in search of more efficacious remedies. Intergroup combinations included condensed tannins/alkaloids and proteases/nitrogen compounds containing plant species in sub experiment one (SEP 1); flavonoids and alkaloids/tannin plant species (SEP 2); and proteases/nitrogen

compound and flavonoid containing plant species (SEP 3). Combined efficacies of SEP 1 related species were not different (p= 0.2760), but high. Synergistic activities were similar (P= 0.3217) and negative. No association occurred between any of alkaloids, condensed tannins or flavonoids with observed efficacy. Multiple regression analysis to seek any relationship among quantified macromolecules with efficacy was not useful. Efficacy of combinations SEP 2 were not different (P= 0.4318). Synergistic means were not different (P= 0.2685). Multiple regression analysis of macromolecules as predictors of observed efficacy was not useful. Observed efficacy of combinations relating to SEP 3 were not different (P= 0.5968) and high, mean (95.8 ± 0.04 %). Webb's synergy for SEP 3 combinations were not different (P= 0.6264) and largely negative. There was no correlation between any of the macromolecules with observed efficacy. Multiple regression analysis of different macromolecules as predictors of observed efficacy was not significant. All synergistic means were negative. Crude extracts of all combinations exhibited potent anthelmintic activity, but could not be attributed to any specific macromolecule(s). This is suggestive that, there are wide ranging biochemical interactions that retain anthelmintic activity and other relevant traits, but may be different from those in combinations in preceding section carrying similar macromolecules. Evidently, there is more to the active principles involved than has been examined in the current study, warranting a more detailed study in the succeeding chapter.

All sixteen selected plant species were analysed for phytochemical composition using GC/MS, in search of anthelmintic traits and other related phytochemicals. Phytochemicals identified belonged to aldehydes, amines, sulphur compounds, nitrogen compounds, ketones, aliphatic acids, benzenoids, alcohols, lactones, amides, alkaloids, furans and esters. A mean procedure was used to determine mean, standard deviation, sum, minimum and maximum biochemical content in Nano grams. Reference to previous screening and related bodies of work identified and profiled phytochemicals with anthelmintic and other related biological activities. Fourty six phytochemicals had antibacterial activity, 42 antioxidant activity, 38 antifungal activity, 24 antiviral activity, and 13 anthelmintic activity. Allotment of thirteen anthelmintic related phytochemicals according to occurrence in selected plant species indicated that 2 plants had one, 6 plants had two, four plants had three phytochemicals, and 4 plants four phytochemicals. It is most plausible that anthelmintic and other related biological activities exerted by plant species are

closely linked to some phytochemical(s). How identified biochemicals influence observed combined efficacy, simple and Webb's synergy was the subject of of the following chapter.

This study evaluated identified phytochemical molecules of all sixteen plant species, for their different relationships or influences on observed anthelmintic efficacy, simple and Webb's synergies. Pearson correlation coefficient was run to explore association of phytochemical candidates with observed efficacy, simple synergy and Webb's synergy. Multiple regressions were also run to explore influence of various phytochemicals on efficacy, simple synergy and Webb's synergy, by conducting 10 searches to identify any of such influences. Some phytochemicals exerted positive influence and included benzofuran, 2,3-dihydro; pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-; 2 propenamide; phytol; 5-hydroxymethylfurfural; furfural) and others negative influences. Some phytochemicals selected to exerted positive influence, did so on both observed efficacy and simple synergy. Both the correlation matrix and multiple regression relationships pointed to more consortia of phytochemical action. It is most likely that many other phytochemical molecules must have been involved in anthelmintic activities observed from plant-plant combinations, but might not have been picked up in the analysis within slated time.

Demonstrating *in vivo*, two pairs of plant species combination *Allium cepa* and *Vernonia amygdalina* (COMBP1) and, *Ananas comosus* and *Carica papaya* (COMBP2) crude extract in 70 % ethanol were used to drench sheep *in vivo* at 5 g, 10 g, 15 g and 20 g dry matter vegetative material equivalent at 50 : 50 weight for weight. Two measurements were adopted to evaluate dose efficacy; nematode egg shed decline in the first and egg hatch and larval recovery in the second. Egg count were done pre-dosing and at the end of weeks 1, 2, 3 and 4 post treatment. Egg hatch and larval recovery were done pretreatment, and on days 1, 14 and 28 post treatment. A positive control was used for both experiments. Sheep treated with COMBP1 had similar weight prior to dosing, and different weights at end of trial ($P< 0.05$). Egg count per gram pre-treatment were similar, while post treatment counts for weeks 1, 2 and 3 were similar, and those of week 4 post treatment for all treatments were high ($P< 0.05$). Initial sheep weight for COMBP2 for all treatments were not different. Final weight of sheep allotted to treatments 2, 3 and 4 were not different ($P> 0.05$), but higher than that of dose 1. Weight changes for all treatments were not different. Control, dosages 2 and 3 had negative changes, while dosages 1 and 4 experienced positive weigh changes. Egg count per gram for all sheep allotted to treatments before drenching

were not different. Epgs' for weeks' 1 and 2 post drench for all treatment doses were not different Meanwhile, epgs' for weeks' 3 and 4 for all treatment doses were different ($P< 0.05$). Egg hatch and larval recovery for all treatment doses for COMBP1 plant species combinations one day post treatment and day 14, for all treatment doses were not different. Oppositely, egg hatch and larval recovery for all treatment doses on day 28 were high ($P< 0.0.5$). Egg hatch and larval recovery only increased slightly over time. Similarly, interaction between treatment doses and time did not result to any remarkable changes in egg hatch and larval recovery for COMBP1. Egg hatch and larval recovery for combination *Ananas comosus* and *Carica papaya* (COMBP2), for all treatment doses 24 hours post drench were not different. Egg hatch and larval recovery for all treatment doses on day 14 were also similar. Egg hatch and larval recovery for all treatment doses on day 28 were high ($P< 0.05$). Egg hatch and larval recovery increased for all treatment doses of COMBP2 with time ($P< 0.0219$). Interaction between treatments and time resulted to increased egg hatch and larval recovery ($P= 0.0166$) for all treatment doses.

9.2 Conclusion

In vitro evaluation of anthelmintic activity of all selected plant species in the current study reaffirmed their anthelmintic traits. Following *in vitro* combination anthelmintic phytotherapy, it was established that:

- There was enhanced efficacy relative to individual species. Combinations involving plant species designated as sharing similar primary classes of anthelmintic principles, had minimum observed efficacy 94.3 ± 5.88 %. On the other hand, intergroup combinations among plant species possessing dissimilar primary putative anthelmintic principles, yielded a minimum efficacy of 95.5 ± 0.25 %. Ultimately, observed efficacy of both combinations was almost similar, suggesting apparent absence of major biochemical anthelmintic antagonism. Plant combinations in phytoanthelmintic therapy are strongly recommended irrespective of the biochemical anthelmintic candidates.
- Combinations yielded high efficacies.
- Simple synergies resulting from combinations were mostly positive, while Webb's synergy were largely negative.

- Alkaloids, condensed tannins and flavonoid content of individual plant species constituting combinations were largely different.
- Alkaloids, condensed tannins and flavonoid content of selected plant species did not associate with observed efficacy.
- Thirteen phytochemicals from the current study were profiled to exert anthelmintic activity, and included benzofuran, 2,3 dihydro; glutarimide; butyrolactone; 2(3H)-furanone; benzyl nitrile; 2-ethyl-5-methlpyrazine; pyridine, 3-(1-methyl-2-pyrrolidinyl)-,(S)-; methylamine, N,N-dimethyl-; 2-propenamide; acetamide; phytol; 5-hydroxymethylfurfural; and furfural.
- Multiregression analysis highlighted some phytochemicals outside of those highlighted above to exert positive influence on observed combined efficacy, and included 2,5-dimethyl-4-hydroxy-3(2H)-furanone; acetic acid; all; 2,5-dimethylpyrazine; 2-butanone, 4-(4-hydroxy-3-methoxymethoxyphenyl)-; 2(3H)-furanone dihydro-3-hydroxy-4,4-dimethyl; 1-hydroxy-2-propanone; dihydro-4-hydroxy-2-(3H)-furanone; 2,3-butanediol; 2,6-dimethylpyrazine; hexanal; Vanillin; 5-hydroxymethylfurfural; and propanoic acid. Oppositely, other identified phytochemicals outside of those known to exert anthelmintic activity were found from multiregression analysis to exert negative influence on anthelmintic activity, and included Dimethylsulphuroxide; 4-Hydroxy-butanoic acid; 1-(1H-pyrrol-2-yl)-ethanone; Z-1-(-butenyl) aziridine; phenylethyl alcohol; 1-butanol, 3-methyl-, acetate; 2,5-dihydro-1H-pyrrole; 2-furancarboxaldehyde 5-methyl; 5-methyl-2-furanmethanol; 5-hydroxymethylfurfural; azabicyclo[3.2.1]octane-3-carbonitrile, 8-methyl; and 1-butanol, 3-methyl-, acetate. Active biochemicals identified to exert either positive or negative influence on anthelmintic activity, are said to be an inherent trait of healing plant species that enable them to play this crucial role.
- *In vivo* dosing with two selected plant species combinations showed strong signs of good efficacy, but would likely have worked better if initial dosing were closely followed by a second for all the treatment doses to improve crude extract/parasite interaction. Interaction of treatment doses with time, showed waning anthelmintic activity as either of epg or egg hatch and larval recovery increased with time.

9.3 Recommendations

The challenge of chemical anthelmintic failure is evident, and a complete switch to any particular

treatment method will be likened to transfer of most problems encountered. Chemical anthelmintic therapy is known to exert high efficacy, but requires viable options to drastically reduce their frequency of application and the negative effects produced. These negative effects include pressure of selection for resistance by nematode parasites from too frequent dosing, residual drugs in animal products and environmental pollution. Phytoanthelmintic therapy offers these advantages from the point of view of its biological nature, and therefore is an important option. Enhanced phytoanthelmintic therapy via plant-plant combinations could be hugely beneficial, granted that combinations in the current study, yielded high observed efficacy. Most combinations should be tested, refined, adopted, licensed and placed at disposal of livestock farmers. Chemical anthelmintic therapy will therefore be applied when parasite load is critically high. Though the current study has not had any cases of outright biochemical antagonism, attention should be accorded to it in subsequent research.

9.4 Further research

- Identification, sourcing, constitution of more plant species combinations and further test for *in vivo* efficacy should be ear marked for research because current work has shown enormous prospects.
- Phytochemicals identified from selected plant species as novel bioactive anthelmintic molecules should be screened for it.
- Initial dosing of animals with plant-plant combinations should be monitored and closely followed by a second dose to explore parasite-crude extract interaction relative to anthelmintic healing activity.
- The mechanism involved in killing for most plant species combinations, especially because more biochemical principles are pooled together in combinations is an important area for research.
- Other livestock species like monogastrics with natural nematode parasite infection should also be tested for combined anthelmintic phototherapy.
- Biochemicals exerting negative influence on anthelmintic activity, should be screened for their mode of action.

.

References

Abbott, W. S., 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18, 265-267.

Abd El-Wahab, A. H. F., 2012. Synthesis, reactions and evaluation of the antimicrobial activity of some 4-(p-Halophenyl)-4H-naphthopyran, Pyranopyrimidine and Pyranotriazolopyrimidine derivatives. Pharmaceuticals 5(7), 745-757.

Abdalla, M. M., Abdel-Wahab, B. F., Amr, A. E., 2009. Synthesis and serotonin antagonist and antianxiety activities of pyrrolidine derivatives from 4-hydrazinyl-1-p-substituted phenyl-2, 5-dihydro-1H-pyrrole-3-carbonitriles. Monatshefte für Chemie 140, 129–137.

Abu-Hashem, A. A., Hussein, H. A. R., Aly, A. S., Gouda, M. A., 2014. Synthesis of benzofuran derivatives via different methods. Synthetic Communications 44, 2285–2312.

Abu-Youssef, M. A. M., Langer, V., Öhrström, L., 2006. Synthesis, a case of isostructural packing, and antimicrobial activity of silver(I)quinoxaline nitrate, silver(I)(2,5-dimethylpyrazine) nitrate and two related silver aminopyridine compounds. Dalton Transaction 21, 2542–2550.

Adamu, H., Endeshaw, T., Teka, T., Kife, A., Petros, B., 2006. The prevalence of intestinal parasites in paediatric diarrhoeal and non-diarrhoeal patients in Addis Ababa Hospitals, with special emphasis on opportunistic parasitic infections and with insight into the demographic and socio-economic factors. Ethiopian Journal of Health Development 20, 39-46.

Ademola, I. O., Akanbi, A. I., Idowu, S. O., 2005. Comparative nematicidal activity of chromatographic fractions of *Leucaena leucocephala* seed against gastrointestinal sheep nematodes. Pharmaceutical Biology 43, 599–604.

Adongo, O. S., 2013. Medicinal plants of Chuka community in Tharaka Nithi County, Kenya and some of their selected essential elements. Master thesis in applied and analytical chemistry, Kenyatta University.

Agarwal, M. C., Banerjee, P.S., 2007. Problems confronting helminthic diseases of domestic animals in India. Journal of Parasitological Diseases 31, 3–13.

Aguilar, J., Roy, D., Ghazal, P., Wagner, E., 2002. Dimethyl sulfoxide blocks herpes simplex virus-1 productive infection in vitro acting at different stages with positive cooperativity. Application of micro-array analysis. BioMed Central Infectious Diseases 2(9). http://doi.org/10.1186/1471-2334-2-9

Aherne, S.A., O'Brien, N. M., 2002. Dietary flavonols: chemistry, food content, and metabolism. Nutrition, 18(1), 75-81.

Ahmad, B., Amin, I., Akram, T., Muneeb- Ur-Rehman, Ahmad, S., Saiema Rasool, S., Arif, A., Farooq, A., Muzamil, S., Hussain, S., Mir, M., 2016. Zingerone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) protects against acetaminophen induced hepatotoxicity in Wistar rats via alleviation of oxidative stress and inflammation. Asian Journal of Animal and Veterinary Advances 11(12), 832-839.

Ahmad, B., Rehman, M. U., Amin, I., Arif, A., Rasool, S., Bhat, S. A., Afzal, I., Hussain, I., Bilal, S., Mir, M., 2015. A review on pharmacological properties of Zingerone (4-(4-hydroxy-3-methoxyphennyl)-2-butanone). The Scientific World Journal volume 2015. Article identity 816364, doi: 10.1155/2015/816364.

Ahmad, S., Rao, H., Akhtar, M., Ahmad, I., Hayat, M. M., Iqbal, Z., Nisar-ur-Rahman, 2011. Phytochemical composition and pharmacological prospectus of *Ficus bengalensis* Linn. (Moraceae)- A review. Journal of Medicinal Plants Research 5(28), 6393-6400.

Ahmed Subeh, D. A., 2011. Anti-Methicillin-Resistant *Staphylococcus aureus* and anti-methicillin-Sensitive *Staphylococcus aureus* effects of *Calligonum Comosum* l'her. methanolic extract. Asean University Network Inter-Library Online (AUNILO).

Ahmed, M., 2013. Integrated control of gastrointestinal nematodes of sheep using plant extracts and bio-control agents. PhD thesis, Animal Science, University of KwaZulu-Natal p102.

Ahmed, M., Laing, M. D., Nsahlai, I. V., 2013. In vitro activity of crude extracts of selected medicinal plants against *Haemonchus contortus* from sheep. Journal of Helmintology 87(2), 174-179.

Ahmed, M., Laing, M. D., Nsahlai, I. V., 2014. In vivo effect of selected medicinal plants against gastrointestinal nematodes of sheep. Tropical Animal Health and Production 46, 411-417.

Aires, A., Mota, V. R., Saavedra, M. J., Rosa, E. A. S., Bennett, R. N., 2009. The antimicrobial effects of glucosinolates and their respective enzymatic hydrolysis products on bacteria isolated from the human intestinal tract. Journal of Applied Microbiology 106, 2086–2095.

Akhtar, M. S., Iqbal, Z., Khan, M. N., Lateef, M., 2000. Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo-Pakistan sub-continent. Small Ruminant Research 38, 99107.

Akula, R., Ravishankar, G. A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling and Behaviour 6, 1720-1731.

Alam, M. M., Husain, A., Hasan, S. M., Khanna, S., Shaquiquzzaman M., 2010. 3-arylidene-5-(4-isobutylphenyl)-2(3H)-furanones: a new series of anti-inflammatory and analgesic compounds having antimicrobial activity. Journal of Enzyme Inhibition and Medicinal Chemistry 25(3), 323-330. doi: 10.3109/14756360903179435.

Al-Amiery, A. A., Kadhum, A. A. H., Mohamad, A. B., 2012. Antifungal and antioxidant activities of Pyrrolidone Thiosemicarbazone complexes. Bioinorganic Chemistry and Applications Volume 2012. Article ID 795812, 6 page doi:10.1155/2012/795812

Alawa, C. B. I., Gefu, J. O., Chiezey, P. N., Abdu, P. A., Magaji, S. O., Eduvie, L. O., Adeyinka, I. A., 2000. Screening of *Vernonia amygdalina* for anthelmintic properties. Proceedings of an international workshop on Ethnoveterinary Practices. National Animal Production Research Institute, Amadu Bello University, Zaria, Nigeria.

Alawa, J. P., Jokthan, G. E., Akut, K., 2002. Ethnoveterinary medicinal practice fot ruminants in the subhumid zone of Northern Nigeria. Preventive Veterinary Medicine 54, 79 – 90.

Alcaide, B., Almendros, P., Alonso, J. M., Ferna´ndez, I., Go´mez-Campillosa, G., Torresd, M. R., 2014. A gold-catalysed imine–propargylamine cascade sequence: synthesis of 3-substituted-2,5- dimethylpyrazines and the reaction mechanism. Chemical Communication 50, 4567—4570.

Alemayehu, K., Houdijk, J. G. M., Tolkamp, B. J., Anthanasiadou, S., Kyriazakis, I., 2009. Consequences of infection pressure and protein nutrition on peripaturent resistance to *Teladorsagia circumcincta* and performance in ewes. Veterinary Parasitology 165, 7887.

Al-Huqail, A. A., Elgaaly, G. A., Ibrahim, M. M., 2015. Identification of bioactive phytochemical from two Punica species using GC–MS and estimation of antioxidant activity of seed extracts. Saudi Journal of Biological Sciences. http://dx.doi.org/10.1016/j.sjbs.2015.11.009

Al-Majeed, M. I. A., Al-Ghizawi, G. J., 2016. Isolation and Identification of alkaloidic extract of *Capparis spinose* L buds and study of its cytoxicity and antibacterial activity. Journal of Natural Sciences Research 6(6), 122-130.

Alonso-Díaz, M. A., Torres-Acosta, J. F. J., Sandoval-Castro, C. A., Hoste, H., Aguilar-Caballero, A. J., Capetillo-Leal, C. M., 2009. Sheep preference for different tanniferous tree fodders and its relationship with in vitro gas production and digestibility. Animal Feed Science and Technology 151, 75-85.

Alonso-Díaz, M. A., Torres-Acosta, J. F. J., Sandoval-Castro, C. A., Hoste, H., 2011. Comparing the sensitivity of two in vitro assays to evaluate the anthelmintic activity of tropical tannin rich plant extracts against *Haemonchus contortus*. Veterinary Parasitology 181, 360–364.

Alvarez, L., Domingue, G., Moreno, L., Ceballos, L., Bistoletti, M., Don-adeu, M., Lanusse, C., 2012b. Testing high osfendazole doses to treat cysticercosis in pigs a safety assessment in: Proceedings of 12th international congress of the European association for Veterinary Pharmacology and Toxicology, Noordwijkerhout. The Netherlands, pp 201-202 (abstract only).

Alvarez, L., Entrocasso, C., Lifschitz, A., Manazza, J., Ceballos, L., Borda, B., Lanusse, C., 2010. Albendazole failure to control resistant nematodes in lambs: lack of effect of fasting-induced improvement on drug absorption. Journal of parasitology 96, 1204-1210.

Alvarez, L., Suarez, G., Ceballos, L., Moreno, L., Lanusse, C., 2012a. Dose-dependent systemic exposure of albendazole metabolites in lambs. Journal of Veterinary Pharmacology and Therapeutics 35, 365-372.

Amarante, A. D., Bricarello, P. A., Rocha, R. A., Gennari, S. M., 2004. Resistance of Santa Ines, Suffolk and Ile de France sheep to naturally acquired gastrointestinal nematode infections. Veterinary Parasitology 120, 91–106.

Amarante, A. F. T., Craig, T. M., Ramsey, W. S., El-Sayed, N. M., Desouki, A. Y., Bazer, F. W., 1999. Comparison of naturally acquired parasite burdens among Florida Native, Rambouillet and crossbreed ewes. Veterinary Parasitology 85, 61–69.

Ancheta, P. B., Dumilon, R. A., Venturina, V. M., Cerbito, W. A., Dobson, R. J., Le Jambre, L. F., Villar, E. C., Gray, G. D., 2004. Efficacy of benzimidazole anthelmintics in goats and sheep in the Philippines using a larval development assay. Veterinary Parasitology 120, 107–121.

Anderson, N., Dash, K. M., Donald, A. D., Southcott, W. H., Waller, P. J., 1978. Epidemiology and control of nematode infections. In: Donald, A. D., Southcott, W. H., Dineen, J. K. (Eds.). The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia. CSIRO, Australia, pp. 23 – 51.

Anderson, N., Martin, P. J., Jarret, R. G., 1988. Mixtures of anthelmintics: a strategy against resistance. Australian Veterinary Journal 65, 62-64.

Anderson, R. C., 2000. Nematode Parasites of Vertebrates. Their Development and Transmission. 2nd edition. Wallingford, Oxon OX10 8DE, UK: CAB International.

Andronicos, N., Hunt, P., Windon, R., 2010. Expression of genes in gastrointestinal and lymphatic tissues during parasite infection in sheep genetically resistant or susceptible to *Trichostrongylus colubriformis* and *Haemonchus contortus*. International Journal of Parasitology 40, 417–429.

Ankers, P., Itty, P., Zinntag, J., Trawally, S., Pfister, K., 1998. Biannual anthelmintics treatment in village Djallonke sheep in the Gambia: effects on productivity and profitability. Preventive Veterinary Medicine 34, 215225.

Anon, 1994. Ethnoveterinary medicine in Asia: An information kit on traditional animal health care practices. International institute of rural reconstruction, Silang, Cavite, Philippines

Artho, R., Schnyder, M., Kohler, L., Torgerson, P. R., Hertzberg, H., 2007. Avermectin-resistance in gastrointestinal nematodes of Boer goats and Dorper sheep in Switzerland. Veterinary Parasitology 144, 68–73.

Arthur, P. F., Archer, J. A., Herd, R. M., 2004. Feed efficiency in beef cattle: overview of recent Australian research and challenges for the future. Australian Journal of Experimental Agriculture 44, 361-369.

Asif Husain, Maaz, M., Ahmad, A., Khan, S. A., 2015. Evaluation of anthelmintic activity of di/trisubstiuted 3-arylidene-5-(biphenyl-4-yl)- 2(3h)-furanones. Indo American Journal of Pharmaceutical Sciences 2 (10), 1437-1440.

Asif, M., 2014. Chemical Characteristics, Synthetic Methods, and Biological Potential of Quinazoline and Quinazolinone Derivatives. International Journal of Medicinal Chemistry 395637. http://doi.org/10.1155/2014/395637.

Athanasiadou, S., Kyriazakis, I., 2004. Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. Proceedings of the Nutrition Society 63, 631-639.

Athanasiadou, S., Houdijk, J., Kyriazakis, I., 2008. Exploiting synergism and interactions in the nutritional approaches to parasite control in sheep production systems. Small Ruminant Research 76, 2-11.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R. L., 2000a. Consequences of long term feeding with condensed tannins on sheep parasitized with *Trichostrongylus colubriformis*. International Journal for Parasitology 30(9), 1025-1033.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R. L., 2000b. Effects of short term exposure to condensed tannins on adult *Trichostrongylus colubriformis*. The Veterinary Record 146(25), 728-732.

Athanasiadou, S., Kyriazakis, I., Jackson, F., 2006. Can plant secondary metabolites have a role in controlling gastrointestinal nematode parasitism in small ruminants? In: Sandoval-Castro, C. A., Hovell, F. D. DeB., Torres-Acosta, J. F. J., Ayala-Burgos, A. (Eds.), Herbivores, the Assessment of Intake, Digestibility and the Roles of Secondary Compounds. Nottingham University Press, Nottingham, UK, pp. 197–207.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R. L., 2000. Effect of short term exposure to condensed tannins on adult *T. colubriformis*. Veternary Record 146, 728–732.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R. L., 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. Veterinary Parasitology 99, 205–219.

Athanasiadou, S., Tzamaloukas, O., Kyriazakis, I., Jackson, F., Coop, R. L., 2005. Testing for direct anthelmintic effects of bioactive forages against *Trichostrongylus colubriformis* in grazing sheep. Veterinary Parasitology 127, 233-243.

Avan, I., 2011. Synthesis and biological activity of novel furan and 1h-pyrrole containing heterocyclic compounds as hiv-1 fusion inhibitors. Master Thesis, Graduate School of the University of Florida.

Azando, E. V. B., Hounzangbé-Adoté, M. S., Olounlade, P. A., Brunet, S., Fabre, N., Valentin, A., Hoste, H., 2011. Involvement of tannins and flavonoids in the in vitro effects of *Newbouldia laevis* and *Zanthoxylum zanthoxyloïdes* extracts on the exsheathment of third-stage infective larvae of gastrointestinal nematodes. Veterinary Parasitology 180, 292-297.

Babu, K. S., Li, X. C., Jacob, M. R., Zhang, Q., Khan, S. I., Ferreira, D., Clark, A. M., 2006. Synthesis, antifungal activity, and structure activity relationships of Coruscanone A Analogs. Journal of Medicinal Chemistry 49(26), 7877–7886.

Bain, R. K., 1999. Irradiated vaccines for helminth control in livestock. International Journal for Parasitology 29, 185 - 19 1.

Baker, R. L. B., Reynolds, L., Kassi, A. L., Rege, J. E. O., Bekelye, T., Mukassa-Mugerwa, E., Rey, B., 1994. Prospects for breeding for resistance to endoparasites in small ruminants in Africa- a new ILCA research programme. Small Ruminant Research and Development in Africa. Proceedings of the Second Biennial Conference of the African Small Ruminant Research Network (p. 223). ILRI (aka ILCA and ILRAD).

Baker, R. L., 1998. Genetic resistance to endoparasites in sheep and goats. A review of genetic resistance to gastrointestinal nematode parasites in sheep and goats in the tropics and evidence for

resistance in some sheep and goat breeds in sub-humid costal Kenya. Animal Genetic Resource Information 24, 13–30.

Bakunzi, F. R., Nkomo, L. K., Motsei, L. E., Ndou, R. V., Nyirenda, M., 2013. A survey on anthelmintic resistance in nematode parasites of communally grazed sheep and goats in a rural area of North West Province, Republic of South Africa. Life Science Journal 10, 391–393.

Baloyi, M. A., Laing, M. D., Yobo, K. S., 2011. Biological control of gastrointestinal nematodes of small ruminants, using *Bacillus turingiensis* (Berliner) and *Clonostchys rosea* (Schroers). MSc. thesis. University of KwaZulu Natal, Pietermaritzburg, South Africa.

Bambou, J. C., Arquet, R., Archimède, H., Alexandre, G., Mandonnet, N., González-García, E., 2009. Intake and digestibility of naïve kids differing in genetic resistance and experimentally parasitized (indoors) with *Haemonchus contortus* in two successive challenges. Journal Animal Science 87, 2367–2375.

Banks, D. J. D., Singh, R., Barger, I. A., Pratap, B., Le Jambre, L. F., 1990. Development and survival of infective larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* in a tropical environment. International Journal Parasitology 20, 155 - 160.

Barbour, E. K., Al Sharif, M., Sagherian, V. K., Habre, A. N., Talhouk, R. S., Talhouk, S. N., 2004. Screening of selected indigenous plants of Lebanon for antimicrobial activity. Journal of Ethnopharmacology 93, 1–7.

Barger, I. A., 1995. Control of nematodes in the presence of anthelmintic resistance-Australian experience. Proceedings of the 35th Sheep & Beef Cattle. Publication no. 165, pp. 87-92. Veterinary Continuing Education, Massey University, Palmerston North.

Barger, I. A., 1998. Control by management. Veterinary Parasitology 72, 493 - 500.

Barger, I. A., 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. International Journal for Parasitology 29, 41 – 47.

Barker, I. K., 1975. Location and distribution of *Trichostrongylus colubriformis* in the small intestine of sheep during the prepatent period, and the development of villus atrophy. Journal of Comparative Pathology 85(3), 417 – 426.

Barker, I. K., Titchen, D. A., 1982. Gastric dysfunction in sheep infected with *Trichostrongylus colubriformis*, a nematode inhabiting the small intestine. International Journal of Parasitology 12(4), 345 – 356.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005. Effects of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology 131(4), 531-538.

Barry, T. N., McNabb, W. C., 1999. The implication of condensed tannins on the nutritive value of temperate forages fed to ruminants. British Journal of Nutrition 81, 263-272.

Bartram, D. J., Leathwick, D. M., Geurden, T., Taylor, M. A., Maeder, S. J., 2012. The role of combination anthelmintic formulations in the sustainable control of round worms of sheep. Veterinary Parasitology 186, 151-158.

Behnke, J. M., Buttle, D. J., Stepek, G., Lowe, A., Duce, I. R., 2008. Developing novel anthelmintics from plant cysteine proteinases. Parasites & Vectors 1(29), 1-18.

Behnke, J. M., Chiejina, S. N., Musongong, G. A., Nnadi, P. A., Ngongeh, L. A., Abonyi, F. O., Fakae, B. B., 2011. Resistance and resilience of traditionally managed West African Dwarf goats from the Savanna zone of northern Nigeria to naturally acquired trypanosome and gastrointestinal nematode infections. Journal of Helminthology 85, 80–91.

Bennett, R. N., Wallsgrove, R. M., 1994. Secondary metabolites in plant defence mechanisms. Tansley Review No. 72. New Phytologist 127, 671-633.

Besier, B., 2007. New anthelmintics for livestock: the time is right. Trends in Parasitology 23(1), 21-24.

Beveridge, I., Pullman, A. L., Phillips, P. H., Martin, R. R., Barelds, A., Grimson, R., 1989. Comparison of the effects of infection with *Trichostrongylus colubriformis*, *T. vitrinus* and *T. rugatus* in Merino lambs. Veterinary Parasitology 32(2 – 3), 229 – 245.

Bhanushali, M., Zhao, C. G., 2012. Organocatalyzed enantioselective aldol reaction of 1H-pyrrole-2,3-diones. Tetrahedron Letters 52(3), 359–362.

Bidkar, A., Ghadiali, M., Patel, C., Manoj Aswar, M., Sanghai, D., Adate, P., 2012. Anthelmintc activities of the crude extracts of *Allium cepa* bulbs and *Elletatria cardomomum* seeds. Research Journal of Pharmaceutical, Biological and Chemical science 1(2), 50-58.

Boersema, J. H., Pandey, V. S., 1997. Anthelmintic resistance of trichostrongylids in sheep in the highveld of Zimbabwe. Veterinary Parasitology 68, 383–388.

Bohan, D. A., Kocipal-Abyazan, R., 1974. Flavonoids and condensed tannins from leaves of *Vaccinium vaticulatum* and *V. calycinium*. Pacific Science 48, 458-163.

Bondock, S., El-Azab, H., Kandeel, E. M., Metwally, M. A., 2013. Efficient Synthesis of New Functionalized 2-(Hetaryl) thiazoles, Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry 43(1), 59-71.

Boonchird, C., Flegel, T. W., 1982. In vitro antifungal activity of eugenol and vanillin against *Candida albicans* and *Cryptococcus neoformans*. Canadian Journal of Microbiology 28(11), 1235-1241.

Brandt, H. D., Osuch, E., Mathibe, L., Tsiba, P., 1995. Plants associated with accidental poisoned patients presenting at Ga-Rankuwa hospital, Pretoria, Suid Afrikaanse. Tydskrif vir wetenskop 91, 5759.

Budak, H. N., Guzel-Seydim, Z. B., 2010. Antioxidant activity and phenolic content of wine vinegars produced by two different techniques. Journal of the Science of Food and Agriculture 90, 2012-2026.

Burke, J. M., Miller, J. E., 2002. Relative resistance of Dorper crossbred ewes to gastrointestinal nematode infection compared with St. Croix and Katahdin ewes in the southeastern United States. Veterinary Parasitology 109, 265–275.

Burke, J. M., Miller, J. E., Olcott, D. D., Olcott, B. M., Terrill, T. H., 2004. Effect of copper oxide wire particles dosage and feed supplementation level on *Haemonchus contortus* infection in lambs. Veterinary Parasitology 123, 2335–243.

Burke, J. M., Morrical, D., Miller, J. E., 2007. Control of gastrointestinal nematodes with copper oxide wire particles in a flock of lactating Polypay ewes and offspring in Iowa, USA. Veterinary Parasitology 146, 372-375.

Butts, C. T., Zhang, X., Kelly, J. E., Roskamp, K. W., Unhelkar, M. H., Freites, J. A., Tahir, S., Martin, R. W., 2016. Sequence comparison, molecular modelling, and network analysis predict structural diversity in cysteine proteases from the Cape sundew, *Drosera capensis*. Computational and Structural Biotechnology Journal 14, 271–282.

Byaruhanga, C., Okwee-Acai, J., 2013. Efficacy of albendazole, levamisole and ivermectin against gastro-intestinal nematodes in naturally infected goats at the National Semi-arid Resources Research Institute Serere, Uganda. Veterinary Parasitology 19, 183–186.

Cabaret, J., 2003. Animal health problems in organic farming: subjective and objective assessments and farmers' actions. Livestock Production Science 80, 91108.

Cabo, M. L., Braber, A. F., Koenraad, P. M., 2002. Apparent antifungal activity of several lactic acid bacteria against *Penicillium discolor* is due to acetic acid in the medium. Journal of Food Protection 65(8), 1309-16.

Cala, A. C., Chagas, A. C. S., Oliveira, M. C. S., Matos, A. P., Borges, L. M. F., Sousa, L. A. D., Souza, F. A., Oliveira, G. P., 2012. In vitro Anthelmintic effect of *Melia azedarach L*. and *Trichilia claussenii C*. against sheep gastrointestinal nematodes. Experimental Parasitology 130, 98–102.

Capleton, A. C., Courage, C., Rumsby, P., Holmes, P., Stutt, E., Boxall, A. B. A., Levy, L. S., 2006. Prioritising veterinary medicines according to their potential indirect human exposure and toxicity profile. Toxicology Letters 163, 213–223.

Cazar, M. E., Schmeda-Hirschmann, G., Astudillo, L., 2005. Antimicrobial Butyrolactone I derivatives from the Ecuadorian Soil Fungus *Aspergillus terreus* Thorn. var terreus. World Journal of Microbiology and Biotechnology 21, 1067–1075.

Chandra, R. K., 1983. Nutrition and immune responses. Canadian Journal of Physiology and Pharmacology 61, 290–294.

Chang, A., 2011. Chemotherapy, Chemoresistance and the changing treatment landscape for NSCLC. Lung Cancer 71, 3-10.

Chartier, C., Etter, E., Hoste, H., Pors, I., Koch, C., Dellac, B., 2000. Effect of copper oxide needles for the control of nematode parasites in dairy goats. Veterinary Research Communications 24, 389–399.

Chaudary, F. R., Khan, M. F. U., Qayyum, M., 2007. Prevalence of *Haemonchus contortus* in naturally infected small ruminants grazing in the Potohar area of Pakistan. Pakistan Veterinary Journal 27(2), 73–79.

Che, C. T., Wang, Z. J., Chow, M. S. S., Lam, C. W. K., 2013. Herb-Herb Combination for therapeutic enhancement and advancement: theory and future perspectives. Molecules 18, 5125-5141.

Cheng, C. L., Liu, Q. Y., Chen, L. H., Jin, W. Z., Si, S. Y., Li, D. D., 2006. Isolation, structure determination and biological activity of a new glutarimide antibiotic, S632A3. Journal Asian Natural Product Research 8(1-2), 55-60.

Chiang, Y. M., Chaung, D. Y., Wang, S. Y., Kuo, Y. H., Tsai, P. W., Shyur, L .F., 2004. Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa*. Journal of Ethnopharmacology 95, 409–419.

Chiejina, S. N., Behnke, J. M., Musongong, G. A., Nnadi, P. A., Ngongeh, L. A., 2010. Resistance and resilience of West African Dwarf goats of the Nigerian savannah zone exposed to experimental escalating primary and challenge infections with *Haemonchus contortus*. Veterinary Parasitology 171, 81–90.

Chiezey, N. P., Gefu, J. O., Jagun, A. G., Abdu, P. A., Alawa, C. B. I., Magaji, S. O., Adeyinka, I. A., Eduvie, L. O., 2000. Evaluation of some Nigerian plants for anthelmintic activity in young cattle. Proceedings of an international workshop on Ethnoveterinary Practices. National Animal Production Research Institute, Amadu Bello University, Zaria, Nigeria.

Choi, S., Chung, M-H., 2003. A review of the relationship between *Aloe vera* components and their biologic effects. Seminars in Integrative Medicine 1(1), 53-62.

Coffey, L., Hale, M., Terrill, T. H., Mosjidis, J. A., Miller, J. E., Burke, J. M., 2007. Tools for managing internal parasites in small ruminants: *Sericea lespedeza*. http://www.attra.ncat.org/attra-pub/sericea lespedeza.html accessed on 26/08/2014.

Colditz, I. G., Warson, D. L., Gray, G. D., Eady, S. J., 1996. Some relationships between age, immune responsiveness and resistance to parasites in small ruminants. International Journal for Parasitology 26, 869-877.

Coop, R. L., Holmes, P. H., 1996. Nutrition and parasite interaction. International Journal for Parasitology 26(8 – 9), 951-962.

Coop, R. L., Kyriazakis, I., 1999. Nutrition–parasite interaction. Veterinary Parasitology 84, 187 –204.

Coop, R. L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends in Parasitology 17(7), 325 - 330.

Corre, J., Lucchini, J. J., Mercier, G. M., Cremieux, A., 1990. Antibacterial activity of phenethyl alcohol and resulting membrane alterations. Research in Microbiology 141(4), 483-497.

Cox, J., 1999. The nature conservation importance of dung. British Wildlife 11, 28-36.

Dabur, R., Chhillar, A. K., Yadav, V., Kamal, P. K., Gupta, J., Sharma, G. L., 2005. In vitro antifungal activity of 2-(3,4-dimethyl-2,5- dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate, a dihydropyrrole derivative. Journal of Medical Microbiology 54, 549–552.

Daciana I., Băra I. I., 2007. Plant products as antimicrobial agents. Analele Ştiinţifice ale Universităţii, Alexandru Ioan Cuza, Secţiunea Genetică şi Biologie Moleculară: Vol. VII, 151-156.

Dakkak, A., Fioramonti, J., Bueno, L., 1981. Haemonchus contortus third-stage larvae in sheep: kinetics of arrival into the abomasum and transformation during rumino-omasal transit. Research in Veterinary Science 31, 384-385.

Dalton, J. P., Mulcahy, G., 2001. Parasite vaccines – a reality? Veterinary Parasitology 98, 149-167.

Danøe, R., Bøgh, H. B., 1999.Usage of herbal medicine against helminths in livestock. An old tradition gets its renaissance. World Animal Review 93, 60-67.

Datta, F. U., Nolan, J. V., Rowe, J. B., Gray, G. D., 1998. Protein supplementation improves the performance of parasitized sheep fed a straw based diet. International Journal Parasitology 28(8), 12691278.

Davyt, D., Fernandez, R., Suescun, L., Alvaro, V., Mombru, W., Saldan˜a, J., Domı´nguez, L., Coll, J., Fujii, M. T., Manta, E., 2001. New Sesquiterpene Derivatives from the Red Alga *Laurencia scoparia*. Isolation, Structure Determination, and Anthelmintic Activity. Journal of Natural Products, 64(12), 1552-1555.

De Clercq, E., Holy, A., 1979. Antiviral activity of aliphatic nucleoside analogues: structure-function relationship. Journal of Medicinal Chemistry 22(5), 510-513.

de la Chevrotiere, C., Bishop, S. C., Arquet, R., Bambou, J. C., Schibler, L., Amigues, Y., Moreno, C., Mandonnet, N., 2012. Detection of quantitative trait loci for resistance to gastrointestinal nematode infections in Creole goats. Animal Genetics 43, 768–775.

de Moraes, J., de Oliveira, R. N., Costa, J. P., Junior, A. L. G., de Sousa, D. P., Freitas, R. M., Allegretti, S. M., Pinto, P. L. S., 2014. Phytol, a diterpene alcohol from chlorophyll, as a drug against neglected tropical disease Schistosomiasis mansoni. PLOS Neglected Tropical Diseases 8(1), e2617. https://doi.org/10.1371/journal.pntd.0002617

De, S., Sanyal, P. K., 2009. Biological control of helminth parasites by predatory fungi. Vet Scan 4(1), 18.

DeRosa, A. A., Chirgwin, S. R., Fletcher, J., Williams, J. C., Klei, T. R., 2005. Exsheathment of *Ostertagia ostertagi* infective larvae following exposure to bovine rumen contents derived from low and high roughage diets. Veterinary Parasitology 129, 77-81.

Desrues, O., Mueller-Harvey, I., Pellikaan, W. F., Enemark, H. L., Thamsborg, S. M., 2017. Condensed tannins in the gastrointestinal tract of cattle after Sainfoin (*Onobrychis viciifolia*) intake and their possible relationship with anthelmintic effects. Journal of Agricutural and Food Chemistry 65(7), 1420-1427.

Devendra, C., 2001. Small ruminants: Imperatives for productivity enhancement improved livelihoods and rural growth. A review. Asian-Australasian. Journal of Animal Science 14(10), 1483-1495.

Devendra, C., Solaiman, S. G., 2010. Perspectives on goats and global production. In: Solaiman, S. G. Goat Science and production, Wiley - Blackwell, pp 3 –19.

Devi, S. S., Nandhini, P., Tripathi, H., Hari, R., 2016. Antioxidant activity of combined ethanolic extract of *Pisonia grandis* and *Cardiospermum halicacabum*. International Journal of Pharmaceutical Sciences Review and Research 39(1), 95-100.

Dhar, D.N., Sharma, R.L., Bansal, G.C., 1982. Gastrointestinal nematodes in sheep in Kashmir. Veterinary Parasitology 11, 271–277.

Dhivare, R. S., 2016. Synthesis and biological study of pyrazoles, amino-pyrimidines and malononitriles derived from cyclic imides. Ph.D. thesis, Department of Chemistry, Shri Jagdish Prasad Jhabarmal Tibrewala University, Vidyanagari, Jhunjhunu, Rajasthan – 333001.

Diplock, A. T., Charleaux, J. L., Crozier-Willi, G., Kok, F. J., Rice Evans C., Roberfroid, M., Stahl, V., Vina-Ribes, J., 1998. Functional food science and defence against reactive oxidative species, British Journal of Nutrition 80, 77-112.

Diris, M. N., Basri, A. M., Metali, F., Ahmad, N., Taha, H., 2017. Phytochemicals and antimicrobial activities of *Melastoma malabathricum* and *Melastoma beccarianum* leaf crude extracts. Research Journal of Photochemistry 11, 35-41.

Dobson, R. J., Besier, R. B., Barnes , E. H., Love, S. C., Vizard, A. Bell, K., LeJambre, L. F., 2001. Principles for the use of macrocyclic lactones to minimize selection for resistance. Australian Veterinary Journal 79, 756-761.

Domingues, L. F., Giglioti, R., Feitosa, K. A., Fantatto, R. R., Rabeloc, M. D., Oliveirac, M. C., Becharaa, G. H., Pereira de Oliveiraa, G., Barioni Junior, W., Chagas, A. C., 2013. In vitro and in vivo evaluation of the activity of pineapple (*Ananas comosus*) on *Haemonchus contortus* in Santa Inês sheep. Veterinary Parasitology 197, 263–270.

Domke, A. V. M., Chartier, C., Gjerde, B., Höglund, J., Leine, N., Vatn, S., Stuen, S., 2012. Prevalence of anthelmintic resistance in gastrointestinal nematodes of sheep and goats in Norway. Parasitology Research 111, 185–193.

Donald, A. D., 1994. Parasites, animal production and sustainable development. Veterinary Parasitology 44, 3544.

Donald, A. D., Southcott, W. H., Dineen, J. K., 1978. The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia. Commonwealth Scientific and Industrial Research Organisation: Melbourne.

Dorman, H. J. D., Deans, S. G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88(2), 308–316.

Dove H., 2010. Ingestive Behaviour, Diet Selection, and Feed Intake. In: Solaiman, S.G. *Goat Science and Production*, Wiley-Blackwell, p179-182.

Drachevaa, L. V., Dorozhkoa, E. V., Avramchuka, O. A., Korotkovaa, E. I., Ryzhkovab, E. P., Li, H., Danilovab, I. V., 2009. Voltammetric study of the antioxidant activity of Propionic acid bacteria in liquid cultures. Moscow University Biological Sciences Bulletin 64(4), 157–160.

Duke, J. A., 1992. Handbook of phytochemical constituents of GRAS herbs and other economic plants. CRC Press, Boca Raton, FL., pp 654.

Dwinger, R. H., Agyemang, k., Kaufmann, K., Grieve, A. S., Bah, M. L., 1994. Effect of trypanosome and helminth infections on health and reproductive parameters of village N'Dama cattle in the Gambia. Veterinary Parasitology 54, 353365.

Dwivedi, A., Dwivedi S., Sitoke A. K., Patel, R., Jhade, D., 2009. Anthelmintic Activity of a Polyherbal Preparation. Ethnobotanical Leaflets 13, 259-62.

Echovska´, L. Cˇ., Cejpek, K., Konecˇny´, M., Velı´sˇek, J., 2011. On the role of 2,3-dihydro-3,5-dihydroxy-6-methyl-(4H)-pyran-4-one in antioxidant capacity of prunes. European Food Research Technology 233, 367–376.

Efferth, T., Koch, E., 2011. Complex interaction between phytochemicals. The multi-target therapeutic concept of phytotherapy. Current Drug Targets 12(1), 122-132.

Eguale, T., Giday, M., 2009. In vitro anthelmintic activity of three medicinal plants against *Haemonchus contortus*. International Journal of Green Pharmacy 3, 29-34.

Eklund, T., 1983. The antimicrobial effect of dissociated and undissociated sorbic acid at different pH levels. Journal of Applied Bacteriology 54(3), 383-389.

El-Wakil, E. A., El-Sayed, M. M., Abdel-Lateef, E. E., 2015. GC-MS Investigation of Essential oil and antioxidant activity of Egyptian White Onion (*Allium cepa* L.) International Journal of Pharma Sciences and Research 6(3), 537-543.

Emery, E. L., 1996. Vaccination against worm parasites animals. Veterinary parasitology 64, 3145.

Entrocasso, C., Alvarez, L., Manazza, J., Lifschitz, A., Borda, B., Virkel, G., Mottier, L., Lanusse, C., 2008. Clinical efficacy assessment of the albendazole-ivermectin combination in lambs parasitized with resistant nematodes. Veterinary Parasitology 155, 249-256.

Estell, R. E., 2010. Coping with shrub secondary metabolites by ruminants. Small Ruminant Research 94, 1–9.

Eysker, M., Ploeger, H. W., 2000. Value of present diagnostic methods of gastrointestinal nematode infection in ruminants. Parasitology 102(07), S109S119.

Falzon, L., O'Neill, T., Menzies, P., Peregrine, A., Jones-Bitton, A., Mederos, A., 2014. A systematic review and meta-analysis of factors associated with anthelmintic resistance in sheep. Preventive Veterinary Medicine 117, 388-402.

Farias, M. T., Bordin, E. L., Forbes, A. B., Newcomb, K., 1997. A survey of resistance to anthelmintics in sheep stud farms in Southern Brazil. Veterinary Parasitology 72, 209214.

Fatima, T., Sajid, M. S., Jawad-ul-Hassan, M., Siddique, M. R., Iqbal, Z., 2014. Phytomedicinal value of *Moringa oleifera* with special reference to antiparasitics. Pakistan Journal of Agricultural Science 15(1), 251-262.

Feng, S., Zeng, W., Luo, F., Zhoa, J., Yang, Z., Sun, Q., 2010. Antibacterial activity of organic acids in aqueous extracts from pine needles (*Pinus massoniana* Lamb.). Food Science and Biotechnology 19(1), 35–41.

Fernandez, B., Vimont, A., Desfossés-Foucault, E., Daga, M., Arora, G., Fliss, I., 2017. Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. Food Control 78, 350-356.

Fernando, W. G. D., Ramarathnam, R., Krishnamoorthy, A. S., Savchuk, S. C., 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology & Biochemistry 37, 955–964.

Flefel, E. M., Abdel-Mageid, R. E., Tantawy, W. A., Ali, M. A., Amr, A. E. G. E., 2012. Heterocyclic compounds based on 3-(4-bromophenyl) azo-5-phenyl-2(3H)-furanone: Anti-avian influenza virus (H5N1) activity. Acta Pharmaceutica 62, 593–606.

Fleming, F. F., Yao, L., Ravikumar, P. C., Funk, L., Shook, B. C., 2010. Nitrile-Containing Pharmaceuticals: Efficacious Roles of the Nitrile Pharmacophore. Journal of Medicinal Chemistry 53(22), 7902–7917.

Fleming, S. A., Craig, T., Kaplan, R. M., Miller, J. E., Navarre, C., Rings, M., 2006. Anthelmintic resistance of gastrointestinal parasites in small ruminants. Journal of veterinary internal medicine *20*(2), 435-444.

Fokialakis, N., Cantrell, C. L., Duke, S. O., Skaltsounis, A. L., Wedge, D. E., 2006. Antifungal Activity of Thiophenes from *Echinops ritro*. Journal of Agricultural and Food Chemistry 54(5), 1651–1655.

Fortes, F. S., Kloster, F. S., Schafer, A. S., Bier, D., Buzatti, A., Yoshitani, U. Y., Molento, M. B., 2013. Evaluation of resistance in a selected field strain of *Haemonchus contortus* to ivermectin and moxidectin using the Larval Migration on Agar Test. Pesquisa Veterinária Brasileira 33, 183–187.

Foster, J. G., Cassida, K. A., Turner, K. E., 2011. In vitro analysis of the anthelmintic activity of forage chicory (*Cichorium intybus* L.) sesquiterpene lactones against a predominantly *Haemonchus contortus* egg population. Veterinary Parasitology 180, 298-306.

Fox, N. J., Marion, G., Davidson, R. S., White, P. C. L., Hutchings, M. R., 2012. Livestock Helminths in a Changing Climate: Approaches and Restrictions to Meaningful Predictions. Animals 2, 93 - 107.

Fukai, S., Tanimoto, S., Maeda, A., Fukuda, H., Okada, Y., Nomura, M., 2009. Pharmacological activity of compounds extracted from persimmon peel (*Diospyros kaki* Thunb.). Journal of Oleo Science 58(4), 213-219.

Gabriel, S., Phiri, I. K., Dorny, P., Vercruysse, J., 2001. A survey on anthelmintic resistance in nematode parasites of sheep in Lusaka, Zambia Onderstepoort. Journal of Veterinary Research 68, 271–274.

Gardini, F., Lanciotti, R., Caccioni, D. R. L., Guerzoni, M. E., 1997. Antifungal activity of Hexanal as dependent on its vapour pressure. Journal of Agricultural and Food Agricultural Chemistry 45(11), 4297-4302.

Geary, T. G., Hosking, B. C., Skuce, P. J., Samson-Himmelstjerna, G., Maeder, S., Holdsworth, P., Pomroy, W., Vercruysse, J., 2012. World Association for the Advancement of Veterinary Parasitology (W. A. A. P.) Guiddeline: anthelmintic combination product targeting nematode infections of ruminants and horses. Veterinary Parasitology 190, 306-316.

Geerts S., Gryseels B., 2000. Drug resistance in human helminths: current situation and lessons from livestock. Clinical Microbiology *Reviews* 13, 207–222. DOI: 10.1128/CMR.13.2.207-222.2000.

Getachew, T., Dorchies, P., Jacquiet, P., 2007. Trends and Challenges in The Effective and Sustainable Control of *Haemonchus contortus* in Sheep: Review. Parasite 14, 3–14.

Ghaidaa, J. M., Al-Jassani, M. J., Hameed, I. H., 2016. Anti-bacterial, Antifungal Activity and Chemical Analysis of *Punica grantanum* (Pomegranate peel) Using GC-MS and FTIR Spectroscopy. International Journal of Pharmacognosy and Phytochemical Research 8(3), 480-494.

Ghaneian, M. T., Ehrampoush, M. H., Jebali, A., Hekmatimoghaddam, S., Mahmoudi, M., 2015. Antimicrobial activity, toxicity and stability of phytol as a novel surface disinfectant. Environmental Health Engineering and Management Journal 2(1), 13–16.

Ghisalberti, E. L., 2002. Secondary metabolites with antinematodal activity. In: Atta-ur-Rahman (Ed.), .In: Studies in Natural Products Chemistry, vol. 26. Elsevier Science BV, pp. 425–506.

Gibson, T. E., 1980. Factors influencing the application of anthelmintics in practice. Veterinary Parasitology 6, 241-245.

Gill, J. H. Lacey, E., 1998. Avermectin/Milbemycin resistance in trichostrongyloid nematodes. International Journal for Parasitology 28, 863-877.

Githiori, B. J., Athanasiadou, S., Thamsburg, S. M., 2006. Use of plants in novel approaches for control of gastrointestinal helminths in livestock with emphasis on small ruminants. Veterinary parasitology 139(3), 308320.

Githiori, J. B., Hoglund, J., Waller, P. J., Baker, R. L., 2004. Evaluation of anthelmintic properties of some plants used as livestock dewormers against *Haemonchus contortus* infections in sheep. Veterinary Parasitology 129, 245–253.

Göker, H., Tuçbilek, M., Ayhan, G., Altanlar, N., 1998. Synthesis of some new benzimidazole carboxamides and evaluation of their antimicrobial activity. 11 Farmaco 53, 415-420.

Gołezbiowski, M., Cerkowniak, M., Urbanek, A., Dawgul, M., Kamysz, W., Bogus, M. I., Sosnowska, D., Stepnowski, P., 2013. Antimicrobial activity of untypical lipid compounds in the cuticular and internal lipids of four fly species. Journal of Applied Microbiology 116, 269-287.

Good, B., Hanrahan, J. P., Crowley, B. A., Mulcahy, G., 2006. Texel sheep are more resistant to natural nematode challenge than Suffolk sheep based on faecal egg count and nematode burden. Veterinary Parasitology 136, 317–327.

Graham, K., Graham, E. A., Towers, G. H. N., 1980. Cercaricidal activity of phenyl heptatriyne and -terthienyl naturally occurring compounds in species of Asteraceae Compositae. Canadian Journal of Zoology 58, 1955–1958.

Gray, G. D., 1997. The use of genetically resistant sheep to control nematode parasitism. Veterinary Parasitology 72, 345–366.

Grime, A. S., Nirmal, S. A., Bhalke, R. D., Chavan, M. J., 2008. Aalgesic, CNS depressant and anthelmintic activity of *Sarcostema viminale.* Iranian Journal of Pharmacology and Therapeutics 7(2), 152-156.

Grønvold, J., Henriksen, S. A., Larsen, M., Nansen, P., Wolstrup, J., 1996. Aspects of biological control with special reference to arthropods, protozoans and helminths of domesticated animals. Vetetrinary Parasitology 64, 47-64.

Gross, S. J., Ryan, W. G., Ploeger, H. W., 1999. Anthelmintic treatment of dairy cows and its effect on milk production. The Veterinary Record 144, 581587.

Grosvenor, P. W., Supriono, A., Gray, D. O., 1995. Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. Journal of Ethnopharmacology 45, 97-111.

Gruner, L., Aumont, G., Getachew, T., Brunel, J. C., Pery, C., Cognie, Y., Guerin, Y., 2003. Experimental infection of Black Belly and INRA 401 straight and crossbred sheep with trichostrongyle nematode parasites. Veterinary Parasitology 116, 239–249.

Gruner, L., Bouix, J., Brunel, J. C., 2004. High genetic correlation between resistance to *Haemonchus contortus* and *Trichostrongylus colubriformis* in INRA 401 sheep. Veterinary Parasitology 119, 51–58.

GüIçin, İ., 2011. Antioxidant activity of food constituents: an overview. Archives of Toxicology 86, 345-391.

Gulcin, I., Kufrevioglu, O. I., Oktay, M., *Buyukokuroglu*, M. E., 2004. Antioxidant, antimicrobial and antiulcer and analgesic activities of nettle (*Urtica dioica* L.). Journal of Ethnopharmacology 90, 205-215.

Gurnani, N., Gupta, M., Mehta, D., Bhupendra Kumar Mehta, K., 2016. Chemical composition, total phenolic and flavonoid contents, and in vitro antimicrobial and antioxidant activities of crude extracts from red chilli seeds (*Capsicum frutescens* L.). Journal of Taibah University for Science 10, 462–470.

Halloran, B. P., Ferguson, V. L., Simske, S. J., Burghardt, A., Venton, L. L., Majundar, S. A., 2002. Changes in bone structure and mass in male with advancing age in the male C57BL/6J mouse. Journal of Bone and Mineral Research 17(6), 1044-1050.

Haman, J. C., 2007. Materia Medica in Veterinary herbal medicine, chapter 24, 457-672.

Hameed, I. H., Altameme, H. J., Mohammed, G. J., 2016. Evaluation of antifungal and antibacterial activity and analysis of bioactive phytochemical compounds of *Cinnamomum zeylanicum* (Cinnamon bark) using Gas chromatography-Mass spectrometry. Oriental Journal of Chemistry 32(4), 1769-1788.

Hammond, J. A., Fielding, D., Bishop, S. C., 1997. Prospects for plant anthelmintics in tropical veterinary medicine. Veterinary research communications 21(3), 213-228.

Harborne, J. B., 1973. Phytochemical methods. Chapman Hall, London, p. 278.

Harborne, J. B., 1986. Nature, distribution, and function of plant flavonoids. In: Cody, V., Middleton, E., Harborne, J. B., eds. Plant flavonoids in biology and medicine: biochemical, pharmacological, and structure–activity relationships, Vol 213. New York: Alan R. Liss, p15.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dopson, A. P., Ostfeld, R. S., Samuel, M. D., 2002. Climate warming risks for terrestrial and marine biota. Science 296, 2158 – 2162.

Hassan, A. S., 2014. The Antibacterial Activity of Dimethyl Sulfoxide (DMSO) with and without of Some Ligand Complexes of the Transitional Metal Ions of Ethyl Coumarin against Bacteria Isolate from Burn and Wound Infection. Journal of Natural Sciences Research 4(19), 106-111.

Hassan, R., El-Kadi, S., Sand, M., 2015. Effect of some organic acids on some fungal growth and their toxins production. International Journal of Advances in Biology 2(1), 1-11.

Hazen, K. C., 2013. Influence of DMSO on antifungal activity during susceptibility testing in vitro. Diagnostic Microbiology and Infectious Disease 75(1), 60-63.

Hein, W. R., Harrison, G. B. L., 2005. Vaccines against veterinary helminths. Veterinary Parasitology 132, 217 – 222.

Hennessy, D. R., 1997. Physiology, pharmacology and parasitology. International Journal for Parasitology *27*(2), 145-152.

Henrioud, A. N., 2011.Towards sustainable parasite control practices in livestock production with emphasis in Latin America. Vetetrinary Parasitology 180, 211.

Higgins, S. I., Kantelhardt, J., Scheiter, S., Boerner, J., 2007. Sustainable management of extensively managed savanna rangelands. Ecological Economics 62(1), 102 - 114.

Hoffman, B., Hoelzl, J., 1988. New chalcones from *Bidens pilosa*. Planta Medica 54, 52–54.

Holmes, P. H., 1993. Interactions between parasites and animal nutrition: the veterinary consequences. Proceedings of the Nutrition Society 52, 113-120.

Hördegen, P., Hertzberg, H., Heilmann, J., Langhans, W., Maurer, V., 2003. The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. Veterinary Parasitology 117, 51-70.

Hosseiny Davarani, S. S., Nematollahi, D., Shamsipur, M., Najafi, N. M., Masoumi, L., Ramyar, S., 2006. Electrochemical oxidation of 2, 3-dimethylhydroquinone in the presence of 1, 3-dicarboyl compounds. The Journal of Organic Chemistry 71(5), 2139-2142.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S. M., Hoskin, S. O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends in Parasitology 22(6), 253-261.

Hoste, H., Torres-Acosta, J. F. J., Aguilar-Caballero, A. J., 2008b. Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? Parasite Immunology 30, 79-88.

Hoste, H., Torres-Acosta, J. F., Alonso-Diaz, M. A., Brunet, S., Sandoval-Castro, C., Adote, S. H., 2008a. Identification and validation of bioactive plants for the control of gastrointestinal nematodes in small ruminants. Tropical Biomedicine 25(1), 56-72.

Hotez, P. J., Molyneux, D. H., Fenwick, A., Kumaresan, J., Ehrlich Sachs, S., Sachs, J., Savioli, L., 2007. Control of neglected tropical diseases. New England Journal of Medicine 357(1018), 1027.

Houdijk, J. G. M., Kyriazakis, I., Jackson, F., Huntley, J. F., Coop, R. L., 2005. Effect of protein supply and reproductive status on local and systemic immune response to *Teladorsagia circumcincta* in sheep. Veterinary Parasitology 129, 105117.

Hrckova, G., Velebny, S., 2013. Pharmacological potential of selected natural compounds in the control of parasitic diseases. SpringerBriefs in Pharmaceutical Science and Drug Development p 38.

Husain, A., Alam, M. M., Hasan, S. M., Yar, M. S., 2009. 2(3H)-furanones and 2(3H)-pyrrolones: synthesis and antimycobacterial evaluation. Acta Poloniae Pharmaceutica ñ Drug Research 66(2), 173-180.

Hussain, A., Khan, M, N., Iqbal, Z., Sajid, M. S., 2008. An account of the botanical anthelmintics used in traditional veterinary practices in Sahiwal district of Punjab, Pakistan. Journal of Ethnopharmacology 119, 185-190.

Inayama, S., Mamoto, K., Shibata, T., Hirose, T., 1976. Structure and Antitumor Activity Relationship of 2-Arylidene-4-cyclopentene- 1,3-diones and 2-Arylideneindan- 1,3-diones. Journal of Medicinal Chemistry 19(3), 433-436.

Iqbal, Z., Lateef, M., Akhtar, M. S., Ghayur, M. N., Gilani, A. H., 2006. In vivo anthelmintic activity of Ginger against gastrointestinal nematodes of sheep. Journal of Ethnopharmacology 106(2), 285-287.

Ivashchenko, A. V., Yamanushkin, P. M., Mit'kin, O. D., Kisil', V. M., Korzinov, O. M., Vedenskii, V. Yu., Leneva, I. A., Bulanova, E. A., Bychko, V. V., Okun', I. M., A. A. Ivashchenko, A. A., Ivanenkov, Ya. A., 2014. Synthesis and Antiviral Activity of Substituted 2,4-bis-aminomethyl-5-hydroxy-1H-indole-3-carboxylic Acid Ethyl Esters and their Derivatives. Pharmaeutical Chemistry 48(9), 569–581.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammed, G., Khan, M. N., Afaq, M., 2006. Anthelmintic resistance: the state of play revisited. Life sciences 79(26), 2413-2431.

Jabbar, A., Zaman, M. A., Iqbal, Z., Yaseen, M., Shamin, A., 2007. Anthelmintic activity of *Chenopodium* (L) and *Caessalpinia crista* (L) against trichostrongylid nematodes of sheep. Journal of Ethnopharmacology 144(1), 86-91.

Jackson, F., Coop, R. L., 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology 120, S95–S107.

Jackson, F., Miller, J., 2006. Alternative approaches to control-Quo vadit? Veterinary parasitology 139, 371384.

Jackson, F., Bartley, D., Bartley, Y., Kenyon, F., 2009. Worm control in sheep in the future. Small Ruminant Research 86, 4042.

Jackson, F., Greer, A. W., Huntley, J., McAnulty, R. W., Bartley, D. J., Stanley, A., Stenhouse, L., Stankiewicz, M., Sykes, A. R., 2004. Studies using *Teladorsagia circumcincta* in an *in vitro* direct challenge method using abomasal tissue explants. Veterinary Parasitology 124, 7289.

Jackson, F., Varady, M., Bartley, D. J., 2012. Managing anthelmintic resistance in goats-Can we learn lessons from Sheep? Small Ruminant Research 103, 3-9.

Jat, R. S., Gajbhiye, N. A., 2017. Secondary metabolite production influenced with soil fertility and irrigation in medicinal plants, Mandukaparni (*Centella asiatica* L.). National academy science letters 40(2), 87-90.

Jerzykiewicz, M., Cwielaga, I., Jerzykiewicz, W., 2009. The antioxidant and anticorrosive properties of crude glycerol fraction from biodiesel production. Journal Chemical Technology Biotechnology 84, 1196–1201.

Ji, X. Y., Zhong, Z. J., Xue, S. T., Meng, S., He, W. Y., Gao, R. M., Li, Y. H., Li, Z. R., 2010. Synthesis and antiviral activities of synthetic glutarimide derivatives. Chemical and Pharmaceutcal Bulletin 28, 1436-1441.

Jin, Z., 2003. Amaryllidaceae and Sceletium alkaloids. Natural product reports 20(6), 606-614.

Jung, K. H., Yoo, S. K. Moon, S. K., Lee, U. S., 2007. Furfural from pine needle extract inhibits the growth of a plant pathogenic fungus, *Alternaria mali*. Mycobiology 35(1), 39-43.

Just, Z. W., 2007. "Synthesis of 2(3H)-furanones via electrophilic cyclization". Retrospective Theses and Dissertations. 15078. Iowa State University. http://lib.dr.iastate.edu/rtd/15078.

Kabasa, J. D., Opuda-Asibo, J., Ter Meulen, U., 2000. The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. Tropical Animal Health and Production 32, 73-86.

Kadhum, A. A. H., Al-Amiery, A. A., Musa, A. Y., Mohamad, A. B., 2011. The Antioxidant Activity of New Coumarin Derivatives. International Journal Molecular Sciences 12, 5747-5761.

Kaemmerer, K., Butenkotter, S., 1973. The problem of residues in meat of edible domestic animals after application or intake of organophosphate esters. Residue Review 46, 1.

Kahiya, c., Mukaratirwa, S., Thamsborg, S. M., 2003. Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. Veterinary Parasitology 115(3), 265-274.

Kahn, L. P., Woogate, R. G., 2012. Integrated parasite management: Products for adoption by the Australian sheep industry. Veterinary parasitology 186(12), 5864.

Kahn, L. P., Knox, M. R., Gray, G. D., Lea, J. M., WalkdenBrown, S. W., 2003. Enhancing immunity to nematode parasite in single bearing Merino ewes through nutrition and genetic selection. Veterinary Parasitology 112, 211-225.

Kalt, F. R., Cock, I. E., 2014. Gas chromatography-mass spectroscopy analysis of bioactive petalostigma extracts: Toxicity, antibacterial and antiviral activities. Pharmacognosy Magazine 10 supplement 1, S37-S49.

Kamal, M., Shakya, A. K., Jawaid, T., 2011. Benzofurans: a new profile of biological activities. Internatioal Journal of Medicine and Pharmaceutical Science 1, 1-15.

Kamaraj, C., Rahuman, A. A., Elango, G., Bagavan, A., Zahir, A. A., 2011. Anthelmintic activity of botanical extracts against sheep gastrointestinal nematodes, *Haemonchus contortus.* Parasitology Research 109, 37–45.

Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., Weber, S. S., Wenger, A., Wieland-Berghausen, S., Goebel, T., Noelle Gauvry, T., Pautrat, F., Skripsky, T., Olivier Froelich, O., Komoin-Oka, C., Bethany Westlund, B., Ann Sluder, A., Ma¨ser, P., 2008. A new class of anthelmintics effective against drug resistant nematodes. Nature 452, 177180.

Kanazawa, A., Ikeda, T., Endo, T., 1994. Synthesis and antimicrobial activity of dimethyl- and trimethyl-substituted phosphonium salts with alkyl chains of various lengths. Antimicrobial Agents and Chemotherapy 38(5), 945-952.

Kaplan, R. M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends in Parasitology 20(10), 477-481.

Kaplan, R. M., Burke, R. M., Terrill, T. H., Miller, J. F., Getz, W. R., Mobini, S., Valencia, E., Williams, M. J., Williamson, L. H., Vatta, A. H., 2004. Validation of FAMACHA© eye colour chart for detecting clinical anaemia in sheep and goats on farms in the southern United States. Veterinary Parasitology 123, 105–120.

Kaplan, R. N., Vidyashankar, A. N., 2012. An inconvenient truth: Global warming and anthelmintic resistance. Veterinary parasitology 186, 7078.

Kasaian, J., Asili, J., Iranshahi, M., 2016. Sulphur-containing compounds in the essential oil of Ferula alliacea roots and their mass spectral fragmentation patterns. Pharmaceutical Biology 54(10), 2264-2268.

Katen, S. P., Chirapu, S. R., Finn, M. G., Zlotnick, A., 2010. Trapping of hepatitis B virus capsid assembly intermediates by phenylpropenamide assembly accelerators. ACS Chemical Biology 5(12), 1125–1136.

Kaufmann, J., 1996. Parasitic infections of Domestic Animals: A Diagnostic Manual. Library of Congress, Washington, USA.

Kaur, P., Wakode, S. R., 2016. Antimicrobial properties of Benzimidazole and Mannich bases of Benzimidazole: A review. International Journal of Science and Research 5(3), 762-772.

Kerboeuf, D., Riou, M., Guegnard, F., 2008. Flavonoids and related compounds in parasitic disease control. Mini-Review, Medicinal Chemistry 8,116–128.

Kettle, P. R., Vlassoff, A., Reid, T. C., Hotten, T. C., 1983. A survey of nematode control measures used by milking goat farmers and anthelmintics resistance on farms. New Zealand Veterinary Journal 31(8), 139143.

Ketzis, J. K. Vercruysse, J., Stromberg, B., Larsen, M., Athanasiadou, S., Houdijk, J., 2006. Evaluation of efficacy expectations for novel and non-chemical helminth control strategies in ruminants. Veterinary Parasitology 139, 321 – 335.

Kim, B. S., Moon, S. S., Hwang, B. K., 1999. Isolation, antifungal activity, and structure elucidation of the glutarimide antibiotic, streptimidone, produced by *Micromonospora coerulea*. Journal Agricultural and Food Chemistry 47(8), 3372–3380.

Kim, J. H., Chan, K. L., Mahoney, N., Campbell, B. C., 2011. Antifungal activity of redox-active benzaldehydes that target cellular antioxidation. Annals of Clinical Microbiology and Antimicrobials 10(23). http://doi.org/10.1186/1476-0711-10-23

Kim, M. K., Nam, P. W., Lee, S. J., Lee, K. G., 2014. Antioxidant activities of volatile and non-volatile fractions of selected traditionally brewed Korean rice wines. Journal of Institute of Brewing & Distilling 120, 537-542.

Kishimoto, N., Kakino, Y., Iwai, K., Mochida, K., Fujita, T., 2005. In vitro antibacterial, antimutagenic and anti-influenza virus activity of caffeic acid phenethyl esters. Biocontrol Science 10(4), 155-161.

Klongsiriwet, C., Quijada, J., Williams, A. R., Mueller-Harvey, I., Williamson, E. M., Hoste, H., 2015. Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed tannins. International Journal for Parasitology: Drugs and Drugs Resistance 5, 127-134.

Knight, T. L., Moss, R. A., Fraser, T. J., Rowarth, J. S., Burton, R. N., 1996. Effect of pasture species on internal parasites of lambs. Proceedings of the New Zealand Grassland Association 58, 59-62.

Knox, D. P., 2000. Development of vaccines in gastrointestinal nematodes. Parasitology 120, S43-S 61.

Knox, D. P., Smith, S. K., Smith, W. D., Murray, J. M., Redmond, D. L., 1995. Vaccines against helmintic parasites. International Patent Application No. WO 95;26402.

Knox, M. R., 2002. Effectiveness of copper oxide wire particles for *Haemonchus contortus* control in sheep. Australian Veterinary Journal 80, 224–227.

Knox, M. R., Besier, R, B., Le Jambre, L. F., Kaplan, R. M., Torres-Acosta, J. F. J., Miller, J., Sutherland, I., 2010. Novel approaches for the control of helminth parasites of livestock VI: Summary of discussions and conclusions. Veterinary Parasitology 181, 143149.

Knox, M. R., Torres-Acosta, J. F. J., Aguilar-Caballero, A. J., 2006. Exploiting the effect of dietary supplementation of small ruminants on resilience and resistance against gastrointestinal nematodes. Veterinary Parasitology 139, 385 – 393.

Koga, T, Moro, K., Matsudo, T., 1998. Antioxidative behaviors of 4-Hydroxy-2,5-dimethyl-3(2H)-furanone and 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone against lipid peroxidation. Journal. Agricultural and Food Chemistry 46(3), 946–951.

Krecek, R. C., Waller, P. J., 2006. Towards the implementation of the "basket of options" approach to helminth parasites control of livestock: Emphasis on the tropics and subtropics. Veterinary Parasitology 139. 270-282.

Kubola, J., Siriamornpun, S., 2008. Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts in vitro. Food Chemistry 110, 881–890.

Kumar, A., Mishra, A. K., 2015. Synthesis and antimicrobial activity of some new diphenylamine derivatives. Journal of Pharmacy & Bioallied Sciences 7(1), 81–85.

Kumar, N., Rao, T. K. S., Varghese, A., Rathor, V. S., 2012. Internal parasite management in grazing livestock. Journal of parasitic Diseases 37(2), 151-157.

Kumar, R., Joshi, Y. C., 2008. Synthesis and antimicrobial, antifungal and anthelmintic activities of 3H-1, 5-benzodiazepine derivatives. Journal of the Serbian Chemical Society 73(10), 937-943.

Kumaraswamy, M. N., Chandrashekhar, C., Shivakumar, H., Mathias, D. P., Mahadevan, K. M., Vaidya, V. P., 2008. Synthesis and activity evaluation of 2-(naphtho [2, 1-b] furan-2-yl-carbonyl)-3, 5-disubstituted-2, 3—dihydro-1H-pyrazoles. Indian Journal of Pharmaceutical Sciences 70(6), 175.

Kumsa, B., Abebe, G., 2009. Multiple anthelmintic resistance on a goat farm in Hawassa (southern Ethiopia). Tropical Animal Health Production 41, 655–662.

Kundu, S., Roy, S., Lyndem, L. M., 2014. Broad spectrum anthelmintic potential of Cassia plants. Asian Pacific Journal of Tropical Biomedicine 4(S1), S436-S441.

Kurita, N., Miyayi M., Kurane, R., Takahara, Y., 1981. Antifungal Activity of Components of Essential Oils. Agricultural and Biological Chemistry 45(4), 945-952.

Kyaw, B. M., Arora, S., Lim, C. S., 2012. Bacterial antibiotic-phytochemical combinations against methicillin resistant *Staphylococcus aureus.* Brazilian Journal of Microbiology 43(3), 938-945.

Lans, C., 2007. Comparison of plants used for skin and stomach problems in Trinidad and Tobago with Asian Ethnomedicine. Journal of Ethnomedicine and Ethnobiology 3(3), 1-12.

Lanusse, C., Alvarez, L., Lifschitz, A., 2014. Pharmacological knowledge and sustainable anthelmintic therapy in ruminants. Veterinary Parasitology 204, 18-33.

Lanusse, C., Lifschitz, A., Alvarez, L., 2015. Basic and clinical pharmacology contribution to extend anthelmintic molecules lifespan. Veterinary Parasitology 212, 35-46.

Lapshina, L. A., Reunov, A. V., Nagorskaya, V. P., Shestak, O. P., Novikov, V. L., 2010. A study of the antiviral activity of cyclopentene β,β′-triketone disodium salt in the leaves of two

Nicotiana tabacum L. cultivars infected with tobacco mosaic virus. Biology. Bulletin of the Russian Academy of Science 37, 537.

Larsen, M., 1999. Biological control of helminths. International Journal of Parasitology 29, 139–146.

Larsen, M., 2000. Prospects for controlling animal parasites by predacious micro fungi. Parasitology 120, S121–S131.

Larsen, M., 2006. Biological control of nematode parasites in sheep. Journal of Animal Science 84, E133–E139.

Lawrence, C. E., Leathwick, D. M., Rhodes, A. P., Jackson, R., Heuer, C., Pomroy, W. E., West, D. M., Waghorn, T. S., Moffat, J. R., 2007. Management of gastrointestinal nematode parasites on sheep farms in New Zealand. New Zealand Veterinary Journal 55, 228-234.

Le Jambre, L. F., Racliff, L. H., Whitlock, J. H., crofton, H. D., 1970. Polymorphism and egg-size in the sheep nematode, *Haemonchus contortus*. Proceedings of the Society for the study of Evolution 24, 625–631.

Learmount, J., Stephens, N., Boughtflower, V., Barrecheguren, A., Rickell, K., 2016. The development of anthelmintic resistance with best practice control of nematodes on commercial sheep farms in the UK. Veterinary Parasitology http://dx.doi.org/10.1016/j.vetpar.2016.09.006.

Leathwick, D. M., Besier, R. B., 2014. The management of anthelmintic resistance in grazing ruminants in Australasia—Strategies and experiences. Veterinary Parasitology 204, 44-54.

Leathwick, D. M., 2012. Modelling benefits of new class of anthelmintic in combination. Veterinary Parasitology 186, 93-100.

Leathwick, D. M., Pomroy, W. E., Heath, A. C., 2001. Anthelmintic resistance in New Zealand. New Zealand Veterinary Journal 49, 227-235.

Leathwick, D. M., Waghorn, T. S., Miller, C. M., Candy, P. M., 2012. Managing the anthelmintic resistance-use of a combination anthelmintic and leaving some lambs untreated to slow the development of resistance to ivermectin. Veterinary Parasitology 187, 285-294.

Lee, J. P., Moon, S. S., Hwang, B. K., 2003. Isolation and antifungal and antioomycete activities of Aerugine Produced by *Pseudomonas fluorescens* Strain MM-B16. Applied Environmental Microbiology 69(4), 2023–2031.

Leng, R. A., 1990. Factors affecting the utilization of 'poor-quality' forages by ruminants particularly under tropical conditions. Nutritional Research Reviews 2, 277 – 303.

Levine, N. D., 1968. Nematode Parasites of Domestic Animals and of Man. Minneapolis, Minnesota. Burgess Publishing Company.

Li, Y., Miller, J. E., Franke, D. E., 2001. Epidemiological observations and heterosis analysis of gastrointestinal nematode parasitism in Suffolk, Gulf Coast Native and crossbred lambs. Veterinary Parasitology 98, 273–283.

Liguori, L., Califano, R., Albanese, D., Raimo, F., Crescitelli, A., Di Matteo, M., 2017. Chemical Composition and Antioxidant Properties of Five White Onion (*Allium cepa* L.) Landraces. Journal of Food Quality, vol. 2017, 9 pages. Article ID 6873651, doi:10.1155/2017/6873651.

Lind, H., Broberg, A., Jacobsson, K., Jonsson, H., Schnürer, J., 2010. Glycerol enhances the antifungal activity of dairy propionibacteria. International Journal of Microbiology, volume 2010. Article ID 430873, 1-9.

Lindahl, I. L., Colglazier, M. L., Enzie, F. D., Turner, J. H., Whitmore, G. E., Wilson, R. L., 1970. Effect of management systems on the growth of lambs and development of internal parasitism: Field trials with lambs on soilage and pasture involving medication with N. F. and purified grades of phenothiazine. Journal of Parasitology 56, 991–999.

Longbottom, D., Redmond, D. L., Russell, M., Liddell, S., Smith, W. D., Knox, D. D., 1997. Molecular cloning and characterization of an aspartate proteinase associated with a highly protective gut membrane protein complex from adult *Haemonchus contortus*. Molecular and Biochemical Parasitology 88, 63–72.

López-Aroche, U., Salinas-Sánchez, D. O., Mendoza de Gives, P., López-Arellano, M. E., Liébano-Hernández, E., Valladares-Cisneros, G., Ariás-Ataide, D. M., Hernández-Veláquez, V., 2008. *In vitro* nematicidal effects of medicinal plants from the Sierra Huautla, Biosphere

reserve, Morelos, Mexico against *Haemonchus contortus* infective larvae. Journal of helminthology 82, 25-31.

Lorimer, S. D., Perry, N. B., Foster, L. M., Burgess, E., 1996. A nematode larval motility inhibition assay for screening plant extracts and plant products J. Agric. Food Chem. 44, 2842-2845.

Luoga, W., Mansur, F., Buttle, D. J., Duce, T. R., Carnett, M. C., Behnke, J. M., 2012. The anthelmintic efficacy of papaya latex in a rodent-nematode model is not dependent on fasting before treatment. Journal of Helmintology 86, 311-316.

Lyantagaye, S. L., 2011. Ethnopharmacological and phytochemical review of *Allium* species (sweet garlic) and *Tulbaghia* species (wild garlic) from Southern Africa. Tanzania Journal of Science 37, 58-72.

Maaz, M., Ahmad, A., Khan, S. A., Husain, A., 2015. Biological evaluation of 2-arylidene-4-(methoxyphenyl)but-3-en-4-olides. Journal of Biomedical and Pharmaceutical Research 4(5), 32-35.

Macedo, I. T. F., Bevilaquaa, C. M. L., de Oliveiraa, L. M. B., Camurça-vasconcelos, A. L. F., Vieira, Luiz da S., Oliveiraa, F. R., Queiroz-Juniora, E. M., Toméa, Adriana da R., Nascimento, N. R. F., 2010. Anthelmintic effect of Eucalyptus straigeriana essential oil against goat gastrointestinal nematodes. Veterinary Parasitology 173, 9398.

Madalaa, N. E., Piater, L., Duberya, I., Steenkamp, P., 2016. Distribution patterns of flavonoids from three Momordica species by ultra-high performance liquid chromatography quadrupole time of flight mass spectrometry: a metabolomic profiling approach. Revista Brasileira de Farmacognosia 26, 507–513.

Madhavi, K., Sree Ramya, G., 2017. Synthesis, antioxidant and anti-inflammatory activities of Ethyl 2-(2-cyano-3- (substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates. Asian Journal Pharmaceutical and Clinical Research 10(7), 95-100.

Mahboobi, S., Eichhorn, E., Winkler, M., Sellmer, A, Möllmann, U., 2008. Antibacterial activity of a novel series of 3-bromo-4-(1H-3-indolyl)-2,5-dihydro-1H-2,5-pyrroledione derivatives – An extended structure–activity relationship study. European Journal of Chemistry 43(2), 633-656.

Maingi, N., Bjørn, H., Thamsborg, S. M., Dangolla, A., Kyvsgaard, N. C., 1996. Worm control practices on sheep farms in Denmark and implications for the development of anthelmintic resistance. Veterinary Parasitology 66(1-2), 39-52.

Makkar, H. P. S., 2006. Chemical and biological assays for quantification of major plant secondary metabolites. In: Sandoval-Castro, C. A., DeB Hovell, F. D., Torres-Acosta, J. F. J. and Ayala-Burgos (Eds.), Herbivores, assessment of intake, digestibility and the roles of secondary compounds. Nottingham University Press, Nottingham, pp. 235-249.

Makkar, H. P. S., Francis, G., Becker, K., 2007. Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. The Animal Consortium 1(9), 1371-1391.

Makovitzki, A., Avrahami, D., Shai, Y., 2006. Ultrashort antibacterial and antifungal lipopeptides. Proceedings of the National Academy of Sciences of the United States of America 1039430, 15997-16002.

Malan, F. S., Van Wyk, J. A., Wessels, C. D., 2001. Clinical evaluation of anaemia in sheep: early trials. Onderstepoort Journal of Veterinary Research 68, 165-174.

Manado, C. H. M., Carencro, P. L. S., Affonso, P. R. A. M., Souza Jr, A. A. O., Sarmento, J. L. R., 2009. Growth curves in Dorper sheep crossed with the local Brazilian breeds, Morada Nova, Rabo Largo, and Santa Inês. Small Ruminant Research 84, 16-21.

Mander, M., Mander, J., Breen, C. M., 1996. Promoting the cultivation of indigenous plants for markets: Experience from KwaZulu-Natal, South Africa. INR Occasional paper No. 167. Institute of Natural Resources, Pietermaritzburg.

Mandonnet, N., Aumont, G., Fleury, J., Arquet, R., Varo, H., Gruner, L., Bouix, J., Khang, J. V., 2001. Assessment of genetic variability of resistance to gastrointestinal nematode parasites in Creole goats in the humid tropics. Journal Animal Science 79, 1706–1712.

Maphosa, V., Masika, P. J., 2010. Ethnoveterinary uses of medicinal plants: A survey of plants used in the ethnoveterinary control of gastrointestinal parasites of goats in the Eastern Cape, South Africa. Pharmaceutical Biology 48(6), 697-702.

Mares, D., Romagnoli, C., Rossi, R., Carpita, A., Ciofalo, M., Bruni, A., 1994. Antifungal activity of some 2,2':5',2''-terthiophene derivatives. Mycoses 37(9-10), 377-383.

Marley, C. L., Cook, R., Keatinge, R., Barrett, J., Lampkin, A. H., 2003. The effect of birdfoot trefoil (Lotus corniculatus) and Chicory (Cichorium intybus) on parasite intensities and performance of lambs naturally infected with helminth parasites. Veterinary Parasitology 112(1), 147-155.

Marson, C. M., 2011. New and unusual scaffolds in medicinal chemistry. Chemical Society Review 40, 5514–5533.

Marwat, S. K., Fazal-Ur-Rehman, Khan, M. A., Ahmad, M., Zafar, M., Ghulam, S., 2011. Medicinal Folk recipes used as traditional phytotherapies in district Dera Ismail Khan, KPK, Pakistan. Pakistan Journal of Botany 43(3), 1453-1462.

Masika, R. J., Van Averbeeke, W., Sonandi, A., 2000. Use of herbal remedies by small-scale farmers to treat livestock diseases in Central Eastern Cape Province, South Africa. Journal of the South African Veterinary Association 71, 87-91.

Matika, O., Nyoni, S., Van Wyk, J. B., Erasmus, G. J., Baker, R. L., 2003. Resistance of Sabi and Dorper ewes to gastro-intestinal nematode infections in an African semi-arid environment. Small Ruminant Research 47, 95–102.

McCorkle, M., Mathias, E., van Schillhorn Veen, T. W., 1996. Ethnoveterinary research and development. IT studies in indigenous knowledge and development intermediate technology publications. Southampton Row, London, pp 1–23.

McGaw, J., Eloff, J. N., 2005. Screening of sixteen poisonous plants for antibacterial, anthelmintics and cytotoxic activity in vitro. South African Veterinary Association 76, 302306.

McGaw, J., Eloff, J. N., 2008. Ethnoveterinary use of South African plants and scientific evaluation of their medicinal properties. Journal of Ethnopharmacology 119, 559574.

McGaw, L. J., Jäger, A. K., van Staden, J., 2000. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72, 247-263.

McKellar, Q. A., Jackson, F., 2004. Veterinary anthelmintics: old and new. Trends in Parasitology 20(10), 456 – 461.

McMahon, C., Bartley, D. J., Edgar, H. W. J., Ellison, S. E., Barley, J. P., Malone, F. E., Hanna, R. E. B., Brennan, G. P., Fairweather, I., 2013. Anthelmintic resistance in Northern

Ireland (I): prevalence of resistance in ovine gastrointestinal nematodes, as determined through faecal egg count reduction testing. Veterinary Parasitology 195, 122–130.

Mederos, A. E., Ramos, Z., Banchero, G. E., 2014. First report of monepantel *Haemonchus contortus* resistance on sheep farms in Uruguay. Parasites and Vectors 7, 1-4.

Meenakshisundaram, A., Harikrishnan, T. J., Thavasi, A., 2017. Anthelmintic evaluation of Cucurbita pepo against gastrointestinal nematodes of sheep. Indian Journal of Animal Research 51(3), 518-524.

Menghini, L., Leporini, L., Scanu, N., Pintore, G., La Rovere, R., Di Filippo, E. S., Pietrangelo, T., Fulle, S., 2011. Journal of biological regulators and homeostatic agents 25(1), 27-35.

Michel, J. F., 1985. Strategies for the use of anthelmintics in livestock and their implications for the development of drug resistance. Parasitology 90, 621-628.

Miller, J. E., 1996. Controlling goat parasites in the Southeast. In: Proceedings of the Southeast Regional Meat Goat Production Symposium: Meat Goat Production in the Southeast—Today and Tomorrow, 21–24 February 1996, A&M University, Tallahassee, Florida, pp. 80–82.

Miller, J. E., Bahirathan, M., Lemarie, S. L., Hembry, F. G., Kearney, M. T., Barras, S. R., 1998. Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep with special emphasis on relative susceptibility to *Haemonchus contortus* infection. Veterinary Parasitology 74, 55–74.

Miller, J. E., Bishop, S. C., Cockett, N. E., McGraw, R. A., 2006. Segregation of natural and experimental gastrointestinal nematode infection in F2 progeny of susceptible Suffolk and resistant Gulf Coast Native sheep and its usefulness in assessment of genetic variation. Veterinary Parasitology 140, 83–89.

Min, B. R., Hart, S. P., 2003. Tannins for suppression of internal parasites. Journal of Animal Science 81 (E. S2), E102–E109.

Min, B. S., Na, M. K., Oh, S. R., Ahn, K. S., Jeong, G. S., Li, G., Lee, S. K, Joung, H., Lee, H. K., 2004. New furofuran and butyrolactone lignans with antioxidant activity from the stem bark of *Styrax japonica*. Journal of Natural Products 67(12), 1980-1984.

Mirdeilami, S. Z., Barani, H., Mazandarani, M., Heshmatic, Gh. A., 2011. Ethnopharmoclogical survey of medicinal plants in Maraveh Tappeh region, north Iran. Iranian Journal of plant physiology 1(2), 327-338.

Miyazawa, M., Usami, A., 2014. Character impact odorants from mushrooms [*Pleurotus citrinopileatus*, *Pleurotus eryngii* var. ferulae, Lactarius hatsudake, and Hericium erinaceus (Bull.: Fr.) Pers.] used in Japanese traditional food. Nagoya Gaknin University 50(2), 1–24.

Mnayer, D., Fabiano-Tixier, A. S., Petitcolas, E., Hamieh, T., Nehme, N., Ferrant, C., Xavier Fernandez, X., Chemat, F., 2014. Chemical composition, antibacterial and antioxidant activities of six essentials oils from the Alliaceae family. Molecules 19, 20034-20053.

Mohamed, H. A., Abdel-Latif, E., Abdel-Wahab, B. F., Awad, G. E. A., 2013. Novel antimicrobial agents: fluorinated 2-(3-(Benzofuran-2-yl) pyrazol-1-yl) thiazoles. International Journal of Medicinal Chemistry, vol. 2013, Article ID 986536, 6 pages. doi:10.1155/2013/986536

Mohammad Asif Iqbal, C., Satyendra, D., Apurba, T., Patel, M., Monika, K., Girish, K., Mohan, S., Saravanan, J., 2012. Synthesis and Antimicrobial screening of some Novel Substituted Thiophenes. Hygeia Journal for drugs and medicines 4(1), 112-118.

Molan, A. L., Waghorn, G. C., Min, B. M., McNabb, W. C., 2000. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration in Vitro. Folia Parasitologica 47, 39-44.

Montazeri, N., Oliveira, A. C. M., Himelbloom, B. H., Leigh, M. B., Crapo, C. A., 2013. Chemical characterization of commercial liquid smoke products. Food Science and Nutrition 1(1), 102-115.

Morales-López, J., Centeno-Álvarez, M., Nieto-Camacho, A., López, M. G., Pérez-Hernández, E., Pérez-Hernández, N., FernándezMartínez, E., 2017. Evaluation of antioxidant and hepatoprotective effects of white cabbage essential oil, Pharmaceutical Biology 55(1), 233-241.

Morgan, E. R., Lundervold, M., Medley, G. F., Shaikenov, B. S., Torgerson, E. J., Milner-Gulland, 2006. Assessing risk of disease transmission between wildlife and livestock: The Saiga antelope as a case study. Biological Conservation 131. 244254.

Morris, C. A., 1998. Response for selection to disease resistance in sheep and cattle in New Zealand and Australia. In: Proceedings of the sixth World Congress on Genetics Applied to Livestock Production 27, 295–302.

Mortensen, L. L., Williamson, L. H., Terrill, T. H., Kircher, R. A., Larsen, M., Kaplan, R. M., 2003. Evaluation of prevalence and clinical implications of anthelmintic resistance in gastrointestinal nematodes in goats. Journal of American Veterinary Medical Association 223, 495–500.

Mottier, M. D., Prichard, R. K., 2008. Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on *Haemonchus contortus*. Pharmacogenet. Genomics 18, 129-140.

Mrityunjay Banerjee, Banerjee, R., Kumar, H. K. S., 2012. Medicinal significance of furan derivatives: a review. International Journal of Review in Life Sciences 2(1), 7-16.

Mugambi, J. M., Bain, R. K., Wanyangu, S. W., Ihiga, M. A., Duncan, J. L., Murray, M., Stear, M. J., 1997. Resistance of four sheep breeds to natural and subsequent artificial *Haemonchus contortus* infection. Veterinary Parasitology 69, 265–273.

Muir, J. P., 2011. The multi-faceted role of condensed tannins in goat ecosystem. Small Ruminant Research 98, 115-120.

Mukaratirwa, S., Charakupa, R., Hove, T., 1997. A survey of anthelmintic resistance on ten sheep farms in Mashonaland East Province, Zimbabwe. Journal of South African Veterinary Association 68, 140–143.

Mulcahy, G., O'neil, S., Donelly, S., Dalton, J. P., 2004. Helminths at mucosal barriers-interaction with the immune system. Advanced Drug Delivery Reviews 56, 853-868.

Mwamachi, D. M., Audho, J. O., Thorpe, W., Baker, R. L., 1995. Evidence for multiple anthelmintic resistance in sheep and goats reared under the same management in coastal Kenya. Veterinary Parasitology 60, 303–313.

Nair, J. J., Machocho, A. K., Campbell, W. E., Brun, R., Viladomat, F., Cordina, C., Bastida, J., 2000. Alkaloids from *Crinum macowanii*. Phytochemistry 54(8), 945-950.

Nalawade, T. M., Bhat, K., Sogi, S. H. P., 2015. Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. Journal of International Society of Preventive and Community Dentistry 5(2), 114–119.

Nansen, P., Larsen, M., Roepstor, A., Grùnvold, J., Wolstrup, J., Henriksen, S. A., 1996. Control of *Oesophagostomum dentatum* and *Hyostrongylus rubidus* in outdoor-reared pigs through daily feeding with the microfungus *Duddingtonia flagrans*. Parasitology Research 82, 580-584.

Ndagijimana, M., Vallicelli, M., Cocconcelli, P. S., Cappa, F., Patrignani, F., Lanciotti, R., Guerzoni, M. E., 2006. Two 2[5H]-Furanones as possible signaling molecules in *Lactobacillus helveticus*. Applied and Environmental Microbiology 72(9), 6053–6061.

Negi, J. S., Bisht, V. K., Bhandari, A. K., Singh, P., Sundriyal, R. C., 2011. Chemical constituents and biological activities of the genus Zanthoxylum: A review. African Journal of Pure and Applied Chemistry 5(12), 412-416.

Nejad, S. M., Özgüneş, H., Başaran, N., 2017. Pharmacological and toxicological properties of Eugenol. Turkish Journal of Pharmaceutical Sciences 14(2), 201-206.

Newman, M. B., Arendash, G. W., Shytle, R. D., Bickford, P. C., Tighe, T., Sanberg, P. R., 2002. Nicotine's oxidative and antioxidant properties in CNS. Life Science 71(24), 2807-2820.

Newton, S. E., Munn, E. A., 1999. The development of vaccines against gastro-intestinal nematodes, particularly *Haemonchus contortus*. Parasitology today 15(3), 116122.

Ngarmsak, M., Delaquis, P., Toivonen, P., Ngarmsak, T., Ooraikul, B., Mazza, G., 2006. Antimicrobial activity of vanillin against spoilage microorganisms in stored fresh-cut mangoes. Journal of Food Protection 69(7), 1724-1727.

Nirmale, S. A., Shelke, S. M., Gagare, P. B., Jadhav, P. R., Dethe, P. M., 2007. Antinociceptive and anthelmintic activity of *Canna indica.* Natural product research 21(12), 1042-1047.

Nogareda, C., Mezo, M., Uriarte, J., Lloveras, J., Cordero Del Campillo, M., 2006. Dynamix of Infestation of Cattle and Pasture by Gastrointestinal Nematode Parasites in an Atlantic Temperate Environment. Journal of Veterinary Medicine, B53, 439 - 444.

Ntalli, N. G., Vargiu, S., Menkissoglu-Spiroudi, U., Caboni, P., 2010. Nematicidal Carboxylic Acids and Aldehydes from Melia azedarach Fruits. Journal of Agricultural and food Chemistry 58, 11390–11394.

O'Connor, L. J., Walkden-Brown, S. W., Kahn, L. P., 2006. Ecology of the free-living stages of major trichostrongylid parasites of sheep. Veterinary Parasitology 142(1/2), 1–15.

Odoi, A., Gathuma, J. M., Gachuiri, C. K., Omore, C., 2007. Risk factors of gastrointestinal nematode parasite infections in small ruminants kept in smallholder mixed farms in Kenya. BMC Veterinary Research 3, 6.

Olivier, D. K., 2012. Ethnobotany and chemistry of South African traditional tonic plants. Ph. D. thesis in Botany, Faculty of Science, University of Johannesburg.

Omoruyi, B. E., Afolayan, A. J., Bradley, G., 2014. Chemical composition profiling and antifungal activity of the essential oil and plant extracts of *Mesembryanthemum edule* (L.) bolus leaves. African Journal of Traditional Complementary Alternative Medicine 11(4), 19-30.

Ortu, E., 2015. Anthelmintic evaluation of selected phytochemicals in dairy ewes. Doctoral thesis, Università Degli Studi Di Sassari, Scuola Di Dottorato Di Ricerca, Scienze e Biotecnologie dei Sistemi Agrari e Forestali e delle Produzioni Alimentari, Indirizzo Scienze e Tecnologie Zootecniche.

Ortu, E., Sanna, G., Scala, A., Pulina, G., Caboni, P., Battacone, G., 2016. In vitro anthelmintic activity of active compounds of the fringed rue *Ruta chalepensis* against dairy ewe gastrointestinal nematodes. Journal of Helminthology. Doi: 10.1017/S0022149x16000419.

Osbourn, A. E., Lanzotti, V. (Eds) 2009. Plant derived Natural Products, Synthesis, Function, and Application. Springer, Verlag, Preface.

Over, H. J., Jansen, J., Van Olm, P. W., 1992. Distribution and impact of helminth diseases of livestock in developing countries. FAO animal production and health paper 96, Lelystad, Netherlands.

Owens, F. N., Dubeski, P., Hanson, C. F., 1993. Factors that alter the growth and development of ruminants. Journal of Animal Science 71, 3138-3150.

Owokotomo, I. A., Ekundayo, O., Oladosu, I. A., Aboaba, S. A., 2012. Analysis of the essential oils of leaves and stems of *Crassocephalum crepidioides* growing in South Western Nigeria. International Journal of Chemistry 4(2), 34-37.

Ozaraga, B. P., Ozaraga, M. S. I., Barrios, M. B., 2015. Ethnobotanical Dewormer Composition for Free Range Native Chickens. Mindanao Journal of Science and Technology 13, 12-19.

Özden, S., Atabey, D., Yıldızb, S., Gökera, H., 2005. Synthesis and potent antimicrobial activity of some novel methyl or ethyl 1H-benzimidazole-5-carboxylates derivatives carrying amide or amidine groups. Bioorganic & Medicinal Chemistry 13, 1587–1597.

Pala, K., Reddy, S. M., Reddy, B. N., Pathak, M., Reddy, C. N., 2016. Synthesis and Biological evaluation of novel (4-chlorophenyl) ((1R, 3r, 5S)-3- (phenyl amino)-8-aza-bicyclo [3.2.1] octan-8-yl) methanone derivatives. Der Pharma Chemica 8(14), 67-72.

Palani, S., Raja, S., Kumar, R. P., Selvaraj, R., Kumar, B. S., 2011. Evaluation of phytoconstituents and anti-nephrotoxic and antioxidant activities of *Monochoria vaginalis*. Pakistan Journal of Pharmaceutical Sciences 24(3), 293-301.

Paolini, V., Bergeaud, J. P., Grisez, C., Prevot, F., Dochies, P., Hoste, H., 2003b. Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. Veterinary Parasitology 113(3), 253-261.

Paolini, V., Frayssines, A., De La Farge, F., Dorchies, P., Hoste, H., 2003a. Effects of condensed tannins on established populations and on in coming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. Veterinary Research 34(3), 331-339.

Papachristou, T. G., Nastis, A. S., Mathur, R., Hutchings, A. R., 2003. Effect of physical and chemical plant defences on herbivory: implications for Mediterranean shrub land management. Basic and Applied Ecology 4, 141156.

Papadopoulos, E., Himonasa, C., Coles, G. C., 2001. Drought and flock isolation may enhance the development of anthelmintic resistance in nematodes. Veterinary parasitology 97, 253-259.

Pasricha, V., Satpathy, G., Gupta, R. K., 2014. Phytochemical & Antioxidant activity of underutilized legume *Vicia faba* seeds and formulation of its fortified biscuits. Journal of Pharmacognosy and Phytochemistry 3(2), 75-80.

Patani, G. A., LaVoie, E. J., 1996. Bioisosterism: A Rational Approach in Drug Design. Chemical Reviews 96(8 3149), 3147−3176.

Paulitz, T., Nowak-Thompson, B., Gamard, P., Tsang, E., Loper, L., 2000. A novel antifungal furanone from *Pseudomonas aureofaciens*, a biocontrol agent of fungal plant pathogens. Journal of Chemical Ecology 26(6), 1515-1524.

Pavia, C. S., Pierre, A., Nowakowski, J., 2000. Microbial activity of nicotine against a spectrum of bacterial and fungal pathogens. Journal of Medical Microbiology 49, 675-676.

Pena˜, M. T., Miller, J. E., Horohov, D. W., 2004. Effect of dexamethasone treatment on the immune response of Gulf Coast Native lambs to *Haemonchus contortus* infection. Veterinary Parasitology 119, 223–235.

Peng, L. X., Zou, L., Wang, J. B., Zhao,J. L., Xiang, D. B., Zhao, G., 2015. Flavonoids, Antioxidant activity and aroma compounds analysis from different kinds of tartary buckwheat tea. Indian Journal of Pharmaceutical Sciences 77(6), 661–667.

Perry, B. D, Randolph, R. F., McDermott, J. J., Sones, K. R., Thornton, P. K., 2002. Investing in Animal Health Research to Alleviate Poverty. International Livestock Research Institute (IlRI) Nairobi Kenya, p. 148.

Perry, B. D., Randolph, T. F., 1999. Improving the assessment of the economic impact of parasitic diseases and their control in production animals. Veterinary Parasitology 84, 145-168.

Perry, L.M., Metzger, J., 1980. Medicinal plants of East and Southeast Asia: attributed properties and uses. MIT Press, Cambridge, Massachusetts and London, pp 620.

Phaechamud, T., Mahadlek, J., Charoenteeraboon, J., Choopun, S., 2012. Characterization and antimicrobial activity of N-Methyl-2-pyrrolidone-loaded Ethylene Oxide-Propylene Oxide Block Copolymer Thermosensitive Gel 74(6), 498-504.

Pharmaceutical Society of Great Britain. Department of Pharmaceutical Sciences, 1965. **British veterinary codex**, 1965: Prepared in the Department of Pharmaceutical Sciences and published by the direction of the Council of the Pharmaceutical Society of Great Britain. London: Pharmaceutical Press.

Pharmaceutical Society of Great Britain. Department of Pharmaceutical Sciences, 1953. **British veterinary codex**, 1965: Prepared in the Department of Pharmaceutical Sciences and published by the direction of the Council of the Pharmaceutical Society of Great Britain. London: Pharmaceutical Press.

Piedrafita, D., Raadsma, H. W., Gonzalez, J. and Meeusen, E., 2010. Increased production through parasite control: can current breeds of sheep teach us new lessons? Trends in Parasitology 12(26), 568573.

Ploeger, H. W., Kloosterman, A., 1993. Gastrointestinal nematode infections and weight gain in the dairy replacement stock: first-year calves. Veterinary Parasitology 46, 223241.

Politeo, O., Jukié, M., Miloš, M., 2007. Chemical composition and antioxidant activity of free volatile aglycones from Laurel (Laurus nobilis L.) Compared to Its Essential Oil. Croatica Chemica Acta 80(1), 121-126.

Porter, L. J., Hrstich, L. N., Chan, B. G., 1986. The conversion of procyanidins and prodelphinidins to cyanidins and delphinidins. Phytochemistry 25, 223-230.

Pour, M., Spulák, M., Balsánek, V., Kunes, J., Buchta, V., Waisser, K., 2000. 3-Phenyl-5-methyl-2H,5H-furan-2-ones: tuning antifungal activity by varying substituents on the phenyl ring. Bioorganic and Medicinal Chemistry Letters 10(16), 1893-1895.

Prichard, R. K., 1990. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. International Journal of Parasitology 20, 515 – 523.

Prichard, R. K., Hall, C. A., Kelly, J. D., Martin, I. C. A., Donald, A. D., 1980. The problem of anthelmintic resistance in nematodes. Australian Veterinary Journal 56(5), 239–251.

Pyun, M. –S., Shin, S., 2006. Antifungal effects of the volatile oils from Allium plants against Trichophyton species and synergism of the oils with ketoconazole. Phytomedicine 13, 394-400.

Quijada, J., Fryganas, C., Ropiak, H. M., Ramsay, A., Mueller-Harvey, I., Hoste, H., 2015. Anthelmintic activity against *Haemonchus contortus* or *Trichostrongylus colubriformis* from small ruminants are influenced by structural features of condensed tannins. Journal of Agricultural and Food chemistry 63(28), 6246-6354.

Rajendiran, S., Park, G., Yoon, S., 2017. Direct Conversion of Propylene Oxide to 3-Hydroxy butyric acid using a Cobalt carbonyl ionic liquid catalyst. Catalysts 7, 228.

Rami, C., Patel, L., Patel, C. N., Parmar, J. P., 2013. Synthesis, antifungal activity, and QSAR studies of 1,6-dihydropyrimidine derivatives. Journal of Pharmacy & Bioallied Sciences 5(4), 277–289.

Rampadarath, S., Puchooa, D., Ranghoo-Sanmukhiya, 2014. A comparison of polyphenolic content, antioxidant activity and insecticidal properties of Jatropha species and wild *Ricinus communis* L. found in Mauritius. Asian Pacific Journal of Tropical Medicine 7(S1), S384-S390.

Rana, S., Blowers, E. C., Tebbe, C., Contreras, J. I., Radhakrishnan, P., Smitha Kizhake, S., Zhou, T., Rajule, R. N., Arnst, J. L., Munkarah, A. R., Rattan, R., Natarajan, A., 2016. Isatin derived spirocyclic analogues with α-Methylene-γ- butyrolactone as anticancer agents: A structure–activity relationship study. Journal of Medicinal Chemistry 59(10), 5121–5127.

Rangaswamy, J., Kumar, H. V., Harini, S. T., Naik, N., 2017. Functionalized 3-(benzofuran-2-yl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole scaffolds: A new class of antimicrobials and antioxidants. Arabian Journal of Chemistry 10(S2), S2685-S2696.

Razavi-Rohani, S. M., Griffiths, M. W., 1999. Antifungal effects of sorbic acid and propionic acid at different pH and NaCl conditions. Journal of Food Safety 19, 109-120.

Refaat, J., Kamel, M. S., Ramadan, M. A., ALI, A. A., 2012. Crinum; An endless source of bioactive principles: A review. Part 1- Crinum alkaloids: Lycorine-type alkaloids. International Journal of pharceutical Sciences and research 3(7), 1883–1890.

Ress, S. B., Harborne, J. B., 1985. The role of sesquiterpene lactones and phenolics in the chemical defence of the chicory plant. Phytochemistry 24, 2225-2231.

Revathi, P., Jeyaseelansenthinath, T., Thirumalaikolundhusubramaian, P., 2014. Preliminary Phytochemical screening and GC-MS analysis of ethanolic extract of mangrove plant-*Bruguiera cylindrica* (Rhizho) L. International Journal of Pharmacognosy and Phytochemical Research 6(4), 729-740.

Rialch, A., Vatsya, S., Kumar, R. R., 2013. Detection of benzimidazole resistance in gastrointestinal nematodes of sheep and goats of sub-Himalayan region of northern India using different tests. Veterinary Parasitology 198, 312–318.

Rice-Evans, C. A., Miller, N, J., Paganga, G., 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2(4), 152-159.

Rochfort, S., Parker, A. J., Dunshea, F. R., 2008. Plant bioactives for ruminant health and productivity. Phytochemistry 69, 299-322.

Roeber, F., Jex, A. R., Gasser, R. B., 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. Parasites and Vectors 6(153), 1-13.

Rose, H., Caminade, C., Bolajoko, M. B., Phelan, P., van Dijk, J., Baylis, M., Williams, D., Morgan, E. R., 2016. Climate-driven changes to the spatio-temporal distribution of the parasitic nematode, *Haemonchus contortus*, in sheep in Europe. Global Change Biology 22, 1271–1285.

Ross, I. A., 2003. Medicinal plants of the world: Chemical constituents, traditional and modern medicinal uses. Humana Press Inc. 2nd ed. Totowa New Jersey. pp. 33-102.

Rossanigo, C. E., Gruner, L., 1994. Relative effect of temperature and moisture on the development of strongyle eggs to infective larvae in bovine pats in Argentina. Veterinary Parasitology 35(3), 317–325.

Rout, P. K., Chauhan, K. K., Matika, O., Bishop, S. C., 2011. Exploring the genetic resistance to natural gastrointestinal nematode infection in Indian goats. Veterinary Parasitology 180, 315–322.

Ryssel, F., Kloeters, O., Gemman, G., Schafer, Th., Wiedemann, G., Oehlbauer, M., 2009. The antimicrobial effect of acetic acid—An alternative to common local antiseptics? Burns 35(5), 695-700.

Saddiqi, H. A., Iqbal, Z., Khan, M. N., Muhammad, G., 2010. Comparative resistance of sheep breeds to *Haemonchus contortus* in a natural pasture infection. International Journal Agricultural Biology 12, 739–743.

Saeed, M., Iqbal, Z., Jabbar, A., Masood, S., Babar, W., Saddiqi, H. A., Arshad, M., 2010. Multiple anthelmintic resistance and the possible contributory factors in Beetal goats in an irrigated area (Pakistan). Research in Veterinary Science 88, 267–272.

Saikachi, H., Matsuo, J., 1966. Synthesis of the Furan Derivatives. XXXIV. Preparation of 2, 3-Bis-(5-nitro-2-furyl)pyrazine derivatives. Yakugaku Zasshi 86(10), 927-932.

Saito, N., Suzuki, F., Sasaki, K., Ishida, N., 1976. Antiviral and interferon-inducing activity of a new glutarimide antibiotic, 9-Methylstreptimidone. Antimicrobial Agents and Chemotherapy 10(1), 14-19.

Sajid, M., McKerrow, J. H., 2002. Cysteine proteases of parasitic organisms. Molecular and Biochemical Parasitology 120, 1–21.

Salahuddin, Shaharyar, M., Mazunder, A., 2017. Benzimidazoles: A biologically active compounds. Arabian Journal of Chemistry 10, S157-S173.

Salat, K., Librowski, T., Moniczewski, A., Stanisz-Wallis, K., Wieckowski, K., Malawska, B., 2012. Analgesic, antioedematous and antioxidant activity of γ-butyrolactone derivatives in rodents. Behavioural Pharmacology 23(4), 407-416.

Sampaio, B. L., Edrada-Ebel, R., Da Costa, F. B., 2016. Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*: a model for environmental metabolomics of plants. Scientific Reports 6, 29265. Doi. Org/10.1038/srep29265.

Sánchez-Oritz, B. L., Sánchez-Fernández, R. E., Duarte, G., Lappe-Oliveras, P., Maciás-Rubalcava, M. L., 2016. Antifungal, anti-oomycete and phytotoxic effects of volatile organic compounds from the endophytic fungus *Xylaria* sp. Strain PB3f3 isolated from *Haematoxylon brasilettto*. Journal of Applied Microbiology 120, 1313-1325.

Sangster, N. C., Dobson, R. J., 2002. Anthelmintic resistance. In Biology of nematodes (Lee, D. L., 2002), pp531-567, Taylor and Francis.

Sanmartín-Suárez, C., Soto-Otero, R., Sánchez-Sellero, I., Méndez-Álvarez, E., 2011. Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. Journal of Pharmacological and Toxicological Methods 63(2), 209-215.

Santoyo, S., Plaza, M., Jaime, L., Ibañez, E., Reglero, G., Señorans, F. J., 2010. Pressurized liquid extraction as an alternative process to obtain antiviral agents from the edible microalga Chlorella vulgaris. Journal of Agricultural Food and Chemistry 58(15), 8522-8527.

Sargison, N. D., 2016a. Keys to solving health problems in small ruminants; Athelmintic resistance as a threat to sustainable nematode control. Small Ruminant Research 142, 11-15.

Sargison, N., 2016b. New worming guidelines: Quarantine anthelmintic treatments. United Kingdom Veterinary 11(5), 57-61.

SAS, 2000. Statistical Analysis System user guide (Version 8). SAS Institute Inc., SAS Campus Drive, Cary, N. C., USA.

Sasaki, O., Tani, S., 1997. Chemical and biological control of land dwelling Leech *Heamadipsa japonica.* Medical Entomology and Zoology 48, 303-309.

Savioli, L., Albonico, M., Engels, D., Montresor, A., 2004. Progress in the prevention and control of schistosomiasis and soil-transmitted helminthiasis. Parasitology International 53, 103-113.

Sawant, R., Kawade, D., 2011. Synthesis and biological evaluation of some novel 2-phenyl benzimidazole-1-acetamide derivatives as potential anthelmintic agents, Acta Pharmaceutica 61, 353–361.

Saxena, M., Saxena, J., Nema, R., Singh, D., Gupta, A., 2013. Phytochemistry of medicinal plants. Journal of Pharmacognosy and Phytochemistry 6(1), 168-182.

Sayers, G., Hanrahan, J. P., Good, B., Ryan, M., Angles, J.M., Sweeney, T., 2005. Major histocompatibility complex DRB1 locus: its role nematode resistance in Suffolk and Texel sheep. Parasitology 131, 1–7.

Schillhorn van Veen, T. W., 1997. Sense or nonsense? Traditional methods of animal parasitic disease control. Veterinary Parasitology 71, 177–194.

Schwab, W., 2013. Natural 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol®). Molecules 18, 6936-6951.

Scogings, P. F., Dziba, L. E., Gordon, I. J., 2003. Leaf chemistry of wooden plants in relation to seasons, canopy retention and goat browsing in a semiarid subtropical savannah. Austral Ecology 29, 278-286.

Scogings, P. F., Hattas, D., Skarpe, C., Hjältén, J., Dziba, L., Zobolo, A., Rooke, T., 2015. Seasonal variations in nutrients and secondary metabolites in semi-arid savannas depend on year and species. Journal of Arid Environments 114, 54-61.

Scott, I., Pomroy, W., Kenyon, P., Smith, G., Adlington, B., Moss, A., 2013. Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Veterinary Parasitology 198, 166-171.

Sendl, A., Chen, J. L., Jolad, S. D., Stoddart, C., Razhon, E., Kernan, M., Nanakorn, W., Balick, M., 1996. Two new Naphthoquinones with antiviral activity from *Rhinacanthus nasutus*. Journal of Natural Products 59(8), 808-815.

Shaik, S. A., Terrill, T H., Miller, J. E., Kouakou, B., Kannan, G., Kaplan, R. M., Burke, J. M., Mosjidis, J. A., 2006. *Sericea lespedeza* hay as a natural deworming agent against gastrointestinal nematode infection in goats. Veterinary Parasitology 139, 150–157.

Shakya, K. P., Miller, J. E., Horohov, D. W., 2009. A Th2 type of immune response is associated with increased resistance to *Haemonchus contortus* in naturally infected Gulf Coast Native lambs. Veterinary Parasitology 163, 57–66.

Shalaby, H. A., 2013. Anthelmintic Resistance; How to overcome it? Iranian Journal of Parasitology 8(1), 18-32.

Shehab, W. S., Sherif, M. H., Assy, M. G., 2016. Synthesis of some 3-indolyl heterocycles and evaluation of its antimicrobial activity. Iranian Journal of Organic Chemistry 8(3), 1851-1858.

Shirey, K. A., Nhu, Q. M., Yim, K. C., Roberts, Z. J., Teijaro, J. R., Farber, D. L., Blanco, J. C., Vogel, S. N., 2011. The anti-tumor agent, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), induces IFN-β-mediated antiviral activity in vitro and in vivo. Journal of Leukocyte Biology 89(3), 351-357.

Shrestha, B., Banerjee, J., Yadav, P. K., Gupta, A. K., Khanal, H., 2016. Comparison of anthelmintic activity Bizaryl benzyl piperazine and Benzimidazole linked piperazine derivatives. International Journal of Pharmaceutical Sciences and Research 7(4), 1547-1555.

Silici, S., Kutluca, S., 2005. Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. Journal of Ethnopharmacology 99, 69–73.

Simelane, M. B. C., Lawal, O. A., Djarova, T. G., Opoku, A. R., 2010. *In vitro* antioxidant and cytotoxic activity of *Gunnera perpensa* (Gunneraceae) from South Africa. Journal of Medicinal Plants Research 4(21), 2181-2188.

Singh, G. S., Tabane, T. H., 2015. Synthetic approaches to small and medium size heterocycles in aqueous media. In: Green Synthetic Approaches for Biologically Relevant Heterocycles, P 163-184. Ed. Brahmachari, G. Elsevier, Amsterdam, Netherlands.

Singh, M. K., Lakshman, M. K., 2009. A Simple Synthesis of Nitriles from Aldoximes1. The Journal of Organic Chemistry 74(8), 3079–3084.

Singh, R., Mehta, A., Mehta, S., 2011. Anthelmintic activity of rhizome extracts of *Curcuma longa* and *Zingiber officinale* (Zingiberaceae). International Journal of Pharmacy and Pharmaceutical Sciences 3 (S2), 236-237.

Singh, S., Gupta, S. K., 2010. A survey of anthelmintic resistance in gastrointestinal nematodes in sheep of Haryana. Haryana veterinarian 49, 2528.

Singh, U. P., Pandey, V. N., Wagner, K. G., Singh, K. P., 1990. Antifungal activity of ajoene, a constituent of garlic (*Allium sativum*). Canadian Journal of Botany 68(6), 1354-1356.

Singh, U. P., Sarma, B. K., Mishra, P. K., Ray, A. B., 2000. Antifungal Activity of Venenatine, an Indole Alkaloid Isolated from *Alstonia venenata*. Folia Microbiology 45(2), 273-176.

Sishu, R., Yonathan, M., Seyoum, A., Asre, A., 2005. Radical scavenging activity of volatile oils of herbs traditionally used to spice cooking butter in Ethiopia 23, 7-14.

Sissay, M. M., 2007. Helminth parasites of sheep and goats in Eastern Ethiopia: Epidemiology, and Anthelmintic Resistance and its Management. PhD thesis (Agricultural Science), Swedish University of Agricultural Science, Uppsala, Sweden.

Sissay, M. M., Asefa, A., Uggla, A., Waller, P. J., 2006. Anthelmintic resistance of nematode parasites of small ruminants in eastern Ethiopia: exploitation of refugia to restore anthelmintic efficacy. Veterinary Parasitology 135, 337–346.

Sissay, M. M., Uggla, A., Waller, P. J., 2007. Prevalence and seasonal incidence of nematode parasites and fluke infections of sheep and goats in Eastern Ethiopia. Tropical Animal Health and Production 39, 521531.

Smidt, D., 1997. Brugen af urter i graesmarken til husdyr (Use of herbs in pasture) KVL, M. Sc. Thesis.

Solaiman, S. G., Owens, F. N., 2010. Digestive physiology and nutrient metabolism. In: Solaiman. S. G. Goat science and production, Wiley-Blackwell pp 157-178.

Solaiman, S. G., 2010. Feeds and feeding management. In: Solaiman, S. G. Goat science and production, Willey-Blackwell, pp 193-215.

Soulsby, E. J., 1982. Helminths, Arthropods and Protozoa of Domesticated Animals. 7th edition. Edited by B. Tindall, Baltimore, Williams and Wilkins, London, UK. p1-?.

Stahla-Beek, H. J., April, D. G., Saeedi, B. J., Hannah, A. M., Keenan, S. M., Geiss, B. J., 2012. Identification of a novel antiviral inhibitor of the Flavivirus guanylyltransferase enzyme. Journal Virology 86(16), 8730–8739.

Stamatiou, G., Foscolos, G. B., Fytas, G., Kolocouris, A., Kolocouris, N., Pannecouque, C., Witvrouw, M., Padalko, E., Neyts, J., De Clercq, E., 2003. Heterocyclic Rimantadine analogues with antiviral activity. Bioorganic & Medicinal Chemistry 11, 5485–5492.

Stear, M. J., Bishop, S. C., Mallard, B. A., Raadsma, H., 2001. The sustainability, feasibility and desirability of breeding livestock for disease resistance. Research in veterinary science, 71, 17.

Stear, M. J., Doligaska, M., Donskow-Schmelter, 2007. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 139-151.

Stepek, G., Behnke, J. M., Buttle, D. J., Duce, L. R., 2004. Natural plant cysteine proteinases as anthelmintics. Trends in Parasitology 20, 322-327.

Stepek, G., Buttle, D. J., Duce, I. R., Low, A., Behnke, J. M., 2005. Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus*, *in vitro*. Parasitology 130(2), 203-211.

Strobel, G. A., Dirkse, E., Sears, J., Markworth, C., 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology 147, 2943-2950.

Stuart G., Stuart Sandiago A., Sandiago-Flores M. L., 2012. Philippine Alternative Medicine, www.stuartxchange.org/Tabako.html. Retrieved from the internet 24/01/2015.

Su, G., Zheng, L., Cui, C., Yang, B., Ren, J., Zhao, M., 2011. Characterization of antioxidant activity and volatile compounds of Maillard reaction products derived from different peptide fractions of peanut hydrolysate. Food Research International 44, 3250–3258.

Surikova, O. V., Mikhailovskii, A. G., Mikhailovskii, B. Y., Yusov, A. S., 2017. Synthesis and anthelmintic and insecticidal activities of 1-R-3-Methyl-6-Methoxy-7-(P-Propoxy)-3,4-Dihydroisoquinoline Hydrochlorides. Pharmaceutical Chemistry Journal 50(12), 794–797.

Sutherland, I. A., Leathwick, D. M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends in Parasitology 27(4), 176-181.

Sutherland, I., Scott, I., 2010. Gastrointestinal nematodes of sheep and cattle Biology and Control. 1st edition, Willey-Blackwell, United Kingdom.

Svetaz, L. A., Di Liberto, M. G., Zanardi, M. M., Suárez, A. G., Zacchino, S. A., 2014. Efficient production of the flavoring agent Zingerone and of both (R)- and (S)-Zingerols via green fungal biocatalysis. Comparative antifungal activities between enantiomers. International Journal of molecular Sciences 15, 22042-22058.

Swargiary, A., Daimari, A., Daimari, M., Basumatary, N., Narzary, E., 2016. Phytochemicals, antioxidant, and anthelmintic activity of selected traditional wild edible plants of lower Assam. Indian Journal of Pharmacology 48(4), 418–423.

Swarnkar, C. P., Singh, D., 2011. Role of quarantine in management of anthelmintic resistance in strongyle worms of sheep. Indian Journal of Small Ruminants 18, 95–99.

Sykes, A. R., Coop, R. L., 1976. Intake and utilization of food by growing lambs with parasitic damage to the small intestine caused by daily dosing with *Trichostrongylus colubriformis* larvae. The Journal of Agricultural Science 86(3), 507 - 515.

Sykes, A. R., 1994. Parasitism and production in farm animals. Animal Production Science 59, 155172.

Tagboto, S., Townson, S., 2001. Antiparasitic properties of medicinal plants and other naturally occurring products. Advances in Parasitology 50, 199–295.

Tai, A., Sawano, T., Yazama, F., Ito, H., 2011. Evaluation of antioxidant activity of vanillin by using multiple antioxidant assays. Biochimica et Biophysica Acta 1810(2), 170-177.

Talvik, H., Christersen, C. M., Joachik, A., roepstorff, A., Bjorn, H., Nansen, P., 1997. Prepatent periods of different Oesophagostomum spp. isolates in experimentally infected pigs. Parasitology Research 83, 563–568.

Tandon, V., Yadav, A. K., Roy, B., Das, B., 2011. Phytochemicals as cure of worm infections in traditional medicine systems. Emerging Trends in Zoology, p351–378.

Tariq, k. A., Chishti, M. Z., Ahmad, F., Shawl, A. S., 2009. Anthelmintic activity of extracts of *Artemisia absinthium* against Ovine nematodes. Veterinary Parasitology 160, 8388.

Taylor, M. A., Coop, R. L., Wall, R. L., 2007. Veterinary Parasitology 3rd edition. Blackwell Publishing Ltd, 9600 Garsington Road OX42DQ, UK.

Temberly, S., Lahlou-Kassi, A., Rege J. E., Sovani, S., Diedhou, M. L., Baker, R. L., 1997. The epidemiology of nematode infections of sheep in a cool tropical environment. Veterinary Parasitology 70, 129 – 141.

Terrill, T. H., Kaplan, R. M., Larsen, M., Samples, O. M., Miller, J. E., Gelaye, S., 2001. Anthelmintic resistance on goat farms in Georgia: efficacy of anthelmintics against gastrointestinal nematodes in two selected goat herds. Veterinary Parasitology 97, 261–268.

Terrill, T. H., Waghorn, G. C., Woolley, D. J., McNabb, W. C., Barry, T. N., 1994. Assay and digestion of ^{14}C-labelled tannins in the gastrointestinal tract of sheep. British Journal of Nutrition 72, 467-477.

Thamsborg, S. M., Roepstorff, A., Larsen, M., 1999. Integrated and biological control of parasites in organic and conventional production systems. Veterinary parasitology 84, 169186.

Thamsborg, S. M., Roepstorff, A., Nejsum, P., Mejer, H., 2010. Alternative approaches to control of parasites in livestock: Nordic and Baltic perspectives. Acta Veterinaria Scandinavica 52(supplement), S27.

Thangam, R., Suresh, V., Rajkumar, M., Vincent, J. M., Gunasekaran, P., Anbazhagan, C., Kaveri, K., Kannan, K., 2013. Antioxidant and in vitro anticancer effect of 2-Pyrrolidinone rich fraction of *Brassica oleracea* var. capitata through induction of apoptosis in human cancer cells. Phytotherapy Research 27, 1664–1670.

Tharachand, C., Selvaraj, C. I., Abraham, Z., 2015. Comparative evaluation of anthelmintic and antibacterial activities in leaves and fruits of *Garcinia cambogia* (Gaertn.) Desr. and *Garcinia indica* (Dupetit-Thouars) Choisy. Brazilian Archives of Biology and Technology 58(3), 379-386.

Thompson, J. L., Hinton, M., 1997. Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. British Poultry Science 38(1), 59-65.

Thorp, M. A., kruger, J., Oliver, S., Nilssen, E. L., Pescott, C. A., 1998. The antibacterial activity of acetic acid and Burow's solution as topical otological solution. Journal of Laryngology and Otology 112, 925-928.

Togashi, N., Shiraishi, A., Nishizaka, M., Matsuoka, K., Endo, K., Hamashima, H., Inuo, Y., 2007. Antibacterial activity of long-chain fatty alcohols against Staphylococcus aureus. Molecule 12, 139-148.

Tolstorozhev, G. B., Skornyakov, I. V., Belkov, M. V., Shadyro, O. I., Brinkevich, S. D., Samovich, S. N., 2012. Hydrogen bonds and antiviral activity of benzaldehyde derivatives. Journal of Applied Spectroscopy 79(4), 645-650.

Torres-Acosta, J. F. J., Hoste, H., 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. Small Ruminant Research 77, 159 – 173.

Trombetta, D., Saija, A., Bisignano, G., Arena, S., Caruso, S., Mazzanti, G., Uccella, N., Castelli, F., 2000. Study on the mechanisms of the antibacterial action of some plant α,β-unsaturated aldehydes. Letters in Applied Microbiology 35, 285–290.

Tsotetsi, A. M., Njiro, S., Katsande, T. C., Moyo, G., Baloyi, F., Mpofu, J., 2013. Prevalence of gastrointestinal helminths and anthelmintic resistance on small-scale farms in Gauteng Province, South Africa. Tropical Animal Health Production 45, 751–761.

Udaweediye, L. R. R. P., Ginigandarage, M. S. W. S., 2016. Bioactive compounds and antioxidant activity of *Bunchosia armeniaca*. World Journal of Pharmacy and Pharmaceutical Sciences 5(10), 1237-1247.

Ugwu, D. I., Okoro, U. C., 2014. Synthesis, characterisation and antifungal activities of [4-Methylphenylsulphonamido]-N-(Pyridin-2 Yl) acetamide derivatives. Medical Chemistry 4(2), 330-333.

Ullah, I., Khan, A. L., Ali, L., Khan, A. R., Waqas, M., Hussain, J., Lee, I. J., Shin, J. H., 2015. Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by *Photorhabdus temperata* M1021. Journal of Microbiology 53(2), 127-33.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M., Jennings, F. W., 1996. Veterinary Parasitology, 19 – 164, 2nd edition, Blackwell Science

Vagenas, D., Jackson, F., Russel, A. J. F., Merchant, M., Wright, I. A., Bishop, S. C., 2002. Genetic control of resistance to gastrointestinal parasites in crossbred cashmere producing goats: response to selection, genetic parameters and relationships with production traits. Animal Science 74, 199 – 208.

Van den Brom, R., Moll, L., Kappert, C., Vellema, P., 2015. *Haemonchus contortus* resistance to monepantel in sheep. Veterinary Parasitology 209, 278-280.

Van Dijk, J., Sargison, N. D., Kenyon, F., Skuce, P. J., 2010. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal 4, 377–392.

Van Wyk, B-E., Wink, M., 2004. Medicinal plants of the world. Briza publications, Pretoria.pp1-?

Van Wyk, J. A., 2001. Refugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepooort Veterinary Research 68, 55-67.

Van Wyk, J. A., 1990. Occurrence and dissemination of anthelmintic resistance in South Africa, and management of resistant worm strains. In: Boray, J. C., Martin, P. J., Roush, R. T. (Eds) Resistance of Parasites to Antiparasitic Drugs. Round Table Conference held at the VIIth International Congress of Parasitology Paris, August 1990: p103-113.

Van Wyk, J. A., Bath, J. F., 2002. The FAMACHA© system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. Veterinary Research 33, 509–529.

Van Wyk, J. A., Hoste, H., Kaplan, R. M., Besier, R. B., 2006. Targeted selective treatment for worm management–how do we sell rational programs to farmers? Veterinary Parasitology 139, 336-346.

Van Wyk, J. A., Malan, F. S., Bath, G. F., 1997. Rampant anthelmintics resistance in sheep in South Africa-what are the options? In: Van Wyk, J. A., Van Schalkwyk, P. C., (Eds). Managing Athelmintic Resistance in Endoparasites. Workshop held at the 16th International conference of the WAAVP 10-15 August, Sun City, South Africa, pp51-63.

Vanimisetti, H. B., Andrew, S. L., Zajac, A. M., Notter, D. R., 2004. Inheritance of faecal egg count and packed cell volume and their relationship with production traits in sheep infected with *Haemonchus contortus*. Journal of Animal Science 82, 1602–1611.

Várady, M., Papadopoulos, E., Dolinská, M., königová, A., 2011. Anthelmintic resistance in parasites of small ruminants: sheep versus goats. Helminthologia 48(3), 137-144.

Velioglu, Y. S., Mazza, G., Gao, L., Oomah, B. D., 1998. Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. Journal of Agricultural and Food Chemistry 46, 4113−4117.

Vercruysse, J., Claerbout, E., 2001. Treatment of helminth infection in cattle: Defining the threshold. Veterinary Parasitology 98, 195214.

Vercruysse, J., Charlier, J., Dorny, P., Claerebout, E., 2006. Diagnosis of helminth in cattle: were we wrong in the past? In: proceedings of the 24th world buiatrics congress Nice, France. www.ivis.org/proceeding/wbc2006/vercruysse.pdf.

Verma, M., Bansal, Y. K., 2015. Screening and evaluation of bioactive components of *Hedychium coronarium* j. koenig in nature grown and *in vitro* regenerated plants by GC-MS analysis. World Journal of Pharmacy and Pharmaceutical Sciences 4(4), 1729-1747.

Vieira, L. S., Cavalcante, A. C. R., Pereira, M. F., Dantas, L. B., Ximenes, L. J. F., 1999. Evaluation of anthelmintic efficacy of plants available in Ceara state, North-east Brazil, for the control of goat gastrointestinal nematodes. Revue de Medecine Veterinaire 150, 447–452.

Vignoli, J. A., Viegas, M. C., Bassoli, D. G., Benassi, M. T., 2014. Roasting process affects differently the bioactive compounds and the antioxidant activity of arabica and robusta coffees. Food Research International 61, 279–285.

Villalba, J. J., Provenza, F. D., 2009. Learning dietary choice in herbivores. Range land Ecology and Management 62(5), 399-406.

Voigt, K., Scheuerle, M., Hamel, D., 2012. Triple anthelmintic resistance in *Trichostrongylus* spp. in a German sheep flock. Small Ruminant Researh. 106, 30–32.

Volkovoy, V. A., Sevrukov, O. V., Kolisnyk, S. V., Derkach, N. V., Kryzhna, S. V., Ostapets, M. O., 2017. VÌSNIK FARMACÌÏ 3 (91), 61-65.

Von Samson-Himmelstjerna, G., Blackhall, W., 2005. Will technology provide solution for drug resistance in veterinary helminths? Veterinary Parasitology 132, 223239.

Wafa, G., Amadou, D., Larbi, K. M., Héla, E. F. O., 2014. Larvicidal activity, phytochemical composition, and antioxidant properties of different parts of five populations of *Ricinus communis L*. Industrial Crops and Products 56, 43-51.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. Animal Feed Science and Technology 147, 116-139.

Waghorn, T. S., Reynecke, D. P., Oliver, A-M. B., Miller, C. M., Vlassoff, A., Koolard, J. P., Leathwick, D. M., 2011. Dynamics of the free-living stages of sheep intestinal parasites on pasture in the North Island of New Zealand. 1. Patterns of seasonal development. New Zealand Veterinary Journal 59, 279–286.

Waller, P. J., 1997b. Nematode parasite control of livestock in the Tropics/Subtropics: the need for novel approaches. International Journal of Parasitology 21(10), 1193 - 1201.

Waller, P. J, Bernes, G., Thamsborg, S. M., Sukura, A., Richter, S. H., Ingebrigtsen, K., Hoglund, J., 2001. Plants as de-worming agents of livestock in the Nordic countries: historical perspective, popular beliefs and prospects for the future. Acta Veterinaria Scandinavica 42, 31–44.

Waller, P. J., 1997c. Anthlemintic resistance. Veterinary Parasitology 72, 391412.

Waller, P. J., Chandrawathani, P., 2005. *Haemonchus contortus*: parasite problem no. 1 from tropics to polar circle. Problems and prospects of control based on epidemiology. Journal of Tropical Biomedicine 22(2), 131-137.

Waller, P. J., 1986. Anthelmintic resistance in nematode parasites of sheep. Agricultural zoology reviews 1, 333-373.

Waller, P. J., 1997a. Sustainable helminth control of ruminants in developing countries. Veterinary Parasitology 71, 195 - 207.

Waller, P. J., 1999. International approaches to the concept of integrated control of nematode parasites of livestock. International Journal Parasitology 29, 155 – 164.

Waller, P. J., 2004. Management and control of nematode parasites of small ruminants in the face of total anthelmintic failure. Tropical Biomedicine 21(2), 7 - 13.

Waller, P. J., 2006a. Sustainable nematode control strategies for ruminant livestock by grazing management and biological control. Animal Feed Science Technology 126 (2-3), 277-289.

Waller, P. J., 2006b. From discovery to development: Current industry perspectives for the development of novel methods of helminth control in livestock. Veterinary parasitology 139(13), 114.

Waller, P. J., Bernes, G., Thamsborg, S. M., Sukura, A., Richter, S. H., Ingebrigtsen, K., Höglund, J., 2001. Plants as de-worming agents of livestock in the Nordic countries: historical perspective, popular beliefs and prospects for the future. Acta Veterinaria Scandinavica 42, 31–44.

Waller, P. J. Chandrawathani, P., 2005. *Haemonchus contortus*: Parasite problems and prospects for control based on epidemiology. Tropical Biomedicine 22(2), 131 - 137.

Waller, P. J., Thamsborg, S. M., 2004. Nematode control in "Green ruminant production systems" Trends in Parasitology 20(10), 493-497.

Wang, D. C., Sun, S. H., Shi, L. N., Qiu, D. R., Li, X., Wei, D. S., Zhang, Y. M., Qin, J. C., 2015. Chemical composition, antibacterial and antioxidant activity of the essential oils of Metaplexis japonica and their antibacterial components. International Journal of Food Science and Technology 50, 449–457.

Wang, D. H., Tao, W. Y., 2009. Antitumour activity in vitro and volatile components of metabolites from mycobacteria Stigmatella WXNXJ-B. African Journal of Microbiology Research 3(11), 755-760.

Wang, G-X., Zhou, Z., Jiang, D-X., Han, J., Wang, J-F., Zhao, L-W., 2010. In vivo anthelmintic activity of five alkaloids from *Macleaya microcarpa* (Maxim) Fedde against *Dactylogyrus intermedius* in *Carassius auratus*. Veterinary Parasitology 171, 305-313.

Wang, H., Zhao, M., Yang, B., Jiang Y., Rao, G., 2008. Identification of polyphenols in tobacco leaf and their antioxidant and antimicrobial activities. Food Chemistry 107, 1399-1406.

Wang, X. J., Wang, M., Wang, J. D., Jiang, L., Wang, J. J., Xiang, W. S., 2010. Isolation and identification of novel macrocyclic lactones from *Stretomyces avermitilis* NEAU1069 wih acaricidal and nematocidal activity. Journal of Agriculture and Food Chemistry 58(5), 2710-2714.

Wanyangu, S. W., Mugambi, J. M., Bain, R. K., Duncan, J. L., Murray, M., Stear, M. J., 1997. Response to artificial and subsequent natural infection with *Haemonchus contortus* in Red Maasai and Dorper ewes. Veterinary Parasitology 69, 275282.

Watt, J. M., Breyer-Brandwijk, M. G., 1962. The medicinal and poisonous plants of Southern Eastern Africa. E and S Livingstone LTD, Edinburgh.

Webb, J. L., 1963. Effect of more than one inhibitor. Enzyme and Metabolic inhibitors, volume 1. Academic Press, New York, 66-79 and 487-512.

Wei, A., Mura, K., Shibamoto, T., 2001. Antioxidative activity of volatile chemicals extracted from beer. Journal Agricultural and Food Chemistry 49 (8), 4097–4101.

Wenk, C., 2003. Herbs and botanicals as food additives in monogastric animals. Asian-Australian Journal of Animal Science 16(2), 282-289.

Werne, S., Perler, E., Maurer, V., Probst, J. K., Hoste, H., Drewek, A., Heckendorn, F., 2013. Effect of Sanfoin (*Onobrychis viciifolia*) and faba bean (*Vicia faba*) on the periparturient rise in ewes infected with gastrointestinal nematodes. Small Ruminant Research 113(2-3), 454-460.

Winkler, C., Frick, B., Schroecksnadel, K., Schennach, H., Fuchs, D., 2006. Food preservatives sodium sulphide and sorbic acid suppress mitogen-stimulated peripheral blood mononuclear cells. Food and Chemical Toxicology 44(12), 2003-2007.

Wlostenholme, A. J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G., Sangster, N. C., 2004. Drug resistance in veterinary helminths. Trends in Parasitology 20, 469-476.

Wu, Y., Wang, D., Gao, Y., Feng, J., Zhang, X., 2016. New α-methylene-γ-butyrolactone derivatives as Potential Fungicidal Agents: Design, Synthesis and Antifungal Activities. Molecules 21(130); doi: 10.3390/molecules21020130

Xinhua, M. A., Tianjiao, Z. H. U., Qianqun, G. U., Rui, X. I., Wei, W., Delai, L. I., 2014. Structure and antiviral activities of butyrolactone derivatives isolated from *Aspergillus terreus* MXH-23. Oceanic and Coastal Sea Research 13(16), 1067-1070.

Yanagimoto, K., Lee, K. G., Ochi, H., Shibamoto, T., 2002. Antioxidative activity of heterocyclic compounds found in coffee volatiles produced by maillard reaction. Journal Agricultural and Food Chemistry 50(19), 5480–5484.

Yang, J. F., Yang, C. H., Liang, M. T., Zi-Jie Gao, Z. J., Wu, Y. W., Chuang, L. Y., 2016. Chemical Composition, Antioxidant, and Antibacterial Activity of Wood Vinegar from *Litchi chinensis*. Molecules 21, 1150; doi: 10.3390/molecules21091150

Yang, X. N., Khan, I., Kang, S. C., 2015. Chemical composition, mechanism of antibacterial action and antioxidant activity of leaf essential oil of *Forsythia koreana* deciduous shrub. Asian Pacific Journal of Tropical Medicine 8(9), 694–700.

Yar, M. S., Abdullah, M. M., Husain, A., Bhakht, M. A., De Clercq, E., 2009. Synthesis and evaluation of in vitro antiviral activity of 2-[3-(substituted phenyl)-4,5-dihydro-1H-5-pyrazolyl] benzofuran-3-yl chloride derivatives. Journal of Enzyme Inhibition and Medicinal Chemistry 24(4), 949–956.

Yeap, S. K., Ho, W. Y., Beh, B. K., Liang, W. S., Ky, H., Hadi, A., Yousr, N., Alitheen, N. B., 2010. *Vernonia amygdalina*, an ethnoveterinary and ethnomedical used green vegetable with multiple bioactivities. Journal of medicinal plants research 4(25), 2787-2812.

Younie, D., Thamsborg, S. M., Ambrosini, F., Roderick, S., 2004. Grassland management and parasite control. In: Vaarst, M. (ed) Animal health and welfare in organic agriculture. CABI, Wallingford, pp 309–324.

Yu, X., Zhao, M., Hu, J., Zeng, S., Bai, X., 2012. Influence of pH on the formation and radical scavenging activity of volatile compounds produced by heating glucose with histidine/tyrosine. European Food Research Technology 234, 333–343.

Zajac, A. M., 2006. Gastrointestinal nematodes of small ruminants: Life cycle, anthelmintics, and diagnosis. Veterinary Clinics: Food Animal Practice 22(3), 529-541.

Zandi, K., Taherzadeh, M., Yaghoubi, R., Tajbakhsh, S., Rastian, Z., Fouladvand, M., Sartavi, K., 2009. Antiviral activity of *Avicennia marina* against herpes simplex virus type 1 and vaccine strain of poliovirus (An in vitro study). Journal of Medicinal Plants Research 3(10), 771-775.

Zhao, J., Davis, L. C., Verpoorte, R., 2005. Elicitor signal transduction leading to production of plant metabolites. Biotechnology Advances 23, 283-333.

Zhou, X., Seto, S. W., Chang, D., Kiat, H., Razmovski-Naumovski, V., Chan, K., Bensoussan, A., 2016. Synergistic effects of Chinese herbal medicine: a comprehensive review of methodology and current reseaech. Frontiers in Pharmacology 7, 201. Doi. 10.3389/fpher.2016.00201

Zvinorova, P. I., Halimani, T. E., Muchadeyi, F. C., Matika, O., Riggioe, V., Dzama, K., 2016. Breeding for resistance to gastrointestinal nematodes – the potential in low-input/output small ruminant production systems. Veterinary Parasitology 225, 19–28.

Appendix

Appendix 1. 1: Biochemical composition of alkaloid/tannin and flavonoid containing plant species as determined using GC/MS from crude ethonolic extracts of vegetative material in Nano grams/gram (Ng/g)

Compound class & different molecules	*Aloe vb.* (Ng/g)	*Crinum m.* (Ng/g)	*Gunnera p.* (Ng/g)	*Nicotiana t.* (Ng/g)	*Sarcostema v.* (Ng/g)	*Vernonia a.* (Ng/g	*Zingiber o.* (Ng/g)	*Zizyphus m.* (Ng/g)
Aldehyde								
Hexanal	0	0	0	0	0	0	10.37	0
furfurals	1.10	0	4.76	0	0	0	0	0
2-Furancarboxaldehyde, 5-methyl-	0	1.74	0.87	0	0	0	0	0
5-Hydroxymethylfurfural	14.91	2.35	25.59	0	3.74	0	0	0
Amine/amide								
Methylamine,N,N-dimethyl	0	0	0	0	0.80	0	0	0
Acetamide	0	1.57	0	0	0	0	1.57	0
2-Propenamide	0	0	0	0	0	0	1.70	0.52
Nitrogen Compound								
2,6-diemthylpyrazine	0	0	0	0	0	0	5.19	0
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	0	0	0	144.27	6.43	0	0	0
2-Ethyl-5-methylpyrazine	0	0	0	0	0	0	0	1.84
8-Azabicyclo[3.2.1]otane-3-carbonitrile, 8-methyl-	0	0	0	0	0	0	0	6.65
Ketone								
1-hydroxy-2-propanone	4.73	3.26	1.24	3.46	1.52	1.48	5.19	8.82
1-(1H-pyrrol-2-yl)-ethanone	0	2.85	0	0	1.18	0	1.37	0
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	0.40	0.76	0	0	1.08	0	1.53	2.87
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-	0	0	0	0	0	2.12	0	0
2,3-dihydro-3,5-dihydroxy--6-methyl-4H-pyran-4	14.47	32.71	14.12	2.46	13.31	14.90	6.64	23.16
Dihydro-4-hydroxy-2-(3H)-furanone	0	0.80	0	0	0.40	0	0	0
Aliphatic acid								
Acetic acid	24.95	17.34	19.30	159.24	67.25	30.56	66.34	133.46

Compound class & different molecules	***Aloe vb.* (Ng/g)**	***Crinum m.* (Ng/g)**	***Gunnera p.* (Ng/g)**	***Nicotiana t.* (Ng/g)**	***Sarcostema v.* (Ng/g)**	***Vernonia a.* (Ng/g**	***Zingiber o.* (Ng/g)**	***Zizyphus m.* (Ng/g)**
Propionic acid	0	0	0	0	0	0	0.30	0.76
Sorbic acid	0	0	0	0	4.14	0	0	0
Benzenoid								
Benzaldehyde	0	0	0	0	0	2.92	0	0
Phenylethyl alcohol	0	1.49	1.11	0	0	1.45	0	0
2-Methoxy-4-vinylphenol	0	0.43	0	0	0.89	0	1.49	3.73
Vanillin	0	0	0	0	0	0	0.77	0
2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-	0	0	0	0	0	0	5.75	0
Alcohols								
2,3-butanediol	0	0	0	6.06	0	0	0	7.70
2-Furanmethanol	3.60	1.80	1.67	3.28	2.13	0.01	1.19	3.10
5-methyl-2-Furanmethanol	0	0	0	1.47	0	0	0	0
2,7-Octadiene-1,6-diol, 2,6-dimethyl-	2.26	0	0	0	0	0	0	0
Glycerin	0	0	0	2.84	0	8.32	0	0
Phytol	0	0	0	0	0	0	0	4.92
Sulphur compounds								
Dimethyl Sulfoxide	0	2.56	0.86	5.24	0	1.16	0	0
Lactone								
Butyrolactone	0	0	3.74	6.42	0	2.76	0.59	0
2-Pyrrolidinone	0	1.40	1.24	0.48	0	2.76	0.74	0.44
2-Hydroxy-gamma-butyrolactone	1.52	0	0	0	0.73	0	0	0
Furan								
Benzofuran, 2,3-dihydro	0	0	0	0	0	7.99	0.89	2.98
No name								
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a			0.45	0	0	4.01	0	0
Total biochemical concentration	67.94	71.06	74.95	335.22	102.33	80.44	105.87	202.28

Aloe vb. = Aloe van balenii; *Crinum m.= Crinum macowanii*; *Gunnera p.= Gunnera perpensa*; *Nicotiana t.= Nicotiana tabacum*; *Sarcostema v.= Sarcostema viminale*; *Vernonia a.= Vernonia amygdalina*; *Zingiber o.= Zingiber officinale*; *Zizyphus m.= Zizyphus mucronata*; Ng= Nano gram; g= gram

Appendix 1. 2: Biochemical composition of proteases and nitrogen compound containing plant species as determined using GC/MS from crude ethonolic extracts of vegetative material in Nano grams/gram

Compound class & different molecules	*Allium c*. (Ng/g)	Ananas c. (Ng/g)	*Bidens p*. (Ng/g)	*Carica p.*(Ng/g)	*Ricinus c*. (Ng/g)	*Trema o*. (Ng/g)	*Urtica d*. (Ng/g)	*Zanthozylum c*. (Ng/g)
Aldehyde								
furfurals	50.35	0	0	0	5.17	0	0	0
2-Furancarboxaldehyde, 5-methyl-	0	0	0	0	3.05	0	0	0
5-Hydroxymethylfurfural	304.04	0	0	0	15.25	0	0	0
Amides								
Acetamide	0	3.67	1.61	1.33	0	0	0	0
2-propenamide	0.54	3.61	0.82	0	0	7.86	0	0
Nitrogen compound								
2,5-Dimethylpyrazine	0	2.23	0	0	0	1.15	1.26	0
2,5-dihydro-1H-pyrrole	0	0	1.41	1.87	0	0	0	0
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-,(s)-	0	0	0	0	4.59	0	0	0
Benzyl nitrile	0	0	0	9.94	0	0	0	0
2-Ethyl-5-methylpyrazine	0	0	0	0	0	0	0	1.20
Ketone								
1-hydroxy-2-propanone	24.96	9.08	5.97	3.75	2.67	5.32	5.62	13.82
4-Cyclopentene-1,3-dione	0	0	0	0	0	0	0	0.89
4-Methyl-5H-furan-2-one	0	0	0	0	0	2.01	0	0
2(3H)-furanone	3.24	0	0	0	0	0	0	0
1-(1H-pyrrol-2-yl)-ethanone	6.55	1.04	0.85	0.77	1.30	0	0	0

Compound class & different molecules	***Allium c*. (Ng/g)**	**Ananas c. (Ng/g)**	***Bidens p*. (Ng/g)**	***Carica p*.(Ng/g)**	***Ricinus c*. (Ng/g)**	***Trema o*. (Ng/g)**	***Urtica d*. (Ng/g)**	***Zanthozylum c*. (Ng/g)**
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	8.93	6.09	1.77	2.79	1.36	1.42	0	1.30
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	430.01	36.71	19.51	24.58	35.14	13.03	9.13	15.49
Dihydro-4-hydroxy-2-(3H)-furanone	4.70	1.76	0	0	1.75	0	0	0
Alipphatic acids								
Acetic acid	229.69	374.09	63.88	35.78	30.89	99.74	38.03	84.05
Propanoic acid	0	0	0	0	0	0	0	1.12
4-hydroxy-butanoic acid	0	0	1.43	0	0	0	0	1.09
Benzenoids								
Benzeneacetaldehyde	4.35	4.72	6.74	0	7.01	0	0	0
2-Methoxy-4-vinylphenol	3.64	2.96	1.30	3.89	1.63	0	0	0.77
Alcohols								
2,3-butanediol	340.76	0	0	0	0	0	0	0
2-furanmethanol	36.07	2.02	1.20	1.07	3.84	0.79	1.35	3.52
5-methyl-2-Furanmethanol	5.81	0	0	0	0.44	0	0	0
Glycerin	0	0	0	0	3.58	0	0.01	4.19
Phytol	0	0	0	0	0	1.80	0.82	0
Sulphur compounds								
3,4-Dimethylthiophene	3.53	0	0	0	0	0	0	0
1-propenyl methyl disulfide	4.19	0	0	0	0	0	0	0
Dimethyl trisulphide	2.19	0	0	0	0	0	0	0
Dimethylsulphuroxide	0	0	0.39	3.17	2.25	0.60	1.08	0

Compound class & different molecules	***Allium c*. (Ng/g)**	**Ananas c. (Ng/g)**	***Bidens p*. (Ng/g)**	***Carica p*.(Ng/g)**	***Ricinus c*. (Ng/g)**	***Trema o*. (Ng/g)**	***Urtica d*. (Ng/g)**	***Zanthozylum c*. (Ng/g)**
Lactones								
Butyrolactone	0	4.83	1.87	7.06	0	1.04	0.39	0
2-Pyrrolidinone	0	1.11	1.04	1.33	2.07	0.27	0	0.23
2-Hydroxy-gamma-butyrolactone	3.54	1.08	0	0	0.90	0	0	1.76
Alkaloids								
Z-1-(1-butenyl) aziridine	0	0	0	0	0	5.53	0	0
Glutarimide	0	0	0	0	0	5.56	0.37	0
Furans								
Benzofuran, 2,3-dihydro	2.33	15.32	0.72	5.68	0	1.67	0	2.99
Ester								
1-Butanol, 3-methyl-, acetate	0	0	0	0	0	0	0	37.37
No name								
(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	0	0	0.68	0.42	0	0	0	0.48
Total mass of biochemical	1469.42	469.62	111.19	103.43	122.89	147.76	58.06	170.27

Allium c.= *Allium cepa*; *Ananas c*.= *Ananas comosus*; *Bidens p*.= *Bidens pilosa*; *Carica p*.= *Carica papaya*; *Ricinus c*.= *Ricinus communis*; *Trema o*.= *Trema orientalis*; *Urtica d*.= *Urtica dioica*; *Zanthozylum c*.= *Zanthozylum capense*; Ng= nano gram; g= gram