University of KwaZulu-Natal

Integrated control of gastrointestinal nematodes of sheep using plant extracts and biocontrol agents

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Thesis Summary

Infection of small ruminants by gastrointestinal nematodes (GIN) is a major health concern because they cause substantial economic losses, especially in the tropics and sub-tropics. For many years, control of GIN has been based upon use of anthelmintics. However, there is now a global challange because mutant GIN individuals can tolerate most of the widely used anthelmintics. Therefore, alternative control measures are needed. The objective of the study was to screen a number of plant species for their anthelmintic effects, and to evaluate selected strains of *Bacillus thuringiensis* (Berliner) and *Clonostachys rosea* (Schroers) for activity against sheep GIN. Subsequently, the combined treatments would test a dual control strategy for nematodes by using a combination of plant extracts with biocontrol agents.

Ethanol extracts of 25 plant species were screened for their anthelmintic effects against *Haemonchus contortus* (Rudolphi 1803). Extracts of each plant were used *in vitro* at various concentrations (10, 20 and 30%) to treat 10 day faecal cultures. Five plants with high efficacies (*Ananas comosus* L. Merr., *Aloe ferox* Mill., *Allium sativum* Linn., *Lespedeza cuneata* Dum. Cours. and *Warburgia salutaris* Bertol.f. Chiov*)* were selected for further investigation, using ethanol, dichloromethane and water extracts at four concentrations (2.5, 5, 10 and 20%). Ethanol was the most effective solvent. Larval counts decreased as a result of increasing extract concentrations. An ethanolic extract of *Lespedeza cuneata* caused more than 70% mortality at all concentrations.

In an *in vivo* study, the five plants *A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris* extracts were compared to a positive Control (Equimax®, a modern anthelmintic based on abamectin and praziquantel). Gender, eggs count (EPG_0) and initial body weights were used in assigning sheep (24 females and 24 males) to six groups. Each group was randomly assigned a treatment. Plant extracts were applied as an oral dose (100 mg kg^{-1} BW), one dose per week per animal for 42 days (Phase 1). Subsequently, the same sheep were dosed for three consecutive days with the same treatments, keeping them in the same groups (Phase 2). Rectal faecal samples were taken for counting of eggs per gram of faeces (EPG) and L_3 larvae per gram (LPG) in faecal cultures. With application of plant extracts, the EPG count decreased with time ($P<0.001$), and the impact of the plant extracts increased ($P<0.001$) with

time. Two extracts, from *A. comosus* and *L. cuneata,* were the most effective in Phase 1 (58% and 61% reduction of EPG, respectively,), and in Phase 2 (77% and 81% reduction of EPG, respectively).

In a study on potential biocontrol agents, two strains of *Bacillus thuringiensis* (Bt) and one of *Clonostachys rosea f. rosea* (*C. rosea*), and compared with a diatomaceous earth (DE) product for their anthelmintic activity in sheep. *Bacillus thuringiensis* and *C. rosea* were fed to sheep at a rate of 1g kg^{-1} BW, and DE was fed at 2% of sheep diet. The biocontrol treatments had no effect on EPG ($P>0.05$), but reduced GIN larvae per gram (LPG) ($P<0.001$) in faecal culture. Efficacy varied with time ($P<0.001$). By Day 7 *Bt, C. rosea* and DE had caused mortalities of GIN of 75.7, 86.9 and 60.6%, respectively. In addition, the efficacy of feeding 1g kg^{-1} BW of *C. rosea* chlamydospores to sheep every day, every second day and every third day was tested. Daily feeding of fungal chlamydospores reduced LPG (a count of 12±1.67 GIN larvae) ($P<0.001$) more than feeding them the biocontrol agent every second day (39±0.77) or third day (58±1.77). By Day 12, feeding the biocontrol agent to sheep every day, every second day, or every third day caused mortality of GIN larvae of 90, 63 and 49%, respectively.

Four dietary levels (treatments) of *C. rosea* (0.25g (F1), 0.5g (F2), 1g (F3) and control (C) of *C. rosea* product kg-1 BW) were tested. Treatments were each mixed with a complete diet and fed to sheep once daily for 10 weeks, according to body weights. Increased doses of the biocontrol agent reduced LPG ($P<0.001$), larval development (LD) ($P<0.001$), and increased efficacy ($P<0.001$). On Day 70, F1, F2, F3 and the Control controlled LD by 33.3, 72.3, 89.4 and 2.6%, respectively. *Clonostachys rosea* was effective in reducing third stage larvae (L_3) on pastures significantly ($P<0.001$) by Day 63 and Day 70.

Ethanolic extracts of *A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris* all reduced egg production by GIN parasites of sheep. Feeding sheep cultured chlamydospores of a biocontrol fungus, *Clonostachys rosea,* reduced counts of nematode larvae in sheep; and 1g *C. rosea* chlamydospores kg^{-1} BW daily was enough to reduce nematode infective larvae, therefore reducing the degree of pasture contamination. An initial trial showed that the combination of the two treatments of an *A. comosus* extract and *C. rosea* chlamydospores was

more effective than either treatment on its own in controlling gastrointestinal nematodes in sheep. A long-term trial is being undertaken currently to confirm this finding.

Declaration

The research described in this thesis was carried out in the Discipline of Animal and Poultry Science, School of Agricultural, Earth and Environmental Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus. The research carried out according to UKZN animal ethics policy (Reference: 076/10/Animal). The work was under the supervision of Prof. Mark Laing and co-supervision of Prof. Ignatius Nsahlai.

This is to declare that this thesis is the result of my own investigation, except where acknowledged, and has not been presented in any previous application for a degree purpose.

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Signed…… Date……

I, Prof. Mark Laing, the supervisor, approved the release of this thesis for examination.

Signed…… Date…….

I, Prof. Ignatius Nsahlai, the co-supervisor, approved the release of this thesis for examination.

Signed…… Date…….

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Thesis Outputs

Published Articles

1. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing,** 2012. *In vitro* anthelmintic activity of crude extracts of selected medicinal plants against *Haemonchus contortus* of sheep. Journal of Helminthology pp 1-6 online.
2. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing,** 2011. *In vivo* effect of plant species on gastrointestinal nematodes of sheep. Published in the proceeding of the Eighth International Symposium on the Nutrition of Herbivores, 6^{th}-9^{th} September, 2011, Wales, UK, page 399.

Articles Submitted for Publication

1. Mawahib Ahmed, Ignatius Nsahlai and Mark Laing, 2012. *In vivo* effect of selected medicinal plants against gastrointestinal nematodes of sheep. Submitted to Journal of Helminthology.

Conference Abstract

1. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing,** 2011. Anthelmintic activity of plant extracts against gastrointestinal nematodes of sheep. Southern Africa Society of Animal Science, 44^{th} Biannual Congress, 11-14 July 2011, Stellenbosch University, Eastern Cape Province, SA.

Articles in Preparation

1. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing**. The effects of *Bacillus thuringiensis*, *Clonostachys rosea f. rosea* and diatomaceous earth on gastrointestinal nematodes of sheep.

2. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing**. The ability of a fungus, *Clonostachys rosea f. rosea,* to reduce infective larvae of gastrointestinal nematodes in sheep faeces: a dose time interval study.

3. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing.** The effects of a fungus, *Clonostachys rosea f. rosea,* on nematode parasites of sheep in a grazing trial.

4. **Mawahib Ahmed, Ignatius Nsahlai and Mark Laing.** A new control strategy for nematodes of sheep using a fungus, *Clonostachys rosea f. rosea,* and an extract of *Ananas comosus*.

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Thesis Introduction

Background

Diseases caused by gastrointestinal nematodes (GINs) in livestock are a major production constraint, causing economic losses, especially in small ruminants in the tropics and subtropics (Odoi *et al.,* 2008). The infection of sheep and goats by nematodes is rampant in most African countries, where the environmental conditions are conducive to nematode growth and transmission (Fikru *et al.,* 2006). In addition, in this region, there is lack of veterinary health care, poor pastures and the quantities of nutritious feed consumed do not cover the nutritional requirements of animals (Leng, 1991). Manipulation of animal nutrition can increase the ability of animals to withstand the adverse effect of parasitism (Coop and Kyriazakis, 2001).

In South Africa, nematode infection is a serious veterinary health challenge and the existing means are adequate for addressing the problem (Van Wyk *et al.,* 1999). In general, GINs reduce productivity of small ruminants by means of lowering fertility, reduction in milk production and depressed weight gains when feed intake is reduced (Butter *et al*., 2001). In addition, loss of endogenous protein and anorexia reduce immunity. Treatment expenses are high. In worse situations, death may result from critical infections (Fikru *et al.,* 2006; Hale, 2006). The level of nematode infection depends mainly upon the age of the host, the breed, the parasite species involved, and the epidemiological patterns, which include husbandry practices and the physiological status of the animals (Tembely *et al.,* 1997). The most highly pathogenic GIN species is *Haemonchus contortus* Rudolphi (1803), which is widely distributed in the tropics and suptropics.

Currently, the conventional control strategy for GIN infections is the use of synthetic anthelmintic drugs (Waller, 1994; Pomroy *et al.,* 2002; Crawford *et al.,* 2006). Five classes of modern synthetic anthelmintics are known, each having a different mode of action (Stepek *et al*., 2004; Kaminsky *et al.,* 2008). However, several problems associated with the use of these anthelmintics have been observed worldwide (Prichard, 1994). Development of resistance by

the parasites to the available drugs is widespread, and alternative control measures are needed. Consumers of animal by-products have also become cautious of possible contamination of the products as a result of drugs' residual effects. Another issue is that small-scale farmers are being pushed out of the livestock industry by the unaffordable cost of drugs. Thus, studies seeking alternative methods for controlling GINs are now under development. These include improved grazing management strategy (Barger, 1999), improved animal breeding (Mitreva *et al.,* 2007), vaccination (Smith, 1999), supplementing feed with plants containing anthelmintic properties (Stepek *et al.*, 2004) and biological control (Larsen, 1999).

The effect of supplementing animal feed with plants containing anthelmintic properties depends upon the availability of that plant, its palatability and selective behaviour of animals. However, many plants have shown potential to control GINs in animals such as *Sericea lespedeza* (Min *et al.,* 2005), *Ananas comosus, Carica papaya, Ficus* spp. (Stepek *et al.,* 2004) and *Allium sativum* (Singh *et al.,* 2009). Their anthelmintic properties are attached to different active ingredients and their concentration in the plants. However, some of these active ingredients have been associated with adverse reactions when fed on by livestock. For example, tannins, the perceived active substance in *Acacia* spp., when fed to livestock in large quantities, reduce voluntary intake and digestibility (Min *et al.,* 2005).

Biological control has shown positive results in controlling GINs. Gronvold *et al.* (1996) defined biological control as the control of parasitic organisms by the use of naturally living antagonists to reduce the parasite loads to sub-economic threshold levels. Research on *Duddingtonia flagrans* (Cooke) has shown that this fungus could be used successfully to control nematodes. Most published research on the biocontrol of livestock nematodes has centered on this predacious fungus. Additionally, research on the bacterium *Bacillus thuringiensis* (Berliner) (Bt) has shown that some strains of Bt have potential to control nematodes of both plants and animals (Larsen, 1999).

Objectives

The overall goal of the study was to develop a new control strategy, by integrating plant extracts with biocontrol agents for the control of GIN parasites in sheep.

The specific objectives of the study were:

(i) *In vitro* screening of selected plant extracts for activity against nematode parasites.
(ii) *In vivo* field evaluation of the best plant extracts.
(iii) *In vivo* determination of dose frequency of the best plant extracts.
(iv) *In vivo* screening for efficacy of potential biological agents.
(v) *In vivo* determination of dose levels and frequency of application of the best biocontrol agents.
(vi) *In vivo* combination of the best plant extract and best biocontrol agent.

The thesis is structured as follows:

- A review of the literature relevant to the research process (Chapter 1).

- *In vitro* anthelmintic effect of selected plant extracts against gastrointestinal nematodes of sheep (Chapter 2).

- *In vivo* effect of selected plant extracts activity against gastrointestinal nematodes of sheep (Chapter 3).

- The effects of *Bacillus thuringiensis*, *Clonostachys rosea f. rosea* and diatomaceous earth on gastrointestinal nematodes of sheep (Chapter 4).

- The effects of chlamydospores of *Clonostachys rosea f. rosea* on nematode parasites of sheep in a grazing trial (Chapter 5).

- Integration of the best plant extracts with the best biocontrol agents to control nematodes of sheep *in vivo* (Chapter 6).

- A thesis overview and recommendations for further research.

Chapters 2-6 are written as discrete research papers, ready for publication, a format adopted by the University of KwaZulu-Natal. Owing to this, there will be some overlapping of content and references.

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Chapter 1

Literature review

Parasitic helminths with its very wide distribution, has become a very important production constraint in sheep farms in tropical, subtropical and temperate regions worldwide. Various internal and external factors determine the survival of parasitic helminths and hence the development of the disease in the animal. In general, control of helminths infestation in sheep relies heavily on anthelmintic treatments. However, the indiscriminate use of these drugs has led to the widespread emergence of drug resistant strains of parasites. For this reason, development and use of various parasite control methods such as grazing management, botanical extracts and biological agents is very necessary, with or without moderate use of anthelmintics. The ultimate goal of such control programs is to enhance productivity, while minimising risks regarding drug resistance and consumer and environmental concerns. This review attempts to highlight parasitic helminths in small ruminants, in particular gastrointestinal nematodes (GIN), anthelmintic, anthelmintic resistance and alternative approaches to control the disease with emphasis on botanical extracts and biological control.

1.0 Helminths of small ruminants

Parasitic helminths of sheep (*Ovis aries* L.) and goats (*Capra hircus* L.) belong to the phylum *Platyhelminths* (flatworms) and *Nemathelminths* (roundworms and their relatives) (Soulsby, 1982). The former phylum, *Platyhelminths*, has two classes, Cestoda (tapeworms) such as Moniezia spp. and Trematoda (flukes) such as *Dicrocoelium* spp. Members of the family *Taeniidae* are the most important cestode parasites of small ruminants (Denegri *et al.*, 1998). These include larval stages of *Echinococcus granulosus* Batsch (1786), *Taenia hydatigena* Pallas (1776), *Taenia ovis* Cobbold (1869) and *Taenia multiceps* Leske (1780) (Sissay, 2007). On the other hand, parasitic trematode species in small ruminants belong to the subclass *Digenea* (Mehlhorn, 2008). Main species of *Digenea* in Africa are liver flukes, *Fasciola hepatica* Linnaeus (1758), *Fasciola gigantica* Cobbold (1855) and rumen flukes (*paramphistomes*), *Paramphistomum* spp. (Soulsby, 1982). *Nemathelminths* phylum is the main parasites that influence livestock farms (Githiori, 2004). The phylum includes many

superfamilies of veterinary importance, including: *Trichostrongyloidea*, *Strongyloidea*, *Metastrongyloidea*, *Ancylostomatoidea*, *Rhabditoidea*, *Trichuroidea*, *Filarioidea*, *Oxyuroidea*, *Ascaridoidea* and *Spiruroidea* (Sissay, 2007). In small ruminants, gastrointestinal nematodes are important members of the order *Strongylida*, which contains *Trichostrongyloidea*, *Strongyloidea*, *Metastrongyloidea* and *Ancylostomatoidea*, but most of them belong to the superfamily *Trichostrongyloidea.* Small ruminants are infected with a group of these strongylid nematodes, causing parasitic gastroenteritis (Zajac, 2006). Details of gastrointestinal nematodes life cycle, infections and treatments are discussed below.

1.1 Gastrointestinal nematode

Gastrointestinal nematodes are the most damaging parasites (Waller, 1997) in small ruminants (Mulugeta *et al.*, 1989) and are a worldwide problem identified as the most serious health problem in small ruminant production (Chenyambuga *et al.*, 2009). In Africa, particularly, the infection in most cases is a member of the order Strongylid. These include: *Haemonchus contortus* Rudolphi (1803), and *Trichostrongylus* spp. such as *Trichostrongylus axei* Cobbold (1879), *Trichostrongylus colubriformis* Giles (1892) and *Trichostrongylus vitrinus* Looss (1905) (Sissay, 2007). Hansen and Perry (1994) reported other species of lesser important such as: *Nematodirus spathiger* Railliet (1896), *Cooperia curticei* Railliet (1893), *Bunostomum trigonocephalum* Rudolphi (1808), *Gaigeria pachycelis* Railliet and Henry (1910), *Oesophagostomum* spp.

The nematode life cycle (Figure 1.1) may be direct or may include an intermediate host. Sexes are usually separated (male and female). However, the economically important gastrointestinal parasites (Strongylid) in sheep and goats have direct life cycles, with no intermediate host (Sissay, 2007). Mature parasites (worms) breed inside the host and lay eggs which are shed in faeces. After eggs pass out of the host, they hatch into first-stage larvae (L_1), which grow then moult into second-stage larvae (L_2) under appropriate conditions of temperature and humidity. Larvae need moisture to develop and move. During this time, larvae (L_2) feed on bacteria, and then moult into infective larvae (L_3), which migrate out of faeces and up blades of grass. The time for development from egg to infective larvae can be as short as 7-10 days, especially during summer. Further, when an animal (sheep or goat) grazes, it may ingest parasitic larvae

along with the grass. Normally the L_3 stage larvae moult into fourth-stage larvae (L_4) within 2-3 days, then after a further 10-14 days, they moult into young adult parasites (Smyth, 1962; Soulsby, 1982; Hale, 2006; Coffey *et al.*, 2007). The time from ingestion of infective larvae to adult laying eggs, called the pre-patent period, vary among nematode. For example; the life cycle for *H. contortus* is 18-21 days (LeJambre *et al.*, 1970), 15-28 days for *Nematodirus* spp. (Lindahl *et al.*, 1970), 20-25 days for *Trichostrongylus* spp. (Kaufmann, 1996), 6-7 days for *Oesophagostomum* spp (Talvik *et al.*, 1997) and 15-20 days for *Cooperia* spp.(Kaufmann,1996).

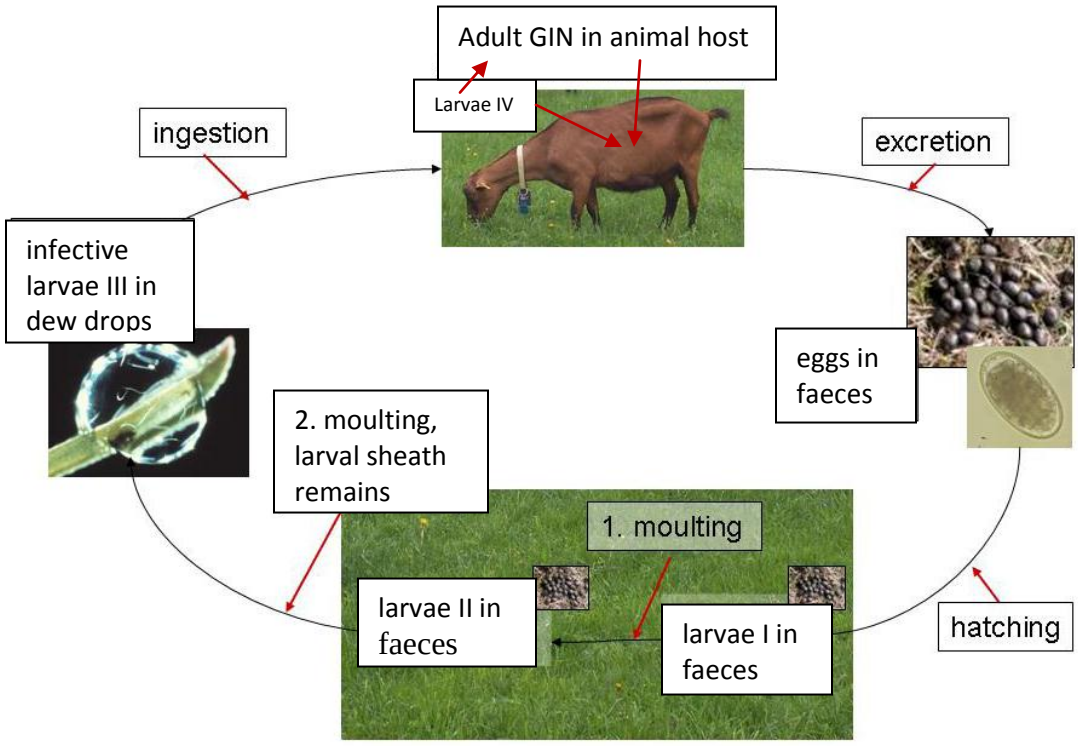

Figure 1.1: Life cycle of gastrointestinal nematodes of small ruminants. (after Scheuerle, 2009).

Development of nematode eggs and transmission of nematode infective stage are influenced by climatic conditions such as temperature, rainfall, humidity and soil moisture (Tembely *et al.*, 1997; Menkir *et al.*, 2006). In tropical and subtropical areas, the environmental factors are favourable for development and survival of nematode stages. However, variation in optimum requirements is noticeable among gastrointestinal nematode species. Sissay (2007) reported

that the development of *Trichostrongylid* larvae generally occurs in a temperature range of approximately 10–36 °C and the optimal humidity requirement for free living stage development of most species is 85%. Notably, *H. contortus*, the most important nematode in small ruminants, develops more rapidly in summer (Altaif and Yakoob, 1986). Infective stage of nematode can survive for weeks to months on the pasture depending on the weather conditions and the nematode species (Stromberg and Averbeck, 1999). On pastures the L_3 stage can survive days to months in the dry season (Sissay, 2007). When the rainy season arrives, larval activity increases rapidly (Tembely, 1998). Generally, the combined effects of these weather factors have great impact on the prevalence of infective larvae in pastures. This is has influence on the prevalence of worm burden in animals (Agyei, 1997; Tembely *et al.*, 1997; Tembely, 1998; Viassoff *et al.*, 2001; Waller *et al.*, 2004; Sissay *et al.*, 2007). In addition, Teklye (1991) stated that the climatic situation in Sub-Saharan Africa is widely suitable for the growth of infective nematode larvae.

Livestock nematodes have economic consequences when their numbers rise under suitable conditions (Table 1.1; Houdijk and Athanasiadou, 2003; Hale, 2006). Nematodes usually live in the digestive system of host animals, affecting the absorption and/or retention of minerals especially phosphorus (Coop and Kyriazakis, 2001). Effects of nematode parasites on the host manifest themselves as loss of condition, rough coat, diarrhoea, bottle jaw, anaemia and death (Hale, 2006). Moreover, clinical signs such as diarrhoea, weight loss and loss of appetite can be indicators of nematode infection, especially in young animals (Sloss and Kemp, 1978).

Diagnoses of nematode infection in the host can be done by examination of the host faeces for parasitic eggs and larvae (Sloss and Kemp, 1978). Demonstration of parasite eggs or larvae in the faeces or faecal culture provides evidence that the host is infected, but does not indicate the level of infection (Hansen and Perry, 1994). Heavy nematode infection is indicated by finding more than a thousand eggs per gram (EPG) of faeces. Between 200 and 800 EPG of faeces would count as a moderate nematode infection. However, the level of infection as interpreted from faecal egg or larvae counts are variable in each country or region, according to weather conditions and the nematode's specific pathogenicity (Lindqvist *et al.*, 2001).

Table1.1: Effects of nematode parasites on ruminants

Nematode	Host	Organ	Effect
Haemonchus contortus	sheep and goats	abomasum	feeding on the host's blood, causing anaemia
Trichostrongylus cirumcincta	sheep and goats	abomasum	reduces feed intake
Nematodirus battus	sheep and goats	small intestine	dehydrates the animal
Trichostrongylus colubriformis	sheep and goats	small intestine	reduces feed efficiency
Trichostrongylus vitrinus			
Haemonchus placei	cattle	abomasum	anaemia
Ostertagia ostertagi	cattle	abomasum	reduces feed efficiency
Cooperia oncophora, *Cooperia Punctata*	cattle	small intestine	reduces feed efficiency and affect protein metabolism

(Houdijk and Athanasiadou, 2003; Hale, 2006).

Holmes (1993) reported that parasitism by nematodes results in the loss of host protein to nematodes; the proteins represent plasma and frequently erythrocytes, exfoliated epithelial cells and mucus. Furthermore, Parkins and Holmes (1989) reported losses of blood proteins to nematodes using radio-isotopic techniques to be about 10% of the total blood volume per day. Nematode infections are usually associated with hypoalbuminaemia and, in some cases, with anaemia. Nematode parasites also affect digestion, energy and nitrogen utilization in the parasitized animal and reduce the performance of the host and feed consumption (Holmes *et al.*, 1986). A reduction in feed intake is a primary effect of nematode parasitism, but the explanation is not clear (Parkins and Holmes, 1989). One suggestion is that abdominal pain resulting from tissue damage at the site of infection may be responsible (Fox, 1997). Other

effects are changes in pH of the gut contents (Simcock *et al.*, 2006), changes in the flow rate of digesta, changes in protein/energy ratios of absorbed nutrients (Parkins and Holmes, 1989; Holmes, 1993), and changes in secretion of gastrointestinal hormones cholecystokinin and gastrin (Fox, 1997). However, the control of nematode parasites in animals is based on the use of chemical and non-chemical drugs.

1.2 Chemical control of nematodes

Anthelmintics are the most popular way to treat nematode infections (Waller, 1994; Pomroy *et al.*, 2002; Mickael *et al.*, 2003; Gillian *et al.*, 2004; Crawford *et al.*, 2006). Kohler (2001) noted that the availability of safe, broad spectrum anthelmintics has helped to reduce the incidence of nematode infections. Generally, anthelmintic groups are effective against both the immature and mature stages of virtually all of the important gastrointestinal nematodes, as well as many other intestinal parasites (Kohler, 2001). A majority of modern anthelmintics exert their effects in three biochemical and physiological areas. The known target sites are solely proteins and include ion channels, enzymes, structural proteins and transport molecules (Kohler, 2001). There are also a number of ways that the anthelmintics are administered, such as orally or injectable. The rotation of anthelmintics between the active groups is also purported to increase efficacy, and rotation between animal species has been reported to decrease resistance (Barger, 1999). In general, broad-spectrum anthelmintics are divided into 2 broad types; a) Short acting products like moxidectin and closantel (repeat the treatment after 3 weeks) and b) long acting products as albendazole or ivermectin (repeat the treatment after 100 days) (Leathwick *et al*., 2001).

Thiabendazole, introduced in 1961, was the first successful helminthic product with low mammalian toxicity (Kaplan, 2004), followed by the anthelmintic groups, levamisole and ivermectin.

1.2.1 Benzimidazoles

Benzimidazole is a class of products used mainly to treat nematode infections in humans and animals (Stepek *et al.*, 2004). Benzimidazole structures are diverse, based on chemical substitutions at different positions of the benzimidazole nucleus (Nagawade and Shinde, 2006). Chemically, the benzimidazoles are heterocyclic aromatic organic compounds. This

bicyclic compound consists of the fusion of benzene and imidazole. The most prominent benzimidazole compound in nature is N-ribosyl-dimethylbenzimidazole, which serves as an axial ligand for cobalt in vitamin B_{12}. The Benimidazole group includes many members such as mebendazole, flubendazole, fenbendazole, oxfendazole, oxibendazole, albendazole, albendazole sulfoxide, thiabendazole, thiophanate, febantel, netobimin, triclabendazole, netobimin, albendazole, and triclabendazole. These benzimidazoles affect microtubules of the worm (Kohler, 2001), by binding to B-tubulin, which inhibit the polymerization of tubulin and the formation of microtubules. The lack of microtubules inhibits many cellular functions such as transport, cell division, neural transmission, and cell differentiation, ultimately leading to cell death in the worm (Prichard, 2005).

BenZimidazoles have greater activity in gut of small ruminants than in large ruminants. BenZimidazole metabolism in large ruminants is more extensive than in small ruminants. The European Medicines Agency reported in 2004 that, in ruminants, benzimidazoles go to the rumen directly, then slowly into the remainder of the gastro-intestinal tract; so there is no need to repeat the dose for greater efficacy expet for goats. In goats, is essential to repeat the doses for graeter efficiency (European Medicines Agency, 2004). Absorption of fenbendazole is slow in ruminants, but is more rapid in monogastrics (Table 1.2). Fenbendazole is eliminated mostly by the faecal route. The liver is the main target tissue in all species tested. The metabolism of fenbendazole, febantel and oxfendazole are similar in most species that have been investigated (European Medicines Agency, 2004).

Table 1. 1: Comparative of fenbendazole characters between the animal species

Character	**Experimental animals**	**Time**
Remaining in host blood after treatment	rat	8 hours
	rabbit	8 hours
	dog	24 hours
	sheep	2-3 days
	cattle	7 days
Half-life for plasma elimination	rat	6 hours
	rabbit	13 hours
	dog	15 hours
	sheep	2-3 days
	cattle	5-7 days

(European Medicines Agency, 2004).

1.2.2 Imidazothiazoles and Tetrahydropyrimidine

Imidazothiazoles are chemical products discovered in 1965, which are used as anthelmintics to treat worm infestations in both humans and animals. As most commercial preparations, imidazothiazoles are used as dewormers for cattle, pigs, and sheep. However, it has also gained prominence among aquarists as an effective treatment for camallanus roundworm infestations in freshwater tropical fish.

Imidazothiazoles are nicotinic drugs. They affect the nervous system of worms; consequently, they cause sustained muscle contraction, leading to paralysis in nematodes and other parasites. Levamisole, tetrahydropyrimidines (e.g., pyrantel and morantael) and some other structurally related compounds are the main nicotinic drugs (Kohler, 2001). Imidazothiazoles are rapidly absorbed from the digestive tract, metabolized in the liver and eliminated in the urine largely as metabolites in 2 days. They are found in many formulations (suspension, paste, drench, or tablets), used as broad spectrum anthelminthic. Imidazothiazoles in ruminants are highly effective against the common adult gastrointestinal nematodes and lungworms, and many larval stages. However, pyrantel activity is limited to adult gastrointestinal nematodes (Merck and Co., Inc., 2008).

1.2.3 Macrocyclic lactones

Macrocyclic lactones are class of anthelmitic used mainly to control nematode and arthropod parasites. Macrocyclic lactones family includes avermectin, doramectin, abamectin, milbemycin, and moxidectin. However, avermectin is widely used as an anthelmintic in veterinary medicine and to treat onchocerciasis or river blindness in humans (Cully *et al.*, 1994). Avermectin and milbemycin compounds are closely related members of macrocyclic lactones. Both are chemicals produced through fermentation by soil actinomycetes from the genus *Streptomyces* and have similar spectra of activity (Hennessy and Alvinerie, 2002).

Macrocyclic lactones in general bind to glutamate and gamma-amino butyric acid (GABA)-gated (chloride channels) causing a hyper-polarization of nerve or muscle cells, leading to paralysis and killing the parasite (Prichard, 2001). In addition, macrocyclic lactones are broad-

spectrum and exert antiparasitic activity at low dose levels against many immature nematodes and arthropods. However, the commercially available products are primarily excreted in faeces and kill non-target dung insects. Decomposition of ivermectin in faeces or soil is slow, especially in winter (Merck and Co., Inc., 2008).

1.2.4 Other anthelmintics

There are other active ingredients represented by other common anthelmintics in the market such as Salicylanilides (closantel). This anthelminthic interferes with the proton gradient in parasite's mitochondria, which, in turn, inhibits the generation of ATP by the parasite (Vanden Bossche, 1985). Recent results using nuclear magnetic resonance (31P-NMR) to measure effect of closantel on fluke intrategumental pH, suggest that closantel is a membrane-active molecule that is capable of affecting a number of helminth biochemical and physiological processes (Pax and Bennett, 1989). Aminoacetonitrile also is a class of anthelmintics (kaminsky *et al.,* 2008), known as a simple compound containing both nitrile and amino groups. This compound is commercially available as the chloride and sulfate salts (e.g. Monepantel). This anthelmintic acts as nematode specific causing a spastic paralysis and rapid expulsion from the host (kaminsky *et al.,* 2008). In addition, spiroindoles are modern class of anthelmintic (e.g. derquantel). Spiroindoles have adverse effect against a range of gastrointestinal roundworms. There is a lack of information on how this class of medicine works. Likewise, oxamniquine and related compounds, their target activity and mode of action have been less extensively investigated (Kohler, 2001). However, nematode resistance against anthelmintics has developed, making the control of nematodes quite problematic. The use of the above chemicals either independently or in combinations has led to a reduction in the ability of chemicals to reduce the parasites burden in livestock. This resistance to chemical drugs is discussed below, and coping strategies are reported.

1.3 Anthelminthic resistance

Anthelminthic resistance is the ability of helminth parasites in a population to survive anthelminthic treatment that used to be effective against the same species and stage of infection at the same dose rate (Kaplan, 2004; Coffey *et al.*, 2007). The first anthelmintic resistance problem occurred in *H. contortus* (barber pole worm) of sheep, then in

cyathostomins (small strongyles) of horses (Kaplan, 2004). The resistance was reported against phenothiazine and thiabendazole in the early 1960s (Kaplan, 2004). Later, gastrointestinal nematode resistance was reported against all drugs within the three major classes of anthelmintics (Besier and Love, 2003).

A survey on 59 farms indicated that on 90% of the sheep farms in South Africa nematode were resistant to drugs from at least one of the anthelmintic groups. About 40% of these farms showed multiple resistance to compounds from anthelmintic groups (Van Wyk and Van der Merwe, 1993). Prichard (2005) reported that the challange is rampant in gastro-intestinal nematodes of ruminants and horses. The European Medicines Agency (2006) confirmed that resistance to anthelmintics is a global problem in sheep, goats and horses and is a rising problem in cattle (Table 1.3), but information on anthelmintic resistance in other animal species is currently more limited.

A number of studies have addressed the relationship between the nutritional status of the host and its ability to regulate a parasite infection (Coop and Kyriazakis, 2001). In addition, the age, breed and the reproductive state of the animal can influence resistance development. Generally, the speed with which anthelmintic resistance develops is due to the number of genetic polymorphism in the population, the initial frequency of resistance-contributing alleles, the number of genes involved and the complexity of the resistance mechanisms, the biology of the nematode, whether the resistance gene(s) are dominant or recessive in expressing the resistance phenotype, anthelmintic treatment coverage, the relative reproductive fitness of the wild-type (susceptible) and resistant genotype in the absence and presence of anthelmintics, treatment frequency, and any other management practices which may impact on parasite transmission and drug dosage (Prichard, 2005).

Drug resistance can happen in a number of ways (Wolstenholme *et al.*, 2004). There may be a change in the molecular target, so that the drug no longer recognizes the target, or a change in the nematode metabolism that inactivates or removes the drug or that prevents its activation, or a change in the distribution of the drug in the target. For example, Kohler (2001) reported that benzimidazoles alter the structure of B-tubulin isotypes in the parasites, nicotinic agonists alter

target structure and/or arrangement of nicotinic acetylcholine receptors (nAChR) subunits in the nematode body, and macrocyclic lactones alter target structure of glutamate-gated chloride channels (GluCl).

1.4 Other problems of anthelmintics use

Anthelmintic treatments are too expensive for many small scale farmers (SSF), who cannot afford the purchase price of these drugs. In addition, livestock GIN may be resistant to these drugs. Public awareness of food contamination with chemicals has resulted in consumer pressure to reduce drug residues in meat and meat products. Benzimidazole, imidazothiazole and ivermectin all leave residues in meat, milk and their products. In 2009 De and Sanyal reported that ivermectin is excreted in faeces in sufficient quantity to have a detrimental effect on invertebrates that usually degrade dung heaps, and hence on organisms higher up the food chain. Thus, other means of gastro-intestinal nematode control have been explored such as using of copper wire particles, grazing management, breeding for host resistance, vaccination, using plants extracts and biological control, which are all discussed below.

Table 1. 2: Worldwide reported cases of anthelmintic resistance in ruminants

Country	BZ	LEV	ML	Reference
Argentina	+	+	+	Eddi *et al.*, 1996
Australia	+	+	+	Overend *et al.*, 1994
Belgium	+	-	-	Geerts, 1995
Brazil	+	+	+	Echevarria *et al.*, 1996
Denmark	+	+	+	Maingi *et al.*, 1996
England	+	+	+	Coles, 2005
France	+	+	-	Chartier *et al.*, 1998; 2001
Germany	+	-	-	Scheuerle *et al.*, 2009
Greece	+	-	-	Papadopoulos *et al.*, 2001
India	+	+	-	Gill, 1996
Ireland	+	-	-	O'Brien *et al.*, 1994
Italy	+	-	-	Cringoli *et al.*, 2007
Kenya	+	+	+	Mwamachi *et al.*, 1995
Malaysia	+	+	+	Chandrawathani *et al.*, 2004b
Country	**BZ**	**LEV**	**AVM**	
Netherlands	+	+	+	Borgsteede *et al.*, 1996
New Zealand	+	+	+	Leathwick *et al.*, 2001
Paraguay	+	+	+	Maciel *et al.*, 1996
Scotland	+	+	+	Jackson *et al.*, 1992
Slovak	+	-	+	Čerňanská *et al*., 2006
South Africa	+	+	+	Van Wyk *et al.*, 1997
Spain	+	-	-	Requejo-Fernández *et al.*, 1997
Switzerland	+	-	+	Schnyder *et al.*, 2005
Tanzania	+	-	-	Bjorn *et al.*, 1990
Turkey		+		Tinar *et al.*, 2005
Uruguay	+	+	+	Nari *et al.*, 1996
USA	+	+	+	Terrill *et al.*, 2001
Zambia	+	-	+	Gabrie *et al.*, 2001
Zimbabwe	+	+	-	Boersema and Pandey, 1997

BZ: Benzimidazoles, LEV: Imidazothiazoles, ML: Macrocyclic lactones and AVM: Avemectins. + = resistance has been reported, - = resistance has not been reported.

1.5 Alternatives to anthelminthic

1.5.1 Copper wire particles

This method is mainly efficient against *H.contortus* and much less against the other nematode speacies (Soli *et al.*, 2010). In general, copper oxide reduces parasite load in lambs. However, copper treatments do not appear effective in mature sheep (Burke *et al.*, 2005). To estimate the safe dose of copper oxide wire in controlling nematodes, Burke *et al.* (2007) found that a dose of copper wire less than 0.5 g kg-1 BW limited the risk of copper toxicity. It was effective in reducing nematode eggs per gram of faeces in kids, while 5 g kg-1 BW of copper oxide was effective in older goats. An important observation of these authors was that copper oxide does not appear to be effective in controlling larvae of *H. contortus.* The mode of action of copper wire particles to control internal parasites is poorly understood. Copper has a direct effect on internal parasites and/or that copper may help to improve the host immune system (Hale, 2006).

1.5.2 Grazing management

Grazing management procedure including field rotation and rotation between animal species can decrease resistance and to assist in the control of the parasites (e.g., Barger, 1999). Schoenian (2009) reported that rotational grazing does not help to control gastrointestinal parasites unless the pasture rest periods are long enough (more than 70 days). This makes it an expensive method of control because it requires more land and fewer animals (Athanasiadou *et al.*, 2001a; Gillian *et al.*, 2004). However, in tropical condition, a rigourous application of grazing management system can be efficient to reduce infection especially with *H. contortus* (e.g., Barger *et al.*, 1994).

1.5.3 Breeding for host resistance

Breeding for resistance to nematodes is a viable option (Mitreva *et al.*, 2007). In cattle, efforts focus on generating quantitative trait loci that contain genes affecting resistance to nematodes (Sonstegard and Gasbarre, 2001). Furthermore, in sheep numerous studies have shown similar correlation between host genetics and nematode egg per gram values (McEwan, 1998). In regard of host resistance to nematode infection, the current view is generally that the

phenomenon is multigentic, not monogentic (Bishop and Morris, 2007).Crawford *et al.* (2006) assumed that the gene responsible for parasite resistance is near the end of chromosome 8 in nematodes, this measured in adults Trichostrongylus spp. in the abomasum and small intestines.

1.5.4 Vaccination strategy

Vaccination strategies usually focus on the use of antigens derived from the intestine of nematodes (Smith, 1999) and from the surface of nematodes gut (Knox and Smith, 2001). These antigens stimulate host immune responses as a result of infection (Knox and Smith, 2001). Currently vaccines are acting directly against potentially susceptible antigens on the parasite (Smith, 1999). The most promising vaccine against *H. contortus* involves "hidden gut" antigens. These antigens are derived from the gut of the nematode and would stimulate the synthesis of antibodies when administered to the animal (Miller and Horohov, 2006). Vaccines against other nematode species have largely focused on using antigens found in worm somatic tissue and secretory products (Jasmer and McGuire, 1996). Over all, deeper understanding of both the important antigenic characteristics, and mechanisms utilized by nematodes to survive host immune responses are both indispensable (Smith, 1999). Despite promising results and ongoing research over the years, a commercial product is yet to be produced.

1.5.5 Nutrition

Nutrition has an influence on host resistance to helminth infections (Besier and Love, 2003). The nutritional status of the host can influence the pathogenesis of parasitic infection. Well-nourished animals generally withstand parasitism better than those less adequately fed (Adewoga *et al.*, 2010). Specifically, protein supplementation reduces faecal egg counts, expelling of adult worms, and improves host performance (Datta *et al.*, 1998). Moreover, Coop and Holmes (1996) reported that protein supplementation increases the rate of acquisition of immunity, and increases resistance to re-infection. This has been associated with an enhanced cellular immune response in the gastro-intestinal mucosa. Nutritional regimes that minimise the impact of worm infections and enhance the immune response require investigation and integration into sustainable control strategies (Besier and Love, 2003).

1.5.6 Botanical extracts

Botanical extracts maybe used as an alternative anthelmintic strategy (Stepek *et al.,* 2004). A wide range of plants and plant extracts have been used traditionally for the treatment of helminth infections of humans (Waller *et al.*, 2001). Globally, medicinal plants are found and frequently used in Africa, China, India, Japan, Pakistan, Thailand and particularly in South Africa (Mukhtar *et al.*, 2008). However, Indian and Chinese traditional medicine are recognized as the oldest and most developed, respectively (Gurib-Fakim, 2006). In South Africa, Van Wyk *et al.* (1997) highlighted that approximately 3000 botanical plants are used for medicinal purpose by indigenous traditional South African healers. Generally, South Africa has experienced an increase in ethnopharmacology research due to recognition of the economic value of available botanicals. This has resulted in the investigation of many South African plants for bioactivities such as anthelmintic activities (McGaw *et al.*, 2000). Some popular and widely used South African medicinal plant species include *Aloe ferox* Miller (Maphosa *et al.*, 2010) and *Peltophorum africanum* Sonder (Bizimenyera, 2008). Plants extracted using different methods, however, water extraction considered the main method used in traditional medicine (Sparg *et al.,* 2002). Table 1.4 lists *in vitro* studies around the world, evaluating botanical extracts with the potential to control livestock nematodes.

1.5.6.1. Mechanisms of botanical extracts

The mode of action of some botanical extracts is poorly understood. However, there are reported mechanisms for some. For example, Gillian *et al.* (2004) reported that plants such as *Ananas comosus* Merr, *Carica papaya* L. cv. Rathna and *Ficus* spp contain products that can digest the cuticle of nematode, leading to their death. The active ingredients of these plants are proteolytic enzymes of the papaya, the enzyme ficin from fig trees and cysteine proteinases of pineapples. These enzymes have been used in medicine. Papain from papaya is used as anti-inflammatory substance. Chymopapain is used to treat prolapsed intervertebral discs with a similar success rate to surgery. The enzyme ficin from *Ficus* spp is also used as an anti-inflammatory agent (Stepek *et al.,* 2004). Fresh pineapple juice was found to possess an enzyme, bromelain, which is similar to ficin. Gillian *et al.* (2004) showed that proteolytic enzymes from the genus *Ficus* have anthelmintic action.

Allium sativum Linn contains a sulphuric compound that has anthelminthic effects (Singh *et al.*, 2009). Ferri *et al.* (2003) isolated another sulphuric compound from *A. sativum* blub called Ajoene. Ajoene inhibits protein prenylation and arterial smooth muscle cell proliferation in parasites, which would be fatal (Ferri *et al.*, 2003).

Extracts of leaves and seeds of neem (*Azadirachta indica* A. Juss) trees also have anthelmintic effects in small ruminants (Thomas *et al.*, no date; Costa *et al.*, 2006). Akhtar (1999) reported that neem contains several chemicals (e.g. azadirachtin, nimbocinol and nimbin) which are responsible for the nematicidal properties. *In vivo* trial using neem leaves in goats showed 100% reduction in faecal egg counts (Radhakrishnan *et al.*, 2007). When leaves of the neem tree were fed to parasitized sheep, no anthelmintic effect was however recorded against *H. contortus* (Githiori, 2004).

Another plant-based nematode control method lies on grazing of plants containing condensed tannins (CT) (Min *et al.*, 2003). Growing these plants for hay or grazing, to be used as a natural de-worming agent, may be a cost-effective, environmentally friendly alternative to the exclusive use of chemical anthelmintics by small ruminant producers (Shaik *et al.*, 2004). Generally, CT are phenolic compounds found in forage legumes, trees and stems (Barry and McNabb, 1999). Condensed tannins are widely distributed in legume pasture species such as *Lotus corniculatus* L. cv. Grassland Goldie and in several *Acacia* spp (Degen *et al.*, 1995), and *Lespedeza cuneata* Dum.Cours (Min *et al.*, 2004). Condensed tannins have either beneficial or detrimental effects on ruminants, that depending upon their level of intake and their structures (Min *et al.*, 2003). Low to moderate level of CT can improve animal performance (Waghorn and Shelton, 1997; Athanasiadou *et al.*, 2001b; Min *et al.*, 2003). Levels of CT less than 50 mg kg^{-1} BW in feed can reduce the risk of bloat, increase the uptake of essential amino acids, enhance the production of milk and wool, and be effective against gastrointestinal parasites (Athanasiadou *et al.*, 2001b). However, higher levels of CT could decrease voluntary feed intake, digestibility of fibre in the rumen and animal growth (Rojas *et al.*, 2006).

The manner in which condensed tannins affect nematode parasites can be classified as a direct

or an indirect effect. A direct effect of CT might be mediated through CT–nematode interactions affecting physiological functions of gastrointestinal parasites (Rochfort *et al.*, 2008; Hoste *et al.*, 2012). Condensed tannins can also react directly by interfering with egg hatching, and larval development to infective larval stage (Min and Hart, 2003). This reduces pasture contamination and infective larvae ingestion, which in itself might provide adequate control of gastrointestinal parasites. Condensed tannins also have the ability to bind with proteins in nematode walls, making them inactive or killing them (Athanasiadou *et al.*, 2001a). Non-direct effects of CT appear to be improving protein nutrition by binding to plant proteins in the rumen to prevent microbial degradation; this increases protein flow to the duodenum. Min and Hart (2003) have shown improved protein nutrition decreases parasite infection by enhancing host immunity.

Table 1. 3: Some botanical extracts used *in vitro* to control gastrointestinal nematodes in animals around the world

Plant family	Plant scientific name	Country of Use	Method of Preparation	Claims	References
Asphodelaceae	*Aloe ferox*	South Africa	Leaf extract	Effective	Maphosa *et al.,* 2010
Liliaceae	*Allium sativum*	Pakistan	Water extract of the garlic bulb	Effective	Iqbal *et al.,* 2001
Myrsinaceae	*Myrsine africana*	Kenya	Dried fruits and leaves	Ineffective even with the highest dose	Githiori *et al.,* 2002
Fabaceae	*Lespedeza cuneata*	USA	Dried ground hay	Reduction of nematodes	Min *et al.,* 2003; 2004
Caricaceae	*Papaya carici*	Not stated	Latex extract	Effective	Adu *et al.,* 2009
Moraceae	*Ficus spp.*	Not stated	Juice extract	Effective	Gillian *et al.,* 2004
Bromeliaceae	*Ananas comosus*	Not stated	Juice extract	Effective	Gillian *et al.,* 2004
Bromeliaceae	*Ananas comosus*	Pakistan Philippines	Raw powder	Ineffective	Hördegen *et al.,* 2003.
Zingiberaceae	*Zingiber officinale*	Pakistan	Ethanol extract of Rhizomes	Effective	Iqbal *et al.,* 2001
Meliaceae	*Azadirachta indica*	Bangladesh	Ethanol extract from leaves	Effective	Sujon *et al.,* 2008
Anacardiacae	*Spondias mombin*	Nigeria	Aqueous and ethanolic crude extract	Effective	Ademola *et al.,* 2005

Plant family	Plant scientific name	Country of Use	Method of Preparation	Claims	References
Fabaceae	*Hedysarum coronarium*	Newzerland	Condensed tannin extracts in aqueous solution	Effective	Niezen *et al.,* 2002.
Leguminosae	*Tephrosia vogelli*	Ethiopia	Ethanol extract of leaves	Effective	Siamba *et al.,* 2007
Fabaceae	*Lotus corniculatus*	USA	Condensed tannin extracts in aqueous solution	Effective	Min *et al.,* 1999
Cucurbitaceae	*Curcurbita mexicana*	Not stated	Ethanol extract of whole fruit	Effective	Iqbal *et al.,* 2001
Fabaceae	*Lotus pedunculatus*	USA	Condensed tannin extracts in aqueous solution	Effective	Molan *et al.,* 2000
Amaranthaceae	*Halothamnus somaliensis*	Ethiopia	Crude preparation of roots powder	Effective	Dawo and Tibbo, 2005
Leguminosae	*Ornobrychis viciafolia*	France	Aqueous acetone extract	Effective	Paolini *et al.,* 2003
Fabaceae	*Caesalpinia crista*	Pakistan Philippines	Aqueous ethanol extract	Ineffective	Hördegen *et al.,* 2003.
Compositae	*Vernonia anthelmintica*	Pakistan Philippines	Raw powder	Ineffective	Hördegen *et al.,* 2003.

Plant family	Plant scientific name	Country of Use	Method of Preparation	Claims	References
Lamiaceae	*Leonotis leonurus*	South Africa	Aqueous extract of leaves	Effective with high doses	Maphosa *et al.*, 2010
Fabaceae	*Elephantorrhiza elephantina*	South Africa	Aqueous extract of leaves	Effective with high doses	Maphosa *et al.*, 2010
Fumariaceae	*Fumaria parviflora*	Pakistan Philippines	Aqueous ethanolic extract	Ineffective	Hördegen *et al.*, 2003.
Mimosaceae	*Albizia anthelmintica*	East Africa, Kenya, Sweden, Ethiopia, Uganda	Heat treated or soaked in water	Ineffective	Minja, 1994; Desta, 1995; Grade and Longok, 2002
Fabaceae	*Peltophorum africanum*	South Africa	Ethanol extract of dried leaves	Effective	Bizimenyera, 2008
Cucurbitaceae	*Momordica charantia*	Bangladesh	Ethanol extract of dried leaves	Effective	Sujon *et al.*, 2008
Canellaceae	*Warburgia salutaris*	South Africa	Acetone extract of dried leaves	Effective	McGaw and Eloff, 2005
Fabaceae	*Acacia karoo Acacia nilotica*	Zimbabwe	dried leaves	Effective	Kahiya *et al.*, 2003

1.5.7 Biological control

Over the last 20 years, there has been growing interest and involvement in research on biological control of GIN nematodes of livestock (Larsen, 1999). In general, biological control of nematodes has gone from being something considered to be a novelty of basic research interest to an area of applied research, with the potential to become an important, integrated element and sustainable strategies to control nematodes in livestock (Larsen, 2006). Biological control is the use of living microorganisms introduced into an environment to control a target organism and thereby reduce the population of the target to a threshold below which it no longer causes clinical problems and economic losses (Thamsborg *et al.*, 1999). De and Sanyal (2009) defined biological control as the activity of natural enemies which maintain a host population at levels lower than would occur in the absence of the enemies. These enemies include classical, un-exploited organisms and genetically modified organisms (Waller and Faedo, 1996). Biological control can be divided into two major categories: natural and applied. Natural biological control is influenced by native or co-evolved natural enemies in the environment without human intervention. Applied biological control involves the repeated application of cultured biological control agents (De and Sanyal, 2009).

Biological control might be different from other methods of nematode parasite control in livestock, which are directed at the parasitic stages within the host. However, biological control is targeted at the free-living stages on pasture (Waller, 2006). This means that biological control focuses on the faecal deposits in which eggs, L_1, L_2 and L_3 larval stages are found. The entire philosophy of using biological control agent against GIN nematodes in animals is to reduce the number of infective stages that are available to be picked up by grazing susceptible individuals of the different species of livestock (Larsen, 1999; Waller, 2006). This reduction in larval stages on herbage will subsequently prevent the build-up of worm burdens in hosts, which otherwise would cause subclinical or clinical responses, in particular in young animals (Larsen, 1999). Biological control of nematodes in livestock aims to establish a condition where grazing animals are exposed to a low number of infective larvae. Natural immunity in the animals will tolerate these low levels.

Biological control has the ability to reduce the level of nematode parasitism in livestock (Epe *et al.*, 2009). Reduced larval numbers in cultures of sheep faeces has been reported as a result of the application of biocontrol agents (Waller *et al.*, 2001). An important issue is that biocontrol agents have no negative effects on the environment (Gronvold *at al.*, 1996), however the effect on animals requires investigation. Furthermore, biocontrol agents are free of chemicals, thus producers can capitalize on the residue-free, organic meat products that are in high demand from consumers and command a premium price (Waller, 2006).

Several natural enemies of nematodes have been used in the past. Others have been suggested as potential candidates, such as earthworms, viruses, soil amoeba, protozoa, collembolans, mites, fungi, and bacteria (Larsen, 1999; Thamsborg *et al.*, 1999).

1.5.7.1 Earthworms

Earthworms are a vital component of the soil ecosystem. Earthworm populations consume large volumes of soil and organic matter, including animal faeces (Gronvold *at al.*, 1996). During feeding, they consume nematodes present in soil and faeces. The effect of earthworms on nematode parasites could be direct or indirect. Some earthworm actively feed on nematodes by catching the prey with their oral styles (Waller and Faedo, 1996). However, these earthworms are only active in moist habitats, which limit their biological control potential. Notable, the role of earthworms in relation to pasture infectivity is very complex. They may reduce transmission by degrading faecal pats but they may also prolog the survival of larvae by taking them into the soil.

1.5.7.2 Viruses

Cases of livestock nematodes infected by nematopathogenic viruses have been reported (Stifling, 1991). However, viruses cause abnormal behaviour in plant parasitic nematodes such as *Meloidogyne* and *Tylenchorhynchus* spp. (Mankau, 1981). One challenge is that it is difficult to recognize viral infection in microscopic nematodes, or differentiate sick nematodes from those that are diseased for some other reason (Waller and Faedo, 1996). Consequently, viral pathogens against free living stages of animal parasitic nematodes are unlikely to be developed as commercial products.

1.5.7.3 The soil amoeba, *Theratromyxa weberi* Zwillenberg

Theratromyxa weberi Zwillenberg can ingest nematode parasites of livestock (Sayre and Wergin, 1979). It traps the nematode and digests it within a day. However, Gronvold *at al.* (1996) indicated that *T. weberi* and other amoebae have limited biological control capacity because they are slow-moving compared with nematodes, and they are sensitive to low soil water potentials, conditions under which nematodes may survive.

1.5.7.4 Protozoa

Protozoa have been used as biological control agents for nematodes (Canning, 1973) and for insects control (Lacey *et al.*, 2001). Most promising species seem to be those that produce spores which attack nematodes following ingestion. However, this ingestion is prevented in many nematode species by the narrow bore of their style canal (Waller and Faedo, 1996). For this reason, protozoa might not offer a high class opportunity for biological control of nematode parasites of livestock.

1.5.7.5 Micro-arthropods

A number of soil invertebrates have been tested for their potential as biological control of nematode parasites. Micro-arthropods such as collembolans and mites may be effective options (Akhtar and Malik, 2000). The mechanisms by which collembolans and mites affect nematodes have not yet been determined. Lysek (1963) noted that collembolans and mites are capable of consuming *Ascaris suum* Goeze (1782) eggs.

1.5.7.6 The fungus *Duddingtonia flagrans* Cooke

Use of nematophagous fungi may be a way to reduce pasture contamination and parasite populations (Larsen, 1999; Epe *et al.*, 2009). A number of fungal species have shown anthelmintics properties (Epe *et al.*, 2009). These fungi are able to attract and kill the developing larval stages of parasitic nematodes in a faecal environment (Table 1.5), either as a primary source of nutrients or as a supplement to a saprophytic existence (Waller *et al.*, 2006). According to their morphology, functional characteristics and the way they capture nematode parasites, these fungi are divided into three main classes: predacious, endo-parasitic and egg-destroying fungi (Waller and Faedo, 1996).

The fungus *Duddingtonia flagrans* Cooke is one of the fungi that traps nematodes and has shown much promise as a biological agent (Larsen, 1999). This fungus belongs to a heterogeneous group of fungi in the Deuteromycetes family (Waller *et al.*, 2006). Specifically, *D. flagrans* is a predacious fungus that produces adhesive three dimensional hyphal networks during development, which trap the larval stages of nematodes (Campos *et al.*, 2008).

This fungus has shown its ability to survive through the digestive system of ruminants as a resistant spore (chlamydospore) form (Manueli *et al.*, 1999) and has demonstrated its potential to consistently and significantly reduce the numbers of infective *Trichostrongyle* larvae and most of the economically important gastrointestinal parasites, when applied in animals feed as fungal spores (Larsen, 1999). Knox and Faedo (2001) reported a reduction in nematode larvae count and an improvement in live weight gains in sheep fed a supplement containing *D. flagrans* chlamydospores compared to untreated sheep.

The dosage of *D. flagrans* spores which affect nematode parasites negatively has been reported in many studies. For example, a fungal dose of 5g of grain/sheep per day for 2 consecutive days reduced larval numbers in faecal culture in an *in vitro* study (Waller *et al.*, 2001). In addition, Larsen *et al.* (1998) demonstrated that sheep fed a dose of 5×10^5 *D. flagrans* chlamydospores per day resulted in a substantial (more than 80%) reduction in the number of infective larvae derived from nematode eggs in faeces. Moreover, dosing calves with 10^6 *D. flagrans* chlamydospores per kg BW daily was sufficient to significantly reduce herbage infectivity and subsequently infection levels (Sarkunas *et al.*, 2000). Another study in the equine field reported that dosage of 2×10^6 chlamydospores per kg BW were effective in reducing numbers and transmission of equine nematode L_3 stages from faecal pats to surrounding herbage (Baudena *et al.*, 2000). More importantly, feeding of daily dosages of 2.5×10^4 to 5×10^5 *D. flagrans* chlamydospores per kg BW can control larval stages of *H. contortus* in sheep (Peña *et al.*, 2002).

Duddingtonia flagrans traps the free-living larval stages, which include eggs, L_1 and L_2 stages within the faecal deposit, and infective third-stage larvae on pastures. Faedo *et al.* (1998)

reported that *D. flagrans* can survive passage through the gastrointestinal tract in the host and might be effective in trapping larvae present in faeces, thereby reducing pasture larval population. Larsen *et al.* (1991) reported that *D. flagrans* can produce large numbers of thick-walled spherical chlamydospores that survive unfavourable conditions in the gut. Consequently, chlamydospores are administered orally and deposited in faeces. Larsen *et al.* (1995) proposed that these chlamydospores produced by *D. flagrans* germinate in the host faeces, forming specialized, three-dimensional networks that trap the parasite larvae and prevent nematode infective stages from developing. However, Faedo and Krecek (2002) pointed out that fungi such as *D. flagrans* do not affect the populations of gastrointestinal nematodes within the host, and therefore they are not curative.

1.5.7.7 The fungus *Clonostachys rosea f. rosea* Schroers

The fungus is also known as *Gliocladium roseum* Bain., belongs to the family *Bionectriaceae*, and the Order *Hypocreales*. It is widely distributed, facultative saprophyte in the soil (Schroers *et al.*, 1999). *Clonostachys rosea f. rosea* is a predacious fungus has a potential to control nematodes of plants and animals (Zhang *et al.*, 2008). The fungus has shown its ability to survive through the digestive system of ruminants as chlamydospore form (Baloyi *et al.*, 2012). The fungus also is able to attack many organisms, including other fungi, bacteria, insects and nematode parasites because it produces a wide range of volatile organic compounds. For example, *C. rosea* protects plants against *Botrytis cinerea* by suppressing spore production (Li *et al.*, 2004). Its hyphae grow inside the conidia of *Botrytis cinerea*. In addition, a strain of *C. rosea*, IK726, isolated from barley roots infected with *Fusarium culmorum* was evaluated for its biological activity (Knudsen *et al.*, 1995). The strain IK726 was found to be effective against seed-borne diseases of cereals under field conditions. Moreover, Jensen *et al.* (2000) reported that IK726 strain of *C. rosea* controlled *Alternaria radicina* Meier, Drechsler and Eddy on carrot seeds.

The mode of action of *C. rosea* as biological control agent is not well understood. However, mycoparasitism, substrate competition, enzymatic activity and induced resistance are all thought to play a role (Lübeck *et al.*, 2002). Production of secondary metabolites might provide antibiotic effect as is known for other biocontrol fungi (Roberti *et al.*, 2008).

Clonostachys rosea f. rosea conidia attach to the cuticle of nematodes, germinate and produce germ tubes, which then penetrate the host body, and kill the nematode (Zhang *et al.*, 2008). *Clonostachys rosea f. rosea* produces cell wall-degrading enzymes, including chitin, glucan, and cellulose-degrading enzymes (Lübeck *et al.*, 2002; Roberti *et al.*, 2008). The enzymatic hydrolysis of chitin is catalysed by the action of three types of chitin-hydrolysing enzymes belonging to glycosyl hydrolase families. These enzymes digest the cell walls of ascomycetes and basidiomycetes, as well as insect skeletons (Mamarabadi *et al.*, 2009).

1.5.7.8 The bacterium *Bacillus thuringiensis* Berliner

Bacillus thuringiensis Berliner is a gram positive bacterium, found in various ecological niches such as soil, plant surfaces and dust from stored-products (Maagd *et al.*, 2003). *Bacillus thuringiensis* is closely related to *Bacillus cereus* Frankland and Frankland, a soil bacterium, and *Bacillus anthracis* Koch, the cause of anthrax. The three organisms are mainly different in their plasmids (Helgason *et al.*, 2000). Like other members of the genus, all three are aerobes capable of producing endospores.

Bacillus thuringiensis produces crystals of proteinaceous insecticidal δ-endotoxins (crystal proteins or Cry proteins) (Ito *et al.*, 2006). Cry toxins have specific toxin effects against insect species of the orders *Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera,* and *Mallophaga* as well as other organisms such as nematodes, mites and protozoa (Maagd *et al.*, 2003). *Bacillus thuringiensis* has received considerable attention because of its utility for control of insect pests in agriculture and insect vectors of human diseases (Maagd *et al.*, 2003; Quesada-Moraga *et al.*, 2004). Swadener (1994) explained that there are at least 34 subspecies of *B. thuringiensis* (also called serotypes or varieties), and probably over 800 strain isolates (De Barjac and Frachon, 1990). Basically, *B. thuringiensis* strains have been used to control insect pests since the 1920s (Wood, 2002). They are now used as specific insecticides under trade names such as Dipel® and Thuricide®. These products have captured 90-95% of the biopesticide market (Swadener, 1994). The most advantageous character of *B. thuringiensis* products is the selectivity of the protein crystals. However, this is complemented by the many strains available that attack a wide range of specific target pests.

Bacillus thuringiensis is considered safe to people and non-target species, such as wildlife. Its formulations can be used on all food crops, making it relatively easy to exploit commercially. Moreover, *B. thuringiensis* does not have dominant alleles, which could be easy to influence genetically (Raymond *et al.*, 2010). Some *B. thuringiensis* strains can kill nematodes (Larsen, 1999). Ciordia and Bizzell (1961) showed that *B. thuringiensis* toxins could affect free-living stages of *Cooperia* and *Ostertagia* spp., which are parasitic nematodes of cattle. Bone *et al.* (1987) reported that eggs and larvae of *T. colubriformis* could be killed by the toxin of *B. thuringiensis*. In addition, many *B. thuringiensis* isolates have been found to be highly toxic to larval stages and adults of *H. contortus*, *T. colubriformis* and *O. circumcincta in vitro* (Kotze *et al.*, 2005). These studies indicated that *B. thuringiensis* is toxic to both larval stages and adults of economically significant nematodes (Table1.5). These *B. thuringiensis* strains could be utilised as anthelmintics to kill nematodes of the gastrointestinal tract, and/or the free-living larval stages that develop in faeces or on pastures.

Bacillus thuringiensis would be an attractive alternative biocontrol candidate because it has low mammalian toxicity (Siegel, 2001), and it has activity against both eggs and larvae of nematode parasites on pastures (Larsen *et al.* 1998). The spores of *B. thuringiensis* could pass through the intestinal tract of many animal species and subsequently germinate in faecal cultures to form colonies containing proteins toxic to nematodes (Lee *et al.*, 2002).

Research on using *B. thuringiensis* to control the free-living larval stages of nematodes resulted in a significant reduction of existing parasites (Kotze *et al.*, 2005). On the other hand, *B. thuringiensis* spores are slow to germinate and develop crystal toxins to disturb the nematode growth (Grady *et al.*, 2007). Notably freshly hatched larvae are more susceptible to *B. thuringiensis* toxins than older ones. These constraints limit the ability of *B. thuringiensis* isolates to inhibit nematode populations (Grady *et al.*, 2007).

Bacillus thuringiensis acts via its crystal toxin proteins. These crystal toxin proteins are produced when *B. thuringiensis* bacterium develops endospores. Bacteria produced endospores when conditions for bacterial growth are not optimal. These spores are considered

as an inactive stage of the bacterial life cycle. When the conditions improve, the spores germinate.

Swadener (1994) reported that it is the crystal toxin proteins that kill the insects. Consolidated, mixtures of protoxins dissolve in the alkaline gut after they are ingested, and then the digestive enzymes break down the protoxins into the *B. thuringiensis* insecticidal components called the delta-endotoxins. Delta-endotoxins bind to the cells lining the midgut membrane, creating pores in the membrane. Then haemolymph flows into the gut through the pores, killing the pest with "peritonitis". The bacterial spore germinates after the gut membrane is broken. Cells then reproduce and make more spores.

Wei *et al.* (2003) reported that *B. thuringiensis* uses the same mode of action to kill nematodes. Kotze *et al.* (2005) have shown that different *Cry* proteins have variable toxicity towards nematodes. For example, *Cry5B* endotoxin kills *Caenorhabditis elegans* (Marroquin *et al.*, 2000). Some protoxins *Cry5B, Cry6A*, *Cry14A* and *Cry21A* are toxic to a number of free-living stages of nematodes, and *Cry5B, Cry14A* and *Cry21A* were highly toxic to the free-living larval stages of the rodent-parasitic nematode, *Nippostrongylus brasiliensis* (Wei *et al.*, 2003). These studies have confirmed that *Cry* proteins cause damage to the intestine of both nematodes and insects.

1.6 Conclusions

Gastro-intestinal nematodes have been identified as the primary parasites of small ruminants in tropical and sub-tropical areas. Their control has depended upon chemical compounds, but this does not seem to work due to nematode resistance. However, the review has shown that botanical plants and biological control agents could be effective replacements for chemicals when supplemented in the diet of small ruminants.

Table 1. 4: Effect of biological control agents on nematodes

Biocontrol agent	Experimental model	Effect on				Reference
		EPG	LPG	WT	L_3*	
Arthobotrys dactyloides	Goats and Sheep		+			Sandecris *et al.*, 2008
Arthobotrys oligospora	Cattle		+			Gronvold *et al.*, 1985
Catenaria anguillula	Goats and Sheep		+			Sandecris *et al.*, 2008
Clonostachys Rosea	Sheep	None	+	None		Baloyi *et al.*, 2012
Clonostachys Rosea	Plants (+)					Li *et al.*, 2004
Duddingtonia flagrans	Sheep	None	+	None		Peña *et al.*, 2002
Duddingtonia Flagrans	Goats	None	+	+	None	Epe *et al.*, 2009
Duddingtonia Flagrans	Cattle	None	+	None	+	Sarkunas *et al.*, 2000
Duddingtonia flagrans	Horse	None	+		+	Fernández *et al.*, 1997
Drechmeria coniospora	*In vitro*		+			Santos and Charles, 1995
Harposporium anguillulae	Sheep		+			Charles *et al.*, 1996
Bacillus thuringiensis	Sheep	None	+	+		Baloyi, 2011
Bacillus thuringiensis	Cattle	+	+	+		Ciordia and Bizzell, 1961
Bacillus thuringiensis	*In vitro*	+	+			Kotze *et al.*, 2005

EPG= egg per gram of faeces; LPG= larvae per gram of faeces; WT= live weight; * = L_3 from pasture;
+ = reduction in LPG; None= no effect

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Chapter 2[*1]

In vitro anthelmintic activity of crude extracts of selected medicinal plants against *Haemonchus contortus* of sheep

Abstract

Ethanol extracts of 25 plant species were screened for anthelmintic effects against *Haemonchus contortus* (Rudolphi 1803). Ethanol extracts of each plant were used at various concentrations (10, 20 and 30%) to treat 10 day faecal cultures, incubated at 27°C with control cultures which were treated with ethanol for 48 hours. Five plants with high efficacies (*Ananas comosus* L. Merr., *Aloe ferox* Mill., *Allium sativum* Linn., *Lespedeza cuneata* Dum. Cours. and *Warburgia salutaris* Bertol.f. Chiov*)* were selected from the first screening for further investigation using ethanol, dichloromethane and water extracts at 4 concentrations (2.5, 5, 10 and 20%). Ethanol was the most effective solvent. Larval counts decreased with increasing extract concentrations, 10 and 20% of which had similar effects. *Lespedeza cuneata* caused more than 70% mortality in all concentrations. However, there remains a need to assess *in vivo* efficacy of these plants.

2.1 Introduction

Gastrointestinal nematodes (GIN) cause serious economic losses and are the most important factor limiting sheep production worldwide (Kaplan, 2004; Menkir *et al.*, 2006; Abebe *et al.*, 2010). *Haemonchus contortus* is one of the most important nematode parasites of both sheep and goats as adult worms suck blood, causing loss of plasma and protein in the host. Synthetic anthelmintics are currently being used to control GIN (Pomroy *et al.*, 2002). However, the evolution of resistance in GIN species to anthelmintic drugs has been reported in all sheep-producing countries, including South Africa (Van Wyk *et al.*, 1999).

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For this reason, effective alternatives against GIN are needed. Plant extracts with anthelmintic properties are considered one of the most promising alternatives for the control of GIN (Waller *et al.,* 2001; Gillian *et al.,* 2004). Previous studies have shown that many plants and forage crops have anthelmintic effects and in most cases they could be considered as an aid to control GIN in both human and livestock species (Athanasiadou *et al.,* 2001b; Gillian *et al.,* 2004; Singh *et al.,* 2009; Maphosa *et al.,* 2010). However, the biological effects of most of these plants have not been clearly defined.

The objectives of the study were to screen a number of plant extracts for their anthelmintic activity, and to evaluate the effect of different concentrations of these plants on infective larvae stages of *H. contortus* from sheep grazing contaminated pastures.

2.2 Materials and methods

2.2.1 Collection of plants and preparation of extracts

A total of 25 plants (Table 2.1) that are used traditionally to treat helminth infections were selected from available literature (Hördegen *et al*., 2003; Min and Hart, 2003; Fennell *et al.*, 2004; Min *et al*., 2004; Lange *et al*., 2006; Stepek *et al.*, 2006; Mohanlall and Odhav, 2009; Maphosa *et al.,* 2010). Plant material of these species was collected from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg campus, UKZN Research Farm (Ukulinga). Samples of *Ficus* spp. were from a private garden (Pietermaritzburg) and garlic and ginger samples were purchased from a commercial supermarket. Voucher specimens of plants were deposited at the UKZN Herbarium, Pietermaritzburg.

Fresh plant material was washed and then cut into small pieces. All plant material was air-dried followed by drying in an oven (LABCON, Model 5SOE1B, Maraisburg 1700) at 50 °C for 2-5 days. Each plant was then pulverized into a powder using an electric grinder (RETSCH, GmbH and Co.KG, 5657 HAAN1, West-Germany) until it was fine enough to pass through a 1-mm diameter sieve. Powdered samples were preserved in airtight plastic containers under dark conditions at room temperature.

Ten gram samples of ground material from each plant were boiled for 24 hours in 100 ml of each of three solvents, ethanol, dichloromethane and water in a Soxhlet's apparatus. The resulting extracts were transferred to 50 ml test tubes and placed in a waterbath (LABOTEC, Model 101, South Africa) at 50 °C. Extracts were condensed by evaporation of the solvent to a final volume of 30 ml. Condensed extracts were then preserved in airtight glass bottles and stored at 10 °C until required for screening. Two experiments were undertaken to screen plant extracts, one experiment using ethanol extracts of 25 plants followed by a second experiment using five of the most potent plants based on the results of the ethanol extracts.

2.2.2 *In vitro* screening of plant extracts

Faecal samples were collected from 35 randomly selected sheep (Merino) grazing on a contaminated Kikuyu pasture (*Pennisetum clandestinum* Hochst. ex Chiov.) at Ukulinga Research Farm. Rectal faecal samples were taken from sheep by hand and stored in plastic bags. Faeces were pooled and mixed. Sub-samples (5 g) were placed in trays and incubated (MEMMERT, 854 Schwabach, West-Germany) for 12 days at 27 °C. Samples were kept damp by daily watering at 10h00 during the period of incubation. On day 10, ethanol extracts were applied in 10, 20 and 30% (v/v) concentrations. Each concentration was used to treat four trays and there were four controls where only ethanol was used. Both Controls and treated trays were incubated for a further 48 hrs.

The Baermann Technique was used to count *H. contortus* larvae as described by Hansen and Perry (1994). Each faecal culture was placed in double-layer cheesecloth which was tied using a rubber band. Each faecal culture was placed in a funnel (supported by a funnel stand), which was then filled with lukewarm water until water covered the faecal material. The apparatus was left for 24 hr; then 15 ml of fluid was taken from the stem of the funnel and placed into a test tube which was left to stand for 30 min. The supernatant was removed with a Pasteur pipette and a drop of aliquot was transferred to a microscope slide using a Pasteur pipette. A drop of iodine was added- and the slide was covered with a cover-slip. Samples were examined and larvae were counted using 100× magnification.

In the second experiment, five plants with highest efficacy in two concentrations were selected for further *in vitro* screening using three solvent extracts at concentrations 2.5, 5, 10 and 20%. Each concentration was used to treat four trays and there were four controls for each solvent. Both Controls and treated trays were incubated for a further 48 hrs.

2.2.3 Statistical

Nematode mortality was calculated using Abbott's Formula (Abbott, 1925), as follows:

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

where n= number of larvae, T= Treated and Co= Control.

Nematode larvae counts were analysed using General Linear Model procedure of SAS (2000). In Experiment 1, following statistical model was used to analyse larval mortality at specific concentrations:

$$Y_{ij} = \mu + S_i + e_{ij};$$

Where, Y_{ij} = individual observation; μ = overall mean; S_i = effect of plant species; e_{ij} = the error term. In Experiment 2, effect of plant species and concentration on nematode larvae was determined by following model:

$$Y_{ijk} = \mu + S_i + Cj + (S \times C)_{ij} + e_{ijk};$$

Where, Y_{ijk} = individual observation; μ = overall mean; S_i = effect of plant species; Cj = effect of concentration; $(S \times C)_{ij}$ = interaction between plant species and concentration; e_{ijk} = the error term. Regression analysis were done between (a) the efficacy of 10% ethanol extracts (x-axis) and against efficacy of 20 and 30% ethanol extracts (y-axis); (b) the efficacy of water extracts (x-axis) and against the efficacy of dichloromethane and ethanol extracts (y-axis) using excel.

Table 2. 1: Leaves, bulbs (↕) and rhizomes (↕↕) of plant species evaluated for their anthelmintic activity.

Family	Scientific name	Common name
Liliaceae	*Allium sativum*↕	garlic
Asphodelaceae	*Aloe ferox*	bitter Aloe
Bromeliaceae	*Ananas comosus*	pineapple
Caricaceae	*Carica papaya*	papaya
Moraceae	*Ficus benjamina*	weeping fig
Moraceae	*Ficus ingens*	red leaved rock fig
Moraceae	*Ficus carica*	domestic brown fig
Moraceae	*Ficus carica*	domestic white fig
Moraceae	*Ficus indica*	Indian fig (Banyan)
Moraceae	*Ficus lutea*	giant-leaved fig
Moraceae	*Ficus elastica*	rubber tree
Moraceae	*Ficus natalensis*	natal fig
Moraceae	*Ficus sur*	broom cluster fig
Moraceae	*Ficus sycamorus*	sycamore fig
Moraceae	*Ficus ornamental Thai*	fig sp.
Lamiaceae	*Leonotis leonurus*	wild dagga
Moraceae	*Melia azedarach*	syringa
Fabaceae	*Peltophorum africanum*	weeping wattle
Amaryllidaceae	*Scadoxus puniceus*	snake lily
Fabaceae	*Lespedeza cuneata (syn. Sericea lespedeza)*	Chinese bush clover
Leguminosae	*Tephrosia inandensis*	Inanda tephrosia
Canellaceae	*Warburgia ugandensis*	Uganda greenheart
Canellaceae	*Warburgia salutaris*	pepper bark tree
Cucurbitaceae	*Cucumis myriocarpus*	wild cucumber
Zingiberaceae	*Zingiber officinale*↕↕	ginger

2.3 Results

2.3.1 Experiment 1: Screening of ethanol extracts for anthelmintic activity

Ethanol extracts from plant species had varied (P<0.001) effects on *H. contortus* larvae of sheep at 10, 20 and 30% concentrations (Table 2.2). Increasing the concentration of plant extracts caused stronger larvicidal effects on *H. contortus* larvae (Figure 2.1). *Ananas comosus* extracts at various concentrations (10, 20 and 30%) had the highest larval mortality range (98.3-100%), followed by *Lespedeza cuneata* (96.1-100%), *Aloe ferox* (73.9-86.9%), *Allium sativum* (68.0-84.0%) and *Warburgia salutaris* (76.9-80.8%). *Ficus sycamorus* and *Ficus indica* extracts had the lowest larval mortality range (-3.1-6.3%) and (7.4-44.5%), respectively. Other plant species showed a range of larval mortality between 15.6 and 81.5% (Table 2.2).

2.3.2 Experiment 2: Screening of different extracts of five plant species for anthelmintic activity

The efficacy of ethanol, dichloromethane and water extracts of five plants (*Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata* and *Warburgia salutaris*) against *H. contortus* larvae of sheep were studied *in vitro* at different concentrations (Table 2.3). The ethanol, dichloromethane and water extracts killed 75, 70 and 60% of larvae, respectively, at a concentration of 20%. *Lespedeza cuneata* had the highest efficacy causing a mortality of 89.7% with 20% ethanol extract, while *W. salutaris* had the lowest efficacy of 76.7% (Figure 2.2).

Table 2. 2: *In vitro* efficacy (mean± SEM) of ethanol extracts of 25 medicinal plants against *Haemonchus contortus* of sheep.

ıtal plants	Extract concentrations%		
	10	**20**	**30**
Aloe ferox	73.9±4.21	78.3±3.70	86.9±2.91
Allium sativum	68.0±5.74	77.0±5.72	84.0±4.31
Ananas comosus	98.3±3.41	100±2.12	100±1.01
Carica papaya	56.0±3.13	64.0±6.42	76.0±5.15
Ficus benjamina	28.1±3.53	68.8±3.94	78.1±3.55
Ficus ingens	68.8±5.83	75.0±8.02	78.1±5.74
Ficus carica (brown)	15.6±5.12	53.1±7.13	56.3±2.82
Ficus carica (white)	29.6±6.84	62.9±7.50	74.1±7.91
Ficus indica	7.40±3.73	29.6±2.72	44.5±7.04
Ficus lutea	40.0±5.43	53.4±4.22	60.0±6.32
Ficus elastica	66.7±3.15	74.1±8.24	77.8±6.61
Ficus natalensis	62.5±5.72	68.6±5.61	68.8±7.22
Ficus sur	71.9±5.71	75.0±5.61	81.3±5.60
Ficus sycamorus	-3.10±3.41	0.00±1.71	06.3±4.32
Ficus Thai red fruit	50.0±5.53	50.0±2.72	60.0±1.73
Leonotis leonurus	43.5±3.91	56.6±5.12	56.5±6.12
Melia azedarach	48.1±8.74	62.9±6.41	66.7±4.42
Peltophorum africanum	52.2±1.82	60.9±5.22	65.2±4.02
Scadoxus puniceus	15.6±4.63	50.0±4.81	59.4±8.21
Lespedeza cuneata	96.1±4.51	100±1.80	100±1.61
Tephrosia inandensis	24.0±7.33	48.0±5.5	64.0±7.82
Warburgia salutaris	76.9±2.62	80.8±2.92	80.8±3.41
Warburgia ugandensis	74.1±7.81	76.8±5.71	81.5±3.51
Cucumis myriocarpus	48.0±4.32	56.0±3.51	60.0±5.72
Zingiber officinale	56.0±4.21	60.0±4.82	72.0±2.51
F value	242.55	140.43	119.67
P<	0.001	0.001	0.001
RMSE	2.89	2.95	2.92
CV%	5.74	4.67	4.23

CV% = coefficient of variance; RMSE = the root mean square error.

Table 2. 3: *In vitro* efficacy (mean± SEM) of five medicinal plants against *Haemonchus contortus* of sheep using three different solvents

Plant species	Extract concentrations %	Extract		
		Ethanol%	Dichloromethane %	Water%
A. comosus	2.5	68.4±5.72	38.9±4.31	29.5±12.13
(Pineapple)	5	71.9±5.71	60.2±7.11	48.2±14.11
	10	85.7±7.13	82.1±6.71	71.1±9.21
	20	87.9±3.74	84.4±3.62	75±10.12
A. ferox	2.5	64±11.92	39.8±3.74	27.9±5.91
(Bitter Aloe)	5	68.4±7.11	58.3±5.71	48.7±12.31
	10	84.1±7.11	76.2±5.71	69.9±17.13
	20	86.1±3.72	79.9±1.82	72.9±3.52
S. sativum	2.5	66.4±6.61	55.7±8.74	41.5±11.61
(Garlic)	5	69.0±5.63	58.3±5.74	47.5±8.73
	10	79.4±5.74	73.6±2.91	66.2±15.01
	20	81.7±6.73	77.6±3.93	68.6±15.01
L. cuneata	2.5	70.9±5.92	46.7±7.22	31.0±5.62
(Chinese bush clover)	5	79.1±7.22	65.4±12.11	52.6±8.73
	10	86.7±4.31	81.7±1.72	71.9±6.93
	20	89.7±3.73	84.4±2.13	75.7±2.54
W. salutaris	2.5	43.8±5.44	23.3±7.41	08.8±10.52
(Pepper bark tree)	5	57.0±5.32	31.1±7.41	22.8±5.71
	10	74.2±10.91	69.8±12.11	58.6±9.91
	20	76.7±6.33	72.0±4.02	62.3±6.71
F value		40.07	145.09	54.37
P>		0.001	0.001	0.001
RMSE		3.52	2.99	5.18
CV%		4.78	4.86	10.21

CV% = coefficient of variance; RMSE = the root mean square error.

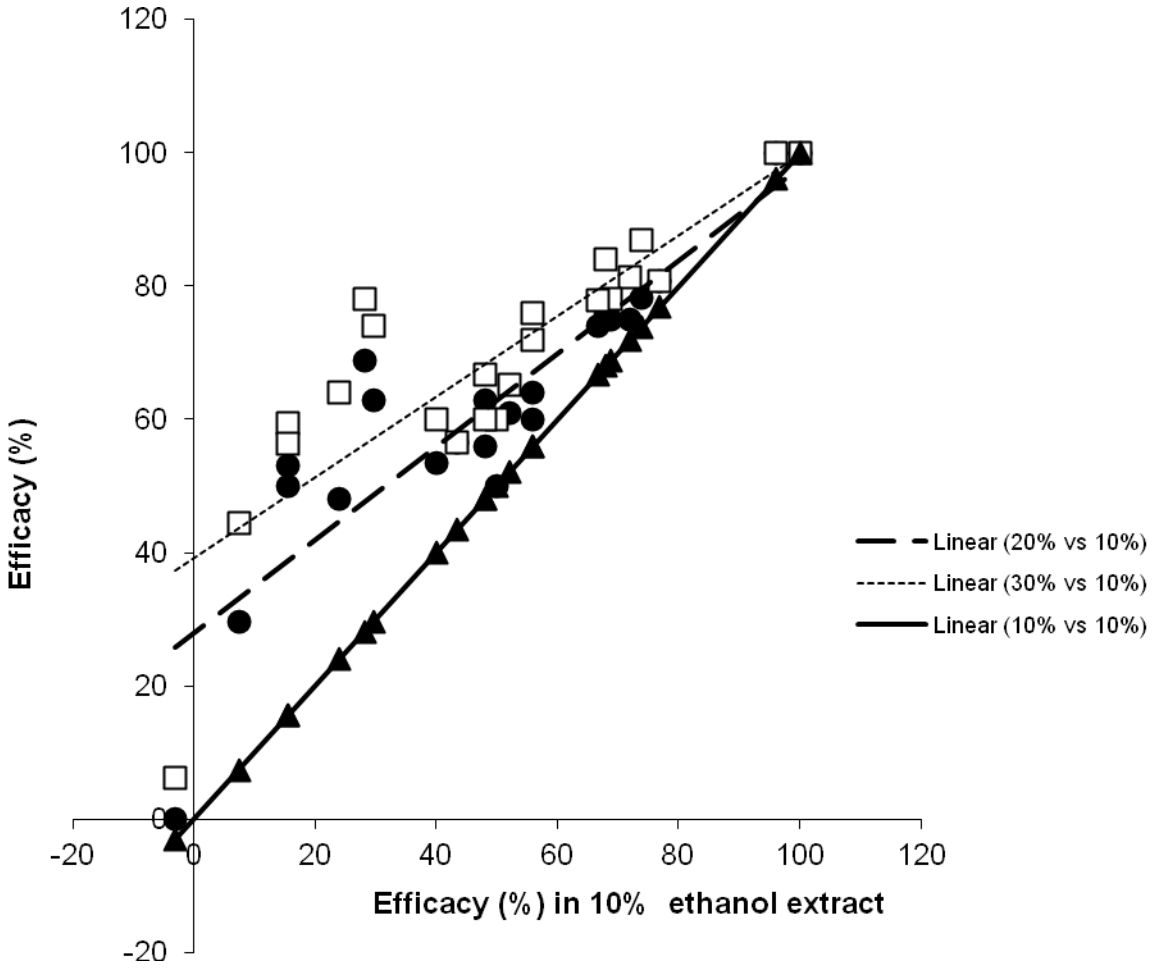

Figure 2.1: *In vitro* efficacy of 25 medicinal plants against *Haemonchus contortus* in sheep at different concentrations (20% vs 10%: y= 0.6971x + 28.058, R^2= 0.7976); (30% vs 10%: y=0.6067x + 39.148, R^2=0.7148); Triangle=10%, Circle=20% and Square=30%.

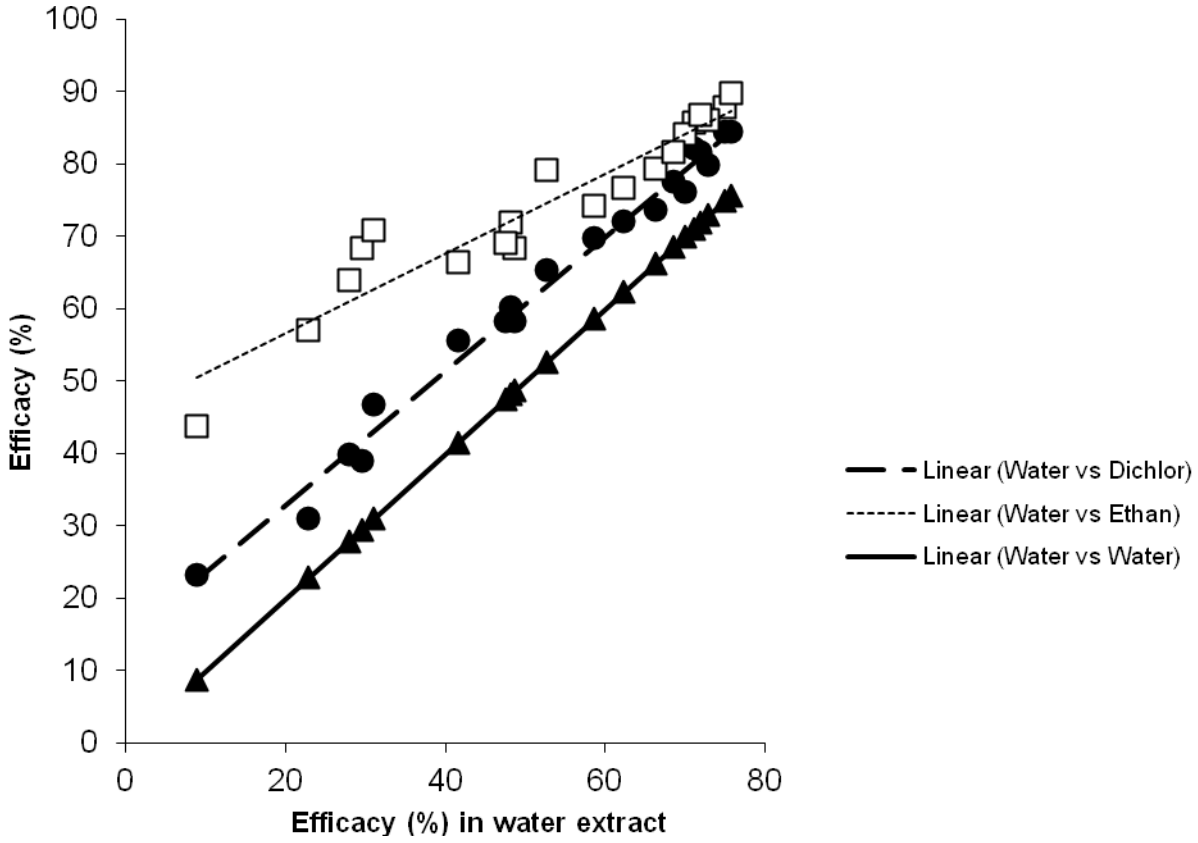

Figure 2.2: *In vitro* efficacy of 5 medicinal plants against *Haemonchus contortus* in sheep of different extracts (Water vs Dichloromethane: y= 0.9265x + 14.295, R^2= 0.9879); (Water vs Ethanol: y=0.5495x + 45.687, R^2=0.8994); Triangle=Water, Circle=Dichloromethane and Square=Ethanol.

2.4 Discussion

A total of twelve families were selected. In the Fabaceae and Canellaceae, two species were investigated, while for Moraceae, twelve species were available for investigation. The *in vitro* screening demonstrated larvicidal effects of ethanol extracts of 25 plants. Maphosa *et al.* (2010) reported that no compound with anthelmintic properties has yet been identified from plants. However, each plant contains secondary compounds with biological activity that could be responsible for the anthelmintic effects shown. For example, cysteine proteinase enzymes in *A. comosus* (Stepek *et al.,* 2004), amino acids, saponins and sterols in *A. ferox* (Mabusela *et al.,* 1990), and tannins in *L. cuneata* (Min *et al.,* 2004) and *Melia azedarach* (Nakanishi *et al.,* 2011) have anthelmintic properties. In addition, fig trees might have unidentified yet active compounds such as the enzyme ficin (Stepek *et al.,* 2004), which may be responsible for various levels of larval mortality. Several compounds such as ajoene in *A. sativum* (Ferri *et al.,* 2003), proteolytic enzyme papain of papaya (Stepek *et al.,* 2004) and alkaloids, saponins and tannins in *L. leonurus* (Bienvenu *et al.,* 2002) would cause larval mortality. The compound muzigadial in species of *Warburgia* possesses antifeedant activity against parasites (Fennell *et al.,* 2004; Mohanlall and Odhav, 2009), whereas polyphenolic compounds are found in *Peltophorum africanum* **(**Bizimenyera *et al.,* 2006).

Ethanol extracts of the five plant species demonstrated strong larvicidal effects against *H. contortus*. *Ananas comosus* and *L. cuneata* showed good larvicidal inhibition even at a concentration of 10%. This agrees with a similar work in which a 10% ethanolic extract of *A. comosus* had an efficacy of 100% against nematode larvae of goats (Sujon *et al.,* 2008). The enzyme bromelain from the stem of *A. comosus* was effective in reducing *H. contortus* larvae by 93% relative to pyrantel tartrate (Hördegen *et al.,* 2006). Bromelain enzyme is known to damage nematode cuticles (Stepek *et al.,* 2006). The effect of *L. cuneata* as a natural anthelmintic has also been reported to drastically reduce nematode levels in small ruminants (Hördegen *et al.,* 2003; Min and Hart, 2003; Min *et al*., 2004; Lange *et al*., 2006). This effect may be due to tannins forming complexes with proteins on the surface of nematodes, thereby

disturbing their metabolism (Ahmed, 2010) and/or tannins binding and disturbing the integrity and growth of nematodes (Niezen *et al.*, 1995).

Aqueous extracts of *A. ferox, A. sativum* and *W. salutaris* inhibited larval development assay of *H. contortus*. In another study, Maphosa *et al.* (2010) reported larvicidal effects of aqueous extracts of *A. ferox* on *H. contortus* of goats. Similar results have been found on water bulb extract of *A. sativum* for killing *H. contortus* of sheep (Iqbal *et al.,* 2001). *Warburgia salutaris* is a traditional medicinal plant used as an anthelmintic in South African traditional medicine (Fennell *et al.,* 2004; Mohanlall and Odhav, 2009). Efficacy of these plant extracts at low concentrations shows that the plants have good anthelmintic activity. However, the active compound is yet to be clearly identified.

To enhance extraction of all biologically active substances in plant material, different solvents were used due to differences in polarity. Water is the main solvent used for plant preparation in traditional medicine (Sparg *et al.,* 2002). Ethanol and dichloromethane solvents have been used regularly to extract potentially bioactive compounds (McGaw and Eloff, 2008). The ethanol extracts were consistently more active than dichloromethane and water extracts (Figure 2.2). Ethanol extracts of the five plants investigated in the second part of the study, at 20%, were highly active, causing over 75% larval mortality. The other crude extracts displayed moderate to weak larvicidal activity. These finding support those of Le Dang *et al.* (2010) who tested several organic solvent extracts of *Chenopodium ficifolium* Smith for insecticidal activity against melon and cotton aphid, *Aphis gossypii* Glover, on cucumber plants. Methanol and ethanol extracts were active (causing more than 80% mortality), while other extracts such as acetone, ethyl acetate and dichloromethane had a lower range between 16-69% (Le Dang *et al.,* 2010).

2.5 Conclusions

Ethanol extracts of five plants (*Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata* and *Warburgia salutaris*) killed larval stages of *H. contortus* of sheep *in vitro*. Future research needs to focus on application of the *in vitro* results to an *in vivo* situation, although *in vivo* efficacy maybe influenced by the physiological status of the host (Githiori *et al.,* 2005).

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Chapter 3*[2]

In vivo effect of selected medicinal plants against gastrointestinal nematodes of sheep

Abstract

Nematode resistance to anthelmintic drugs affects small ruminant production globally. This study evaluated *in vivo* effect of five plant extracts as alternative nematode Control treatments. Gender, eggs count (EPG_0) and initial body weight were used in placing animals (24 females and 24 males, aged 5-18 months) into six groups. Each group was randomly assigned a treatment: Abamectin and Praziquantel (CAP) (positive Control), ethanol extracts of *Ananas comosus* L. Merr. (AC), *Aloe ferox* Mill. (AF), *Allium sativum* Linn. (AS), *Lespedeza cuneata* Dum. Cours. (LC) and *Warburgia salutaris* Bertol.f. (WS). These were applied as an oral dose (100 mg kg^{-1} BW), one dose per week per sheep for 42 days (Phase 1). After, the same sheep were orally dosed for three consecutive days with the same treatments in the same groups (Phase 2). Rectal faecal samples were taken every 7 days up to Day 63. Eggs per gram of faeces were counted in individual rectal samples, L_3 larval stage was counted in faecal cultures, with 4 replicates per group. For plant extracts, EPG decreased ($P<0.001$) with time and efficacy of plant extracts increased ($P<0.001$) with time. *Ananas comosus* and *L. cuneata* treatments had the highest efficacies of 58 and 61%, respectively, in Phase1, and 77 and 81%, respectively, in Phase 2. Continuous treatment with these plants could further reduce nematode parasites and improve host health.

3.1 Introduction

For decades, anthelmintics have been used as the primary control measure for nematode parasites in sheep (Kaplan, 2004). Although anthelmintics have been efficient and work quickly, nematodes have developed resistance in a number of countries such as Scotland

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(Jackson *et al.*, 1992), Australia (Overend *et al.*, 1994), South Africa (Van Wyk *et al*., 1997), New Zealand, (Leathwick *et al.*, 2001), Switzerland (Schnyder *et al.*, 2005) and Italy (Cringoli *et al.*, 2007). In addition to the high cost of anthelmintics, their limited availability in many developing countries (Akhtar and Malik, 2000) makes it clear that control strategies based exclusively on their use are not sustainable. The need for new methods to control livestock nematodes is vital. Currently, screening of medicinal plants for anthelmintic activity as an alternative to synthetic drugs is of increasing interest.

The history of herbal medicine is almost as old as human civilization (Iqbal *et al.*, 2001). Medicinal plants may control the disease and could be both sustainable and environmentally acceptable, if proper harvesting practices are applied (Maphosa *et al.*, 2010). In developing countries, the use of medicinal plants can reduce drug usage, thus enhancing economic self-reliance (Olayiwola, 1993). An estimated 80% of people in developing countries depend on phytomedicine for primary healthcare for both humans and animals (Plotkin, 1992; McCorkle *et al.*, 1996; Ameh *et al.*, 2010). A wide range of plants and plant extracts have been used traditionally as anthelmintic treatments in humans (Waller *et al.*, 2001). For example, plants such as *Ananas comosus* L. Merr, *Carica papaya* L. cv. Rathna, *Azadirachta indica* A. Juss, *Allium sativum* Linn, *Acacia* spp and *Ficus* spp have all been found to possess anthelminthic effects (Akhtar, 1999; Gillian *et al.*, 2004; Singh *et al.*, 2009). In addition, many positive investigations on the use of ethnoveterinary medicines have been made (McGaw and Eloff, 2008), but the challenge is to implement the results practically.

The objective of the current study was to evaluate the effect of ethanol extracts from five medicinal plants *Ananas comosus* L. Merr., *Aloe ferox* Mill., *Allium sativum* Linn., *Lespedeza cuneata* Dum. Cours. and *Warburgia salutaris* Bertol.f. Chiov on egg production by gastrointestinal nematodes (GIN) of Merino sheep, and the hatching and development of GIN eggs of sheep grazing on a contaminated pasture. It was hypothesised that, the ethanol extract of these plants could influence the worms inside the sheep digestive tract negatively resulting in less production of eggs.

3.2 Material and methods

3.2.1 Study area

This study was conducted at the Livestock Section of the University of KwaZulu-Natal Research Farm at Ukulinga outside Pietermaritzburg (KwaZulu-Natal Province) in the subtropical hinterland, which is approximately 700m above sea level. The climate is characterized by an annual rainfall (735mm) which falls mainly between October and April (summer). Maximum and minimum mean annual temperatures are 25.7 and 8.9°C, respectively. Light to moderate frost occurs occasionally in winter.

3.2.2 Experimental design and sheep management

The experiment was designed to estimate the efficacy of plant extracts *in vivo*. Plant extracts prepared according to Ahmed *et al*., (2012). Forty eight sheep (24 males and 24 females), aged 5-18 months, with a mean initial live weight of 33.7 ± 11kg were used. Animals were sorted on the basis of sex, initial eggs per gram (EPG) in faeces and initial live weight, and placed into six groups of eight sheep each. Each group was randomly assigned to one of six treatments: A combination of Abamectin and Praziquantel (CAP) (positive Control), ethanol extract of *Ananas comosus* (AC group), *Aloe ferox* (AF group), *Allium sativum* (AS group), *Lespedeza cuneata* (LC group) and *Warburgia salutaris* (WS group). The control was dosed using hypodermic syringes (2.5ml $10kg^{-1}$) and the plant extracts (100 mg ml^{-1} kg^{-1} BW) were drenched weekly for 6 weeks using a stomach tube. On week 7, plant extracts were applied for three consecutive days (same groups). The Control treatment (CAP) was repeated on Day 35, because the EPG count of the Control group had increased rapidly and one sheep had died with haemonchosis symptoms.

Experimental animals were allowed to graze freely on planted contaminated Kikuyu pasture (*Pennisetum clandestinum* Hochst. ex Chiov.) under the same conditions as other animals on the farm so that they continue their exposure to infective larvae (L_3). Larval contamination of pasture was determined in four paddocks. Herbage samples were collected at the beginning of the experiment to determine L_3 infestation of pasture (Hansen and Perry, 1994). Grass samples (300-600g) were cut in the morning at 0630 to 0700h using a scissor from different locations,

following a W-shape walk in each paddock and placed in plastic bags. Grass samples were transferred into gauze bags of cheesecloth and soaked in water overnight. During the first 3-4 hours, grass bags in water were removed and replaced 5 times then left at room temperature overnight. During the next morning, bags were removed after rinsing with fresh tap water into a bucket. The bucket and contents were allowed to stand for an hour. The top of the supernatant was carefully siphoned off, leaving about one litre. The sediment was poured into a large funnel fastened at the bottom with a clamp onto a stand, and then left to stand for 1 hour after discarding any heavy debris within the first 10 minutes. Sediments (35ml) were taken from the funnel bottom into a beaker and kept in a refrigerator for 30 minutes to cool at 4°C. Third-stage larvae (L_3) were counted under a microscope after addition of 1ml of iodine and 0.2ml of sodium thiosulphate as a counter stain to the cool sediment (Hansen and Perry, 1994). The dry matter content of pasture grass samples was determined and results were expressed as the number of L_3 per kg of herbage dry matter (count multiply by 1000/weight of dry herbage in grams).

3.3.3 Sample collection and parasitological analysis

Faecal samples were taken on Days 0, 7, 15, 21, 28, 35, 42, 49, 56 and 63 post-treatment. Samples were collected rectally, placed in plastic bags bearing the animal's identification number and then conveyed to the laboratory of Animal and Poultry Science where the parasitological analysis was done.

Faecal nematode egg count was done using the McMaster Technique (Hansen and Perry, 1994) and the process was usually completed on the day following the collection day. Fifty six ml of saturated salt solution was added to four grams of faeces in a beaker. The faecal suspension was filtered into another beaker. Both wells of a McMaster counting chamber were filled with the suspension, allowed to stand for 5 minutes, and examined under a light microscope at 100 x magnifications. The number of nematode eggs counted in both wells of the McMaster chamber was multiplied by 50 to get EPG count.

The efficacy of each plant extract was estimated weekly using the following formula, according to Sujon *et al.* (2008):

$$\text{Efficacy} = \frac{\text{EPG prior to treatment} - \text{EPG post treatment}}{\text{EPG prior to treatment}} \times 100$$

However, angular transformation was applied to the values of efficacy (the efficacies values presented as percentage) to normalize homogeneity of variance of data. These data are presented in a table and the untransformed means presented in a graph, which are easier to interpret.

Faeces were also pooled and mixed per group and sub-samples of 8 g each were transferred into four trays and incubated (MEMMERT, 854 Schwabach, West-Germany) for 12 days at 27 °C. Samples were kept damp by watering every day at 10h00 during the period of incubation. The Baermann Technique was used to identify infective larvae (Hansen and Perry, 1994). Under 100x magnification, the samples were examined and the larvae were identified as per Van Wyk *et al.* (2004).

Table 3. 1: Identification infective larvae of livestock nematode

Nematode spp	Total length (µm)	Head	Inside the body	Sheath tail	Other differential features
Haemonchus	650-850	Narrow rounded (bullet-shaped)	16 gut cells	Medium	Tail ending in fine point.
Trichostrongylus	560-796	Tapered head	16 gut cells	Short	Smooth larval tail without filament
Strongyloides	650-850	Bullet-shaped	Oesophagus extends to ½ length of larval body	Absent	Slender body, larval tail notched
Nematodirus	752-1248	Broad, rounded	8 large intestinal cells	Extremely long	Sheath tail filamentous, larval tail notched or lobed.

(Van Wyk *et al.*, 2004).

3.2.4 Statistical analysis

Nematode egg counts were analysed using following model:

$$Y_{ijkl} = \mu + T_i + G_j + L_k + S_l + e_{ijkl;}$$

Where: Y_{ijkm} = individual observation; μ = overall mean; T_i = effect of treatment; G_j = co-variate effect of initial egg count, L_k = co-variate effect of initial live weight, S_l = Effect of gender and e_{ijklm} = the error term, with time being introduced as a repeated measure.

Larvae counts of the faecal culture and transformed efficacy data were analyzed using the General Linear Model (GLM) procedure of SAS (2000) according to the following model:

$$Y_{ijkl} = \mu + T_i + G_j + L_k + S_l + e_{ijkl;}$$

Where: Y_{ijkm} = individual observation; μ = overall mean; T_i = effect of treatment; G_j = co-variate effect of initial egg count, L_k = co-variate effect of initial live weight, S_l = Effect of gender and e_{ijklm} = the error term.

3.3 Results

3.3.1 Larval infestation from the pasture

Larval infestation ranged from 315 to 677 L_3 larvae kg^{-1} of dry herbage, with a mean of 425 ± 174 L_3 larvae kg^{-1} of herbage.

3.3.2 Live-weight gain

Treatments did not affect the final live weight, but trends could be observed (Table 3.2). Sheep given *Warburgia salutaris* had the lowest average daily gain of (-16.7g), while *A. ferox* group had the highest average daily gain of (44.7g).

Table 3. 2: The effect of five plant species on sheep weight

Treatments	Wt_0 (kg)	Wt_{63} (kg)	ADG (g/day)
A. comosus	35.6	36.4	1.1
A. ferox	32.5	35.7	44.7
A. sativum	35.5	37.1	8.6
L. cuneata	31.4	33.3	17.8
W. salutaris	34.1	33.8	-16.7
Abamectin-Praziquantel	33.3	35.8	28.7
F value		0.24	0.28
P<		0.962	0.945
CV%		12.5	21.1

W_0=weight on Day 0, W_{63}=weight on Day 63, ADG=Average daily gain. CV% = coefficient of variance.

3.3.3 Nematode egg counts

Following administration, for sheep on CAP, EPG dropped rapidly to Day 7, beyond which it recovered rapidly until Day 35; then EPG dropped to Day 42 after administering another dose of CAP (Figure 3.1). Treatment with CAP stopped controlling nematode egg counts on Day 56, and the EPG started to increase until Day 63. Eggs (EPG) consistently decreased through time ($P<0.05$) for all plant extracts, LC and AC treatments consistently caused the lowest EPG counts (Figure 3.1).

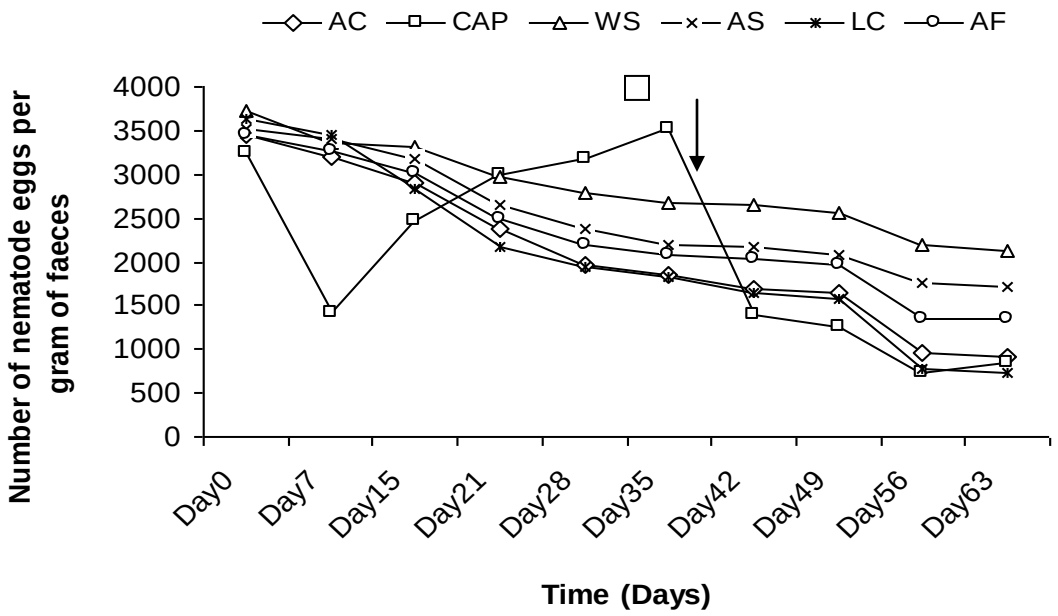

Figure 3.1: The effects of five plant species on gastrointestinal nematodes egg count. P=0.008, SED 294. AC, AF, AS, LC, WS, and CAP stand for *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata, Warburgia salutaris* and. combination of Abamectin and Praziquantel. Arrow indicates time when plant extracts were dosed for three consecutive days and square indicates time of CAP repeated.

3.3.4 Efficacy of treatments

During phase 1, the efficacy of CAP was highest on Day 7 beyond which it consistently decreased until Day 35 (Figure 3.2, Table 3.3). On Days 42 and 49, the efficacy was boosted to a similar level like on Day 7 beyond which it consistently decreased throughout the study. Among plant extracts, *W. salutaris* and *A. ferox* were the least effective, *A. sativum* intermediate and *A. comosus* and *L. cuneata* the most effective treatments. There was no difference ($P>0.05$) among the plant extracts although the effect of time was significant ($P<0.05$).

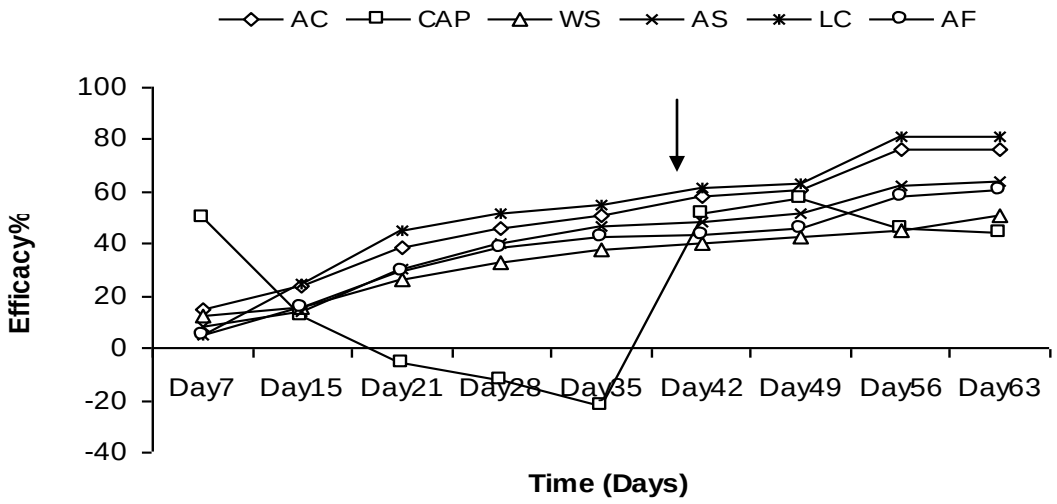

Figure 3.2: The efficacy of five plant species on gastrointestinal nematodes egg count. SED 21.2. AC, AF, AS, LC, WS, and CAP stand for *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata, Warburgia salutaris* and. combination of Abamectin and Praziquantel. Arrow indicates time when plant extracts were dosed for three consecutive days.

3.3.5 Numbers of larvae recovered from faecal cultures

Figure 3.3 shows total number of larvae recovered from faecal cultures. Total larvae decreased over time in a pattern similar to the pattern of EPG. The control group had similar trends for *Haemonchus* and *Trichostrongylus* slightly increased with time. However, within CAP treatment *Strongyloides* and *Nematodirus* spp. recorded the lowest larvae counts (Figure 3.4). These larvae decreased consistently with time for all plant extracts (Figure3.4). There was, however, a small increase for AF extract on Day 35 for *Nematodirus* spp, on Day 56 for *Trichostrongylus* species and on Day 42 for *Strongyloides* spp. The effect of treatments was significant ($P<0.001$).

Table 3. 3: Efficacy (mean ± SEM) of five plant species as anthelmintics

Treatment	Transformed Efficacy% (Angular transformed)								
	Day7	**Day15**	**Day21**	**Day28**	**Day35**	**Day42**	**Day 49**	**Day 56**	**Day 63**
A. comosus	0.46±0.07c	0.47±0.08	0.59±0.05b	0.65±0.04b	0.68±0.04b	0.73±0.04	0.74±0.06	0.82±0.03	0.82±0.04
A. ferox	0.22±0.06a	0.38±0.03	0.54±0.03a	0.60±0.02b	0.63±0.03b	0.74±0.04	0.64±0.05	0.74±0.03	0.75±0.03
A. sativum	0.37±0.05b	0.37±0.08	0.56±0.03a	0.61±0.04b	0.65±0.04b	0.66±0.04	0.69±0.05	0.75±0.04	0.75±0.04
L. cuneata	0.27±0.06a	0.45±0.06	0.65±0.04b	0.69±0.04b	0.71±0.07b	0.75±0.08	0.77±0.06	0.85±0.02	0.85±0.04
W. salutaris	0.41±0.04c	0.41±0.08	0.48±0.05a	0.54±0.06a	0.58±0.06b	0.60±0.06	0.61±0.04	0.69±0.06	0.69±0.07
Abamectin-Praziquantel	0.68±0.04d	0.56±0.04	0.26±0.11a	0.21±0.09a	0.16±0.09a	0.82±0.16	0.76±0.05	0.69±0.06	0.65±0.07
F value	5.6	0.24	7.0	13.3	18.9	1.3	1.4	2.1	1.7
P<	0.001	0.942	0.001	0.001	0.001	0.299	0.243	0.900	0.152
LSD	0.152	NA	0.373	0.338	0.322	NA	NA	NA	NA
CV%	30.7	38.7	27.6	23.8	22.3	15.3	21.6	16.7	18.4

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable
Figures with the same letters are not significantly different at P=0.05.

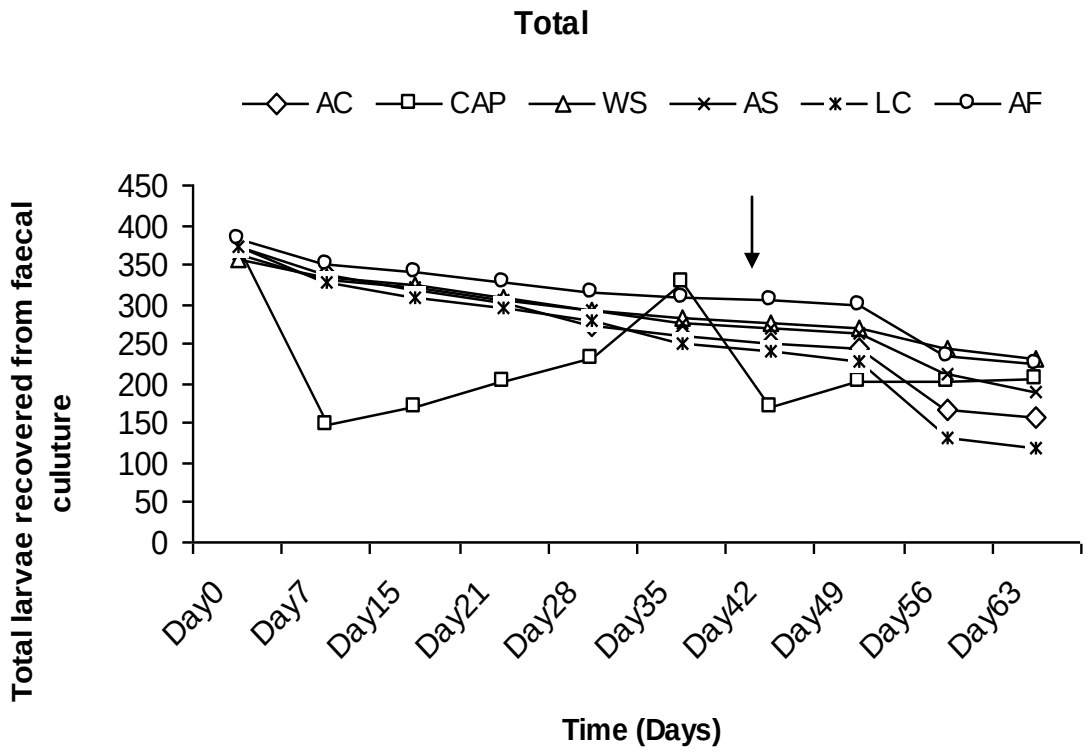

Figure 3.3: Total nematode infective larvae obtained from the faecal culture**.** SED value: 5.85. AC, AF, AS, LC, WS, and CAP stand for *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata, Warburgia salutaris* and. combination of Abamectin and Praziquantel. Arrow indicates time when plant extracts were dosed for three consecutive days.

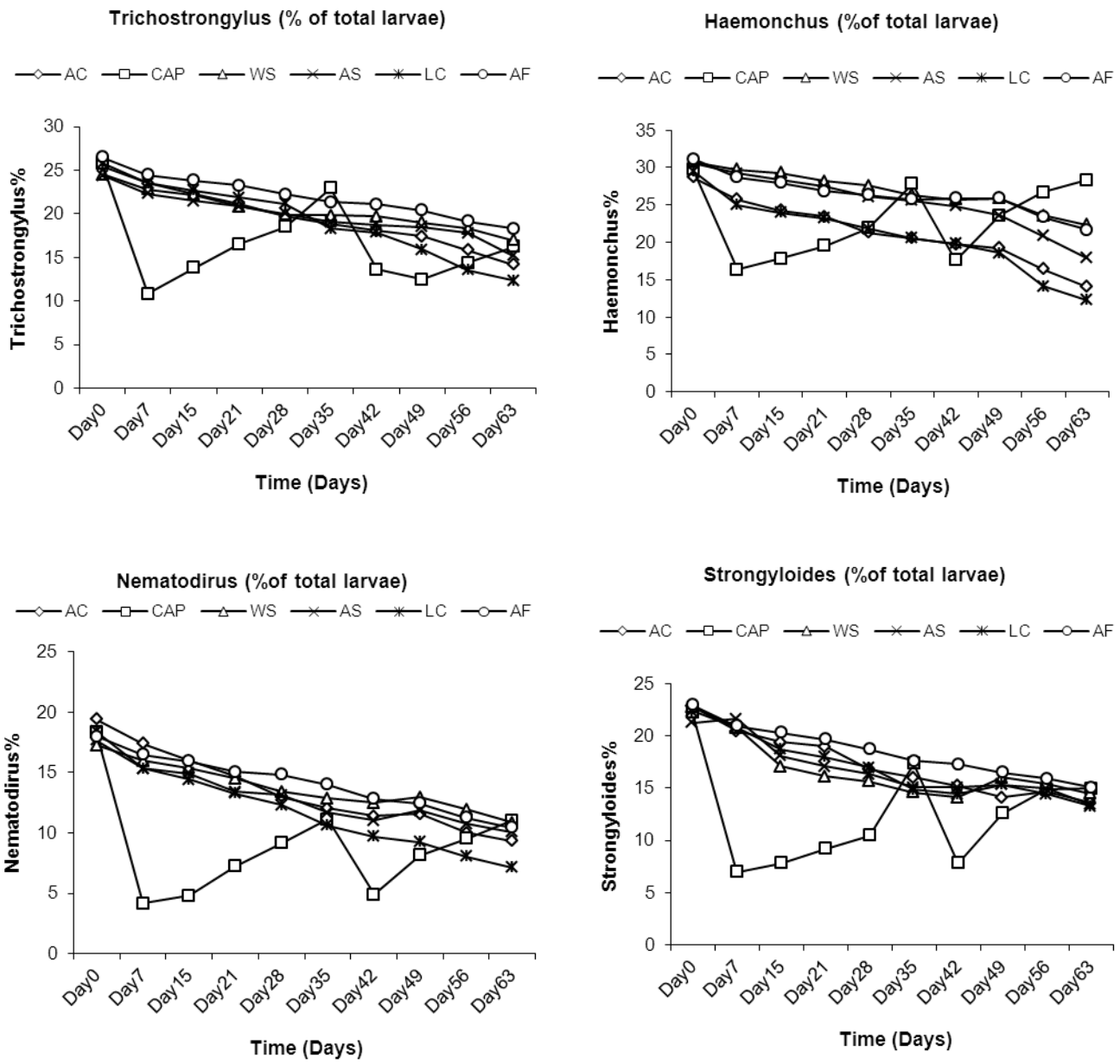

Figure 3.4: Levels of nematode infective larvae obtained from the faecal culture (% of total). SED values were: *Haemonchus spp.* 0.88, *Trichostrongylus spp.* 0.79, *Strongyloides spp.* 0.51 and *Nematodirus spp.* 0.57. AC, AF, AS, LC, WS, and CAP stand for *Ananas comosus*, *Aloe ferox*, *Allium sativum*, *Lespedeza cuneata*, *Warburgia salutaris* and. combination of Abamectin and Praziquantel. Arrow indicates time when plant extracts were dosed for three consecutive days.

3.4 Discussion

The infestation of Kikuyu pastures with L_3 larvae ranged from 315 to 677 L_3 larvae kg^{-1} of dry pasture gran with a mean of 425 ± 174. Previously, Ahmed (2010) found that larval count (L_3 larvae/kg of dry herbage) of Kikuyu pastures at Ukulinga Farm ranged from 115 to 180 with a mean of 138 ± 28.9. Tembely *et al.* (1997) reported that numbers of L_3 larvae from 12 L_3 larvae kg^{-1} of dry herbage recovered from the grass were enough to contaminate the pasture when the weather conditions are stable. This value is high enough to sustain continued re-infection of sheep over time.

In the current study, there was clear evidence that adult nematodes of sheep at Ukulinga farm have resistance to CAP (a commercial anthelmintic), sine EPG increased just after seven days. Notably, dosing with CAP by prescription is supposed to be repeated every three months, indicating that there is nematodes resistance to a commercial product CAP in this farm. This result agrees with Ahmed (2010), who reported nematode resistance in sheep to anthelmintic treatments at Ukulinga Research Farm. Weekly dosage with ethanolic extracts of *A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris* inhibited nematode egg production in sheep at a dosage of 100 mg kg^{-1} BW. Similar observations were reported in goats (Sujon *et al.,* 2008), where ethanolic extracts of *Momordica charanti* Linn., *Azadirachta indica* A. Juss. and *Ananas comosus* L. Merr. at 100 mg kg^{-1} BW dose showed significant nematicidal effects. In cattle, Amin *et al.* (2008) also reported that 100 mg kg^{-1} BW of *A. sativum*, *Curcuma longa* Linn. and *Piper betle* Linn caused a reduction in nematode egg count.

The difference among plant extracts could be due to the presence of different chemicals having variable nematicidal activity. Distinct chemical profiles of five plants have been reported; tannins in *L. cuneata* (Min *et al.,* 2004) which can form complexes with proteins on the surface of nematodes, thereby disturbing their metabolism (Ahmed, 2010). Cysteine proteinases enzymes in *A. comosus* (Stepek *et al.,* 2004) damage nematode cuticles (Stepek *et al.,* 2006), thus killing them. Amino acids, saponins and sterols in *A. ferox* (Mabusela *et al.,* 1990) can disturb protein structure, therefore

affecting growth and repair of nematode body. A compound ajoene in *A. sativum* inhibited protein prenylation (the addition of hydrophobic molecules to a protein) and the proliferation of arterial smooth muscle cells (Singh *et al.,* 2009) in the parasite. A compound, muzigadial, in *W. salutaris* has antifeedant activity against parasites (Fennell *et al.,* 2004; Mohanlall and Odhav, 2009).

Plant efficacies increased weekly owing to the new dosage. This probably suggests that efficacy could be improved by increasing the dose and/or by repeated treatment for a few days (Prichard *et al.,* 1978). This agrees with our findings of increased efficacy due to repeating dosage with plant extracts for three consecutive days. Plant extracts could disturb the metabolism of nematodes when attached and/or enter the nematode's body by forming complexes that are toxic or affecting growth and reproduction of nematodes. However, the mechanism by which these plant extracts can enter the nematodes is yet undefined. Transcuticular circulation is a common means of entry into helminth parasites for non-nutrient and non-electrolyte substances in nematodes (Eguale *et al.,* 2007). This transcuticular circulation is predominant for the uptake of major broad-spectrum anthelmintics such as benzimidazole, levamisole and ivermectin by nematodes, cestode and trematode parasites (Geary *et al.,* 1999). Easier transcuticular absorption of these plant extracts into nematode body could be suggested due to high efficacy obtained from plants.

Dosing sheep with plant extracts for three consecutive days reduced EPG and larval burden from the faecal culture. This is in agreement with Ademola *et al*. (2004), who reported dosing sheep with 500 mg kg^{-1} BW ethanolic extract of Senegal mahogany *Khaya senegalensis* Desr. extract for two consecutive days reduced EPG and warm burden. Minho *et al.* (2008) also reported that an *Acacia molissima* Willd. extract could control nematode parasites in lambs naturally infected with *H. contortus* and *T. colubriformis*. Administration of *A. molissima* extract at 1.6 g kg-1 BW for 2 consecutive days per month in 60 days caused a reduction in EPG and worm burden in the abomasum, but not in the small intestine. In addition, Buttle *et al.* (2011) reported that oral dosing with papaya latex extract (234 μmol active cysteine proteinase), once daily

for three days had a 79.4% efficacy on sheep infected with *H. contortus* and *T. colubriformis*.

Total larvae count followed EPG trends, which increased with time for the Control treatment after Day 7 and only decreased again on Day 42 after a second dosing. Total larvae decreased with time for the plant extracts. This suggests that plant extracts may disturb and/or stop egg development. Declining levels in the larval yield was observed in a study of Molan *et al.* (2000), where extract from several forages inhibited hatching and larval development of *T. colubriformis.* Results from the faecal culture indicated that *H. contortus* was the most prevalent parasite in the experimental sheep. Southcott *et al.* (1976) listed *H. contortus* as a warm climate species. The effect of plants extract on *H. contortus* was reported previously in and out Africa. Maphosa *et al.* (2010) reported that *A. ferox* extract can affect *H. contortus* of goats negatively. Min *et al.* (2003) reported inhibition of egg hatching and larval development of *H. contortus* in goats grazing *L. cuneata.* Iqbal *et al.* (2001) showed that *A. sativum* was effective in killing *H. contortus* larvae of sheep. Hördegen *et al.* (2006) reported that *A. comosus* can kill bovine *H. contortus.* *Warburgia salutaris* was reported as a South African traditional anthelmintic in both humans and animals (Aremu, 2009), thus, there remains a need to know how *W. salutaris* affect nematodes.

In this study, there was increased live weight gain but without any effect among treatments. Ademola *et al.* (2004) reported that the means of terminal live weight of lambs treated with 250 and 500 mg ml^{-1} BW of *K. senegalensis* extract were improved, and no difference between treatments was obtained.

3.5 Conclusions

Ethanolic extract of *A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris* reduced nematode egg production and infective larval yields of sheep. Plant extracts showed no significant effect on body weight gain. However, the findings can be used to design an effective control program strategy for nematode parasites of sheep. Continuous treatment with these plant extracts could further reduce nematode parasites.

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Chapter 4

Effects of two isolates of *Bacillus thuringiensis*, an isolate of *Clonostachys rosea f. rosea*, and a diatomaceous earth product on the control of gastrointestinal nematodes of sheep

Abstract

Nematode parasites have developed resistance to anthelmintics necessitating alternative control strategies. Biological control of gastrointestinal nematodes (GIN) in sheep is a promising non-chemical method. Two experiments were done using Merino sheep. In each experiment, sex, initial egg count per g (EPG) and initial body weight (BW) aided in placing animals into four groups, each of which was randomly allocated to, and fed, with one of four treatments. Experiment 1 evaluated the anthelmintic effects of *Bacillus thuringiensis* Berliner (Bt), *Clonostachys rosea f. rosea* Schroers and diatomaceous earth (DE) against the control of nematodes in sheep. *Bacillus thuringiensis* and *C. rosea* were fed to sheep at a rate of 1g kg^{-1} BW, and DE was fed at 2% of sheep diet. Relative to the control, treatments had no effect ($P>0.05$) on EPG, but reduced ($P<0.001$) larvae per gram (LPG) in faecal culture. Efficacy varied with time ($P<0.001$), and on Day 7, *Bt*, *C. rosea* and DE had efficacies of 75.7, 86.9 and 60.6%, respectively. In Experiment 2, the efficacy of feeding 1g kg^{-1} BW of *C. rosea* chlamydospores to sheep every day, every second day and every third day was tested. Daily feeding of fungal chlamydospores had no effect on EPG ($P>0.05$), but reduced ($P<0.001$) LPG (12±1.67) more than every second day (39±0.77) or third day (58±1.77). On Day 12, feeding daily, every second day and every third day had efficacies of 90, 63 and 49%, respectively. These findings suggest that each of Bt, *C. rosea* isolates and DE products affected nematode larvae, *C. rosea* having the highest effect, and daily use of *C. rosea* is effective for the biological control of nematodes in sheep.

4.1 Introduction

Globally, anthelmintic resistance in nematode population has been a main factor limiting production in small ruminants (Kaplan, 2004; Jabbar *et al.,* 2006; Getachew *et al.*, 2007; Jackson *et al.,* 2009; Ahmed, 2010). Thus, there is a need to develop effective alternative gastrointestinal nematodes (GIN) control strategies. Most of these studies have shown that biological control organisms, such as fungi, could be used to control GIN (Waller, 1999; Larsen, 2006).

Biological control uses natural enemies to reduce nematode larvae on pasture to a level below economic threshold levels (Ketzis *et al.,* 2006; Larsen, 2006). The fungus *Duddingtonia flagrans* (Cooke) has been investigated extensively as a biocontrol agent that targets larval stages of nematode in all livestock species (Faedo *et al.,* 1998; Larsen *et al.,* 1998; Faedo *et al.,* 2000; Waghorn *et al.,* 2003; Chandrawathani *et al.,* 2004a; Waller *et al.,* 2004; Ojeda-Robertos *et al.,* 2008). *Duddingtonia flagrans* fungus reduces nematode parasite populations, thus can serve as anthelmintic (Larsen, 2006).

In addition, other microorganisms such as *Bacillus thuringiensis* (Berliner) have the ability to kill free-living and adult stages of nematodes (Maagd *et al.*, 2003; Kotze *et al.*, 2005). The mechanism that *B. thuringiensis* uses to kill nematodes has been reported by Wei *et al.* (2003) as being the same mechanism involved in controlling insect. *Bacillus thuringiensis* acts via its crystal toxin proteins. After ingestion, the target organism's digestive enzymes break down the protoxins into the *B. thuringiensis* insecticidal components called the delta-endotoxins. These protein toxins bind to highly specific binding sites on the gut lining, which cause pores to form in the gut membrane, leading to peritonitis as the haemolymph pours into the gut cavity, killing the insect or nematode (Swadener, 1994).

The fungus *Clonostachys rosea* (Schroers) has been investigated as a biocontrol agent for pathogenic nematode in plants and therefore its potential to control livestock nematodes by controlling larvae stages (Zhang *et al.,* 2008). Diatomaceous earth is a naturally

occurring siliceous sedimentary mineral compound derived from the microscopic remains of unicellular algae-like plants called diatoms (Bakr, 2010) targeting adult worms inside the host. Diatom particles have sharp edges suggestive of a mechanical mode of action by causing injury to the cuticle of nematodes when they are in contact, leading to dehydration and hence death of the parasite.

The objective of the study was to determine the *in vivo* effect of pre-selected strains of Bt and *C. rosea,* and DE for the control of GIN in Merino sheep. Two experiments were conducted: Experiment 1 tested the efficacy of Bt and *C. rosea* isolates, and DE product in controlling nematodes in sheep; and Experiment 2 determine a dosage rate for *C. rosea,* as *C. rosea* had the highest efficacy in Experiment 1. The hypothesis of this study was that treatment with biocontrol agents and DE will further reduce nematodes in the faeces over that response to a standard chemical antihelmintic programme.

4.2 Materials and methods

4.2.1 Formulation of biocontrol agents

Isolates of *Clonostachys rosea f. rosea* and *Bacillus thuringiensis* were previously obtained from soil at the Livestock Section of Ukulinga farm at the University of KwaZulu-Natal, Pietermaritzburg. These were grown on wheat bran and barley grain, respectively, by Plant Health Products (PHP) (Pty) Ltd[3].

4.2.2 Animals used in Experiment 1

Thirty two sheep (22 females and 10 males) aged 9-18 months with initial body weights of 32.3 ± 8.9kg were used. The initial faecal egg counts were determined in rectal faecal samples. Gender, initial eggs per gram (EPG) of faeces and initial body weight were used to place the sheep into four groups of eight each and then each group was assigned to a treatment randomly. Sheep were already naturally infected with mixed cultures of gastrointestinal nematodes. *Haemonchus contortus* Rudolphi (1803) was 27%

[3] Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, South Africa.

predominant species, others like *Trichostrongylus, Strongyloides, Coopera* and *Nematodirus Spp* were also found (Ahmed, 2010). Sheep were housed in pens (0.90 × 1.50m) in the sheep facility at Ukulinga Research Farm. Each pen was provided with an individual feeder and drinker on wooden slatted flooring. The facility was temperature regulated with large fans.

4.2.3 Feed preparation

Standard sheep diets consisted of the following ingredients: Cottonseed cake (37.5kg), hominy chop (33.3kg), molasses liquid (8.6kg) and Vit. Premix (1.1 kg). *Clonostachys rosea* and *Bacillus thuringiensis* (Bt) products were each added to the standard diet at a rate of 1g kg^{-1} BW. Diatomaceous earth product was added to the standard diet at a rate of 2% for each sheep. These products were thoroughly mixed with the standard diet before being given to sheep. Sheep were fed the standard diet daily between 0700-0900h and then given veldt hay and water *ad libitum*.

4.2.4 Experimental design and sample collection

During a 7-days pre-treatment period, sheep were randomly allocated to individual sheep feeding stalls to acclimatize to handling facilities. At the end of this period, faecal samples were collected and EPG, LPG and LD variables were determined. During the experiment, sheep were assigned to one of four treatments: Standard feed without treatment, 2% DE, Bt at1g kg^{-1} BW per day and *C. rosea* at1g kg^{-1} BW per day. Treatments were mixed with the ration formulation given daily.

Rectal grab samples were collected on Day 0, 2, 4, and 7 during the experiment for nematode egg counts using the McMaster Technique, according to Hansen and Perry (1994), and for larval culture purposes. Larval mortality was determined after 12 days of incubation using the Baermann Technique (Hansen and Perry, 1994). Faeces were cultured per sheep (4 trays replicate) and incubated (MEMMERT, 854 Schwabach, West-Germany) for 12 days at 27 °C.

4.2.5 Animals and experimental design for Experiment 2

For 3 days prior to the study, sheep received a daily supplement of approximately 0.5 kg of diet described previously while having free access to fresh pasture (*Pennisetum clandestinum* Hochst. ex Chiov.) for 2 hours a day in the morning. Thirty two sheep (22 females and 10 males) aged 10-18 months of initial body weight 34.6 ± 7.8kg were sorted on the basis of sex, initial eggs per gram of faeces and initial body weight, and then placed into four groups of eight sheep per group. Each group was then randomly assigned to one of four treatments: An untreated control and three groups dosed with spore at the following rates: 1g kg^{-1}BW (10^8 chlamydospores/g-dry-matter) fed daily, every second day, or every third day. The fungus product was chosen because of the relatively high reduction (87%) of larvae count. Sheep were naturally infected with mixed cultures of GIN.

4.2.6 Sample collection

Over a 15 day period, faeces were collected once in the pre-treatment period and daily during the treatment period, and then continued 2 days post-treatment to estimate EPG. Faecal culture was undertaken to produce and count L_3 larval. Feeding of *C. rosea* product started on Day 4 and ended on Day 10. The McMaster Technique was used for EPG count (Hansen and Perry, 1994) and the Baermann Technique for larval counting from faecal culture (Hansen and Perry, 1994).

4.2.7 Statistical analysis

Efficacy of each treatment was estimated using the following formula, according to Peña *et al.* (2002):

$$\text{Efficacy} = \frac{\text{LPG prior to treatment} - \text{LPG post treatment}}{\text{LPG prior to treatment}} \times 100$$

Larval development (LD) was calculated using a formula proposed by Paraud *et al.* (2005), which is as follows:

$$LD= (LPG/EPG) \times 100$$

where EPG is egg per gram and LPG is larvae per gram.

Data on faecal egg count, larvae count in faecal cultures, larval development and efficacy of treatments were analyzed by using the General Linear Model (GLM) procedure of SAS (2000), according to the following model:

$$Y_{ijkl} = \mu + W_i + T_j + (W*T)_{ij} + G_k + L_l + e_{ijkl;}$$

where: Y_{ijkl} = individual daily observation; μ = overall mean; W_i = daily effect; T_j = effect of treatment; $(W*T)_{ij}$ = interaction between day and treatment; G_k = co-variate effect of initial egg count, L_l = co-variate effect of initial body weight and e_{ijkl} = the error term.

Log transformations were applied to normalize EPG and LPG data. These data are presented in tables together with the untransformed means. The transformed data were analyzed using the same statistical model. The transformed data were also used to calculate LSD values to separate treatment means. Angular transformation was applied to the LD values (percentage) before analysis.

4.3 Results

4.3.1 Effect of treatments on nematode eggs per gram (EPG)

Table 4.1 shows effect of treatments on nematode egg counts and transformed egg count data. None of the treatments had any effect on nematode EPG.

4.3.2 Effect of treatments on nematode larvae per gram (LPG)

Treatment affected nematode larvae counts ($P>0.001$; Table 4.2). Treatment with *C. rosea* resulted in the lowest numbers of larvae, followed by Bt and DE. The control group had the highest count of L_3 larvae.

Table 4. 1: Faecal egg count of nematodes in sheep faeces when treated with *Bacillus thuringiensis*, *Clonostachys rosea* and diatomaceous earth as daily feed additives for 1 week

Treatment	Mean values (± SEM) of eggs per gram of faeces			
	Day0	**Day2**	**Day4**	**Day7**
B. thuringiensis	3413±549	3219±541	3038±524	2831±540
C. rosea	3550±451	3310±391	3310±37	3190±320
D. earth	3413±905	3256±894	3044±909	2850±889
Control	3413±813	3313±810	3156±804	3050±791
F value	0.25	0.31	0.28	0.29
P<	0.860	0.816	0.842	0.830
CV%	62.46	64.84	68.58	71.68
Treatment	**Transformed egg per gram of faeces (Log transformed)**			
	Day0	**Day2**	**Day4**	**Day7**
B. thuringiensis	3.49±0.06	3.47±0.06	3.44±0.07	3.40±0.07
C. rosea	3.48±0.07	3.44±0.07	3.42±0.06	3.39±0.09
D. earth	3.46±0.08	3.43±0.09	3.39±0.09	3.36±0.09
Control	3.47±0.08	3.45±0.09	3.43±0.08	3.41±0.08
F value	0.34	0.45	0.41	0.39
P<	0.794	0.721	0.749	0.762
CV%	5.91	6.09	6.36	6.71

CV% = coefficient of variance; NA=not applicable

Table 4. 2: Larvae of nematodes per gram of sheep faeces when treated with *Bacillus thuringiensis*, *Clonostachys rosea* and diatomaceous earth as daily feed additives for 1 week

Treatment	Mean values (± SEM) of larvae per gram of faeces			
	Day0	**Day2**	**Day4**	**Day7**
B. thuringiensis	113±2.41a	91±1.71a	75±0.78b	57.5±1.18b
C. rosea	118.6±2.99b	89.2±1.28a	56.6±3.32a	39.8±1.01a
D. earth	117.5±2.10b	101.3±1.26b	89.6±0.63c	76.13±1.00c
Control	121.9±1.63b	108.5±2.06c	100.1±2.06d	92.9±0.81d
F value	5.74	27.69	147.57	445.08
P<	0.004	0.001	0.001	0.001
LSD	4.086	6.083	10.106	15.329
CV%	5.18	4.66	4.69	4.00

LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.

4.3.3 Effect of treatments on nematode larvae development (LD)

At Day 4 and Day 7, LD counts were significantly different for the transformed data. On Day 7 the Control had the highest LD of 3.9%, there were significantly fewer larvae developing after treatments with DE at 3.8% and Bt at 2.5%, and *C. rosea* at 2.1% was significantly the best treatment (Table 4.3).

Table 4. 3: Larval development of nematodes in inoculated sheep faeces when treated daily with *Bacillus thuringiensis, Clonostachys rosea* and diatomaceous earth as feed additives for 1 week

Treatment	**Mean values (± SEM) of larval development %**			
	Day0	**Day2**	**Day4**	**Day7**
B. thuringiensis	3.88±0.54	3.32±0.46	2.92±0.39	2.51±0.39
C. rosea	4.71±0.70	4.27±0.64	2.78±0.33	2.12±0.26
D. earth	4.47±0.63	4.11±0.58	4.06±0.61	3.83±0.62
Control	4.49±0.63	4.14±0.57	4.04±0.54	3.94±0.56
F value	0.31	0.60	1.76	2.88
P<	0.817	0.618	0.181	0.559
CV%	38.71	38.60	39.43	43.97
Treatment	**Transformed mean values of larval development % (Angular transformed)**			
	Day0	**Day2**	**Day4**	**Day7**
B. thuringiensis	0.19±0.01	0.15±0.01	0.13±0.01a	0.11±0.01b
C. rosea	0.21±0.01	0.17±0.01	0.11±0.01a	0.08±0.01a
D. earth	0.21±0.02	0.17±0.01	0.16±0.01b	0.14±0.01b
Control	0.21±0.02	0.20±0.01	0.20±0.01b	0.20±0.01c
F value	0.28	2.74	7.67	12.33
P<	0.866	0.132	0.003	0.001
LSD	NA	NA	0.055	0.184
CV%	21.61	22.20	23.01	25.90

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.

4.3.4 Efficacy of treatments

Clonostachys rosea had the highest efficacies throughout and differed (P>0.001) from the rest (Table 4.4). On Day 7, control efficacies were 76, 87, 61, and 24% for *Bt*, *C. rosea*, D. earth and the control, respectively.

Table 4. 4: The efficacy (mean± SEM) of *Bacillus thuringiensis*, *Clonostachys rosea* and diatomaceous earth as feed additives for 1 week as anthelmintics

Treatment	Efficacy in reducing nematode larvae as % of starting point		
	Day2	**Day4**	**Day7**
B. thuringiensis	46.19±1.00c	60.26±0.59c	75.71±1.03c
C. rosea	45.24±2.38c	75.43±3.08c	86.98±0.69d
D. earth	39.26±1.31b	49.19±0.63b	60.61±1.34b
Control	10.81±2.33a	17.74±1.55a	23.73±0.96a
F value	92.60	279.00	610.05
P<	0.001	0.001	0.001
LSD	5.012	5.135	7.056
CV%	13.90	7. 97	5.02

LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.

4.3.5 Effect of *C. rosea* on nematode egg counts

There was no difference in EPG counts (P>0.05) as a result of dosage levels of *C. rosea*. Pre-treatment EPG means for three days were 3075, 3275, 2990 and 3563 EPG for the control, and for dosing sheep with chlamydospores every day, every 2 days and every 3 days, respectively (Table 4.5). Mean EPG on Day 12 of the trial were 2431, 2656, 2040 and 2631, respectively, for these treatments.

4.3.6 Effect of *C. rosea* on count of nematode larvae

Counts of nematode L_3 larvae varied (P<0.001) with treatments (Table 4.6) throughout the time of Exp. 2. On Day 12, the LPG count averaged 96, 12, 39 and 58 for the four treatments, respectively.

4.3.7 Effect of *C. rosea* on nematode larvae development

Feeding *C. rosea* every day resulted in the smallest LD of 0.16% on Day 10, which differed (P<0.01) from dosing every 2 days (1.47%), every 3 days (1.80%) and 5.22% for the Control. On Day 12, LD was 0.51, 2.02, 2.38 and 4.25% for sheep fed on the different frequencies of fungal dosing. Differences were significant (P>0.001) among treatments.

4.3.8 Efficacy of *C. rosea* dosages

Daily feeding of *C. rosea* sustained the highest efficacy and differed (P<0.001) from the rest of the treatments throughout the study, such that on Day 10, the efficacy was 10, 96, 71, and 58% for, dosing sheep with water, or *C. rosea* every day, every 2 days and every 3 days, respectively. On Day 12, efficacies were 11, 90, 63, and 49% for the respective treatments (Table 4.8).

Table 4. 5: Effect of feeding 1gram of *Clonostachys rosea* per kilogram body weight every day, every second day and every third day to nematode-infected sheep on mean (± SEM) number of eggs per gram of faeces

Treatment	**Transformed egg per gram of faeces (Log transformed)**												
	Day0	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**
Daily feeding	3.49±0.05	3.49±0.06	3.49±0.06	3.48±0.06	3.46±0.06	3.44±0.06	3.45±0.06	3.44±0.06	3.43±0.06	3.42±0.06	3.41±0.06	3.39±0.06	3.39±0.06
2^{ed} day feeding	3.46±0.04	3.45±0.04	3.43±0.04	3.42±0.04	3.41±0.05	3.39±0.06	3.38±0.05	3.38±0.05	3.35±0.04	3.33±0.05	3.33±0.04	3.31±0.04	3.29±0.04
3rd day feeding	3.54±0.04	3.53±0.04	3.52±0.04	3.51±0.04	3.49±0.04	3.48±0.04	3.47±0.04	3.46±0.04	3.45±0.05	3.45±0.04	3.44±0.04	3.42±0.05	3.41±0.05
Control	3.46±0.06	3.46±0.06	3.45±0.06	3.44±0.06	3.43±0.06	3.40±0.06	3.39±0.07	3.38±0.07	3.38±0.07	3.37±0.07	3.37±0.08	3.36±0.07	3.35±0.06
F value	0.52	0.43	0.39	0.47	0.35	0.37	0.42	0.38	0.34	0.61	0.54	0.56	0.57
P<	0.670	0.735	0.759	0.703	0.788	0.775	0.738	0.766	0.658	0.613	0.657	0.643	0.611
CV%	3.98	4.13	4.37	4.40	4.53	4.83	4.72	4.69	4.87	4.91	4.89	4.75	0.62

* Feeding of fungus started on Day 1 and ended on Day 10. CV% = coefficient of variance; NA=not applicable.

Table 4. 6: Effect of feeding 1 gram of *Clonostachys rosea* per kilogram body weight every day, every second day and every third day to nematode-infected sheep on mean number of larvae per gram faeces

Treatment	Mean values (± SEM) of larvae per gram of faeces												
	Day0	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**
Daily feeding*	117±2.10b	116±1.78c	98±1.58ab	85±1.91a	74±1.98a	64±1.98a	48±1.77a	35±1.48a	16±1.19a	11±1.14a	4±0.79a	7±0.48a	12±1.67a
2ed day feeding*	109±2.37a	100±1.66a	94±0.89a	88±0.68ab	80±0.68b	74±0.99b	66±1.08b	60±0.97b	53±0.69b	42±0.92b	30±1.16b	34±1.10b	39±0.77b
3rd day feeding*	113±2.42b	106±2.90b	100±2.72b	94±2.39b	87±2.15c	81±2.02c	75±1.84c	68±1.91c	63±1.78c	56±1.43c	47±1.69c	51±1.78c	58±1.77c
Control	119±1.60bc	121±1.60c	121±1.80c	118±2.11c	117±2.11d	116±1.80d	114±1.71d	112±1.69d	111±1.60d	110±1.63d	109±1.65d	108±1.63d	96±12.29d
F value	5.74	16.72	29.77	55.42	97.10	147.03	272.26	402.65	733.85	920.76	999.16	947.29	28.25
P<	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LSD	4.01	5.03	5.06	6.04	5.92	6.89	7.87	7.56	8.53	11.65	13.49	12.87	21.52
CV%	5.18	5.46	5.41	5.80	6.03	6.13	6.18	6.52	6.64	6.81	8.02	7.53	35.77

*Feeding of fungus started on Day 1 and ended on Day 10. LSD=least significant difference; CV% = coefficient of variance

Figures with the same letters are not significantly different at P=0.05.

Table 4. 7: Effect of feeding 1 gram of *Clonostachys rosea* per kilogram body weight every day, every second day and every third day to nematode-infected sheep on the larval development

Treatment	Mean values (± SEM) of larval development %												
	Day0	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**
Daily feeding*	3.95±0.45	3.77±0.49	3.39±0.48b	3.01±0.39	2.69±0.34a	2.47±0.35a	1.85±0.28a	1.36±0.21a	0.66±0.11a	0.45±0.07a	0.16±0.04a	0.31±0.05a	0.51±0.09a
2ed day feeding*	3.86±0.43	3.64±1.42	3.57±0.42c	3.45±0.41	3.26±0.50c	3.19±0.58c	2.93±0.47c	2.60±0.35c	2.45±0.31c	2.05±0.29b	1.47±0.16b	1.69±0.18b	2.02±0.19b
3rd day feeding*	3.41±0.40	3.28±0.41	3.20±0.41a	3.05±0.36	2.92±0.35b	2.79±0.34b	2.66±0.32b	2.48±0.29b	2.32±0.29b	2.08±0.25b	1.80±0.23c	2.02±0.25c	2.38±0.30c
Control	4.49±0.55	4.47±0.52	4.46±0.56d	4.63±0.60	4.69±0.62d	4.90±0.71d	4.91±0.71d	4.95±0.73d	5.13±0.81d	5.19±0.85c	5.22±0.89d	5.15±0.79d	4.25±0.74d
F value	0.91	1.15	1.36	2.75	3.73.03	4.47	7.44	11.00	15.11	16.51	19.01	21.01	12.59
P<	0.452	0.350	0.001	0.064	0.024	0.012	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LSD	NA	NA	0.171	NA	0.172	0.241	0.184	0.103	0.110	0.029	0.251	0.485	0.128
CV%	33.51	34.53	36.65	36.40	38.44	42.98	43.49	44.72	50.60	55.50	62.19	53.21	52.75
Treatment	**Transformed mean values of larval development % (Angular transformed)**												
	Day0	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**
Daily feeding	0.20±0.01	0.19±0.01	0.18±0.01	0.16±0.01	0.16±0.01a	0.13±0.01a	0.12±0.01a	0.08±0.01a	0.06±0.01a	0.04±0.01a	0.03±0.01a	0.05±0.01a	0.06±0.01a
2ed day feeding	0.20±0.01	0.19±0.01	0.19±0.01	0.18±0.01	0.18±0.01a	0.17±0.01b	0.17±0.01b	0.16±0.01b	0.15±0.01b	0.14±0.01b	0.12±0.01b	0.13±0.01b	0.14±0.01b
3rd day feeding	0.19±0.01	0.19±0.01	0.19±0.01	0.18±0.01	0.18±0.01a	0.18±0.01b	0.17±0.01b	0.17±0.01b	0.16±0.01b	0.15±0.01b	0.14±0.01c	0.14±0.01c	0.15±0.01c
Control	0.20±0.01	0.20±0.01	0.20±0.01	0.21±0.01	0.21±0.01b	0.22±0.02c	0.22±0.02c	0.22±0.02c	0.22±0.02c	0.23±0.02c	0.22±0.02d	0.23±0.02d	0.23±0.12d
F value	0.86	1.11	1.31	2.63	3.72	4.62	8.24	13.28	24.25	28.93	40.59	39.45	17.77
P<	0.476	0.363	0.291	0.072	0.025	0.011	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LSD	NA	NA	NA	NA	0.025	0.029	0.032	0.014	0.023	0.052	0.062	0.061	0.044
CV%	17.01	17.55	18.44	18.13	18.74	20.56	20.58	20.72	22.31	23.75	26.49	22.68	26.23

* Feeding of fungus started on Day 1 and ended on Day 10. LSD=least significant difference; CV% = coefficient of variance; NA=not applicable.
Figures with the same letters are not significantly different at P=0.05.

Table 4. 8: The efficacy (mean±SEM) of feeding 1 gram of *Clonostachys rosea* per kilogram body weight every day, every second day and every third day to nematode-infected sheep

Treatment	Efficacy in reducing nematode eggs as % of starting point											
	Day1	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**
Daily feeding*	5.03±1.17b	16.48±1.49d	27.31±1.92c	37.11±1.93d	45.26±2.10d	59.08±1.59d	70.56±1.40d	86.11±1.09d	90.44±1.11d	96.76±0.72d	93.89±0.49d	90.07±1.46c
2^{ed} day feeding*	7.84±0.71c	13.65±1.19c	18.94±1.59b	25.96±1.44c	32.05±1.54c	38.68±1.55c	44.59±1.41c	51.42±1.21c	61.01±1.22c	71.90±1.29c	68.77±0.14c	63.83±0.84b
3rd day feeding*	6.33±1.11bc	11.71±1.25b	17.07±1.33b	23.43±1.47b	28.73±1.37b	34.13±1.31b	39.77±1.23b	44.87±1.10b	51.02±0.98b	58.43±1.24b	55.21±1.40b	49.01±1.41b
Control	-0.52±0.68a	2.15±0.95a	2.74±1.49a	3.66±1.49a	4.98±1.34a	6.32±1.10a	7.86±1.11a	8.58±1.09a	10.13±0.96a	10.44±0.92a	11.25±1.03a	11.48±0.97a
F value	12.65	24.31	40.44	73.86	105.48	245.85	387.19	807.43	986.78	1202.05	1016.25	27.69
P<	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LSD	1.02	1.45	1.49	1.53	2.89	2.59	3.02	4.25	5.99	17.13	10.53	14.82
CV%	63.15	32.84	27.81	20.56	16.84	11.45	9.16	6.67	5.69	5.05	5.43	28.23

* Feeding of fungus started on Day 1 and ended on Day 10. LSD=least significant difference; CV% = coefficient of variance

Figures with the same letters are not significantly different at P=0.05.

4.4 Discussion

Neither Bt, *C. rosea* nor DE given for seven days caused any effect on nematode egg counts, suggesting that none of these agents affected adult GIN inside the host sheep. Consequently, the adult nematodes continued laying eggs at the same rate. Similar observations were reported in trials on another biocontrol fungus, *Duddingtonia flagrans,* in a goat feeding trial (Terrill *et al.,* 2004), where no effect was observed on EPG between treated and untreated animals. Secondly, administration of DE in 150 ml of water to naturally infected goats did not reduce faecal egg counts (Bernard *et al.,* 2009). However, in the case of Bt, our results contrast those of Bone *et al.* (1987) and Meadows *et al.* (1989), who found that Bt produced toxins which were lethal to eggs, L_1, L_2 and L_3 larval stages of *Trichostrongylus colubriformis* (Giles, 1892). Kotze *et al.* (2005) reported that some Bt isolates have a toxic effect on both the larval stages and the adults of *H. contortus in vitro*.

Numbers of L_3 larvae in faecal cultures were depressed by all treatments as a result of biological agents affecting the larval stages of nematodes (Larsen, 1999), demonstrating anthelminthic effects. The fungus *C. rosea* caused the lowest larval count, indicating that its chlamydospores are able to survive through the sheep gut. This finding confirmed the first report representing *C. rosea* as a biological agent against sheep nematodes (Baloyi, 2011). Administration of *C. rosea* as feed additive for seven days reduced nematode L_3 by 84.6%. In addition, the negative effect of *D. flagrans* on larval stages of all important nematode parasites in livestock species were reported (Larsen, 1999), for example, in goats (Terrill *et al.*, 2004), sheep *(*Larsen *et al.*, 1994; Waller *et al.*, 1994; Waller *et al.*, 2001), horses (Larsen, 1996) and cattle (Gronvold *et al.,* 1993). On the other hand, Epe *et al.* (2009) found that there was no clear effect of *D. flagrans* on sheep and goats given the fungus daily for three months. The variation among these studies is probably due to the variation in dose of fungus used, management practice since others reported indoor studies compared to a grazing study by Epe *et al.* (2009), weather condition, animal breed or age. Notably, larval yield of this study was low combaring to others (Larsen, 1996; Terrill *et al.,* 2004).

In the current study, the fungus caused most larvae mortality and numbers of infective larvae declined by the second day following the starting of fungal feeding. This indicates that in order to reduce larval infestation, enough amounts of fungal elements would need to be present in faeces (Larsen *et al.*, 1998). Therefore, the second experiment was designed to assess the frequency of dosage required to produce a substantial and long-lasting reduction in numbers of infective larvae when fungal chlamydospores were delivered at a fixed rate for an extended period.

Feeding with 1g chlamydospores kg^{-1} BW daily, every two days and every three days resulted in reduced larvae numbers in faecal cultures. However, when fungal chlamydospores are provided in the diet every day, there was a growing reduction of L_3 numbers. As with this study, Stear *et al.* (2007) reported that fungi can control nematodes, but their effect is greatest if they are dosed or applied daily. This study demonstrated the advantage of daily continuous administration of *C. rosea* chlamydospores for an extended period of time on the reduction of numbers of infective larvae, reflecting increasing biocontrol efficacy with continuous application.

The highest efficacy was observed in sheep dosed daily with *C. rosea*. Even higher efficacies were observed in goats naturally infected with *H. contortus*, *T. colubriformis* and *Cooperia* spp. when *D. flagrans* was fed every day than every second or third day (Terrill *et al.*, 2004). Notably, the efficacy of *C. rosea* remained high two days after discontinuing feeding of fungal chlamydospores, although control activity dropped slowly for all treatments including the daily feeding.

Using biological agents against GIN is to reduce the number and type of parasites and the number of infective larval stages on pastures. This reduction in infective L_3 stages on herbage will subsequently prevent the build-up of nematode burdens in their hosts, which typically cause subclinical and/or clinical infections, especially in small ungulates (Larsen *et al.*, 1998; Peart, 2002). For these reason, it is essential that a biocontrol agent should pass through the digestive tract of an animal and gets voided with the nematode eggs in the faeces, where it germinates, traps and destroys free living stages of nematodes (Larsen *et al.*, 1998; Thamsborg *et al.*, 1999).

Further research will use *C. rosea* chlamydospores in sustained trials under field conditions, aiming to develop a non-chemotherapeutic form of nematode parasite control.

4.5 Conclusions

Biological agents such as *Bacillus thuringiensis and Clonostachys rosea*, and a commercial diatomaceous earth product all reduced counts of nematode larvae in sheep manure. Daily feeding with 1g *C. rosea* chlamydospores kg^{-1} BW is enough to reduce the numbers of infective L_3 larvae of nematodes, thereby reducing the degree of pasture contamination. Further studies are required to determine how *C. rosea* can be used practically to improve sheep health.

4.6 References

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Chapter 5

Use of a biocontrol agent (*Clonostachys rosea f. rosea*) against sheep nematodes developing on pastures

Abstract

The fungus *Clonostachys rosea f. rosea* (*C. rosea*) has the potential to control gastrointestinal nematodes (GIN) of sheep. A study was conducted using 24 adult Merino sheep (12 females and 12 males, 34.6 ± 9.8kg). sex, eggs count (EPG) and initial body weights were used in placing animals into four groups, and each group was assigned to one of four dietary levels (treatments) of *Clonostachys rosea f. rosea* randomly, which were: (0.25g (F1), 0.5g (F2), 1g (F3) and 0.0g (Control) of *C. rosea* product kg^{-1} BW). Treatments were each mixed with a complete diet and fed to sheep once daily for 10 weeks, according to individual body weights. There was no effect of treatment on egg per gram of faeces (EPG). However, treatments reduced larvae per gram (LPG) from faecal cultures ($P<0.001$), larval development ($P<0.001$), but increased ($P<0.001$) efficacy. On Day 70, Treatments F1 F2, and F3 reduced larval development by 33.3%, 72.3%, 89.4%, whereas the Control treatment was 2.6%, respectively. *Clonostachys rosea* was effective in reducing numbers of third stage larvae (L_3) found in pastures significantly ($P<0.001$) by Day 63 and Day 70. In the present study, daily feeding with *C. rosea* reduced GIN of sheep on pasture.

5.1 Introduction

Development of new control measures against livestock nematodes is a global need due to resistance to all anthelmintic classes (Besier and Love, 2003; Kaplan, 2004; Prichard, 2005; Coffey *et al.*, 2007; Ahmed, 2010). Research on biological control candidates has focused on nematophagous fungi such as *Duddingtonia flagrans* Cooke (Larsen, 1999; Knox and Faedo, 2001; Larsen, 2006; Campos *et al.*, 2008; Epe *et al.*, 2009). Propagules of *D. flagrans* survive passage through the gastrointestinal tract of the host ruminant because it produces thick-walled

chlamydospores (Larsen *et al.,* 1991). The fungus effectively traps nematode larvae present in faeces, thereby reducing the larval population on pasture (Faedo *et al.,* 1998). It has shown much promise against free-living stages of trichostrongylid nematodes in cattle (Larsen *et al.,* 1995). Moreover, in small ruminants, several field studies have reported positive effects of fungal treatment in adult sheep and goats grazing on naturally infested pastures (Faedo *et al.,* 1998; Waghorn *et al.,* 2003; Paraud *et al.,* 2006). Although results from most studies are encouraging, it seems that successful treatments in cattle were not consistently effective on small ruminants (Faessler *et al.,* 2007). For example, Faessler *et al.* (2007) reported the failure of *D. flagrans* to reduce gastrointestinal nematode infections in ewes. Furthermore, large scale production of *D. flagrans* is difficult and expensive, limiting its commercial viability (Morris, MJ, personal communications)[4].

Clonostachys rosea (Schroers) has been suggested as a nematophagous fungus with the potential to control livestock nematodes (Zhang *et al.,* 2008). *Clonostachys rosea* produces cell wall-degrading enzymes, including chitin, glucan, and cellulose-degrading enzymes (Lübeck *et al.,* 2002). Strains of the fungus have the capacity to parasitize livestock nematode (Roberti *et al.,* 2008). Little information is available on the effect of treatment with *C. rosea* chlamydospores on sheep grazing naturally infested pasture. Therefore, the objective of the study was to examine the potential of *C. rosea* as a biological control agent against gastrointestinal nematodes in adult sheep kept under field conditions. The hypothesis of the study, treatment with *C. rosea* could affect the free living stages and further will reduce nematodes.

5.2 Materials and methods

5.2.1 Formulation of a *C. rosea* product

An isolate of *C. rosea* (AB8) was obtained from soils of the Livestock Section of the University of KwaZulu-Natal, Pietermaritzburg and selected for its strong *in vitro* nematicidal capacity (Baloyi, 2011). The strain was grown on double autoclaved millet seed by incubating at 25^0C

[4] Dr. MJ Morris, Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, South Africa.

(Foc 2251, Refrigerated Incubator, VELP® SCIENTIFICA, Usmate, Italy) for three weeks. The fungus was then air dried and ground into a powder using a coffee grinder.

5.2.2 Animals, diets and experiment design

Twenty four sheep (12 females and 12 males) aged 18-24 months, with initial body weights of 42.5 ± 4.8kg at Ukulinga Research Farm were used. Animals were sorted by sex, initial eggs per gram of faeces and initial body weight, and placed into four groups of six sheep each. Each group was then randomly assigned to one of four treatments: 0.25g (F1), 0.5g (F2), 1.0g (F3) and 0.0g (Control) of *C. rosea* product kg^{-1} BW. The product was thoroughly mixed with the standard diet (37.5kg of cottonseed cake, 33.3kg hominy chop, 8.6kg molasses liquid and1.1 kg Vit. Premix) before giving to sheep. Sheep were fed 500g standard diet mixed with treatments daily between 0700-0900h, sheep consumed all their supplement as diet before grazing.

Two sheep each were then randomly accommodated and grazed on same sized paddock of Kikuyu pasture (*Pennisetum clandestinum* Hochst. ex Chiov.) throughout the study period, 12 paddocks in total. Each paddock was 12m × 13m. Sheep were given water *ad libitum*. Sheep were naturally infected with mixed cultures of GIN (Ahmed, 2010), and dosed monthly with a combination of Abamectin and Praziquantel (commercial anthelminthic) to meet ethics requirements.

5.2.3 Sample collection

Sheep were weighed every sampling day. Rectal grab samples were collected at 1000h on Day 0, 14, 28, 42, 56, and 70 for nematode egg counts using the McMaster Technique, according to Hansen and Perry (1994) and for larval culture. Larvae development was determined after 12 days of incubation in an incubator at 27^0C (MEMMERT, 854 Schwabach, Germany) using the Baermann Technique (Hansen and Perry, 1994).

Herbage samples were collected every 21 days for three months from the paddocks for counting third stage larvae (L_3) from pasture using the method of Hansen and Perry (1994). The dry

matter content of pasture grass samples was determined and results were expressed as the number of L_3 per kg of herbage dry matter (the count multiplied by 1000 divided by the weight of dry herbage in grams).

5.2.4 Weather data

An automatic weather station was set up by the Agrometeorology Department in the experimental site at the Ukulinga Research Farm where standard meteorological weather data, including maximum and minimum air temperature, relative humidity and total rainfall were collected. Data were collected every 24 hours then averaged every 2 weeks (Figure 5.1).

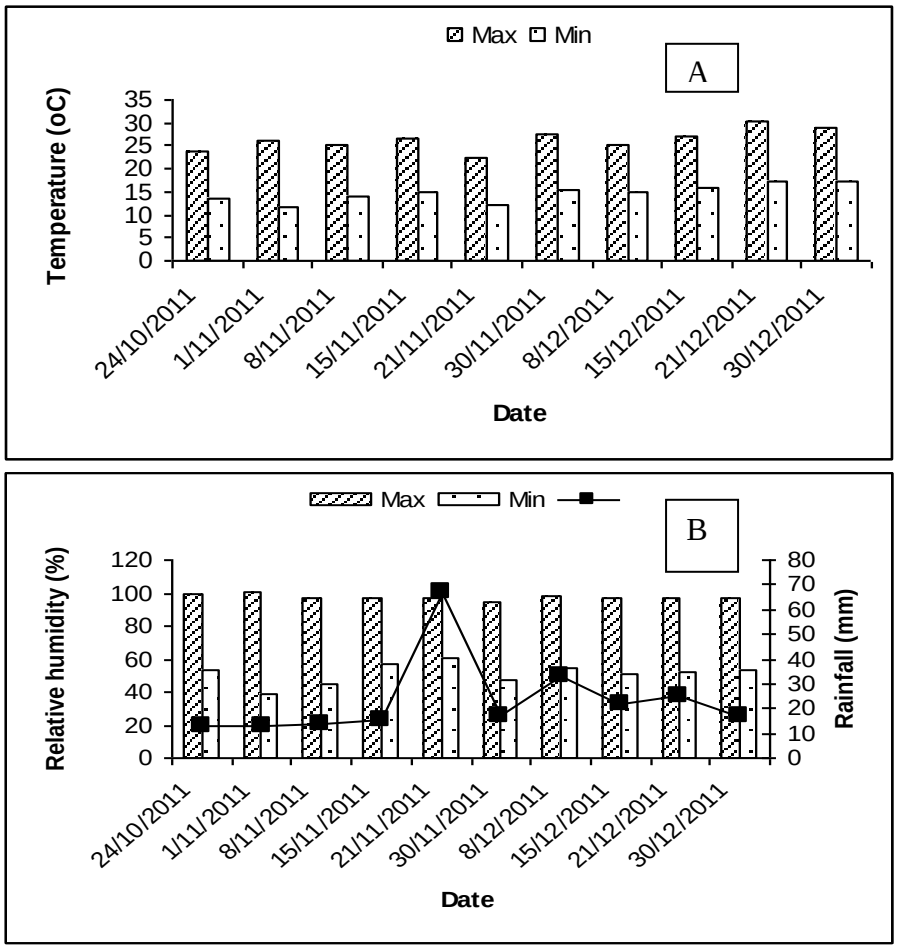

Figure 5.1: (A) Mean maximum and minimum temperatures (◦ C), (B) maximum and minimum relative humidity (%) and total rainfall (mm).

5.2.5 Statistical analysis

Efficacy of each treatment was estimated using the following formula, according to Peña *et al.* (2002):

$$\text{Efficacy} = \frac{\text{LPG prior to treatment} - \text{LPG post treatment}}{\text{LPG prior to treatment}} \times 100$$

Larval development (LD) was calculated using the formula of Paraud *et al.* (2005), which is as follows:

$$LD = (LPG/EPG) \times 100, \text{ as a percentage}$$

where: EPG is the egg per gram, LPG is the larvae per gram.

Data on faecal egg count, L_3 of faecal culture, larval development and treatment efficacy were analyzed using the General Linear Model (GLM) procedure in SAS (2000), according to the following model:

$$Y_{ijklm} = \mu + T_i + G_j + L_k + S_l + e_{ijklm;}$$

Data of L_3 larvae on pasture samples analyzed according to the model:

$$Y_{ij} = \mu + T_i + e_{ij};$$

where: Y_{ijklm} = individual observation; μ = overall mean; T_i = effect of treatment; G_j and L_k =co-variate effects of initial egg count and initial body weight, respectively, S_l = Effect of sex and e_{ijklm} = the error term.

5.3 Result

5.3.1 Body-weight gain

Treatments affected ($P<0.001$) the final live weight of experimental sheep. Sheep given the F3 treatment had the lowest average daily loss, while the Control treatment had the highest average daily gain (Table 5.1).

Table 5. 1: Effect of treating sheep with four levels of *Clonostachys rosea f. rosea* chlamydospores

Treatments	Wt_0 (kg)	Wt_{70} (kg)	ADG (g)
F1	43.1	42.3a	-6.5a
F2	41.8	42.3a	-3.1a
F3	41.9	40.1a	-9.7a
Control (no chlamydospores)	43.3	45.6b	6.1b
F value		9.02	15.93
P<		0.001	0.001
LSD		2.78	2.46
CV%		13.19	7.42

W_0=weight on Day 0, W_{70}=weight on Day 70, ADG=Average daily gain. LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.
F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.3.2 Effect of treatments on nematode eggs per gram (EPG)

The three biocontrol treatments had no effect (P>0.05) on nematode egg counts (Table 5.2).

Table 5. 2: Effect of dietary level of *Clonostachys rosea f. rosea* chlamydospores on faecal egg counts of nematodes in sheep for 10 weeks

Treatment	Mean values (± SEM) of eggs per gram of faeces					
	Day0	Day14	Day28	Day42	Day56	Day70
F1	1617±241	2283±150	2475±305	2450±252	2925±276	3091±242
F2	1679±368	1992±106	2033±208	2216±124	2816±106	3050±155
F3	1675±278	2033v±312	2208±259	2217±316	2950±246	3085±147
Control	1667±159	2625±103	2867±116	3192±152	3158±137	3333±119
F value	0.02	2.68	2.16	3.86	0.65	1.03
P<	0.9964	0.7440	0.1244	0.1249	0.5939	0.3999
CV%	31.33	19.49	25.22	22.85	14.63	10.02

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable.

F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.3.3 Effect of treatments on nematode larvae per gram

The fungus treatments reduced (P<0.001) nematode larvae counts (Table 5.3). On Day 70, the F3 treatment caused the lowest numbers of larvae (mean=13), followed by F2 (mean=36), F1 (83), and then the Control treatment (121).

Table 5. 3: Effect of dietary level of *Clonostachys rosea f. rosea* chlamydospores on larvae per gram of faeces in sheep for 10 weeks

Treatment	Mean values (± SEM) of larvae per gram of faeces					
	Day0	**Day14**	**Day28**	**Day42**	**Day56**	**Day70**
F1	124±2.9	103±1.6b	114±1.4c	100±2.6c	91±2.3c	83±2.3c
F2	129±1.8	89±1.8a	106±1.4b	83±3.0b	61±1.3b	36±1.8b
F3	126±2.9	97±1.2b	76±1.4a	67±1.4a	27±2.5a	13±1.2a
Control	125±1.7	123±1.5c	123±1.7d	120±2.6d	122±1.8d	121±3.1d
F value	0.87	49.02	141.17	83.06	403.93	487.67
P<	0.4754	0.001	0.001	0.001	0.001	0.001
LSD	NA	7.34	4.98	7.34	5.99	6.45
CV%	4.55	3.4	4.98	6.58	6.63	8.47

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.
F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.3.4 Effect of treatments on nematode larvae development (LD)

The control pastures had the highest LD (4.58%) whilst the F3 pastures had the lowest (0.43%), with counts in the F2 and F1 pastures falling in between (Table 5.4). These differences were significant (P<0.001).

Table 5. 4: Effect of dietary level of *Clonostachys rosea f. rosea* chlamydospores on larval development of nematodes of sheep over 10 weeks of treatment

Treatment	Mean values (± SEM) of larval development %					
	Day0	**Day14**	**Day28**	**Day42**	**Day56**	**Day70**
F1	8.49±1.2	5.36±0.3	4.63±0.6	3.81±2.6	3.15±1.3b	2.69±0.8b
F2	8.60±1.2	5.68±0.3	4.45±0.4	3.78±3.0	2.19±0.7ab	1.17±0.8a
F3	7.91±1.1	5.23±1.1	4.05±0.6	3.53±1.4	0.95±0.5a	0.43±0.2a
Control	7.92±1.1	7.07±1.9	5.31±1.3	4.54±2.6	4.90±1.8c	4.58±1.1c
F value	0.12	0.45	0.74	0.61	54.40	191.76
P<	0.9496	0.7171	0.9032	0.0349	0.001	0.001
LSD	NA	NA	NA	0.37	1.25	0.76
CV%	32.25	22.17	31.41	34.07	16.63	12.93

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.
F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.3.5 Third-stage larvae (L_3) on pasture grass

Number of nematode third stage larvae (L_3) recovered from Kikuyu pastures (Table 5.6), were reduced by Day 64 (P<0.05) and Day 84 (P<0.001) as a result of biocontrol treatments. The highest numbers of L_3 larvae were in the Control paddocks, whilst the lowest numbers were counted in the F3 paddocks.

Table 5. 5: Counts of L_3 nematode larvae recovered from Kikuyu paddocks grazed by sheep fed dietary level of *Clonostachys rosea f. rosea* chlamydospores for 10 weeks

Treatment	Mean values (± SEM) of third stage larvae recovered from the pasture				
	Day0	**Day21**	**Day42**	**Day63**	**Day84**
F1	1276±127	1211±166	1254±161	1204±170a	1213±191a
F2	1229±160	1272±126	1227±146	1136±164a	1115±1149a
F3	1595±169	1581±144	1460±137	1225±182a	1090±156a
Control	1482±151	1729±171	1899±189	2051±163b	2064±178b
F value	1.07	1.36	2.13	5.48	4.25
P<	0.4152	0.3237	0.1745	0.0243	0.001
LSD	NA	NA	NA	543	452
CV%	20.72	25.43	25.26	22.82	21.52

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.
F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.3.6 Efficacy of treatment

Differences among treatments increased (P<0.001) as the duration of chlamydospore treatments increased (Table 5.5). The treatment efficacy was highest for Treatment F3 and lowest for the Control. On Day 70, efficacy was 33.31, 72.27, 89.37 and 2.56% for F1, F2, F3 and the control, respectively (Table 5.5).

Table 5. 6: The efficacy (mean±SEM) of dietary level of *Clonostachys rosea f. rosea* fungus as an anthelminthic

Treatment	Efficacy in reducing nematode larvae as % of starting point				
	Day14	**Day28**	**Day42**	**Day56**	**Day70**
F1	8.08±1.44b	16.50±2.39b	18.88±3.59b	26.86±2.39b	33.31±1.46b
F2	17.74±2.07c	30.62±0.81c	35.44±2.13c	52.93±1.65c	72.27±1.46c
F3	22.39±1.58c	39.26±1.60d	46.29±1.55d	78.68±1.95d	89.37±1.01d
Control	1.55±1.29a	1.08±2.22a	3.39±1.43a	1.76±1.15a	2.56±1.93a
F value	33.46	80.62	54.99	260.41	439.65
P<	0.001	0.001	0.001	0.001	0.001
LSD	4.79	5.49	7.49	6.06	5.49
CV%	31.97	20.87	23.90	12.56	9.24

LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.
F1 = 0.25g; F2 = 0.5g; F3 = 1.0g kg^{-1} BW of chlamydospores of *Clonostachys rosea f. rosea*

5.4 Discussion

Treatments did not affect nematode egg count, though at the end of the trial control sheep produced slightly more eggs than the fungus-fed sheep. In addition, clinical symptoms of GIN infection such as diarrhoea and oedema were observed in the Control sheep only. These results are in agreement with those of Baloyi (2011), that treatment with *C. rosea* had no effects on EPG. It also agrees with many other trials with the biocontrol agent *D. flagrans*, which also did not reduce EPG counts (Larsen *et al.*, 1995; Githigia *et al.*, 1997; Larsen *et al.*, 1998; Paraud *et al.*, 2006; Rocha *et al.*, 2007; Braga *et al.*, 2008; Epe *et al.*, 2009). Only Knox and Faedo (2001) reported significant reductions in EPG in lambs fed a supplement containing 2 million *D. flagrans* spores/animal daily for 6 months.

The most efficacious dosage of *C. rosea* in this trial was 1g kg^{-1} BW (10^8 chlamydospores/g-dry-matter), which allowed development rate of 0.43% and an efficacy in killing nematode larvae of 89.37%. This reduction in L_3 counts was translated in safer Kikuyu paddocks 9 week later. Differences in larval recovery from faecal cultures confirmed observations made by others using the fungi *D. flagrans* and *C. rosea*. Waller *et al.* (2001) showed that 5g of *D. flagrans* grain/sheep per day for two successive days was sufficient to virtually eliminate larvae from faecal cultures. Waghorn *et al.* (2003) measured a remarkable kill rate of 93% of nematode larvae when sheep and goats were dosed with 500,000 spores kg^{-1} BW of *D. flagrans* fungus in 2 consecutive days to control three GIN species (*Haemonchus contortus* Rudolphi (1803), *Ostertagia circumcincta* Stadelman (1894) or *Trichostrongylus colubriformis* Giles (1892)), treating juvenile sheep and goats. Similarly, Epe *et al.* (2009) reported that *D. flagrans* reduced the level of L_3 larvae of *Haemonchus* spp from 47.5 to 18.5 % in sheep. Use of *C. rosea* was also effective in reducing the numbers of larvae from faecal cultures when feed at 1g kg^{-1} BW per sheep for 7 days (Baloyi *et al.*, 2012).

Fungal biological agent affected free living stages of nematodes (Larsen, 1999). The study period was characterized by rainy, hot and humid conditions (Figure 5.1), which are ideal for the growth of nematodes, but also for their fungal pathogens. Eysker (1997) reported that warm temperatures and high humidity stimulated larval development on pastures. Gomez-Rincon *et al.* (2006) reported a reduction of L_3 larvae on pasture after daily administration of *D. flagrans* to grazing lambs. Faedo *et al.* (1998) also found a significant reduction of L_3 larvae on pasture when *D. flagrans* was fed to sheep daily. However, biocontrol treatments do not always control livestock nematodes. Epe *et al.* (2009) did not find any difference in larvae counts on pastures when goats and sheep were fed *D. flagrans* spores daily for three months. Rocha *et al.* (2007) also reported a failure of *D. flagrans* to reduce the counts of *Haemonchus* and *Trichostrongylus* larvae on pastures grazed by sheep fed with *D. flagrans* twice a week for 6 months. In both experiments, the animals were naturally infected with GIN and grazed contaminated pastures, also there were sufficiently warm temperatures and humidity both stimulating larval development.

It has been shown that the number of nematode larvae on a pasture depends on the stocking rate of livestock (Thamsborg *et al.,* 1996). The use of *C. rosea* in the study and the number of animals per paddock (2 sheep/paddock for 70 days), could be reflected in the levels of larval development, given a low larval development value of 0.43%. This could explain the difference in results between this study and the above studies of Epe *et al.* (2009) and Rocha *et al.* (2007), as the number of animals per paddock was higher in both of their experiments. That was found to be associated positively with a high numbers of larvae on pastures.

There were significant differences in body weights among treatments, and treatment F3 caused the lowest final body weight. This is puzzling because other researchers have reported no differences in body weights between treated and untreated groups in their experiments on the use of *D. flagrans* to control nematodes (Larsen *et al.,* 1995; Fontenot *et al.,* 2003), and others have reported positive body weight gains in sheep treated with *D. flagrans* (Knox and Faedo, 2001).

5.5 Conclusions

Daily feeding with 0.25-1g *C. rosea* chlamydospores kg^{-1} BW reduced the development of infective larvae of sheep GIN as measured by guantitative larval cultures, and subsequently reduced the counts of L_3 larvae in Kikuyu paddocks. However, the isolates and formulation of *C. rosae* used in this trial reduced sheep body weight, and this negative outcome needs to be understood and solved.

5.6 References

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Chapter 6

Control of nematodes of sheep using chlamydospores of a fungus, *Clonostachys rosea f. rosea,* and an ethanolic extract of a plant, *Ananas comosus*

Abstract

Extracts from pineapple (*Ananas comosus* L. Merr.) has shown potential for the control of nematode parasites, as has a biocontrol fungus, *Clonostachys rosea f. rosea* (Schroers). An experiment was conducted at the Ukulinga Research Farm using 24 Merino sheep (12 females and 12 males, aged 9-18 months) to test a combination of the two natural anthelmintic treatments. Sex, eggs count (EPG_0) and initial live weights were used for placing the animals into four groups. Each group was randomly assigned one of four treatments: (1) 100 mg kg^{-1} BW ethanol extract of *A. comosus* (AC group), (2) 1g of *C. rosea* product kg^{-1} BW (Cr group), (3) a combination of 100 g kg^{-1} BW ethanol extract of AC and 1g of Cr product kg^{-1} BW (ACCR group), and (4) a Control (Co). Sheep were given 450 g dry matter of a standard feed $head^{-1}$ day^{-1} and water ad-libitum. Sheep within a treatment were paired and penned in individual paddocks (12x13m). Rectal faecal samples were taken every 7 days up to Day 42. Eggs per gram of faeces (EPG) in individual rectal samples were counted. Faeces were cultured and third stages (L_3) larval were counted. Grass samples were taken from paddocks and L_3 larvae counted every 21 days up to Day 63. *Ananas comosus* and ACCR treatments reduced ($P<0.001$) EPG. *Ananas comosus, C. rosse* and ACCR reduced ($P<0.001$) larvae per gram (LPG) counts in faecal culture. The efficacy of these treatments increased with time ($P<0.001$) weather for EPG or LPG. *Clonostachys rosea* and ACCR also reduced ($P<0.001$) third stage larvae on pastures by Day 63. It is concluded that a combination of *A. comosus* extract and *C. rosea* is better in nematode control than using them individually.

6.1 Introduction

Gastrointestinal nematodes (GIN) arise from order (*Strongylida*) and are major factors limiting the production of small ruminants. In tropical and sub-tropical areas, *Haemonchus contortus* (Rudolphi 1803) is usually the main species affecting sheep (Fikru *et al.,* 2006), with subclinical infection levels, they cause a reduction in animal growth. At higher levels, *H. contortus* may kill the host animals (Epe *et al.,* 2009). In general, GIN species are primary control by using anthelmintics. However, resistance by GIN species to anthelmintics has been well documented worldwide and in some areas has already reached alarming levels (Maingi *et al.,* 1996; Borgsteede *et al.,* 1996; Zajac and Gipson, 2000; Terrill *et al.,* 2001; Schnyder *et al.,* 2005). In addition, in developing countries, there is limited availability of anthelmintics and their cost is high. For these reasons, alternative GIN control measures are needed.

Use of plants with anthelmintic properties (Stepek *et al.,* 2004) has been proposed as an alternative to the use of chemical drugs, due to their containing natural compounds that can kill nematodes. Many plants have shown potential to control GIN of animals, such as *Ananas comosus* L. Merr, *Carica papaya* L. cv. Rathna and *Ficus* spp. (Stepek *et al.,* 2004). The mechanisms of these plants to reduce GIN are related to their different active ingredients and their concentration in these plants. Numerous studies have confirmed that some plants and plant extracts can reduce nematode egg counts in livestock.

Another control could focus on killing larval stages of GIN species, as such reducing the degree of infestation. Several species of fungi such as *Duddingtonia flagrans* (Cooke) trap and kill developing larval stages of parasitic nematodes in the faecal environment (Larsen *et al.,* 1995). Furthermore, research on *Clonostachys rosea* (Schroers) has demonstrated the potential of this fungus as a biological control agent against insects (Zhang *et al.,* 2008) and its potential to control the free-living stages of parasitic nematodes in livestock (Baloyi *et al.,* 2012). In fact daily feeding with fungal chlamydospores have been successfully used to control parasitic nematodes in small ruminants (Terrill *et al.,* 2001; Baloyi *et al.,* 2012).

However, a combination of the two approaches may be more effective. The objective of this study was to combine an extract of *A. comosus* and conidia of a selected strain of *C. rosea* for the control of nematode parasites of sheep under a typical extensive pasture at Ukulinga Research Farm. It was hypothesised that (a) *A. comosus* extract could affect adult nematodes inside the sheep digestive tract resulting in less production of eggs and (b) *C. rosea* fungus could affect the larval stages of nematde resulting in fewer infective larvae to be ingested by an animal.

6.2 Materials and methods

6.2.1 Pastures, animals, treatments and design

Kikuyu pasture (*Pennisetum clandestinum* Hochst. ex Chiov.) at Ukulinga Research Farm was separated into 12 paddocks and each paddock (12 × 13m) was provided with an individual feeder and drinker. The chemical composition of Kikuyu grass presented in Table 6.1. Sheep were naturally infected with mixed cultures of GIN of which the predominant species was *H. contortus* (Ahmed, 2010). During a one week pre-treatment period, sheep were allowed in paddocks to acclimatize to the handling facilities.

At the end of this period, initial values of faecal egg counts (EPG) and live weight (LW) were determined using 24 sheep (12 females and 12 males) aged 9-18 months with initial body weights of 41.3 ± 5.6kg. Sheep were sorted by sex, initial eggs per gram of faeces and initial weight and placed into four groups of six sheep each. Each group was then randomly assigned to one of four treatments: (1) 100 mg kg^{-1} BW ethanol extract of *A. comosus* (AC group); (2) 1g of *C. rosea* product kg^{-1} BW (CR group); (3) a combination of 100 mg kg^{-1} BW ethanol extract of AC and 1g of CR product kg^{-1} BW (ACCR group); and (4) Control (Co) given standard feed without treatment. Plant extract of AC prepared according to Ahmed *et al*., (2012). *C. rosea* product as mentioned in (section 5.2).

Sheep within a treatment were paired and each pair was accommodated in a paddock chosen randomly making sure that none of the treatments was situated in a contiguous or adjacent paddock. On pasture, each treatment was randomly replicated three times when collecting data on L_3 larvae. Pasture L_3 data were collected for a further 9 week period.

Chemical composition	Kikuyu grass	Standard diet
Dry matter (DM) (g kg^{-1})	900	697
Neutral detergent fibre (g kg^{-1} DM)	722	295
Acid detergent fibre (g kg^{-1} DM)	325	149
Crude protein (g kg^{-1} DM)	153	109
Gross energy (MJ kg^{-1} DM)	13.8	14.9
Ash (g kg^{-1} DM)	80.8	68.7
P (g kg^{-1} DM)	2.7	4.5
Ca (g kg^{-1} DM)	4.0	1.3

Table 6. 1: Chemical composition of Kikuyu grass and standard feed used

6.2.2 Experimental procedures and sample collection

Sheep were was drenched weekly with the *A. comosus* extract for 6 weeks using a stomach tube. The *C. rosea* treatment was given to sheep daily. Sheep were fed on 450g DM of standard feed which consisted of cottonseed cake (37.5kg), hominy chop (33.3kg), molasses liquid (8.6kg) and Vit. Premix (1.1 kg). The standard feed (chemical composition in Table 6.1) was given daily at 0700-0900h, sheep were consuming all the concentrate given. Then sheep were left to graze in the same pasture for 42 days of the experimental period. Water was given *ad libitum*.

Herbage samples were collected in the morning at 0630 to 0700h using a scissor from different locations e.g. 300-600g following W shape in each paddock, on Days 0, 21, 42 and 63 post treatments for counting L_3 nematodes on pasture samples, based on the method of Hansen and Perry (1994). The third stage L_3 nematodes were identified under a microscope after addition of 1ml of iodine to the cool sediment and 0.2ml of sodium thiosulphate as a counter stain (Hansen and Perry, 1994). The DM content of pasture grass was determined and results were expressed as the number of L_3 larvae per kg of herbage dry matter (count multiplied by 1000/weight of dry herbage in grams).

6.2.3 Sample collection

Sheep were weighed weekly. Rectal grab samples were collected on Days 0, 7, 14, 21, 28, 35 and 42 and evaluated for nematode egg count using the McMaster Technique according to Hansen and Perry (1994). Larval mortality was determined in faecal cultures after a 12 day incubation (MEMMERT, 854 Schwabach, West-Germany) using the Baermann Technique

(Hansen and Perry, 1994). Efficacies of treatments were estimated weekly using two formulas due to difference in mode of action between treatments.

1. According to Peña *et al.* (2002):

$$\text{Efficacy}_{L} = \frac{\text{LPG prior to treatment} - \text{LPG post treatment}}{\text{LPG prior to treatment}} \times 100$$

2. According to Sujon *et al.* (2008):

$$\text{Efficacy}_{E} = \frac{\text{EPG prior to treatment} - \text{EPG post treatment}}{\text{EPG prior to treatment}} \times 100$$

Larval development (LD) was calculated using the formula proposed by Paraud *et al.* (2005):

$$LD = (LPG/EPG) \times 100$$

where EPG is the egg per gram, LPG is the larvae per gram.

6.2.4 Statistical analysis

Data on faecal egg counts, L_3 of faecal cultures, larval development and efficacy of treatments were analyzed using the General Linear Model (GLM) procedure in SAS (2000), according to the following model:

$$Y_{ijk} = \mu + T_i + G_j + L_k + e_{ijk};$$

and L_3 larvae on pasture according to the model:

$$Y_{ij} = \mu + T_i + e_{ij};$$

where Y_{ijk} = individual observation; μ = overall mean; T_i = effect of treatment; G_j = co-variate effect of initial egg count, L_k = co-variate effect of initial live weight and e_{ijk} = the error term.

6.3 Results

6.3.1 Third-stage larvae on pasture

Counts of nematode third stage larvae (L_3) recovered from Kikuyu pasture increased for AC and the Control, but reduced for CR and ACCR treatments. The changes were not significant during the first 42 days, however by Day 63 ACCR was lower than CR (P<0.01), and CR was lower than the rest of the treatments (P<0.01) (Table 6.2).

Table 6. 2: Mean values (± SEM) of nematode third stage larvae (L_3) recovered from Kikuyu pasture during the study period

Treatment	**Mean values of third stage larvae recovered from the pasture (L3 Kg^{-1} dry herbage)**			
	Day0	**Day21**	**Day42**	**Day63**
A. comosus	1554±127	1729±171	1899±161	1927±100 a
A. comosus + *C. rosea*	1364±140	1319±115	1256±92	1193±84 c
C. rosea	1596±160	1579±140	1430±127	1419±118 b
Control	1478±151	1865±181	1963±177	1965±163 a
F value	0.35	1.22	2.73	3.25
P<	0.7905	0.3651	0.1139	0.001
LSD	NA	NA	NA	773
CV%	19.95	22.65	21.96	22.08

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.

6.3.2 Effect of treatments on nematode eggs per gram (EPG)

For the Control and CR, EPG increased consistently until the end of the study (Table 6.3). Treatment affected (P<0.001) EPG by Day 35 and Day 42. The treatment ACCR had the lowest mean EPG (725), while the Control treatment had the highest (1741).

Table 6. 3: Effect of *Clonostachys rosea* and *Ananas comosus* on nematodes faecal egg counts (EPG)

Treatment	Mean values (± SEM) of eggs per gram of faeces						
	Day0	**Day7**	**Day14**	**Day21**	**Day28**	**Day35**	**Day 42**
A. comosus	1533±297	1492±289	1400±292	1192±293	1008±269	883±248b	742±233 b
A. comosus + C. rosea	1516±251	1475±251	1308±258	1092±160	958±139	883±136b	725±110 b
C. rosea	1517±136	1525±128	1483±190	1608±127	1650±218	1683±215a	1700±230 a
Control	1500±164	1542±167	1608±166	1608±166	1675±155	1708±144a	1741±150 a
F value	0.01	0.02	0.30	2.13	2.84	4.14	6.56
P<	0.9997	0.9962	0.8238	0.1282	0.0639	0.001	0.001
LSD	NA	NA	NA	NA	NA	659	657
CV%	35.83	31.44	29.22	19.45	26.22	24.05	22.01

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.

6.3.3 Effect of treatments on nematode larvae per gram (LPG) of faeces

Numbers of larvae recovered from faecal cultures increased with time for the Control but reduced consistently for the rest of the treatments (Table 6.4), the effect of treatment attaining significance (P<0.001) from Day14 to the end. On Day 42, ACCR treatment had the lowest larval count (mean=27) followed by CR (mean=32) and AC (mean=47). The pasture of the Control sheep had the highest number of L_3 larvae (122).

Table 6. 4: Effect of *Clonostachys rosea* and *Ananas comosus* on nematodes larvae per gram of faeces (LPG)

Treatment	Mean values (± SEM) of larvae per gram of faeces						
	Day0	**Day7**	**Day14**	**Day21**	**Day28**	**Day35**	**Day 42**
A. comosus	107.7±4.1	102.0±4.6	93.5±4.5b	84.2±3.4b	78.0±3.1b	67.8±1.8b	47.2±1.7b
A. comosus + C. rosea	117.5±6.1	96.0±6.2	79.2±7.7b	69.8±7.9b	58.8±7.7c	39.7±5.9c	27.7±3.4c
C. rosea	107.2±2.7	92.2±4.1	81.3±4.1b	73.3±3.6b	62.2±4.5c	43.5±4.8c	32.7±4.3c
Control	105.5±4.7	109.7±4.5	115.2±3.2a	118.2±3.4a	120.7±3.3a	120.5±3.9a	122.5±3.4a
F value	1.41	2.41	10.31	19.63	32.33	70.88	175.5
P<	0.2704	0.0973	0.001	0.001	0.001	0.001	0.001
LSD	NA	NA	15.16	14.68	14.74	13.05	9.82
CV%	10.27	12.06	13.64	14.11	15.32	15.96	14.19

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.

6.3.4 Effect of treatments on nematode larvae development (LD)

The Control had the highest LD of 7.39% (Table 6.5). Lesser larval development was due to the AC treatment (5.53%) and the CR treatment (2.21%). The lowest LD occurred for ACCR treatment (0.92%). The treatment attained significance from Day 35 to 42.

Table 6. 5: Effect of *Clonostachys rosea* and *Ananas comosus* on nematodes larval development

Treatment	**Mean values (± SEM) of larval development %**						
	Day0	**Day7**	**Day14**	**Day21**	**Day28**	**Day35**	**Day 42**
A. comosus	8.63±1.9	8.19±1.5	8.80±3.4	8.13±3.4	6.79±2.0	6.08±2.1ab	5.53±0.6ab
A. comosus + C. rosea	8.71±1.4	7.31±1.1	6.10±1.4	5.04±1.1	3.33±1.1	1.19±0.7c	0.92±0.7c
C. rosea	7.42±0.8	6.26±0.6	6.21±1.1	4.72±0.4	4.43±0.1	2.94±0.4bc	2.21±0.3bc
Control	7.61±1.1	7.72±1.1	7.69±1.1	7.58±0.9	7.62±0.9	7.41±0.8a	7.39±0.8a
F value	0.25	0.51	0.45	1.27	0.72	5.20	6.16
P<	0.8587	0.6775	0.7225	0.3112	0.5514	0.001	0.001
LSD	NA	NA	NA	NA	NA	3.68	3.53
CV%	30.46	21.19	22.91	34.07	19.26	12.35	10.16

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable. Figures with the same letters are not significantly different at P=0.05.

6.3.5 Efficacy in reducing nematode egg in faeces

Efficacy in reducing nematodes egg output is described in Table 6.6. Treatments had a positive effect (P>0.001) from Day14. The Control had the least efficacy (-18.6%), CR provided an efficacy of 13.2%, while AC had an efficacy of 51.2% and the ACCR treatment had the highest efficacy of 57.6%.

Table 6. 6: The efficacy (mean±SEM) of *Clonostachys rosea* and *Ananas comosus* in reducing nematode eggs in faeces

Treatment	Efficacy in reducing nematode eggs as % of starting point					
	Day7	**Day14**	**Day21**	**Day28**	**Day35**	**Day42**
A. comosus	4.81±0.80	10.47±1.76a	19.27±2.21a	35.75±2.87a	40.96±2.98a	51.21±3.42a
A. comosus + C. rosea	7.83±1.31	6.55±2.71a	27.04±3.31a	39.98±2.6a	47.86±2.64a	57.55±3.03a
C. rosea	-0.97±0.59	4.44±1.11ab	6.71±1.31b	8.71±2.10b	12.74±2.12b	13.24±2.21ab
Control	-2.79±0.58	-7.76±0.46b	-11.18±0.92b	-13.10±1.18b	-16.32±1.92b	-18.56±1.93b
F value	1.37	5.66	15.77	17.16	20.32	31.61
P<	0.082	0.001	0.001	0.001	0.001	0.001
LSD	NA	12.45	15.49	31.50	31.34	32.64
CV%	36.70	24.32	18.42	19.26	17.51	16.42

LSD=least significant difference; CV% = coefficient of variance; NA=not applicable Figures with the same letters are not significantly different at P=0.05.

6.3.6 Nematicidal efficacy in reducing larvae in faecal cultures

Efficacies based on larval counts are presented in Table 6.7. The differences among treatments were significant (P>0.001) from Day 7 to 42. The combination ACCR had the greatest efficacy throughout the study, and the best final efficacy of 76.9%, followed by CR at 69.9, AC at 55.9%, and the Control with an efficacy of -16.7%, indicating that the untreated nematode populations increased by nearly 20%.

Table 6. 7: The efficacy (mean±SEM) of *Clonostachys rosea* and *Ananas comosus* in reducing L_3 larvae in faeces

Treatment	Efficacy in reducing nematode larvae as % of starting point					
	Day7	**Day14**	**Day21**	**Day28**	**Day35**	**Day42**
A. comosus	5.37±0.69	13.23±1.90c	21.79±1.57c	27.47±1.68b	36.79±1.49b	55.89±2.26c
A. comosus + C. rosea	18.50±2.47	33.27±4.51a	41.46±4.99a	50.68±5.28a	66.85±4.27a	76.91±2.19a
C. rosea	14.09±2.48	24.17±2.93b	31.67±2.30b	42.19±3.38a	59.77±3.65a	69.85±3.33b
Control	-4.06±0.89	-9.61±2.17d	-2.45±2.20d	-14.86±2.31c	-14.61±2.45c	-16.68±3.7d
F value	29.45	36.80	58.75	71.53	136.42	243.13
P<	0.001	0.001	0.001	0.001	0.001	0.001
LSD	5.43	8.99	9.03	10.15	9.31	8.14
CV%	23.15	28.94	36.37	31.97	20.77	14.53

LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.

6.3.7 Body-weight gain

All sheep lost weight. The nematicidal treatments significantly affected (P<0.05) the final live weight, and live weight change, of the sheep (Table 6.8). All three nematicidal treatments reduced sheep live weight. Sheep given the CR treatment had the lowest average daily change of -166g, while the Control treatment had the smallest daily weight loss of -105g.

Table 6. 8: Effect of *Clonostachys rosea* and *Ananas comosus* on sheep weight

Treatments	**Wt_0 (kg)**	**Wt_{42} (kg)**	**ADG (g)**
A. comosus	41.8	40.8	-148b
A. comosus + *C. rosea*	40.4	39.4	-146b
C. rosea	41.7	40.5	-166c
Control	41.4	40.8	-105a
F value		9.02	12.37
P<		0.0196	0.001
LSD		1.12	2.36
CV%		13.78	12.03

W_0=weight on Day 0, W_{70}=weight on Day 70, ADG=Average daily gain. LSD=least significant difference; CV% = coefficient of variance. Figures with the same letters are not significantly different at P=0.05.

6.4 Discussion

As a result of the treatments in this study there was a significant difference in L_3 larvae counts on pastures. However, over time the number of larvae on pasture increased for the AC treatment and the Control, but decreased with the ACCR and CR treatments, suggesting a major effect by the CR treatment. Reduced nematodes larvae were reported on natural pastures being grazed by calves treated with *D. flagrans* (Larsen *et al.,* 1995). Use of chlamydospores of *D. flagrans* for two months reduced pasture infectivity and stopped the onset of nematode clinical disease (Larsen *et al.,* 1995). Other trials have shown that nematophagous fungi can reduce numbers of infective larvae on pastures by feeding on the nematodes free living stages, thus reducing the subsequent populations of adult nematodes inside grazing animals (Gronvold *et al.,* 1993; Wolstrup *et al.,* 1994; Githigia *et al.,* 1997).

In accordance with Baloyi *et al.* (2012) the administration of CR did not influence nematode eggs of sheep. It has been established that fungal biocontrol agents do not affect adult nematodes inside the sheep gut, and thus cannot reduce egg output (Larsen *et al.,* 1995; Larsen *et al.,* 1998; Braga *et al.,* 2008; Epe *et al.,* 2009). However, the CR treatment reduced L_3 numbers in faecal cultures. This agrees with others (Peña *et al.,* 2002; Fontenot *et al.,* 2003) reporting that *D. flagrans* is effective in reducing infective larvae (L_3) counts in sheep faeces. In general, fungal treatments affected the propagation of infective larvae are turly preventive and consequently, there is no expectation of immediate results on existing worm burden. In longer perspective, use of fungi should reflect in lower faecal counts. Efficacy of 75% based on LPG count was observed in sheep treated with 1g kg^{-1} *C. rosea* (Baloyi, 2011). In addition, Terrill *et al.* (2004) observed a 94% efficacy nematicidal effect in goats infected with a mixed culture of GIN and fed *D. flagrans* for only eight days. Concerning sheep weight, the CR treatment caused the largest weight loss, with an average daily change of -166g. This weight loss as a result of treatment with CR is similar to observations made by Baloyi (2011).

Consistent with previous observations Ahmed *et al.* (2011), AC reduced EPG. Similarly in goats, extracts of AC can inhibit nematode egg production significantly (Sujon *et al.,* 2008). A reduction of *Trichuris muris* Schrank adult worms was observed when Stepek *et al.* (2006) treated mice with an AC extract. Extracts of *A. comosus* affect nematode due to their content of cysteine proteinases enzymes, which attacks the surface of adult nematodes (Gillian *et al.,* 2004; Stepek *et al.,* 2006). It is also possible that *A. comosus* would affect counts of third stage larvae in faecal cultures, if a portion of the active enzymes survive passage through the gut of the animals. Plant extracts with anthelminthic properties inhibit egg hatching and larval development of nematodes (Molan *et al.,* 2000). Hördegen *et al.* (2006) reported the adverse effect of *A. comosus* on larvae of *H. contortus* in sheep. *Ananas comosus* kills adult nematodes (Ahmed *et al.,* 2011; 2012) and so there would be fewer eggs to develop into larvae.

The efficacy in reducing nematode egg production using plant extracts has been demonstrated *in vitro* and *in vivo* (Githiori *et al.,* 2006). In other studies, Satrija *et al.* (1994) reported 100% efficacy in pigs infected with *Ascaris suum* Goeze (1782), after 7 days treatment with the latex of papaya (*Carica papaya*). An efficacy of 30% based on the EPG has been observed in goats

offered *Acacia karoo* Haines extracts (Kahiya *et al.,* 2003). Moreover, Hördegen *et al.* (2003) reported a 100% control in sheep infected with *H. contortus* and *Trichostrongylus colubriformis* Giles (1892) treated with extract of *Fumaria parviflora* Lam. However, AC decreased body weight change. Githiori (2004) reported no significant body weight gain was observed between treated and Control group in sheep treated with *A. comosus.*

The combination ACCR treatment reduced EPG counts, and numbers of L_3 larvae in faecal culture and in the pastures. The advantage of using both methods together for controlling GIN is that the AC extract will reduce EPG counts, whilst CR will reduce the L_3 larval stages in faeces, and in the pasture. As hypothesized, the efficacy of control of nematodes was highest when both AC and CR were used in a single treatment, ACCR. However, live weight change was negative, and contrary to expectation.

6.5 Conclusions

An ethanol extract of *A. comosus* reduced nematode egg count and infective larvae of sheep. A biological agent, using chlamydospores of *C. rosea,* reduced counts of nematode larvae in sheep faeces and subsequently reduced counts of L_3 larvae on Kikuyu pasture. The combination of the two treatments was more effective than either treatment by itself in reducing EPG, LPG, LD, and L_3 larvae in pastures.

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Chapter 7

Thesis Overview

Gastrointestinal nematodes (GIN) pose a major health threat to sheep and goat production, especially in the tropics and subtropics. On farms where GIN are prevalent, the species *Haemonchus contortus* Rudolphi (1803) usually predominates. Adults of this species attach to the lining of the sheep abomasum (gut), feeding on the host's blood. Consequently, affected animals become emaciated, anaemic, and show signs of severe anaemia before dying. The female of this species is prolific and can produce up to 10,000 eggs per day.

The current methods of GIN control are based on the repeated use of synthetic anthelmintic drugs. However, with increasing exposure by routine dosage of small ruminants, GIN develop resistance anthelmintic drugs (Kaplan, 2004; Prichard 2005; Coffey *et al.*, 2007). Resistance to anthelmintics is demonstrated by the ability of GIN to survive drug treatments that used to be effective against the same species and stage of infection (Coffey *et al.*, 2007). Secondly, consumers of animal products have also become cautious of possible contamination of meat and milk products by residues of drugs. At the other end of the economic scale, small-scale farmers are being pushed out of the small ruminant industry by GIN, and their inability to treat them effectively because of the high price of effective drugs, which then turn out to be of diminishing efficacy as resistance develops against the drugs. Therefore, a search for alternative control measures is underway globally. Dosing sheep with extracts of plants with anthelmintic properties is an ancient strategy (Stepek *et al.,* 2004) that may be affordable to all farmers, including smallholder farmers (Akhtar and Malik, 2000) because most of these plants are readily available in the tropical and sub-tropical region.

Biological control is becoming an important non-chemical option for controlling GIN in animals because biocontrol agents can control a target organism, reducing its population to a level that no longer causes clinical problems and economic losses (Thamsborg *et al.*, 1999). In addition, biocontrol agents have low mammalian toxicity, high efficacy and are naturally occurring, and multiply to a level that matches its target organisms (Larsen, 2006). Thus, biocontrol agents can

avoid the issue of chemical residues in food, and is an attractive option for organic farming. However, there are no commercial biocontrol agents currently available for controlling GIN in livestock anywhere in the world. Consequently, the overall objective of this study was to develop a new control strategy against GIN in sheep using plant extracts and biocontrol agents, with a long term objective of seeing successful products registered for use by farmers.

Ethanol extracts of 25 plant species were screened at various concentrations for their anthelmintic effects against GIN larvae. The extracts caused a range of larval mortality of up to 100%. The five plant species with best efficacies were *Ananas comosus* L. Merr., *Aloe ferox* Mill., *Allium sativum* Linn., *Lespedeza cuneata* Dum. Cours. and *Warburgia salutaris* Bertol.f. Chiov. Mortality ranged from 68 to/or 100%. These extracts were further screened using three solvents: ethanol, dichloromethane and water. Ethanol was the best extractant. *Lespedeza cuneata* extracts caused the highest mortality to nematodes.

In vivo trials were conducted using ethanol extracts of the five selected plants, comparing their performance against a positive Control, which was a commercial drug combination of Abamectin and Praziquantel, using sheep as the test animals. Plant extracts decreased nematode egg counts consistently, and egg counts decreased over time as the duration of dosing increased. *Ananas comosus* and *L. cuneata* treatments caused the highest mortality to nematodes, and were more effective than the positive Control over the duration of the trial. Others have reported similar positive results with sheep orally dosed with a papaya latex extract (Buttle *et al.,* 2011).

The second part of this study examined the anthelmintic effects of isolates of *Bacillus thuringiensis* (Berliner) (Bt) and *Clonostachys rosea f. rosea* (Schroers) (*C. rosea*). *Bacillus thuringiensis* is a bacterium while *C. rosea* is a fungus. *Bacillus thuringiensis* and *C. rosea* isolates were previously isolated from grazing pastures and pens of sheep at the Livestock Section, Ukulinga Research Farm, UKZN, Pietermaritzburg. These isolates were compared to diatomaceous earth (DE) and against negative Control in sheep. Both of the biocontrol agents, and DE did not affect EPG but reduced the numbers of L_3 GIN larvae. *Clonostachys rosea* caused the greatest mortality of the L_3 stage of GIN. Other researchers have found similar outcomes. Baloyi (2011) also reported that a *C. rosea* isolate was pathogenic on GIN larvae of sheep. Similarly, Kotze *et al.* (2005) reported a toxic effect by Bt isolates on nematode larval

stages *in vitro*. Bernard *et al.* (2009) also found that DE reduced the numbers of GIN larvae of goats.

In a dosing frequency study conducted on the use of *C. rosea* to control livestock GIN, a fixed quantity of chlamydospores of *C. rosea* were fed to sheep every day, every second day or every third day. All three treatments were effective at controlling GIN and reduced pasture contamination. Daily use of *C. rosea* was the most effective for the biological control of GIN in sheep compared to dosing them every second or third day. The most effective dosage of *C. rosea* was also determined under field conditions, by testing 0.25, 05 and 1.0g kg^{-1} BW (body weight). Lower dosages were effective in reducing larvae counts of both faecal cultures and grass samples. Numbers of nematode larvae decreased as the fungal dose levels increased. The best dosage tested was 1g of *C. rosea* product kg^{-1} BW per day per sheep. This is agreement with Baloyi *et al.* (2012) who found that 1g of *C. rosea* kg^{-1} BW fed to sheep for 7 days caused larval mortality of 75%.

A combination of these two approaches might be more effective in controlling nematodes of sheep than a single strategy. Nematode control was improved when a combination of an ethanolic extract of *A. comosus*, and chlamydospores of a selected strain of *C. rosea* were used to treat sheep. The extract of *A. comosus* killed adult nematodes, and therefore decreased the number of GIN eggs produced. It may also have had a residual effect that reduced larval development. Chlamydospores of C. *rosea* reduce larvae per gram of faeces (LPG), therefore reducing the numbers of L_3 larvae on pastures, which could be consumed by grazing animals. Consequently, the combination treatment was the most effective treatment at reducing all GIN population measurements: GIN egg counts, larval counts in faeces, larval development and the counts of L_3 stage in pastures. The combination treatment was significantly more effective than using the two treatments individually. However, the integrated treatments caused some loss in the body weights of treated sheep, despite its effectiveness against GIN. Future studies are needed to determine the possible mechanisms affecting sheep body weight changes, to see if the negative effects can be reversed.

7.1 Conclusions

Ethanol extracts of several plants were investigated: Extracts from *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata* and *Warburgia salutaris* killed larval stages of GIN of sheep in *in vitro* trials.

Ethanolic extracts of *A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris* also reduced GIN egg production and counts of infective L_3 larvae when fed to live sheep regularly.

Biological agents such as *Clonostachys rosea* and *Bacillus thuringiensis,* and a diatomaceous earth product, all reduced counts of GIN larvae in sheep faeces, with *C. rosea* being the most effective.

Daily feeding with 1g *C. rosea* chlamydospores kg^{-1} BW was the most effect dose and frequency tested that reduced the numbers of infective larvae of GIN, thereby reducing the degree of pasture contamination.

A combination of two treatments, the *A. comosus* extract and *C. rosea* chlamydospores, was more effective than either of the treatment by themselves in reducing GIN egg count, larval count from faecal cultures, larval development, and counts of L_3 larvae in pastures. It was the most effective treatment as an organic anthelminthic.

7.2 Recommendations

Given the problems of drug resistance by GIN, it is important that new treatments be developed to support or replace failing drug treatments. Given the efficacy of the integrated treatment of an ethanolic extracts of *A. comosus* and chlamydospores of *C. rosea*, it is important that this integrated control strategy is developed as a commercial treatment or treatments, available to both commercial and small scale farmers. Registration trials, toxicity trials, and long term-field trials are needed as a follow up to these results.

7.3 Further research

As these plant extracts (*A. comosus, A. ferox, A. sativum, L. cuneata* and *W. salutaris*) have shown an effect in the laboratory and the field, a combination plant extracts could be tested for greater efficiency.

The biocontrol agent *C. rosea* has proven to be effective under field conditions, but longer trials need to be undertaken to determine the effect of *C. rosea* on nematodes eggs, to determine its effect on body weight and the health of the treated sheep.

The mechanisms of *C. rosea* involved in killing livestock GIN needs to be determined, to find out as to whether it uses the same mechanism as documented on plant nematodes.

The cost of dosing sheep with *A. comosus* extract, and daily feeding of *C. rosea,* need to be addressed relative to the cost of anthelmintics used locally.

The effects of anthelmintics, antibiotics and other medicines fed to sheep on both plant extracts and biocontrol agents need to be studied to determine whether they inhibit one another or improve each other's efficacy.

The plant extracts, the biocontrol agent *C. rosea,* and the combination treatments should be tested on other livestock species to determine their effectiveness against different species of nematodes.

The World Health Organization estimates that more than 2.9 billion of the poorest and most vulnerable people on earth, and especially children, are infected with nematodes, severely compromising their health (Martin *et al*., 2011). In most cases the infected people cannot afford current anthelmintic treatments. An additional problem is that resistance by human nematodes to the commonly used drugs has started to emerge, in parallel to the phenomenon in livestock (Stepek *et al*., 2006). In the longer term, these biological treatments may be useful to apply to this human health issue to supplement the current drug treatments.

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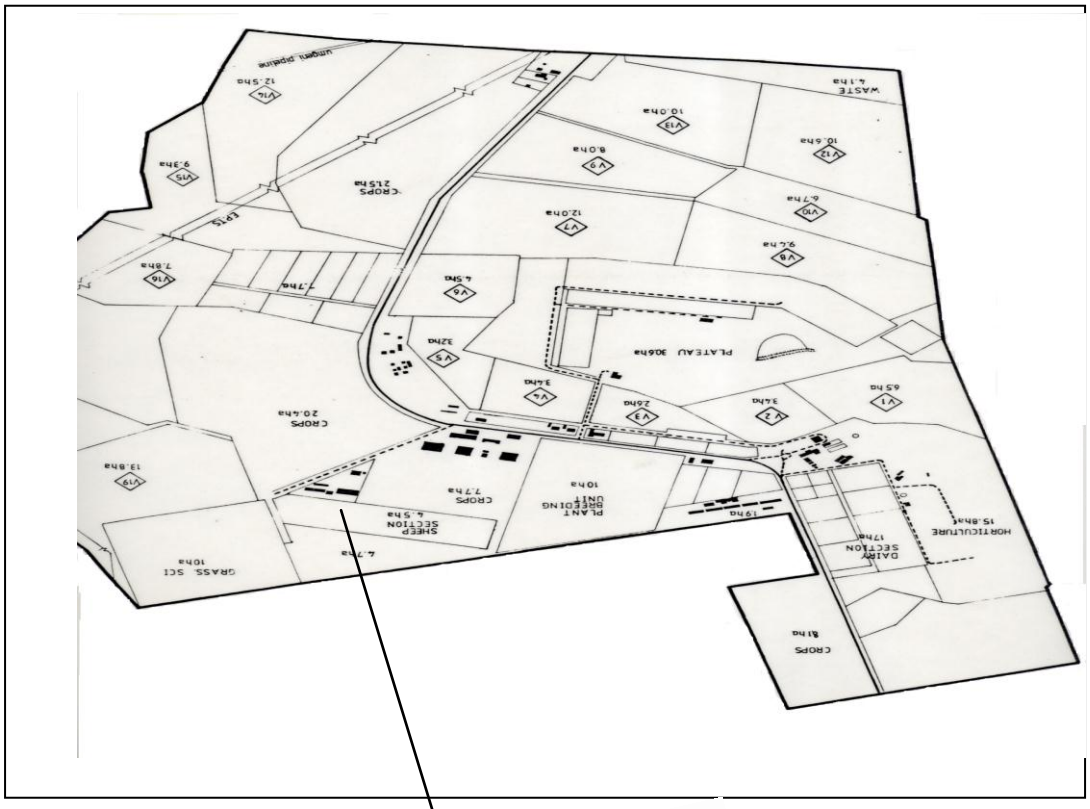
Appendices

Appendix 1

(A)



(B)

Schematic diagram of the (A) Ukulinga Research Farm (B) Livestock Section (experiment site).

Sodium chloride (salt)	400g
Water	1000 ml
Specific gravity :1.200	

Appendix 2 Formulation of the flotation fluid
Saturated Salt Solution:

Add salt until saturation, indicated by the presence of salt at the bottom of the container after stirring for 15 minutes.

Appendix 3
Formulation for other reagents used in diagnostic tests

Iodine Stain:

Iodine re-sublimed crystals	10g
Potassium iodide	50g
Water	1000ml

Dissolve the potassium iodine in the water. Then add and dissolve the iodine crystals.

Sodium Thiosulphate Stain:

Sodium thiosulphate	124.1g
Water	1000ml

Dissolve the Sodium thiosulphate crystals in the water.

Appendix 4: The effects (mean±SEM) of five plant species on gastrointestinal nematodes

Treatment	Transformed egg per gram of faeces (Log transformed)									
	day0	**Day7**	**Day15**	**Day21**	**Day28**	**Day35**	**Day42**	**Day49**	**Day56**	**Day63**
A. comosus	3.4±0.1	3.3±0.1	3.3±0.2	3.2±0.2	3.1±0.1	3.1±0.2	3.0±0.2	3.0±0.2	2.7±0.2	2.6±0.2
A. ferox	3.4±0.1	3.4±0.1	3.4±0.1	3.3±0.1	3.2±0.1	3.2±0.1	3.2±0.1	3.1±0.2	3.0±0.2	3.0±0.2
L. cuneata	3.5±0.1	3.4±0.1	3.3±0.1	3.2±0.1	3.1±0.2	3.1±0.2	2.9±0.2	2.9±0.2	2.6±0.2	2.6±0.2
S. sativum	3.4±0.1	3.4±0.1	3.4±0.1	3.3±0.1	3.2±0.2	3.1±0.2	3.1±0.2	3.1±0.2	2.9±0.2	2.9±0.2
W. salutaris	3.5±0.1	3.4±0.1	3.4±0.1	3.3±0.1	3.3±0.1	3.3±0.2	3.2±0.2	3.2±0.2	3.0±0.2	3.0±0.2
Abamectin-Praziquantel	3.4±0.1	3.1±0.1	3.3±0.1	3.4±0.1	3.4±0.1	3.5±0.1	2.9±0.1	2.9±0.2	2.8±0.1	2.9±0.1
F value	0.05	1.2	0.11	0.48	0.81	1.3	0.47	0.53	0.82	0.88
P<	0.998	0.317	0.989	0.791	0.549	0.296	0.799	0.75	0.54	0.50
LSD	0.317	.339	0.365	0.379	0.393	0.411	0.472	0.501	0.547	0.551
CV%	9.1	10.0	10.8	11.5	12.0	12.7	15.0	16.39	19.12	19.12

egg count