The efficacy of traditionally used *Leucosidea sericea* (Rosaceae) against *Haemonchus contortus* and Microbial pathogens

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Declaration

I declare that this thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor (PhD) has not previously been submitted by me for a degree at this or any other University.That it is my own work in design and in execeution, and that all materials contained herein has been duly acknowledged.

Mathew Adamu

Dedication

This thesis is dedicated to the lovely memory of my beloved late Mother, Mrs Margaret Adamu

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Publications

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Conferences

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Abstract

Haemonchus contortus is globally the most important economic parasite of sheep and is characterised by its ability to induce severe production losses. Current remedies are faced with resistance and the cost of using these treatments is excessive for poorer/smaller scale farmers. To this extent new anthelmintics that are effective against resistant Haemonchus need to be discovered. From previous studies, it has been established that herbal remedies may serve as a source of these alternate remedies. For this study thirteen tree species use traditionally to treat helminthosis were selected for evaluation. These species were: *Brachylaena discolor* DC., *Apodytes dimidiata* E. Mey. ex Arn., *Clerodendrum glabrum* E. Mey., *Clausena anisata* (Wild.) Hook.f. ex Benth., *Cyathea dregei* Kunze, *Heteromorpha trifoliata* (Spreng.) Cham. & Schltdl. var. abyssinica (A.Rich.), *Indigofera frutescens* L.f, *Leucosidea sericea* Eckl. & Zeyh., *Milletia grandis* E.Mey, *Melia azedarach*, *Maesa lanceolata* Forssk., *Strychnos mitis* S. Moore, *Zanthoxylum capense* (Thunb). Harv.

The antiparasitic activity of leaf extracts of the selected plants species were determined using the egg hatch and larval development inhibitory assay recommended by the World Association for Veterinary Parasitology Guidelines. The selected plant species were also evaluated for possible activity against three fungal pathogens *(Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans)* and four bacterial pathogens *(Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa)* using a microplate serial dilution method, antioxidant activity using the TEAC method and cytotoxicity was determined using a MTT colorimetric method. From preferential *in vitro* activity the mechanism of action of the leaf extracts of *L. sericea* was evaluated through the use of transmission and scanning electron microscopy. In vivo activity of the safe leaf extracts of *L.sericea* was also concurrently evaluated for efficacy in a sheep model of haemonchosis. Finally in an attempt at isolating active compound(s), the extracts of *L. sericea* were fractionated into hexane, ethyl acetate, butanol and chloroform and evaluated as for the crude extracts. The best fraction was hereafter selected for isolation of bioactive compounds using open column chromatography.

The extracts of three plant species i.e. *H. trifoliata, M. lanceolata* and *L. sericea* had EC_{50} values of 0.62 mg/ml, 0.72 mg/ml and 1.08 mg/ml respectively for the egg hatch assay. *Clausena anisata* and *C. glabrum* extracts were active with EC50s of 1.08 mg/ml and 1.48 mg/ml respectively. In the larval development assay the *H. trifoliata* extract was the most effective with an EC_{50} of 0.64 mg/ml followed by *L. sericea's* 1.27 mg/ml. Based on the cytotoxicity results *C. anisata* was the least toxic with an LC_{50} of 0.17 mg/ml, while *Cyathea dregei* was the most toxic plant with an LC_{50} of 0.003 mg/ml. The *C. anisata* extract had the best selectivity index with a value of 0.10 and 0.08 for the two assays, followed by *H. trifoliata* and *L. sericea* with values of 0.07, 0.07 and 0.05, 0.04. The *C. dregei* extract had the worst selectivity index with a value of 0.00019 for both assays.

The 13 acetone leaf extracts had good antifungal activities with MIC values as low as 0.02 mg/ml for extracts of *C. anisata* against *A. fumigatus* and 0.04 mg/mL for extracts of *Z. capense, C. glabrum* and *M. grandis* against *A. fumigatus. Clausena anisata* extracts had a reasonable selectivity index (2.65) against *A. fumigatus*. It also had selective activity against *A. fumigatus*, an overall fungicidal activity of 98% and a total activity of 3395 mL/g against *A. fumigatus. Clerodendrum glabrum, Z. capense* and *M. grandis* extracts also had good inhibitory activity of 0.14, 0.09 and 0.28 mg/ml. *Maesa lanceolata* and *L. sericea* with an MIC of 0.02 mg/ml had the best antibacterial activity against *E. faecalis* and *P. aeruginosa. Maesa lanceolata* had a selectivity index of 5.20, 2.60, 2.60 and 1.30 for *P. aeruginosa, E. faecalis, E. coli* and *S. aureus* respectively. *Strychnos mitis* had a selectivity index of 1.08 for *E. coli*.

Following solvent solvent fractionation the ethyl acetate fractions of *L.sericea* with EC_{50} of 0.92 and 0.79 mg/ml against the egg and larvae of *H. contortus* respectively, cytotoxicity of 283.50 mg/ml and selectivity index of 308.15 and 358.86 for the egg hatch and larval development test respectively was the most active extract. Following isolation using open column chromatography, two phloroglucinol derivatives (agrimol A and G) and β-sitosterol were isolated. The egg hatch and larval development activity for agrimol A was 0.52, 0.08 mg/ml and 0.28, 0.11 mg/l for agrimols G and A.

The acetone leaf extracts of *L. sericea* demonstrated only partial activity in artificially infected *H. contortus* sheep. Using an extrapolative pharmacokinetic dosage of 109 mg/kg, a 73.1 % reduction in faecal egg count was achieved. At a dosage of 500 mg/kg a reduction of 83.5 % was achieved. In both cases the difference to control group appeared to be non-significant. No clinical signs of toxicity was observed and no mortality from treatment at this dosage.

The electron microscopy study identified changes such as the alteration and destruction of the cuticle, changes to the hypodermis, vacuoles within the cytoplasm, cytoplasmic degradation, cellular swelling and in a few cases some abnormality within the mitochondria.

This study has confirmed the potential use of a plant *L. sericea* extract in the treatment of Haemonchus infection in sheep. The study also demonstrated the potential of some anthelmintic extract to be an antifungal. Further studies will be required to evaluate the anthelmintic potential of this extract at higher dosages and against resistant field strains.

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List of abbreviations

^{13}C-NMR	Carbon 13 nuclear magnetic resonance
1H NMR	Proton Nuclear Magnetic Resonance spectroscopy,
AADs	Amino-Acetonitrile Derivatives
ABTS	2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AD	*Apodytes dimidiata*
AF	*Aspergillus fumagatus*
ALT	Alanine amino transferase
AR	Anthelmintic resistance
AST	Aspartate amino transferase
ATCC	American typed Culture Collection
BC	Before Christ
BD	*Brachylaena discolor*
BEA	benzene/ethanol/ammonia hydroxide (90:10:1)
C	Cuticle
CA	*Candida albicans*
CA	*Clausena anisata*
CCK	cholecystokinin
CD	*Cyathea dregei*
CEF	chloroform/ethyl acetate/formic acid (5:4:1)
Cfu	Colony forming unit
CG	*Clerodendrum glabrum*
CN	Cryptococcus neoformans
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EC	Effective concentration
EHA	*Egg hatch assay*
ELISA	Enzyme linked immunosorbent assay
EMW	ethyl acetate/methanol/water (40:5.4:5)
EVM	Ethnoveterinary medicine
FAMACHA	Fafa Malan's chart

FCA	Fungicidal activity
FEC	Faecal egg count
GI	Gastrointestinal
GIT	Gastrointestinal tract
HIV/AIDS	Human immune deficiency virus/Acquired immune deficiency syndrome
Ht	Haematocrit
HT	*Heteromorpha trifoliata*
Hy	Hypodermis
IF	*Indigofera frutescens*
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-	Interleukin
INT	p-iodonitrotetrazolium
IR	Infrared
J	Couling constant
JNE	Jacobus Nicholas Eloff
L_1	First stage larva
L_2	second stage larva
L_3	third stage larva
L_4	fourth stage larva
L_5	fifth stage larva
LC	Lethal concentration
LDT	Larval development test
LS	*Leucosidea sericea*
MA	*Melia azedarach*
MA	Mathew Adamu
MAFF	Ministry of Agriculture fisheries and forestry
MEM	Minimal Essential Medium
MG	*Milletia grandis*
MH	Müller–Hinton
MIC	Minimum inhibitory concentration
ML	*Maesa lanceolata*

MTT	3-5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide
Mv	micro villi
NCCLS	National Committee for clinical laboratory standards
Nm	Nano meter
NMR	nuclear magnetic resonance
OsO4	Osmium tetra oxide
P	Parenchyma
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PGE	Parasitic gastroenteritis
PI	Post Infection
Ppm	part per million
PPRR	Periparturient relaxation of resistance
R_f	Retardation factor
SANBI	South Africa National Biodiversity Institute
SE	Standard error
SEM	Scanning electron microscopy
SG	Specific gravity
SI	Selectivity Index
SM	*Strychnos mitis*
TEAC	Trolox equivalent anti-oxidant capacity
TEM	Transmission Electron Microscope
TETFund	Tertiary Education Tax Fund
Th1	T helper type 1
Th2	T helper type 2
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF-a	Tumour necrosis factor-a
TNF-β	tumour necrosis factor β
Trolox	6-hydroxy-2, 5, 7, 8-tetrahydroxyl-chroman-2-carboxylic acid
UV	Ultraviolet
Vmax	maximum absorption frequency

VN   Vinasan Naidoo

WAAVP   World Association for the Advancement of Veterinary Parasitology

ZC   *Zanthoxylum capense*

CHAPTER 1: INTRODUCTION

1.1 Background

Globally, the sheep production industry provides major employment, animal protein, manure and wool for clothing from an estimated one billion animals. To the African small holder farmer, sheep rearing is a store of wealth and a cultural identity which is jealously guided as an inheritance. The sheep industry in South Africa is also a major part of the economy as it worth hundred millions of US dollar, particularly in wool production. Production is however hampered by constraints such as competition for scarce land resources, diseases, of which *Haemonchus* is the single most important constraint to sheep production globally. Currently, there is a widespread resistance to available anthelmintic drug, with the exception of monepantel and derquantel. The development of resistance by *Haemonchus* requires alternative control strategy (MacGlafin et al., 2011). Options available include vaccine development, use of the pathogenic fungus *Duddingtonia flagrans* or the use of plant herbal preparations. Plant herbal preparations have beEN used for treatment of different ailments in animals for a very long time and this constitutes the ever growing field of ethnoveterinary medicine (EVM).

Ethnoveterinary medicine (EVM) comprises a complex system of beliefs, skills, knowledge and practices relating to animal husbandry and general animal care (McCorkle, 1986). This all important field has not received the desired attention until the last thirty years. Currently, several plant species use in ethnoveterinary medicine are been subjected to scientific testing for efficacy and toxicity. In South Africa, only about 13 % of plants used for EVM have been evaluated scientifically for activity and toxicity. This requires more plant extracts to be evaluated for their efficacy and toxicity. This will improve our understanding of EVM and possibly lead to extracts or compounds with potent activity against pathogens that cause diseases in animals. With an estimated 87% of the global medications used against cancer, microbial and parasitic infections being derived from natural products, particularly higher plants the benefits of EVM in primary animal healthcare is clearly evident (Martin et al., 2001).

1.2 Hypothesis

The leaves of the thirteen selected plant species used for this study have bioactive compounds against *Haemonchus contortus.*

1.3 Statement of the problem

Haemonchus contortus is a major cause of diseases in sheep with high morbidity and mortality. Anthelmintic resistance is a serious setback to available anthelmintic. The exploration and bio prospecting of plants with anthelmintic activity is a possible solution to the above mentioned problem.

1.4 Justification of the study

Result generated from this study will add to the increasing data base of knowledge on EVM, there by contributing to the safety and efficacy of plant extracts used in treatment of diseases. This study can also lead to the identification of new therapeutic skeletons from medicinal plants or whole extracts that can be used for treatment of resistant *Haemonchus* strains and other pathogens.

1.5 Aim

The aim of this study is to screen and possibly identify lead compound(s) or plant extracts that may be useful as anthelmintic agents.

1.6 Objectives

The following objectives will be used to help in achieving the overall aim of this study:

❖ Preliminary *in vitro* screening of acetone extracts of thirteen plant species using the egg hatch assay and the larval development test against *Haemonchus contortus* eggs and larvae
❖ Evaluation of the plant extracts for activity against 4 bacteria and 3 fungi pathogens and relationship with anthelmintic activity
❖ Evaluations of the *in vitro* toxicity of the thirteen plant species using Vero cells.
❖ Isolation of pure anthelmintic compounds from the most active plant extracts using bio-guided fractionation.
❖ Establishing the effects of compound(s) isolated from *Leucosidea sericea* on the ultrastructure of *Haemonchus contortus*, using electron microscopy.
❖ Evaluate the activity of the acetone leaf extracts of *L. sericea* in sheep infected with *H. contortus* on ability to reduce faecal egg count and total worm counts.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of sheep in Production

The genus Ovis includes all sheep, with domesticated sheep belonging to the species *Ovis aries*. While the origin of the modern domestic sheep remains uncertain, sheep are believed to be the second species domesticated by ancient man and as a group is made up of more breeds than other domesticated species. Interesting more argument exists on the ancestry and zoological classification of sheep than any other farm animal (Ryder, 1984). A total of 7 distinct wild forms with 40 different varieties have been recognized. Several wild sheep species or subspecies have been proposed as the ancestors of domestic sheep (Ryder, 1984) or are believed to have contributed to specific breeds. Among the wild species that have contributed to the present day modern sheep are the Argali (Ovis ammon) of Central Asia, the Urial (*Ovis vignei*), also of Asia and the Moufflon (*Ovis musimon*) of Asia Minor and Europe (Davendra and McElroy, 1982). Nonetheless nine thousand BC is probably the most widely accepted time of domestication of sheep on the borders of present day Iran – Iraq (Figure, 2.1). From this place of origin sheep spread to the remaining part of the continental masses of Asia, Africa and Europe. The most recent and possibly the greatest migration took place 100-200 years ago, with the settlement of the southern hemisphere by Europeans. They took the Merino breed and the British breeds to South America, South Africa, Australia and New Zealand. Today these places account for 35-40% of the world sheep population (Zygoyiannis, 2006). In South Africa, the Namaque Afrikaner is the oldest breeds of sheep in the area. It appears they were brought into South Africa by the Khoi-Khoin people, who migrated from the central lake area of Africa bringing their sheep with them (Anon, 2010).

Sheep are multiparous animals, kept for various producing purposes of which their primary worth is in meat and wool production, with some regional differences as countries such as Turkey, Iran and southern and central Europe place more value in milk production (Zygoyiannis, 2006). The World total sheep population is estimated at approximately 1.024 billion with Africa contributing 23.8% second only to Asia with 40.6% (Anon, 2005, George and McKellar, 2006). South Africa is second only to Sudan within the Africa continent, with a sheep population of about 25.3 million and 10th on the world ranking (Anon, 2005). Sheep production is an important aspect of South Africa agriculture and makes substantial contribution to the economy. The contributions include granting employment opportunities to thousands, source of clothing and feeding millions with good quality protein. Sheep wool production contributed 1, 616, 701,669 Rands in the 2007/2008 season and 2.27 billion Rands for the 2011/2012 (Anon, 2012).

With the sheep production industry being massive globally, it is not surprising that this industry is faced with numerous constraints. The major challenges to sheep farming amongst others include; high production cost, changing land used pattern, climate change leading to pasture growth decline, and diseases. In South Africa,

diseases are a major constraint to sheep production. Amongst these diseases, the single most important constraint to small ruminant production is helminthiasis (Waller, 2006).

Figure 2.1: Map of the Middle East showing the area where sheep rearing is believed have originated about 9000 BC (shaded grey), currently within the present day Iran-Iraq border (Zygoyiannis, 2006).

2.2 Parasitic gastroenteritis (PGE) complex

Small ruminants are affected by numerous GIT strongylids with the different helminth species contributing to a various extent to the syndrome of parasitic gastroenteritis (PGE). Parasitic gastroenteritis is characterized by inappetance/anorexia, diarrhoea, weight loss, oedema, exsiccosis and anaemia. The major helminth genera of the GIT strongylids contributing to this syndrome are *Haemonchus, Ostertagia, Trichostrongylus, Nematodirus, Cooperia, Bunostomum, Gaigeria, Oesophagostomum* and *Chabertia* (Soulsby, 1986, Taylor et al., 2007) and the trematode genera Fasciola. The major determinant as to which parasite(s) is involved in the PGE syndrome is geographic e.g. *Teladorsagia* and *Nematodirus* species are major cause of disease in temperate climate like Europe in sheep. In the tropics and sub-tropics *Haemonchus* is a major contributor to the PGE syndrome.

PGE causes major economic losses. Current estimates are an annual loss of US $45 to nematode parasite in South African sheep alone (I.G. Horak personal communication: In P.J Waller, 2006). In Australia, PGE cost the sheep industry US $369 million annually or 8.7% of its total market value (Sackett et al., 2006). These losses may be direct or indirect. Direct losses are due to a drop in production (carcass quality and carcass weight) or death of animals; while indirect losses are due to the costs of drugs, labour and drenching equipment required in control strategies (Soulsby, 1986, Kassai, 1999). The losses due to PGE are mainly caused by *Haemonchus contortus* especially in tropical/subtropical regions of the world. This makes *Haemonchus contortus* the singular most important nematode of sheep globally. Thus the understanding of the morphology and life cycle of this parasite is very important to help in the control strategy (Krecek and Waller, 2006).

2.3 *Haemonchus contortus* (Rudolphi, 1803)

2.3.1 Morphology

H. contortus is a unisexual parasite with males reaching sizes of 10-20 mm and the females 18-30 mm. The males are evenly reddish in colour, while the females have the appearance of a barber's pole due to the ovaries that are spirally around the red blood-filled intestine (Soulsby, 1986). This appearance of the females has given the parasites the common name of the Barber's pole worm. Other common names are the stomach worm and wire worm.

Microscopically, the males have an asymmetrical dorsal lobe and barbed spicules, while the females usually have a vulvar flap. Both sexes have cervical papillae and a tiny lancet inside the buccal capsule (Taylor et al 2007).

Figure 2.2: Morphology of male (A) and female (B) *Haemonchus contortus* showing the spicules and the gubernaculum in the male and vulva flap in female (Illustration Courtesy of Estelle Mayhew, University of Pretoria, Department of Education Innovation).

2.3.2 Life cycle

The adult female produces between 5000-10,000 eggs which are passed out within the faeces. The eggs develop in moist conditions in the faeces into the L_1 (First stage larva) and L_2 (second stage larva) juvenile stages over 3 days. The L_2 sheds its cuticle and then develops into the L_3 infective larvae, which subsequently migrate up grass stalks. Sheep get infected during grazing by ingesting L_3 on herbage. After ingestion, the sheath on the L_3 is removed in a process called exsheathment within the rumen and the larvae moult twice in close apposition to the gastric glands to the L_4. Just before the final moult to L_5 the larvae develop the piercing lancet which enables them to obtain blood from the mucosal vessels. The L_5 or immature adult takes a few days to mature and move freely on the surface of the mucosa of the abomasum. The adult female parasite begins to produce eggs and the cycle continues under favourable environmental conditions. The prepatent period, that is the time taken for development from infection until mature female adult parasites start producing eggs, is 2-3 weeks in sheep (Soulsby, 1986, Taylor et al., 2007).
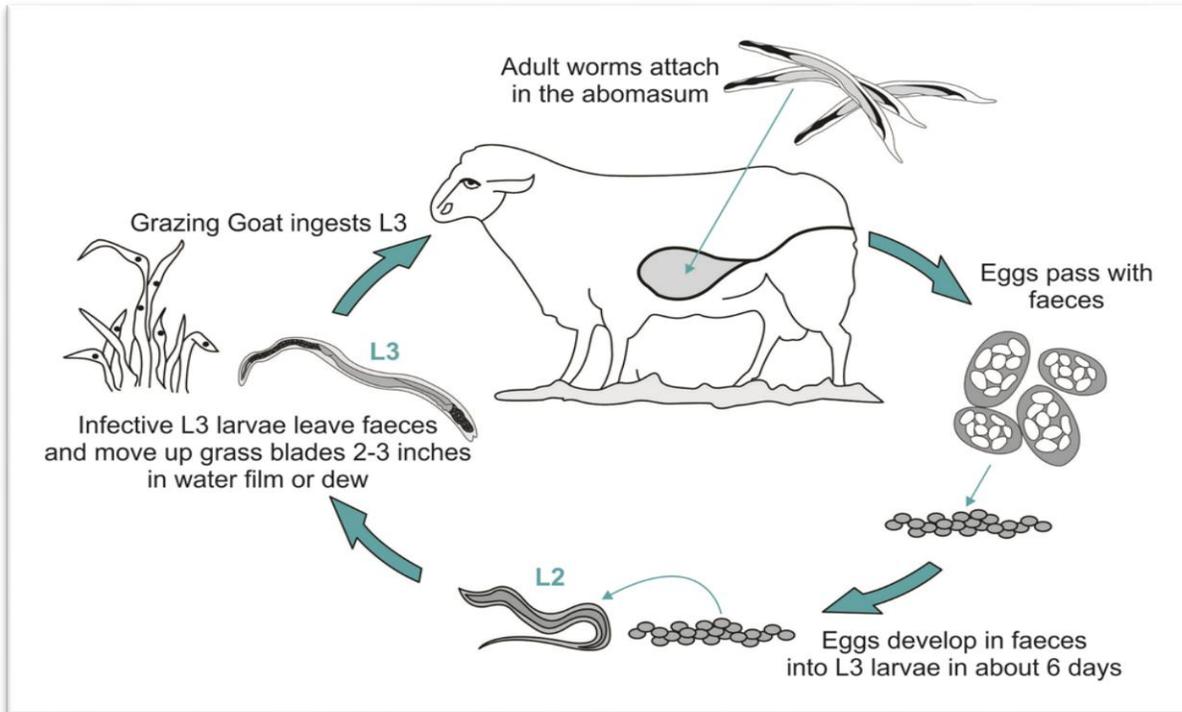
Figure 2. 3: Life cycle of *Haemonchus contortus,* showing sheep ingesting third stage (L3) larvae from pasture, this develops to the fourth and fifth stage larvae in few days in the abomasum. Adult parasite attaches to the abomasum female sucks blood and produces eggs. Eggs are passed out in faeces and develop to first and second stage larvae within 3 days and into the third stage larvae the infective form in about 6 days. (Illustration Courtesy of Estelle Mayhew, University of Pretoria, Department of Education Innovation).

2.3.3 Epidemiology of Haemonchosis

Several factors are involved in the epidemiology of haemonchosis and are briefly discussed below.

2.3.3.1 Environmental factors

Several environmental factors are vital for development and survival of the Haemonchus parasite. They require moderate temperatures within the range of 25-35^{0}C, high humidity and presence of moist ground making the tropics/sub-tropics their favoured environment. Other micro-environmental requirements are soil structure, vegetation type and drainage. Soil types that hold a permanent store of moisture are preferred as it helps egg hatching and subsequent larvae development. Dry climatic condition stops further development. Cold climatic conditions with temperature below 10^{0}C also stop further development of the parasites (Taylor et al., 2007).

2.3.3.2 Nutrition

The plane of nutrition of animals affects their ability to tolerate or succumb to infection with *Haemonchus contortus*. Several studies have reported that well fed animals rarely come down with clinical haemonchosis, with the poorly fed sheep especially on low protein diets being more susceptible (van Houtert et al., 1996). The reason for this is most likely linked to the ability of animals in good condition to better manage the protein and iron loss induced by the parasites (see section 2.3.4 for details).

2.3.3.3 Breed

The ability of sheep to acquire immunity and express resistance against parasitic infection varies considerably among and within breeds (Stear and Murray, 1994). Breeds known to be resistant are the Florida native (Amarante et al., 1999a) and the Gulf coast native (Li et al., 2001) sheep from the south of the United States. The St. Croix and Barbados black belly (Gruner et al., 2003) originating from the Caribbean islands and the red Maasai sheep from Africa (Mugambi et al., 1997). In Brazil, Santa Ines hair sheep demonstrate a greater resistance to GIN including *Haemonchus*, when compared with some sheep breeds of European origin (Amarante et al., 2004, Costa et al., 2007). Unfortunately a major drawback of this innate resistance is that these breeds are lower in productivity when compared with other breeds selected for meat quality.

2.3.3.4 Age, sex and reproductive status

Young sheep are known to be more susceptible to haemonchosis than adult sheep with mortality specifically among young lambs under 6 months being higher (Schallig, 2000). Sex and reproductive status also affect the epidemiology of haemonchosis as pregnant ewes, in their last trimester, shed more eggs than during other periods in their cycle (Jeffcoate et al., 1990). This high egg shedding is termed periparturient relaxation of resistance (PPRR) and is most likely due to hormonal changes associated with pregnancy. The epidemiological significance of the PPRR is that it allows more eggs to be passed out in faeces at the critical time when young lambs are expected on the pasture.

2.3.4 Clinical signs

The clinical signs of haemonchosis are usually non-specific and include various combinations of anorexia, anaemia, hypoproteinaemia, maldigestion, diarrhoea, weight loss, secondary submandibular oedema and ascites from the hypoproteinaemia and reproductive failure from poor condition (Soulsby, 1986, Taylor et al., 2007). In all cases these clinical signs develop as a result of the animal losing blood (red cells, fluid and protein components) into the GIT as a result of the blood feeding activity of the parasites.

2.3.4.1 Anorexia

Reduced appetite or anorexia is considered the major factor contributing to impaired nutrition in parasitized animals (Fox *et al.*, 1989a). In a comprehensive review, Kyriazakis *et al.* (1998) argue that anorexia should be considered a disease-coping strategy as opposed to the detrimental effect of parasitism as there may well not be a single factor behind anorexia. In general feed intake is depressed, in ruminants a number of factors such as reduced flow of digesta, abnormal gut motility, distension of the reticulo-rumen, abomasum or intestine (Grovum and Phillips, 1978, Grovum, 1979) and raised circulating secretin, gastrin and cholecystokinin (CCK) levels (Grovum, 1981). Abomasa parasites alter gut motility (Bueno *et al.*, 1982) and reduce the rate of flow of digesta and abomasa volume (Rowe *et al.*, 1988, Fox *et al.*, 1989a, Dynes *et al.*, 1994) in addition to causing hypergastrinaemia. All the above may therefore be involved in the anorexia of abomasa parasitism of which *Haemonchus* is a major culprit. Possible alternative causes of anorexia include pain and inflammation which are known appetite depressants in humans (Holden and Pakula, 1996).

2.3.4.2 Anaemia

The main cause is the direct lesions to the mucosa induced by the parasites and the resultant loss of blood. With each *H. contortus* adult female parasite ingesting approximately 0.05 ml of blood/helminth/day (Rowe et al., 1988) this leads to a notable blood loss depending on the duration of the infestation, worm burden or a combination thereof. This fall in haematocrit is a common finding in sheep haemonchosis (Gomez-Muñoz et al., 2001, Alunda et al., 2003) and is visible from day 4 post infection (PI) coinciding with the L_{4s} exiting from the mucosa. The fall in haematocrit accelerates with the onset of the prepatent period (3 weeks PI), due to the combined effect of young and adult parasites increased blood demand and blood loss from the traumatic haemorrhagic gastritis (Steer et al., 1995, Howard et al., 1999). The haematocrit values finally peaks in the 7th week PI (Khan et al., 1988). The fall in haematocrit value also coincides with a fall in haemoglobin concentration (5.3-7.7 mg/dl) (Khan et al., 1988) as the loss of blood to the parasites depletes the animals of iron (Fetterer and Rhoads, 1998).

2.3.4.3 Hypoproteinaenia

Reduced plasma protein has been documented as a feature of haemonchosis (Wallace et al., 1996, Amarante et al., 1999a) as a result of the blood loss and haemorrhagic gastritis mentioned above. In addition leakage of proteins to the gastric lumen occurs as a result of the disruption of intracellular unions and increased permeability (Baker et al., 2003) epithelial cell loss, tissue reparation, increase in excretory products released and cellular replacement by immature non-functional cells (Simcock et al., 1999, Hertzberg et al., 2000, Scott et al., 2000, Mulcahy et al., 2004).

The above pathogenic actions directly affect the health of the animal and also indirectly the reproductive ability, although this may go unnoticed if the disease entity is the sub-clinical form.

2.3.4.4 Weight loss

The infection of the abomasum by *Haemonchus* has a notable effect on live weight (Burke and Miller, 2002) and carcass conversion (Wallace et al., 1996). The loss of blood and plasma proteins especially in young animals leads to stunted growth due to poor skeletal growth. The final stages of the disease may be accompanied by emaciation and eventual death of the animal (Taylor, et al., 2007).

2.3.5 Pathology

At necropsy, grossly lesions observed are emaciation, pale mucosa, effusions into body cavities (Quiroz, 1999), degradation of the fat deposits (Taylor et al., 2007), hypertrophy of local lymph nodes (Perez et al., 2001,Dominguez-trano et al., 2003), oedema of the abomasa mucosa with petechial haemorrhages, presence of nodules and the *Haemonchus contortus* adult parasites within the abomasum (Soulsby, 1986). Microscopically, cellular infiltration, dilatation of the gastric glands, ulcers, oedema, haemorrhage and eosinophilia are observed (Balic et al., 2000, Bricarello et al., 2004, Huntley et al., 2004 and Amarante et al., 2005).

2.3.6 Immunity of sheep to *Haemonchus*

The sheep immune system comprises of immunoglobulin (Ig) IgA, IgD, IgE, IgG and IgM produced by plasma cells (Schallig, 2000). IgG1, IgA and IgE are the three immunoglobulin isotypes frequently involved during GI nematode infections in sheep. The immune response in sheep to gastrointestinal nematodes is thought to be mediated by CD 4+ T lymphocytes (CD 4 cells) generated in the mesenteric lymph nodes (Gill et al., 1992b) with other studies demonstrating reduced immunity against *H. contortus* in the presence of reduced CD4+ counts (Gill et al., 1993b, Karanu et al., 1997). The CD 4 cells are precursors of the T helper type 1 (Th1) and T helper type 2 (Th2), which are defined by the cytokines they produced (Mosmann and Coffman, 1989). The Th1 secrete IL-2 (Interleukin 2), Interferon gamma (IFN-γ) and tumour necrosis factor β (TNF-β) resulting in a cell mediated immune response, while, the Th2 secretes IL-3, IL-4, IL-5, IL-6, IL-9 and IL-10 among others. A typical Th2 response is characterized by increase immunoglobulin secretion by B cells, in particular IgG1 and IgE and proliferation of eosinophil's and mast cells (Schallig, 2000).

Following infection, the number of IgA-, IgG- and IgM –containing cells in the abomasum of sheep increases and peak values are found 21 and 28 days post infection (Gill *et al.*, 1992b). IgA containing cells are the most frequently

observed cell types followed by IgG1, suggesting an important role for IgA and IgG1 in the local immune response against haemonchosis. In situations where protection is achieved, the mechanism by which antibodies contribute to immunity against GI nematodes is not completely clear. The antibodies could have a direct effect on the parasite by neutralising or inactivating vital metabolic enzymes of *H. contortus* (Gill *et al.* 1993b) thus leading to dead of the parasites and hence conferring immunity. The conferment of immunity to sheep is also affected by age, as older animals seem to mount a stronger immunity as compared to younger animals. The ability of young lambs less than 6 months to be more susceptible to infection than mature sheep a phenomenon called unresponsiveness or hypo responsiveness is more evident with gastrointestinal nematodes such as *Haemonchus contortus* (Dineen et al., 1978). Lambs have significantly lower proportions of CD4+ and CD8+ lymphocytes (Watson et al., 1994). Also lamb produce less IFN-γ thus making young sheep mount smaller antibody responses than do mature sheep (Colditz et al., 1996). These findings may be responsible for why lambs are in general more susceptible to *Haemonchus* infections than adult sheep.

2.3.7 Diagnosis of Haemonchus

2.3.7.1 Clinical diagnosis

The diagnosis of the different forms of parasitic gastroenteritis in domestic ruminants can be suspected based on a combination of the clinical signs as listed above and the signalment which includes age (as young animals are more prone), season, grazing history and anthelmintic usage. Laboratory confirmation or pathological diagnosis of presence of the parasites is however a prerequisite to differentiate *Haemonchus* from other GIT strongylids. For infections with blood sucking nematodes like *H. contortus* the mucous membranes and the red blood cell values (haematocrit) provide useful tools for diagnosis. In South Africa Fafa Malan's chart (FAMACHA), a colour chart, was developed and is currently in used globally (van Wyk, et al., 1997). The FAMACHA chart categorises the mucous membrane of the eyes into five categories. Category 1 and 2 are safe and denote excellent and good, while category 3 are border line cases. The 4th and 5th categories require treatment as the mucous membranes are usually pale and mucous membrane colour is linked to the packed cell volume (PCV) or haematocrit (ht). This system evolved from the classification of anaemia based on the paleness of the eyes mucous membrane. Pinkish mucous membrane implied haematocrit levels that are higher than 30% and border line cases are slightly pinkish with haematocrit levels of about 25%. Pale mucous membrane are seen with PCV of less than 20% and are usually category 4, while PCV of less than 10% with paper white mucous membrane are category 5 and need immediate treatment.

Monitoring of anaemia through FAMACHA helps to identify sheep that requires treatment and those that do not need thus reducing the cost of treatment. This system also helps to give an early warning sign to avoid severe outbreak

with lethal consequences. For the system to function optimally, animals should be observed routinely every 2-3 weeks in endemic area and weekly especially during the *Haemonchus* season. Animals that receive treatment consistently should be culled as they stand the risk of introducing resistant strain of parasite to the flock. FAMACHA can also be used as a part of an integrated parasite control programme. The main drawback of this method is the influence of other disease conditions like fascioliosis, trypanosomosis, conjunctivitis or environmental conditions like heat, drought, stress and nutritional deficiencies (Bath, 2000).

2.3.7.2 Laboratory Diagnosis

2.3.7.2.1 Faecal egg counts (FEC)

FEC are the most widely used parameter in studies on gastrointestinal nematode infections of ruminants due to its ease of use and the reliance on minimal technology. This is the demonstration of the presence of helminth eggs in the faeces. It provides positive evidence that an animal is infected, but not necessary the level of infection as the counts is dependent on the number of viable females and the quantity of eggs each female is producing. The major drawback of the FEC is that they are less valuable in making clinical judgements, since many factors affects egg production, including species of parasites, host immunity, and stage of infection. The obvious advantage of FEC had been that, it is an easily applicable low technology method.

Several methods are available for the determination of faecal egg counts, but are generally classified as the flotation or the sedimentation methods. The flotation method is most commonly used in veterinary medicine and is based on the principles that parasite eggs are less dense than the flotation medium as such will float to the top where they can be collected for microscopic evaluation. Common flotation salts used are sodium chloride at specific gravity (SG) of 1.20, magnesium sulphate SG of 1.32 and sodium nitrate SG of 1.33, saturated sugar solution SG 1.25 and zinc sulphate. These solutions are easy to prepare, inexpensive and effective in floating most parasites eggs with the exception of trematode and some cestode eggs. The common technique used for quantification of eggs is the modified McMaster test in which 4 g of faeces is mixed with 56 ml of flotation solution to yield a total volume of 60 ml.This is mixed well and strain through coffee strainer and the filtrate is used to fill the two calibrated chambers of the McMaster slides using a pasture pipette with 0.15 ml of the filtrate from the surface. The slide is allowed to settle for a few minutes to allow the flotation process to occur rapidly. The slide is then examined and all eggs within the two chambers are counted and multiplied by 50 to give the number of eggs per gram of faeces.

The sedimentation method is used to isolates eggs of flukes and nematodes whose eggs do not float readily in common flotation solutions. Procedure involves homogenising 3 g of faeces with water and passing the suspension through a coarse mesh sieve of 250µm sieve aperture. Thoroughly wash the material that is retained on the screen,

using a fine water jet and discard the debris. Transfer the filtrate to a conical flask and allow standing for 2 minutes, removing the supernatant and transferring the reminder (approximately 12-15 ml) to a flat bottom tube. After sedimentation for a further 2 minutes, the supernatant is again drawn off. A few drops of 5% methylene blue are added and the sediment is screened using a low power stereomicroscope. Any trematode egg will be readily visible against the pale background.

2.3.7.2.2 Faecal larval cultures

While *Haemonchus* is the most important parasite, in ruminants it is not unusual for animals to have mixed infestations. While this is not a major concern for some species such as *Strongyloides papillosus*, *Toxocara vitulorum*, *Nematodirus* spp., *Trichuris* spp. and *Capillaria* spp. which show a high degree of specificity in terms of shape, *Trichostrongylus* and *Haemonchus* show considerable similarity in shape and specific gravity. In order to differentiate between these two species to genus level faecal larval cultures are needed (MAFF, 1986). Faeces are broken up finely, using a large pestle and mortar or spatula. For a good culture the faeces should be moist and crumbly but not really wet. Very dry faeces should be made moist with water and if very wet vermiculite or animal charcoal, should be added to get the correct consistency. Glass culture dishes or wide mouthed glass jars are then filled with the mixture. The lid replaced and incubated at 27 °C for 7 days when larvae should have reached the infective stage. When an incubator is not available culture can be left for 10-20 days at room temperature. Larvae are then recovered and identification of larvae can be made using keys by Keith (1953), Borgsteede and Hendriks (1974) or MAFF (1986). A setback of larval cultures is that yields are never 100%, the duration of time required for diagnosis and the proportion of larvae developing may differ between species.

2.3.8 Post-mortem examination

This method involves the identification of the worms within the gastrointestinal tract of the animal. After termination, the entire alimentary tract is removed and the individual section evaluated in the laboratory. Within the laboratory the contents are washed into a bucket under running water and total volume made up to 2-4 l. After thorough mixing transfer 10% aliquot to a suitable container and preserve in 10% formalin. Add 2-3 ml of iodine solution to the aliquot sample and count in petri dishes examined under a stereomicroscope (X12 objective).

The identification of parasites is made by using the key for identifications of MAFF (1986) and Taylor *et al.*, (2007). For *H. contortus* the mature adult worms are large (males 10-20 mm long, females 18-30mm) and reddish when fresh, easily seen. The head have a prominent cervical papillae, distance from anterior end and about 3 times diameter between papillae. Females have a linguiform vulvar flap with gravid worms having several hundred eggs,

ovaries curled around the intestine resembling barbers pole. Males have dorsal ray of bursa asymmetric, with spicules barbed near tips (Taylor et al., 2007, Zajac and Conboy, 2012). Alternatively, for large worms, the area of diagnostic importance (usually the anterior and posterior ends of the worm) may be sectioned and mounted in a few drops of clearing solution such as Hoyers solution or lacto phenol. The microscopic slides are then examined under oil immersion with attention to the key identification features listed in MAFF (1986), Zajac and Conboy (2012).

2.3.9 Serology

The use of serological diagnosis is not yet possible for Haemonchus, but may be a future diagnostic aid as commercial ELISA kits for diagnosis of nematodes infections are available for other helminth species.

2.4 Treatment and control

2.4.1 Anthelmintics

Anthelmintic or vermifuge has been used for treatment of worms in man and animals for ages and has a history of near two hundred years (Palmer, 2010). Most of the early treatment involves the use of either metals or plants extracts such as extracts of male fern and thymol, through oil of chenopodium. This later extended to the toxic carbon tetrachloride, tetrachloroethylene and hexylresorcinol. Other dangerous agents used include copper sulphate and nicotine mixture. Piperazine was introduced in 1949 and was really only effective against Enterobius and Ascaris. This was followed by the introduction of niclosamide and thiabendazole in 1960 and 1961 respectively. Pyrantel came into the market in 1966 and was effective against ascaris and hookworms. By 1971 levamisole was introduced, oxantel (1974), mebendazole (1975), praziquantel (1978), albendazole (1980), triclabendazole (1985) and ivermectin (1981) (McKellar and Jackson, 2004). The introduction of ivermectin in 1981 heralded a major breakthrough into the animal health market for anthelmintic. This drug had an excellent broad spectrum activity against nematodes and several parasitic arthropods. However, resistance was reported in the target parasites to each chemical group within years of launch (Carmichael, 1987).

After the introduction of ivermectin, the introduction of new drugs slumped as promising drugs such as cyclodepsipeptides and paraherquamides (Kaplan, 2004) failed to get to the market, while Bayer's much lauded emodepside was effective for use in only cats (Kaminsky et al., 2008). Since ivermectin, it has taken 30 years for the next new classes of broad spectrum anthelmintic to be launched, the amino-acetonitrile derivatives (AADs) represented by monepantel (Kaminsky et al., 2009) and spiroindoles represented by derquantel (Little et al., 2010). A summary of the available anthelmintics are given below and in table 2.1 (Barragry, 1984).

- Benzimidazoles and pro-benzimidazoles: Example of drugs within this group is albendazole, fenbendazole, mebendazole, netobimin, oxfendazole, oxibendazole, triclabendazole. The benzimidazole group acts by binding selectively to β-tubulins of nematodes and other helminths and inhibit microtubules formation. They also interfere with energy production by inhibiting Fumarate Reductase and hence inhibit glucose transport. This lead to starvation and eventual death of the parasite.

- Paraherquamides: example includes emodepside, is a competitive antagonist of postsynaptic calcium activated potassium channel and on presynaptic latrophilins. Interference with synaptic signal transduction at the neuromuscular junction paralyzes gastrointestinal nematodes, and they are either killed or expelled by intestinal peristalsis (Rufener et al., 2010).

- Diphenylsulphides: e.g. bithionol acts by uncoupling oxidative phosphorylation, leading to energy depletion and eventual death of the parasite.

- Isoquinolines: e.g. praziquantel act as agonist at nicotinic acetylcholine receptors of nematodes muscle and cause spastic paralysis. Praziquantel has a selective effect on the tegument of trematodes and increases permeability of calcium.

- Imidazothiazoles examples are levamisole, tetramisole. They act as agonist at nicotinic acetylcholine receptors of nematodes muscle and cause spastic paralysis.

- Macrocyclic lactones such as abamectin, doramectin, eprinomectin, ivermectin, moxidectin. They act by increasing the opening of glutamate gated chloride channels and produce the paralysis of pharyngeal pumping in the parasite.

- Salicylanilides includes among others, closantel, niclosamide, oxyclozanide and rafoxanide. They act by uncoupling oxidative phosphorylation, leading to energy depletion and eventual death of the parasite.

- Tetrahydropyrimidines they include drugs such as oxantel and pyrantel. They are agonist at nicotinic acetylcholine receptors of nematodes muscle and cause spastic paralysis.

- Aminoacetonitrile derivatives (AAD's) with monepantel as the sole drug in this group acts by interfering with nicotinic acetylcholine receptors and their by causing muscle paralysis in the helminth (Kaminsky et al., 2009).

- Spiroindoles represented by derquantel (2-desoxoparaherquamide) (Little et al., 2010)

- Others are clorsulon, nitroxynil, dicyclanil, and organophosphates. The organophosphates act as cholinesterase antagonists.

Table 2. 2: Chemical Groups and Mechanisms of Action of Commonly Used Anthelmintic Agents (Adapted from Barragry, 1984 with modifications)

Group	Chemical Name	Mode of Action in Parasite	Effect
Benzimidazoles	Thiabendazole, Parbendazole, Cambendazole, Mebendazole, Oxibendazole, Fenbendazole, Albendazole, Oxfendazole	Interfere with energy production Inhibit Fumarate Reductase Block Tubulin Synthesis Inhibit Glucose Transport	Starvation of parasite (slow process) Ovicidal
Pro-Benzimidazole	Thiophanate Febantel	Metabolised *in vivo* to Benzimidazole Carbamates	As above.
Imidazothiazoles	Tetramisole, Levamisole	Ganglionic Stimulants	Spastic Paralysis
Tetrahydropyrimidines	Pyrantel, Morantel	Cholinergic Agonists (Ganglionic)	Spastic Paralysis
Salicylanilides	Rafoxanide, Oxyclozanide, Niclosamide	Uncouple Oxidative Phosphorylation	Energy Depletion

Table 2.1 Continue: Chemical Groups and Mechanisms of Action of Commonly Used Anthelmintic Agents (Adapted from Barragry, 1984 with modifications).

Group	Chemical Name	Mode of Action in Parasite	Effect
Diphenylsulphides	Nitroxynil, Niclofolan, Bithionol, Hexachlorophene	Uncouple Oxidative Phosphorylation	Energy Depletion
Organo- phosphate	Trichlorphon, Dichlorvos, Haloxan	Cholinesterase Inhibition	Spastic Paralysis
Piperazines	Piperazine, Diethylcarbamazine	Neuromuscular Hyper polarizers	Flaccid Paralysis
Avermectin	Ivermectin	GABA Potentiation	Flaccid Paralysis
Amino-Acetonitrile Derivatives (AADs)	Monepantel	Interferes with Nicotinic acetylcholine receptors	Body wall muscle Paralysis

2.4.1.1 Problems with current anthelmintic

2.4.1.1.1 Anthelmintic Resistance

Anthelmintic resistance (AR) is defined as genetically transmitted loss of sensitivity of a drug in worm populations that were previously sensitive to the same drug (Kóhler, 2001). The issue of anthelmintic resistance has received more attention by parasitologist than any other topic, especially in sheep. Several authors (Waller et al., 1994, 1995, 1997, 1999, Sangster, 1999, Craig, 1993, Hazelby et al., 1994, Prichard, 1994 and Condor and Campbell, 1995) have made extensive reviews of the subject area. Mechanisms associated with AR against levamisole (Sangster, 1999), the benzimidazoles (Roos et al., 1995) and the Ivermectin (Gill et al., 1998) have been extensively studied. For example, resistance against the benzimidazoles appears to be due to changes in the β-tubulin isotype pattern which results in the loss of high affinity receptor binding sites (Lacey and Gill, 1994). Currently, AR has developed to all classes of broad spectrum anthelmintics with the exception of the most recently introduced drugs which were launched to target multiple resistant *Haemonchus* (Kaminsky et al., 2008). The causes of AR include:

- The frequency of treatment: The repeated treatment of animals with the same anthelmintic usually selects parasites that have innate immunity to the anthelmintic. Since these parasites are not killed on deworming, they continue to produce eggs until such a time that the refugia (Parasite in refugia represent the fraction of the worm population not exposed to the drug when animals are treated) is made predominantly of this resistant species. Thus resistance development may likely occur when the frequency of anthelmintic usage is high. This resistance may extend to other drugs within the same group with a similar mode of action.

- The prepatent period of the parasite: Parasites with short prepatent period impliedly produces many generations of parasites. *H. contortus* with a prepatent period of 3 weeks will led to many generations of parasites within a season. Thus when a resistant worm is present (either innate or from mutation), this resistance development can be rapidly propagated in the refugia due to more rapid spread of the resistance genetic material.

- Percentage of parasite in refugia: Since the refugia represents 99% of the actual population, the number of resistant to non-resistant worms will directly influence how quickly the resistant parasites propagates in an animal model (Martin et al., 1981, van Wyk, 2001)

- Under dosing of animals during treatment: This usually leads to the survival of heterozygous resistant parasites which in turn allows for the accumulation of resistant alleles within the parasite population. Thus under dosing helps in accelerating the process of anthelmintic resistance development (Chartier et al., 2001).The use of fake or adulterated anthelmintics especially in sub-Sahara Africa have also contributes to

AR. This has been reported as a common problem across Africa as use of poorly formulated or adulterated products in markets accessed by small holder farming communities (Monteiro et al., 1998).

Despite the challenge faced due to anthelmintic resistance many farmers however, continue to use anthelmintics as their primary means of nematode management. Report of new cases of anthelmintic resistance is still been reported in the literature except for the new class of anthelmintic the AADs. Recent examples can be cited from the Netherlands (Borgsteede et al., 2007), Switzerland (Artho et al., 2007) and in Scotland, instances of multiple resistance have been confirmed (Sargison et al., 2007). In South Africa, multiple resistance to all known classes of anthelmintic was reported over two decades ago (Arundel, 1985, Malan et al., 1990 and van Wyk et al., 1999). In the absence of new anthelmintic and in the face of resistance, sheep farmers will continue to be affected by declines in the productivity of their flocks and in extreme cases this has threaten the small ruminant industry (Kaplan, 2004). In the face of this problem of anthelmintic resistance several mitigations options have been sought with varied results. These options include the development of vaccine against nematodes, the use of integrated parasite control which encompasses pasture management and anthelmintic treatment, herbal remedies and the introduction of new anthelmintic.

2.5 Alternative Treatment Strategies

Numerous alternative have been proposed or been investigated as alternatives. They are herbal preparations, vaccination, biological control such as use of fungus and grazing management.

2.5.1 Vaccination

Vaccination has been suggested as an alternate approach to chemotherapy. Despite the efforts put into vaccine development no commercial vaccine against *Haemonchus* is available in the market. The search is on-going and may be available in the near future. The major setbacks with the vaccines candidates so far developed are the immunity confers are short lived. Secondly the economic aspect of vaccine production and effect on the sheep when compared to chemotherapy is on the low side. This has failed for the important gastrointestinal (GI) nematode parasites of livestock, *H. contortus* (Capitini et al., 1990). However, efforts at directing response against parasite proteins which are essential to survival are showing some success (Knox and Smith, 2001). An effective vaccine against *H. contortus* has been generated in this manner using a hidden H11 antigen in sheep (Andrews et al., 1995, 1997) (although the H11 antigen was subsequently proposed not to be hidden (Yatsuda et al., 2003). Another vaccine candidate using a membrane-bound thiol Sepharose binding fraction (TSBP) of adult *H. contortus* with

enriched cysteine proteinase activity has led to a significant reduction in worm burden but not FEC (Redmond and Knox, 2004). Scheerlinck et al. (2004) describes how *in vivo* electroporation of the muscle improves immune responses to DNA vaccination in sheep, leading to enhanced humoral responses and immune memory against *H. contortus*. The vaccination of Manchego lambs with a di-peptide fraction of *H. contortus* lead to the lengthening of prepatent periods, significant reduction in FEC, reduced variations in packed cell volume values and significantly lower worm burden at necropsy in comparison to unvaccinated animals (Dominguez-Torano et al., 2000). Similarly, Vervelde et al. (2001) describes lambs protected against the parasitic nematode *H. contortus* after vaccination with excretory/secretory glycoproteins. Vaccination led to a reduction in egg output and increased protection and the authors concluded that hypo responsiveness of lambs to *H. contortus* can be overcome by vaccination with excretory/secretory glycoproteins in a strong T-helper 2 type response-inducing aluminium adjuvant. While, Bakker et al. (2004) evaluated a Dl-dithiothreitol fractionated excretory/secretory products of adult *H. contortus* and demonstrated a high level of protection in vaccinated sheep in which egg output and worm burden were reduced by 52 and 50 per cent respectively.

2.5.2 Grazing management

Grazing management is that system that involves the regulation of pasture consumptive process, primarily through the manipulation of livestock and pasture to meet production goals (Heitschmidt and Stuth, 1991). The major goal of grazing management is to attain production goals at a minimal cost. Grazing management also helps to avoid over and under grazing. This is achieved by proper stocking of animals per unit area. This is also complemented by practices such as rotational grazing that involves the demarcation of pasture field into four or more components and animals rotate either weekly or fortnight. This ensures that pastures are not over grazed. For decades, these various grazing management practices have been the cornerstone of epidemiologically based parasite control strategies in the developed regions of the world. This is not possible among small holders due to cost and managerial capacity required to practice this system of management. Grazing management systems have been developed whereby sheep (or goats) are moved to a new area of pasture after 3–4 days of grazing and are not returned to this area for approximately one month (Gill 1991, Gomez-Muñoz et al., 1998). Not only were they cost efficient and highly effective, particularly when combined with anthelmintic treatment, but they also provided the opportunity for dual livestock species parasite control, such as with sheep/cattle interchange grazing (Gill, 1991). This concept became established in the field of applied veterinary parasitology and is called the 'dose-and-move strategies'. The disadvantage of the dose and move strategies is lack of large expanse of land due to competing demands to the scarce land resources. While the combination of anthelmintic treatment with a move of animals to pastures with low infectivity proved to be highly effective from a parasite control standpoint, it later became apparent in certain localities that these procedures selected strongly for anthelmintic resistance in surviving parasites. However, it has been

shown that not all grazing management strategies to control parasites need to be accompanied by anthelmintic treatment to achieve extremely good levels of parasite control for either young cattle (Amarante et al., 2005, Gill et al., 1993a) or sheep (Gomez-Muñoz et al., 1998). A word of caution also needs to be raised on relying on long-term sheep/cattle interchange systems as there have been reports that parasites primarily of cattle might show an increased ability to infect sheep and cause clinical disease (Gill et al., 1993b). Therefore grazing management alone is not adequate as a solution to helminth control, it should involve anthelmintic medication. Thus the need to search for newer anthelmintic of which plant based anthelmintic is a viable option.

2.5.3 Biological control

In this control strategy, the parasites in the refugia are killed in an attempt to reduce pasture contamination and final infection by grazing animals. For this method, fungal spores that live of the immature nematodes are sprayed onto the field (Flores-Crespo et al., 2003). As the fungi grow they kill the immature stages, thereby breaking the life cycle of nematodes before they have a chance to migrate from dung to the pasture. Field trials of this concept for a range of livestock species, in a variety of geo-climatic regions, have been undertaken over the past decade (Balic et al., 2000, Gomez-Muñoz et al., 2001) with mixed results as some studies report reduced FEC in sentinel animal grazed on treated fields while other have shown poor or indifferent results (Waller et al., 2004b, Knox et al., 2002, Fontenot et al., 2003, Eysker et al., 2006). As a direct consequence of this lack of consistency, research investigating this approach has suffered serious setback in recent years, with no commercially viable product being available.

2.5.4 Herbal preparations

Herbal remedies are also commonly used as dewormers in both the developed and developing countries of the world (Cuquerella et al., 1991, Cuquerella et al., 1994b). Anthelmintic medication has its origin in the use of plant preparations, such as the oil of chenopodium. Herbal remedies will continue to offer a potential source of anthelmintic for man and his livestock. Plants may act as a substitute or compliment chemical anthelmintic due to the global anthelmintic resistant development to all known anthelmintic. Secondly, there is the need to reduce sole reliance on chemical anthelmintic coupled with the concern about synthetic chemical residues in the food chain. It is imperative to seek, identify alternative to chemical anthelmintic for the control of parasites. Plants or herbal products have the potential to offer this alternative. Also, for the resource-poor farmers in developing countries, traditional herbal remedies based on local plants offer an alternative to the expensive and often inaccessible commercial anthelmintic. Thus the search for plants with anthelmintic activity against helminthic parasites is a worthwhile venture.

2.6 Plant selection

For this study thirteen plant species from different genera were selected. These plants were selected from an initial fifty two species, which was reduced to thirteen based on criteria such as sustainability as only tree leaves were used, traditionally documented uses, together with an absence of published information validating their use using *H. contortus* especially were other models were used for screening. The plant species selected are;

2.6.1 *Brachylaena discolor* DC.

Family: Asteraceae

Common names: Coastal Silver-oak, ipahala in Zulu, Kusvaalbos in Afrikans, Mphahla in Northern Sotho.

Figure 2. 4: The leaves and stem of *Brachylaena discolor* in their natural habitat. Picture courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This plant usually attains a height of 4-10 m. The bark is grey to brownish grey, rough, with vertical fissured (Coates-Palgrave, 2002). The leaves are simple and large (5-11 cm long x 1.3 cm wide). It is leathery and glossy dark green above and covered with a silvery-white felt of dense hairs below. The leaves are elliptic with rounded tip and a narrow base that is spirally arranged towards the ends of its branch lets and twigs. *Brachylaena discolor* occurs in coastal woodland, bush and on the margins of evergreen forest from the Eastern Cape to Mozambique. The wood of *B. discolor* is used in the manufacture of boats as it lasts well in water, as well as for fence posts, huts, axles, spokes, implement handles, knobkerries, and long straight branches used to construct roofs of huts (Palmer, 1977;

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Esterhuyse et al., 2001). Suitable branches also make excellent fishing rods, and fire shades are also made from this wood. The leaves are very bitter and unpalatable and are occasionally browsed by nyala, bushbuck, red and blue duiker. The Zulu people used an infusion of the roots as an enema to stop bleeding of the stomach and an infusion of the leaves as a tonic to treat intestinal parasites and for chest pain (Watt and Breyer-Brandwijk, 1962). The ashes of the tree were used by early settlers to provide the alkali needed in soap making. Previous study on this plant was the anthelmintic and antimicrobial activity by McGaw et al., (2000).

2.6.2 *Apodytes dimidiata* E. Mey. ex Arn.

Family: Icacinaceae

Common names: White pear, Wit peer (Afrikaans), UmDakane (Zulu) and sephopha-madi (Northern Sotho).

Figure 2. 5: The leaves and stem of *Apodytes dimidiata* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a small tree that grows up to 5 m in height. In the forest it can reach a height of 20 m. It has an evergreen, glossy, bright green leaves that have a paler green, dull underside. The bark is pale grey and smooth (Coates-Palgrave, 2002). The flowers are frequently produced in striking profusion. The fruit is berry -like, black and flattened. *A. dimidiata* is a protected tree in South Africa. It is in fact one of the best-known forest trees in southern Africa as it is found from Table Mountain in the Cape Peninsula, along the coast through Kwa-Zulu Natal, Gauteng, Swaziland. *A. dimidiata* comprises 3-9% of the total tree population of the Knysna Forest. Two major compounds have been isolated from the bark of this plant, the iridoid genipin and its 10-monoacetate derivative (Drewes *et al.,* 1996). The wood is very hard and was used to manufacture agricultural implements, furniture and in wagon

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construction. This tree is also valued by the Zulu people in traditional medicine. An infusion from the root bark is used as an enema for intestinal parasites in calves (Gerstner, 2001, Bryant, 1966). The leaves are used in the treatment of ear inflammation (Coates-Palgrave, 2002). The molluscicidial property of this plant has been evaluated (Pretorious et al., 1991), while McGaw et al., (2000) reported on the antibacterial and anthelmintic properties.

2.6.3 *Clerodendrum glabrum* E. Mey.

Family: Lamiacae

Common names: Smooth Tinder wood, Gladdetontelhout (Afrikaans) and Ifamu (Zulu).

Figure 2. 6: The leaves and stem of *Clerodendrum glabrum* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a shrub or small to medium sized tree that can attain a height of between 2-10 m. The barks are grey-brown, and roughly fissured with slender erect stems on the main horizontal branches. The leaves are opposite or in whorls of 3, horizontal or dropping from an erect petiole, often folded up along the midrib. The flowers are white, corolla tube 4-10 mm long. The fruits are almost round, 6-10 mm in diameter and cream (Coates-Palgrave, 2002). The leaves are

ingredients in infusions taken in doses of a cupful for intestinal parasites known as ikhambi in Zulu (Hutchings, 1996). The Sotho and Swazi topically applied the leaf decoctions to prevent the development of myiasis (Watt and Breyer-Brandwijk, 1962). The leaf infusions, with bark scraping and milk are used as anthelmintics for calves, dogs and donkeys by the Tswana (Hutchings, 1996). Steroids, terpenes, flavonoids, cyanogenic glycosides and phenolic compounds have been isolated (Shrivastava and Patel, 2007).

2.6.4 *Clausena anisata* (Wild.) Hook.f. ex Benth.

Family: Rutaceae

Common names: Horsewood, Maggot killer (Eng.), Perdepis (Afr.), Unukambiba (Zulu)

Figure 2. 7: The leaves and stem of *Clausena anisata* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a small unarmed tree and grows usually 3-5 m in height but occasionally reaching 10 m. The barks are greyish brown, mottled and smooth. Leaves are imparipinnate, spirally arranged with 10-17 alternate to sub-opposite leaflets including the terminal one (Coates-Palgrave, 2002). Flowers are small about 10 mm in diameter and yellow or white. The fruits are spherical, fleshy, 7 mm in diameter. Roots are used in a tapeworm remedy (Bryant, 1966). Leaves are ingredients in infusions taken in doses of a cupful as parasticides and purgatives. The smoke from the wood is used to fumigate new-born babies and the steam from the boiling leaves is used to clean the body internally (Watt and Breyer-Brandwijk, 1962). The Zulus also used the roots for treatment of pyrexia in children and for minor ailments. Leaves are used for treatment of myiasis, mosquito's repellents, abdominal pain, toothache, gingivitis, rheumatism and gastroenteritis (Hutchings, 1996). The volatile oil of the leaves contains the toxic estragole (Okunade

and Olaifa, 1987). The alkaloids imperatorin and xanthoxyletin, isolated from the root or root bark, have molluscicidal activity (Hutchings et al., 1996). Clausenol a carbazole alkaloid isolated from alcoholic extract of stem-bark of *Clausena anisata* (Willd) Hook (Rutaceae) has shown antibacterial activity (Devi et al 1999; Chakarborty et al 1995)**.** Tetranortriterpenoids have been isolated from the stem bark and roots of *Clausena anisata.* The known compounds were identified spectroscopically as limonin, zapoterin and clausenolide .The novel compounds include clausenolide-l-ethyl ether and Clausenarin **(**Ngadjui *et al* 1989)*.*The glandular leaves have strong smelling particularly when crushed. Many terpenoid, sequiterpenoids and fatty acids have been isolated from pericarps, roots and leaves (Reisch et al., 1985). Recently the antimyiasis activity of this plant has been reported by Mukandiwa et al., (2012b).

2.6.5 *Cyathea dregei* Kunze

Family: Cyatheceae

Common names: Tree fern, Gewon Boomvaring (Afrikanns)

Figure 2. 8: The entire tree of *Cyathea dregei* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a rather large, stout-trunked tree fern from the forests and grasslands. Distribution of this Tree includes South Africa, Madagascar and other parts of East Africa. It grows in areas with temperate to tropical climate such as the Western Cape. Dried roots are used as an anthelmintic (Hutchings et al., 1996). Recently the anthelmintic activity of this plant was evaluated using *C.elegans* (Aremu et al., 2010). The antifungal activity of this plant against fungal pathogen has been reported (Adamu et al., 2012) with some degree of activity.

2.6.6 *Heteromorpha trifoliata* (Spreng.) Cham. & Schltdl. var. abyssinica (A.Rich.)

Family: Apiaceae

Common names: Parsley tree, wildepietersielie (Afrikaans), umbangandlala (Zulu)

Figure 2. 9: The leaves and flower of *Heteromorpha trifoliata* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

The parsley tree has several distinctive features. The bark is smooth and shiny and peels off in horizontal flakes. The leaves are variable in size and shape and may be simple to compound. They are light green to grey-green and may turn yellow and red before falling in autumn. When crushed they smell of parsley or parsnips, hence the common name. The small green or yellow flowers are strong-smelling, inconspicuous and arranged in umbels, i.e. all the stalks arise from the same point. It occurs in wooded grassland, bushveld and on forest margins. This plant has been documented to be used for the treatment of many varied ailments including helminthiasis (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). Previously, antimicrobial activity and COX-I inhibiting activity of *H. trifoliata* have been evaluated (Lundgard et al., 2008, Nkoma and Kambizi, 2009). This plant is fairly widespread in the eastern regions of South Africa, from the southern Cape up through the Eastern Cape and eastern Free State, Kwazulu-Natal, Swaziland into Gauteng, Mpumalanga and Limpopo. It also occurs further north in Africa. The main use of the plant is to treat abdominal pains and intestinal worms in children with an infusion of leaves used as an enema (Palmer and Pitman, 1973). It is also used to treat nervous and mental disorders. A decoction of the root is used to treat shortness of breath, coughs and dysentery (Hutchings, 1996). Smoke from the burning plant is inhaled to treat headaches. The tree is planted with charm intent in every lekhotla (meeting) in Free State, Basuto land. It is

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also planted in the kraals of chiefs in Lesotho to ensure the loyalty of the people (Hutchings, 1996). Anthelmintic and antimicrobial activity has also been evaluated (McGaw et al., 2000, Adamu et al., 2012).

2.6.7 *Indigofera frutescens*

Family: Fabaceae

Common names: River indigo, *kouebos* or *ertjiesbos* (Afrikaans), umnukambiba (Zulu)

Figure 2. 10: The leaves and stem of *Indigofera frutescens* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a species which is found in the Western Cape fynbos and extend to the Cederberg Mountains upto Worcester in South Africa. It has rose to purple flowers in lax racemes and bluish green leaves (Figure 2.10). It forms a stout, leafy shrub up to 2 m tall. It is not often found in cultivation and the name is often confused in the nursery trade with the plant previously known as *Indigofera cylindrica*- now *Indigofera jucunda.* The root bark decoctions are used as anthelmintics for humans and animals, especially for roundworms (Watt and Breyer-Brandwijk, 1962).

2.6.8 *Leucosidea sericea* Eckl. & Zeyh.

Family: Rosaceae

Common names: old wood, umTshitshi by the Zulu's, isiDwadwa by the Xhosa and Ouhout in Afrikaans, mošino (N. Sotho) and Munyonga-tshifumbu (Venda).

Figure 2.11: The leaves and stem of *Leucosidea sericea* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This plant belongs to a large family consisting of approximately 107 genera with approximately 3000 species and nine of these genera are indigenous to South Africa (Jordan, 2000). This plant is used traditionally as a vermifuge (deworming agent) by the local inhabitants (Watt and Breyer-Brandwijk, 1962, Pooley, 1997). This is a small tree of great character which grows in the eastern parts of South Africa. The generic name (*Leucosidea*) is derived from the Greek words for "white or grey appearance", while the species name (*sericea*) is the Latin for "silky" in reference to the silky hairs on the leaves of the tree. The Ouhout is often a straggly shrub or a dense, small, evergreen tree, which grows up to 7m in height (Watt and Breyer-Brandwijk, 1962, Coates-Palgrave, 2002). It is single or multi-stemmed and branches low down. The bark is rough, reddish brown in colour and flakes off to reveal a smooth light brown under-bark. The leaves are alternately arranged, compound and covered with silky, silver hairs. Each leaf possesses 3 to 4 pairs of leaflets. The veins on the leaves are deeply sunken on the upper surface and protrude on the lower surface. The leaves are a dark green colour above and a lighter green colour below. The margins of the leaflets are deeply serrated. When the leaves are crushed they have a strong herb-like smell. The flowers are greenish-yellow in colour, star-shaped, and grow in spikes at the ends of young shoots. The fruits are nut-like and about 3 mm in diameter (Coates-Palgrave, 2002). This tree is usually found growing in dense thickets at altitudes above 1000 metres. It can be found growing in open grassland, along river banks and on wooded, rocky ridges. It is usually found growing in damp conditions, on deep, sandy or clayey and often rocky soil. *Leucosidea sericea* occurs in the Eastern Cape, Lesotho, western KwaZulu-Natal, the eastern Free State, North West, Gauteng, Mpumalanga, and Limpopo provinces, Swaziland and Zimbabwe (Coates-Palgrave, 2002). The flowers and young shoots of this plant are browsed by cattle and goats in spring. It forms dense thickets on overgrazed, eroded or otherwise disturbed areas and can, therefore, become a problem plant on farm lands. The Ouhout produces nectar which is probably utilised by bees and other insects. The wood makes good, durable fence posts in permanently wet soil even though it is soft (Coates-Palgrave, 2002)

Zulu people use a paste made from the crushed leaves of *Leucosidea sericea* for treating ophthalmia (an eye ailment) (Hutchings, 1996). The tree is used by the local people as a charm to protect the inhabitants of homesteads. The wood of this tree burns slowly and produces a lot of smoke like old and decaying wood. This together with the appearance of the flaky bark has given rise to the tree's common name of "oldwood". *Leucosidea sericea* is a frost resistant, evergreen tree, which makes it an ideal species to use to protect less frost hardy plants during winter. Because it is fast growing it can be used to establish an evergreen canopy relatively quickly while slower growing trees can be used around it and ultimately take its place once they have developed sufficiently (Watt and Breyer-Brandwijk, 1962, Coates Palgrave, 2002). The tree can also be used to provide shade in summer if low branches are trimmed off. The Ouhout is a dense plant that can be used to provide a screen against noise, objectionable views and wind. This tree can be very successfully utilised near water sources such as rivers and dams since it enjoys living in damp areas. The leaf of this species have demonstrated anthelmintic activity and aspindinol has been isolated (Bosman et al., 2004; Aremu et al., 2010). The antifungal activity and cytotoxicity of this plant was recently reported (Adamu et al., 2012).

2.6.9 *Milletia grandis* E.Mey

Family: Fabaceae

Common names: Skeels umzimbeet (English); umsambeet (Afrikaans); umSimbithwa (Zulu) umKunye (Xhosa).

Figure 2. 12: The leaves and stem of *Milletia grandis* in their natural habitat. Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

A tree that grows up to 25 m in height, with a spreading crown, but much smaller in shallow soils. The bark is smooth and grey brown on younger branches, flaky when older (Watt and Breyer-Brandwijk, 1962, Coates Palgrave, 2002). Leaves are compound and have 3-7 pairs of opposite, lance-shaped leaflets and are up to 250 mm long and glossy dark green or blue green on top with fine silky hairs on the underside. The attractive flowers which occur in summer are pea-shaped, mauve to purple and held in an upright inflorescence on the ends of the branches (See Figure 2.12). Umzimbeet has a restricted distribution in Kwa-Zulu Natal and the Eastern Cape. The hard wood with its attractive coloration is valued for the manufacture of furniture and small domestic implements. The wood is used for making tough, bicoloured walking sticks which are sold to tourists. It can also be used as a windbreak and harvested at 10-15 years for planks in high rainfall areas (Watt and Breyer-Brandwijk, 1962, Coates Palgrave, 2002). The powdered root can be used as a fish poison, but fish must be boiled before consumption. The ground seed soaked in milk is used as a remedy for roundworm, but with caution as consuming too many seeds is poisonous (Hutchings, 1996). It is reported that a mixture of roots with those of Croton species with one part of lion's fat and one part of python's fat is burnt in the house as a tranquilizer to dispel worries. Other recipes exist for sleep-inducing cures based on the roots. Ground seed can be used as an arrow poison (Palmer and Pitman, 1972). Antifungal activity of acetone extract of *Milletia grandis* has been reported (Adamu et al., 2012).

2.6.10 *Melia azedarach*

Family: Meliaceae

Common names: chinaberry, cape lilac

Figure 2. 13: The leaves and stem of *Melia azedarach* in their natural habitat. Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a deciduous tree that can attain a height of 45 m, with a spreading crown and sparsely branched limbs. The barks are smooth, greenish-brown when young, turning grey and fissured with age. Leaves alternate, 20-40 cm long, pinnate but occasionally tripinnate. The leaflets usually 3-11, serrate and with a pungent odour when crushed. The flowers are showy, fragrant, numerous on slender stalks, white to lilac. Because of the divided leaves, the generic name is derived from the Greek 'melia' (the ash); the specie name comes from the Persian 'azzadirackt' (noble tree). This tree, well known as Persian lilac, is native to India but is now grown in the warmer parts of the world. Under optimal conditions, *M. azedarach* grows fast but some forms in the humid tropics are evergreen. The leaves are used as fodder and are highly nutritious (Coates Palgrave, 2002). Fuel wood is a major use of *M. azedarach* with a calorific value of 5100 kcal/kg. *M. azedarach* wood known as the 'white cedar' of commerce, resembles mahogany and is used to manufacture agricultural implements, furniture, plywood, boxes, pole and tool handle (Coates Palgrave, 2002). Aqueous and alcoholic extracts of leaves and seed is used to control many insect, mite and nematode pests (Hutchings, 1996). However, because they contain toxic components, care is needed in their use. The fruit of *M. azedarach* is highly toxic to warm-blooded animals causing nausea, spasms and in children, even death. This plant is well known for its medicinal uses. Its various parts have anthelmintic, antimalarial, cathartic and emetic properties and are also used to treat skin diseases (Watt and Breyer-Brandwijk, 1962, Hutchings, 1996, Coates Palgrave, 2002). Dried ripe fruit is used as an external parasiticide, some toxic components are found in the seed oil, the oral intake of which may cause severe reactions and even death. Fruit stones make ideal beads and are used in making necklaces and rosaries. Tetranortriterpenes (Botha and Penrith 2008), Triterpenoids, steroids and aromatic compounds have been isolated from this plant (Schulte et *al.,* 1979). Fruits are very poisonous (Coates-Palgrave, 2002). Antifungal and cytotoxicity of acetone extract has been reported (Adamu et al., 2012).

2.6.11 *Maesa lanceolata* Forssk.

Family: Maesaceae

Common names: False assegai (English); Valsassegaai (Afrikaans); Umalunguzalazikhakhona (Zulu); intendekwane (Xhosa); Ligucu/Umbohlobohlo (Swazi); Muunguri (Venda)

Figure 2. 14**: The** leaves and stem of *Maesa lanceolata* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

Maesa lanceolata is a very variable species as it can be a straggling shrub, 2 to 3 m tall, or a small tree with a single stem up to 9 m tall, or a rounded bushy tree with branches almost at ground level. Leaves are spirally arranged, lance-shaped, 40-180 x 25-130 mm, tapering to pointed tips, sometimes with blunt or rounded tips, and simple, pale or dark green, with toothed margins (Coates Palgrave, 2002). Sometimes the leaves are large and thick and resemble those of *Curtisia dentata*, but the leaves of *C. dentata* are opposite, not alternate, as those of the false assegai. The bark is usually smooth and brown (Coates Palgrave, 2002). Flowers are minute, bisexual, sweetly scented, white or yellow, in many-flowered sprays in the axils of leaves and at the ends of branches. It is found throughout the eastern part of Africa, in South Africa from the former Transkei northwards to Arabia, and across to India. Ground-up fruits in milk are given to a child at night as a treatment for ringworm. The roots are used for steaming and as a purgative to remove pimples. Embelin 3 %(Watt and Breyer-Brandwijk, 1962), Dioxybenzoquinone (Arot et *al.,* 2001), Maesanin (Kubo *et al.,* 1987) have been isolated from this plant. Anthelmintic activity of this plant has been reported (Tadesse et al., 2009), while the antifungal activity and cytotoxicity was recently reported (Adamu et al., 2012).

2.6.12 *Strychnos mitis* S. Moore

Family: Strychnaceae

Common names: Yellow Bitter berry, Umnono (Zulu), Umanono (Xhosa)

Figure 2. 15: The leaves and fruits of *Strychnos mitis* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a medium sized to large tree that can attain a height of up to 40 m. The crowns are rounded and occurring in medium altitude moist ever green forests (Coates Palgrave, 2002). The barks are grey to grey –brown and usually smooth very thin branches ascending with lenticels. The leaves are elliptic to ovate, occur 4-11, thin in texture and hairless. The wood is whitish, yellow to light brown, hard and heavy (Coates Palgrave, 2002).This species is currently enlisted among the red list of South African plants. It is not endemic to South Africa but can be found in the following provinces: Eastern Cape, Limpopo, Kwazulu –Natal, Limpopo and Mpumalanga. The following compounds were isolated; Bitter alkaloids, Stryspinolactone, Akagerine, Kribine (Adesogan and Morah, 1981, Oguakwa *et al.,* 1980, Ohiri, et al., 1983, Coates-Palgrave, 2002). Antiplasmodial activity was evaluated among other species (Philippe, et al., 2005). The cytotoxic and antifungal activity was also reported (Adamu et al., 2012).

2.6.13 *Zanthoxylum capense* (Thunb). Harv.

Family: Rutaceae
Common names: small knob wood (English); kleinperdepram (Afr.); umlungumabele (isiXhosa); umnungamabele (isiZulu); monokwane (Sotho); khunugumorupa (Tsonga)

Figure 2. 16**: The** leaves and stem of *Zanthoxylum capense* in their natural habitat.

Courtesy of SANBI (South Africa National Biodiversity Institute) www.plantzafrica.com

This is a small multi-branched tree about 5 m in height, but may reach 15 m under favourable conditions. This is a protected tree in South Africa in terms of the National Forests Act no. 84 of 1998. The bark is smooth with straight dark brown thorns and light to dark grey on older. The knob wood has glossy dark green leaves with clear gland dots in the scalloped margin. They are borne in clusters on short side branches, unevenly compound with 4–8 pairs of leaflets plus a terminal one. They have a strong citrus smell when crushed. The flowers have a sweet smell and are greenish white in colour, with 4 sepals and 4 petals (Watt and Breyer-Brandwijk, 1962, Coates Palgrave, 2002). The fruit is a round splitting capsule up to 5 mm in diameter, covered with glands, green, turning red when ripe, splitting later to reveal a single black, oil-rich seed per capsule. The wood is yellowish and fairly hard.

The small knob wood is distributed from Zimbabwe in the north to the Western Cape of South Africa in the south. It is mostly found in dry to evergreen woodland and on rocky hill slopes, but has adapted to a wide range of habitat. Early records show that this tree was widely used in traditional medicine, mainly for flatulent colic, stomach ache, fever, snake bites, toothache and as a mouthwash. It is an old remedy for epilepsy and numerous other ailments (Watt and Breyer-Brandwijk, 1962, Coates Palgrave, 2002). The Zulu people use the leaves to heal sores (Hutchings, 1996). Infusions or decoctions of the fruits or leaves are used as a carminative medicine (taken to relieve flatulence) and to treat fever (Hutchings, 1996). One teaspoon of fruits or crushed leaves can be taken in one cup of water. Root or bark decoctions are used as mouthwashes (Coates Palgrave, 2002). Tannins have been isolated from this plant (Watt and Breyer-Brandwijk, 1962). Recently, the antifungal activity and cytotoxic potential was evaluated (Adamu et al., 2012).

2.7 General Conclusion

The importance of sheep as a source of protein, employment and a store of wealth to African and its importance globally in production requires continue research aimed at improving the overall health and production. This will help to meet the ever increasing demand for animal protein. The role played by *Haemonchus contortus* in sheep production as a major limitation to health and productivity globally is obvious. This nematode is the single most important constrain to global sheep production. Currently, resistance by *Haemonchus* to available anthelmintics has been reported with the exception of monepantel and derquantel which are expensive and out of reach for the small holder farmer especially in Africa. Therefore there is need to continue to find effective, safer, and cheaper alternative remedies. A possible source will be the study of plants, through selecting plant species that show promising activity *in vitro* and subjecting same for further *in vivo* efficacy and toxicity study that will lead to development of effective and safer drugs against *Haemonchus contortus.*

Chapter 3: Efficacy and toxicity of thirteen plant leaf acetone extracts used in ethnoveterinary medicine in South Africa on egg hatching and larval development of *Haemonchus contortus*

Mathew Adamu, Vinasan Naidoo, Jacobus N Eloff

Preface

The plant species that were selected in chapter 2 were evaluated for activity against the egg and larvae of *Haemonchus contortus*. The EC_{50} was calculated to determine the specie to be used for in depth investigation. We also evaluated the cytotoxicity of the extracts using the MTT method. The text in this chapter was submitted to BMC Veterinary Research and is currently in press.

Abstract

Background: Helminthiasis is a major limitation to the livestock industry in Africa. *Haemonchus contortus* is the singular most important helminth responsible for major economic losses in small ruminants. The high cost of anthelmintics to small farmers, resistance to available anthelmintics and residue problems in meat and milk consumed by humans further complicates matters. The use of plants and plant extracts as a possible source of new anthelmintics or the use of plant extracts has received more interest in the last decade. Our aim was not to confirm the traditional use, but rather to determine activity of extracts.

Methods: Based on our past experience acetone was used as extractant. Because it is cheaper and more reproducible to evaluate the activity of plant extracts, than doing animal studies, the activity of acetone leaf extracts of thirteen plant species used traditionally in ethnoveterinary medicine in South Africa were determined using the egg hatch assay and the larval development test. Cytotoxicity of these extracts was also evaluated using the MTT cellular assay.

Results: Extracts of three plant species i.e. *Heteromorpha trifoliata, Maesa lanceolata* and *Leucosidea sericea* had EC_{50} values of 0.62 mg/ml, 0.72 mg/ml and 1.08 mg/ml respectively for the egg hatch assay. *Clausena anisata*; (1.08 mg/ml) and *Clerodendrum glabrum;* (1.48 mg/ml) extracts were also active. In the larval development assay the *H. trifoliata* extract was the most effective with an EC_{50} of 0.64 mg/ml followed by *L. sericea* (1.27 mg/ml). The activities in the larval development test were generally lower in most plant species than the egg hatch assay. Based on the cytotoxicity results *C. anisata* was the least toxic with an LC_{50} of 0.17 mg/ml, while *Cyathea dregei* was the most toxic plant with an LC_{50} of 0.003 mg/ml. The C. *anisata* extract had the best selectivity index with a value of 0.10 and 0.08 for the two assays, followed by *H. trifoliata* and *L. sericea*

with values of 0.07, 0.07 and 0.05, 0.04. The *C. dregei* extract had the worst selectivity index with a value of 0.00019 for both assays.

Conclusion: The result of this study indicates which species should be further investigated in depth for isolation of compound.

Key words: Anthelmintic, ethnoveterinary, Plant species, *Haemonchus contortus, in vitro, toxicity*

3.1 Background

The parasitic gastroenteritis (PGE) complex is a disease entity caused by helminth parasites belonging to different genera mostly within the class nematoda. Of these, *Haemonchus contortus* is the single most important constraint to sheep production in South Africa [1] with estimated direct and indirect economic losses of US $45 million (I.G. Horak personal communication quoted by Waller [2]. Direct losses are due to a drop in production (carcass quality and carcass weight) or death of animals; while indirect losses are due to the costs of drugs, labour and drenching equipment required in control strategies [3, 4]. The cost of controlling helminth infection in livestock is also very high globally with chemotherapy remaining the most widely used method of treatment.

Unfortunately the excessive use of these drugs when not necessary, in addition to their use at incorrect doses, has resulted in the wide scale emergence of resistance in this parasite. Resistance of *Haemonchus contortus* was first reported in South Africa in 1975, in the benzimidazole group of anthelmintics [5], and was soon followed by successive reports of resistance to the different classes of anthelmintics [6, 7, 8, 9, 10]. The scope of resistance is of major concern when multi-drug resistance *Haemonchus* species no longer responded to the five major anthelmintic groups i.e. benzimidazole, ganglion blockers, macrocyclic lactones, cholinesterase inhibitors and the uncouplers of oxidative phosphorylation [11, 12, 13].

Unfortunately the situation is not getting any better and may not improve in the near foreseeable future with the result that alternative anthelmintic control options need to be developed [16]. Options tried are vaccine development, which while effective, has been bedevilled by antigenic complexity of the parasites [17]. Biological control through the use of the nematode trapping fungus, *Duddingtonia flagrans*, although once again effective, is very complicated to use as it is a pasture treatment mechanism [18, 19]. Therefore the management of the parasites within the animals through the use of medication seems to be the best option. One avenue in which these new treatment agents may be discovered would be to evaluate plant extracts for their ability to treat helminth infection [20, 21, 22].

With South Africa being rich in plant vascular flora, contributing over 10% of the world vascular flora species [23], and these plants may contain chemicals that could manage resistant *Haemonchus* species [24]. More importantly in addition to an extract being active, these plants could lead to the discovery of new chemical

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skeletons that could be further enhanced in the laboratory. The aim of this study was to determine the *in vitro* activity of extracts of plants that are traditionally used to treat helminthic infections. This would then be followed up by the isolation of active compounds from the most active plant extracts.

3.2 Methods

3.2.1 Plant collection

The plants evaluated were selected based on published traditional anthelmintic use of the species in South Africa (Table 1). Leaves of the thirteen plant species were collected in November 2009 at the Pretoria National Botanical Garden where the trees were identified and labelled. Voucher specimens were made and stored at the Pretoria National Herbarium of the South Africa National Biodiversity Institute (SANBI). The leaves were subsequently dried at room temperature in a ventilated room, milled to a fine powder in Macsalab Mill (Model 2000 LAB Eriez®) and stored in closed containers in the dark until used.

3.2.2 Plant extraction

Plant material (1 g) from each species investigated was separately extracted with 10 ml of acetone, (>99% technical grade, Merck) in polyester centrifuge tubes. Acetone was selected based on its superiority as extractant based on a number of parameters [26] including that it extracts compounds with a wide range of polarities. The tube was vigorously shaken for 30 min on an orbital shaker. Tubes were centrifuged at 4000 g for 10 min and the supernatant was filtered using Whatman No.1 filter paper before being transferred into pre-weighed glass containers. The solvent was removed by evaporation under a stream of air in a fume hood at room temperature to produce the dried extract [27]. The extract was reconstituted in 5% DMSO and tested in the assays.

3.2.3 Recovery and preparation of eggs

The helminth eggs were prepared according to the method of the World Association for the Advancement of Veterinary Parasitology (WAAVP) [28] with modification. Eggs used in the study were collected from sheep with a monospecific infection of *H. contortus*. The sheep were housed indoor on concrete floor, fed hay, commercial concentrate pellets and had free access to potable water. The faecal pellets were mashed in a blender to make a relatively liquid suspension (slurry), and filtered through a 400 μm mesh sieve to remove coarse debris. Thereafter, the suspension was serially filtered through sieves of pore sizes from 250, 150, 90, 63 μm, until finally eggs were trapped on the 38 μm pore mesh. The material on the 38 μm mesh was washed into 50 ml centrifuge tubes, resuspended in a magnesium sulphate solution prepared at a specific gravity of 1.10. This was then centrifuged at 1000g for 10 minutes to separate the eggs from other debris. The resultant supernatant was passed through a 38 μm sieve to collect the eggs. The eggs were finally harvested by carefully washing them off

the 38 µm sieve into a 1L conical cylinder with distilled water. The concentration of eggs in an aliquot was counted under a microscope. The egg concentration was subsequently brought to a final concentration of 100 eggs per 0.2 ml.

3.2.4 Egg hatch assay (EHA)

Egg hatch assay (EHA) was conducted according to WAAVP guidelines [28], using the dried plant leaf acetone extract dissolved in 5% dimethyl sulfoxide (DMSO) and albendazole as the positive control. Briefly, an aqueous egg suspension of (0.2 ml containing 100 eggs) was distributed in a 48-flat-bottomed microtitre plate and mixed with 0.2 ml of different concentrations (0.78 to 25 mg/ml) of each plant extract in 5% DMSO to give the final tested concentration of 0.39 to 12.5 mg/ml in 2.5% DMSO. Albendazole was dissolved in 5% DMSO in water and evaluated at various concentrations (0.008 to 25 µg/ml). The plates were incubated at 27°C for 48 h. After the incubation a drop of Lugol's iodine solution was added to each well and the number of larvae and unhatched eggs were counted. The percentage inhibition of egg hatching was calculated. All experiments were undertaken in triplicate on three separate occasions. All results were compared with 5% DMSO as the negative control. In all cases eggs were subjected to 2.5% DMSO due to mixing with the same volume of aqueous suspension.

3.2.5 Larval development test (LDT)

The egg suspension (100 eggs in 150 µl) was placed into 48-well plates, with 20 µl suspension of lyophilised *Escherichia coli* (ATCC 9637) [29], 10 µl amphotericin B (Sigma), 20 µl nutritive media (comprising 0.1 g yeast extract in 0.9 ml normal saline and 0.1 ml Earle's balanced salt solution) and incubated as above for 48 h. After incubation, 200 µl of the test extracts (0.78 to 25 mg/ml) reconstituted in 5% DMSO in water and albendazole were added to the wells (n=3) and further incubated for 5 days. Hereafter, the assay was stopped by addition of one drop of Lugol's iodine solution and the L_1 (first stage larvae), L_2 (second stage larvae) and L_3 (third stage larvae) in each well were counted using an inverted microscope. A percentage inhibition of development to L_3 was calculated using the formula adopted from Coles et al [28] as modified by Ademola and Eloff [30]. All results were compared to 5% DMSO as the negative control. The larvae were subjected to 2.5% DMSO because the extract was mixed with the same volume of aqueous larval suspension.

3.2.6 Cytotoxicity assay using MTT

For the assay Vero monkey kidney cells obtained from a confluent monolayer cells were trypsinised and seeded (0.5×10^3 cells per well) in a 96 well microtitre plate and incubated overnight at 37°C in 5% 200µl minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (VirbacR) and 5% foetal calf serum (Adcock-Ingram). After 24 hours the media was replaced and 200 µl of the extracts (1, 0.1, 0.01, 0.001 mg/ml) and further incubated for 5 days. Viability of cells was determined using the tetrazolium-based colorimetric assay MTT assay (3-5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) described by

Mosmann [31]. In short the media in each well was removed and replaced with fresh media and 30 µl of 5 mg/ml MTT in PBS and subsequently incubated for 4 h. Hereafter the medium was removed and cells washed with PBS, prior to the addition of DMSO (50 µl) to dissolve any formazan crystals present. The absorbance of the wells was measured with a Versamax microplate reader at 570 nm. Different concentrations of berberine chloride (Sigma) were used as a positive control, while wells containing only cells without extracts were the negative control. The percentage cell viability relative to the pure growth was calculated. The LC_{50} values was calculated by determining the concentration of plant extracts resulting in 50% reduction of absorbance compared to untreated cells. Tests were carried out in triplicate and each experiment was repeated three times.

3.2.7 Data analysis

The results generated in this study were recorded using Excel for windows 2003.The EC_{50} and LC_{50} were calculated in Kinetica 5.0 (Thermo) using a sigmoid inhibitory model. The results are presented in the mean EC_{50}/LC_{50} and the standard error of the mean.

3.3 Results

3.3.1 Yield

After extraction with acetone the following yields were obtained: *L. sericea* 6.27%, *A. dimidiata* 6.07%, *Z. capense* (0.81%), *B. discolor* (3.30), *C. glabrum* (1.60), *H. trifoliata* (1.28), *S. mitis* (3.75), *M. lanceolata* (2.79), *I. frutescens* (2.05), *M. azedarach* (2.29), *C. anisata* (3.40), *C. dregei* (2.50) and *M. grandis* (1.24).

3.3.2 Egg hatch assay

The percentage egg hatch inhibition had a dose related response as the concentration increased (Figure 3.1). At the 12.5 mg/ml concentration, all the plants extracts except those from *Brachylaena discolor*, *Clerodendrum glabrum*, *Strychnos mitis* and *Zanthoxylum capense* inhibited egg hatching by 100%. Extracts of three plant species (*Heteromorpha trifoliata, Leucosidea sericea* and *Maesa lanceolata*) led to 100% inhibition at concentrations as low as 3.13 mg/ml. *H. trifoliata* had the best inhibitory activity at 0.39 mg/ml with 36.3% inhibition. The *H. trifoliata* extract had the best EC_{50} with a value of 0.62 mg/ml (Table 3.2), followed by *M. lanceolata* with an EC_{50} of 0.72 mg/ml. Extracts of *C. anisata, C. glabrum, A. dimidiata, B. discolor, M. grandis* and *Z. capense* had a moderate egg hatch activity with EC_{50} values ranging between 1.48-5.70 mg/ml. The plant extracts with the lowest activity were from *C. dregei* and *S. mitis* with EC_{50} of 17.64 mg/ml and 16.56 mg/ml respectively.

3.3.3 Larval development test

Similar to the egg hatch assay, the larval development assay had a linear dose related inhibitory response (Figure 3.2). At concentrations of 12.5 mg/ml extracts of 8 of the 13 plant species led to complete inhibition, while extracts from five plants (*Brachylaena discolor, Clerodendrum glabrum, Cyathea dregei, Strychnos mitis* and *Zanthoxylum capense*) had an activity less than 100%. For *H. trifoliata, L. sericea* and *M. lanceolata* extracts, 100% inhibition was still evident at 6.25 and 3.13 mg/ml. At the lowest concentration tested (0.39 mg/ml) *H.trifoliata* again had the highest activity with 31% inhibition. The EC_{50} of all thirteen plants acetone extracts are presented in Table 2. The EC_{50} of *H. trifoliata* was the best at 0.64mg/ml, followed by *L. sericea* and *M. lanceolata* with 1.27 and 1.68 mg/ml respectively. Both *C. dregei* and *B. discolor* were rather ineffective with the EC_{50} values of 17.93 and 17.23 mg/ml respectively.

3.3.4 Cytotoxicity

The results for the cytotoxicity using the MTT assay are shown in Table 3.2. *Clausena anisata* had the highest LC_{50} (lowest toxicity) of 0.17 mg/ml, followed by *Melia azedarach* at 0.14 mg/ml. *C. dregei* was highly toxic with an LC_{50} of 0.003 mg/ml. Further analysis of the results involves the calculation of the selectivity index (SI) of the plant species based on both assays. The selectivity index is the ratio of the LC_{50} to the EC_{50}. The higher the value of the selectivity index the safer the extract. *C. anisata* was the best for both assays; this was followed by *H. trifoliata* and *L. sericea* for both assays and plant species respectively. The plant species with the worst selectivity index was *C. dregei* for both assays.

3.4 Discussion

The main objective of this study was to establish the inhibitory activity of leaf acetone extracts of the 13 plant species traditionally used for their ability to influence the egg hatching and larval development of *Haemonchus contortus*. Acetone was selected as a suitable extractant due to its ability to extract compounds of a wide polarity range, it's miscible with organic and aqueous solvents and is non-toxic to bacteria and fungi organisms [26]. Acetone is a better extractant for plant secondary compounds than water the common solvent used by rural communities. Because a different extractant was used, these results do not necessarily confirm or dispute the traditional use of plant material by rural pastoralists, as compounds of intermediate polarity may be released by microbial or photo-oxidation processes after extraction. In addition when leaves are fed or particulate material is ingested, it is usually the intermediate to lipid soluble compounds that become bioavailable due to the influence of bile salts or the gastrointestinal tract mucosal barrier preventing the absorption of water soluble compounds [32].

For the evaluation of the results we propose that extracts with EC_{50} above 6 mg/ml should be considered to have weak anthelmintic activity as it is extremely difficult to achieve such high concentration *in* vivo. When this criterion

is applied to the results, the EHA indicate *H. trifoliata, L. sericea* and *M. lanceolata*, with EC_{50s} from 0.62-1.08 mg/ml as potential candidates for further isolation work. These extracts also recorded 100% egg hatch inhibition within the highest concentrations of 12.5, 6.25 and 3.13 mg/ml. While the actives are unknown at this stage, the activity recorded in this study may be attributed to secondary metabolites [33]. The activity shown by *M. lanceolata* in the present study agrees with earlier work that evaluated the anthelmintic property of various parts of this plant on *H. contortus* from Asia [34]. This activity may be due to presence of saponins in the leaf of the plant [35]. Saponins are known to destabilize cell membranes hence increase cell permeability by combining with membranes associated sterols [36].

Leucosidea sericea extracts had a reasonably good EC_{50} value (1.08 mg/ml) in the current study, thus agreeing with a recent study by Aremu et al. [37] who reported a minimum lethal concentration of 0.52 mg/ml for the petroleum ether leaf extract using *Caenorhabditis elegans*. The difference in EC_{50} values may be attributed to the non-pathogenic free living *C. elegans* used in their study compared to the pathogenic *H. contortus* we used. This is evident in the literature where *C. elegans* is criticised as being a poor model due to its non-pathogenic nature [25]. The activity of the extract of *L. sericea* may be due to aspidinol [38], condensed tannins or alkaloids [37]. Condensed tannins have anthelmintic activity with varied possible mechanism of actions [39] most especially its astringent property. This is the first report of good anthelmintic activity of *Heteromorpha trifoliata* and it may be associated with compounds such as fulcarindiol and sarison [40] with antifungal activity from the leaves of this plant. This plant has been used as a vermifuge in horses, by the Xhosa people of South Africa.

The larval development inhibition test yielded similar results to the EHA, *H. trifoliata, L. sericea* and *M. lanceolata*, once again had excellent activity. Most plants extracts had weak activity with EC_{50} of 7 mg/ml and above. The activity of *M. lanceolata* extract confirms activity by Tadesse et al [34] on the same species from a different origin (Ethiopia). The plants extracts generally had better inhibition activity on the eggs than on the larva of the parasite *H. contortus* based on the EC_{50} values recorded. This is contrary to report by Ademola and Eloff [22] were they reported better activity for the larval inhibition compared to the egg hatch assay of some fractionated extracts. The result of this study may be significant as the inhibition of egg hatch is possibly an important method of reducing pasture contamination by the animals during grazing helping in the overall helminth control programme.

In comparison to work undertaken using extracts of other plant species , the activity shown by the three plants with the best activity were in the same order of activities found by Bizimenyera et al., [41] using *Peltophorum africanum* leaf, bark and roots as well as extracts of *Coriandum sativum* on eggs and larval development of *H. contortus* [42]. Bizimenyera et al., [41] recorded an EC_{50} of 0.619 mg/ml and 0.724 mg/ml for the leaf acetone extract for the EHA and LDT assays respectively. This is in the same order with values of 0.62 and 0.64 mg/ml recorded in the EHA and LDT for *H. trifoliata* in the current study. The EC_{50} of *H. trifoliata* was lower than the acetone leaf extracts of *Combretum molle* 0.866 mg/ml and 0.604 mg/ml for the EHA and LDT respectively [22].

The weak activity recorded by *M. azedarach* (EC_{50}=10.96 mg/ml) agrees with Maciel et al., [43] in Brazil were they reported an LC_{50} of 9.18 mg/ml with the leaf ethanol extract of the plant. It is encouraging that despite the difference in geographical location and organic extractant used, similar bioactivity was obtained for related species.

While thee activity from these studies show the potential value of plant extracts in the management of haemonchosis the results need to be interpreted with caution as *in vitro* activity may not automatically translate into *in vivo* efficacy. Factors that still need to be considered are animal factors such as absorption and presystemic elimination [44]. More importantly the potential toxicity of the molecule needs to be considered. For this study we used renal cells in culture as an indicator of toxicity. The cells were specifically selected as the kidneys are one of the main sites of toxicity in animals due to the preferential blood supply of the kidney in addition to their high metabolic capacity. The results of the cytotoxicity study were disappointing as in all cases the extracts were more toxic to the cells than to the parasites.

However the presence of cellular toxicity is not conclusive as numerous factors interplay with toxicity results. In this case, it is possible that the use of an organic extractant may have led to extraction of toxic compounds. For this chemical fractionation and isolation may help separate out the potential toxic compounds. It is also possible that *in vivo* interaction with microsomal and non-microsomal pathways may render the molecule less toxic due to metabolism or simply gut-barrier exclusion may play a role. As a result it is suggested that the most efficacious plants, which had lower toxicity profiles, be evaluated using a dedicated animal model.

In contrast to many authors using organic extractant referred to above, a recent study [45] showed that an aqueous extract of *Markhamia obtusifolia* had double the activity of the acetone extract and that it was slightly less toxic to Vero kidney cells than to *Haemonchus contortus*. These results indicate that in future studies water extracts should also be evaluated for activity and safety. There is also the possibility of using the plant material in the feed directly to control helminths. In an experiment to feed *Cereus jamacara* an invasive plant species grazed by game and used by a commercial farmer to control helminths had only partial success in decreasing helminth load [46].

3.4.1 Conclusion

The documented uses of these plants against helminths have a possible pharmacological basis as indicated by the results of this study. Although the activity of all extracts was several orders of magnitude lower than that of the positive control, high concentrations may be attainable in the animal gut if there are no toxicity problems. It is useful to determine the *in vivo* toxicity of promising extracts to determine if cellular toxicity is a good indicator of *in vivo* toxicity. The most promising plants to continue study were *M. lanceolata, L. sericea* and *H. trifoliata*.

Because substantial work has been done on *M. lanceolata* already we continued with isolating bioactive compounds and evaluating the activity of extracts from *L. sericea* in animal experiments.

Table 3.1: List of plant species used in the investigation, their traditional uses and references. Only the leaves of these plants species were used in the present study.

Plant species	Family	Medicinal uses	Reference
Brachylaena discolor	Asteraceae(267)	Purgatives against intestinal parasites, anthelmintics for calves, sheep and goats	Bryant,1966; Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Zanthoxylum capense	Rutaceae(96)	Gastric and intestinal disorders,anthelmintics, cough, bronchitis, pleurisy	Hutchings, 1996; Bryant, 1966.
Clerodendrum glabrum	Lamiaceae(403)	Intestinal parasites, coughs, fever, and diabetes	Bryant, 1966; Watt and Breyer-Brandwijk, 1962 and Hutching, 1996.
Heteromorpha trifoliata	Apiaceae(491)	Intestinal worms, colic in horses and vermifuge, enemas for abdominal disorders	Bryant, 1966; Watt and Breyer-Brandwijk, 1962, Gerstner, 1938.
Apodytes dimidiata	Icacinaceae(139)	Enemas for intestinal parasites, purgatives, inflammation of the ear	Bryant,1966; Watt and Breyer-Brandwijk, 1962, Gerstner, 1938.
Strychnos mitis	Strychnaceae(73)	Malaria, fevers	Biset,1974
Maesa lanceolata	Maesaceae(615)	Anthelmintics, treatment of wounds and infertility	Watt and Breyer-Brandwijk, 1962.

Table 3.1 Continue**:** List of plant species used in the investigation, their traditional uses and references. Only the leaves of these plants species were used in the present study.

Plant species	Family	Medicinal uses	Reference
Indigofera frutescens	Papilionaceae(675)	Anthelmintics	Watt and Breyer-Brandwijk,1962.
Leucosidea sericea	Rosaceae(288)	Treatment of opthalmia, anthelmintics, astringents and vermifuge	Watt and Breyer-Brandwijk, 1962 and Jacot Guillarmod, 1971.
Melia azedarach	Meliaceae(702)	Effective anthelmintics, emetic, cathartic and treatment of eczema	Watt and Breyer-Brandwijk, 1962;Oliver-Bever 1986;Hutchings, 1996
Clausena anisata	Rutaceae(317)	Anthelmintics, purgatives, rheumatism, fevers and myiasis	Doke and Vilakazi, 1972.
Cyathea dregei	Cyatheaceae(658)	Anthelmintics	Doke and Vilakazi, 1972.
Milletia grandis	Papilionaceae(704)	Anthelmintics and tranquilizers	Watt and Breyer-Brandwijk, 1962 ; Palmer and Pitman 1972

(PRU voucher specimen numbers provided after family names)

Table 3.2: EC$_{50}$ for Egg Hatch assay (EHA) and Larval Development test (LDT) with their corresponding cytotoxicity values and selectivity index (SI) for thirteen plant species

Names of plants	EHA EC$_{50}$±SE	LDT EC$_{50}$± SE	LC$_{50}$	SI EHA	SI LDT
Apodytes dimidiata	5.70±0.23	4.13±0.56	0.00396	0.000695	0.00096
Brachylaena discolor	3.55±0.27	17.23±5.47	0.00752	0.00212	0.00044
Clausena anisata	1.80±0.09	2.07±0.15	0.17186	0.09548	0.08302
Clerodendrum glabrum	1.48±0.07	12.97±2.33	0.04251	0.02872	0.00328
Cyathea dregei	17.64±4.65	17.93±6.66	0.00332	0.00019	0.00019
Heteromorpha trifoliata	0.62±0.02	0.64±0.10	0.04252	0.06858	0.06644
Indigofera frutescens	7.11±1.10	7.58±1.05	0.1044	0.01468	0.01377
Leucosidea sericea	1.08±0.11	1.27±0.07	0.0515	0.04769	0.04055
Maesa lanceolata	0.72±0.05	1.68±0.10	0.01577	0.0219	0.00939
Melia azedarach	6.24±0.20	10.96±1.79	0.14466	0.02318	0.0137
Milletia grandis	5.57±0.33	6.11±1.04	0.05336	0.00958	0.00873
Strychnos mitis	16.56±4.88	16.94±4.71	0.01721	0.00104	0.00102
Zanthoxylum capense	13.26±0.24	13.64±3.44	0.02095	0.00643	0.00153

Note: all values for EHA, LDT and LC$_{50}$ in mg/ml; Albendazole was positive control and recorded 100% inhibition at all concentrations (0.008 to 25 μg/ml) used, while DMSO recorded <10% inhibition
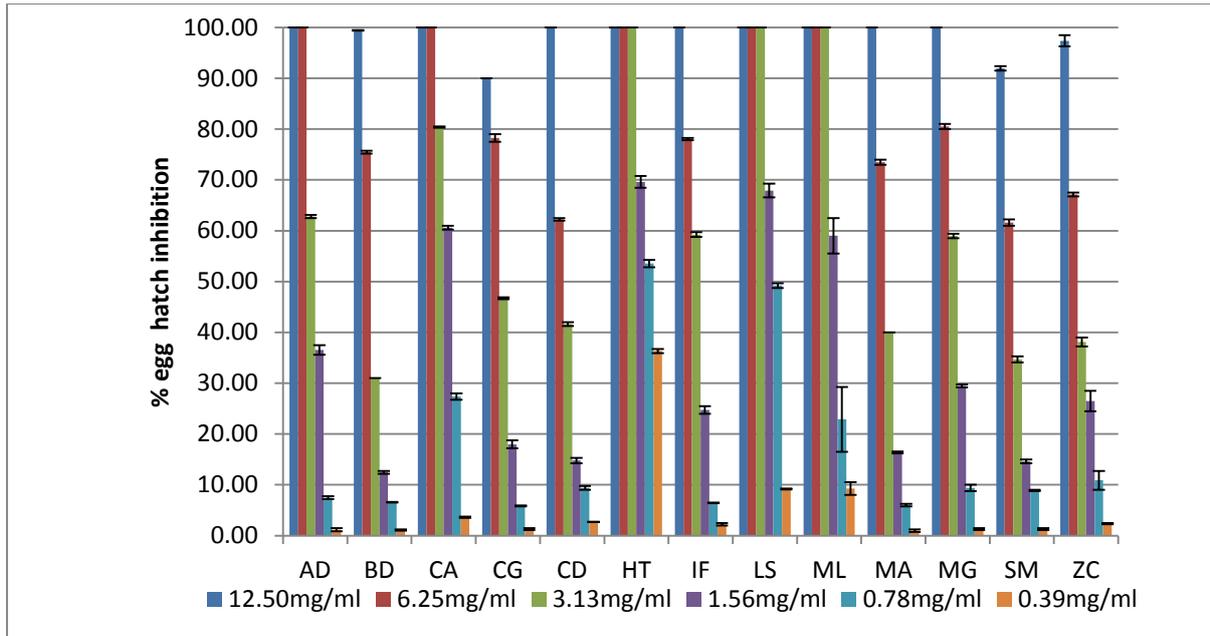
Figure 3.1: Percentage egg hatch inhibition (mean ±SE) of different concentration of acetone leaf extracts from 13 plant species (*AD; A. dimidiata; BD;B. discolor, CA; C. anisata, CG; C. glabrum, CD; C. dregei, HT; H. trifoliata, IF; I. frutescens, LS; L. sericea, ML; M. lanceolata, MA; M. azedarach, MG; M. grandis, SM; S. mitis, ZC; Z. capense)* Albendazole was positive control and recorded 100% inhibition at all concentrations (0.008 to 25 μg/ml) used.
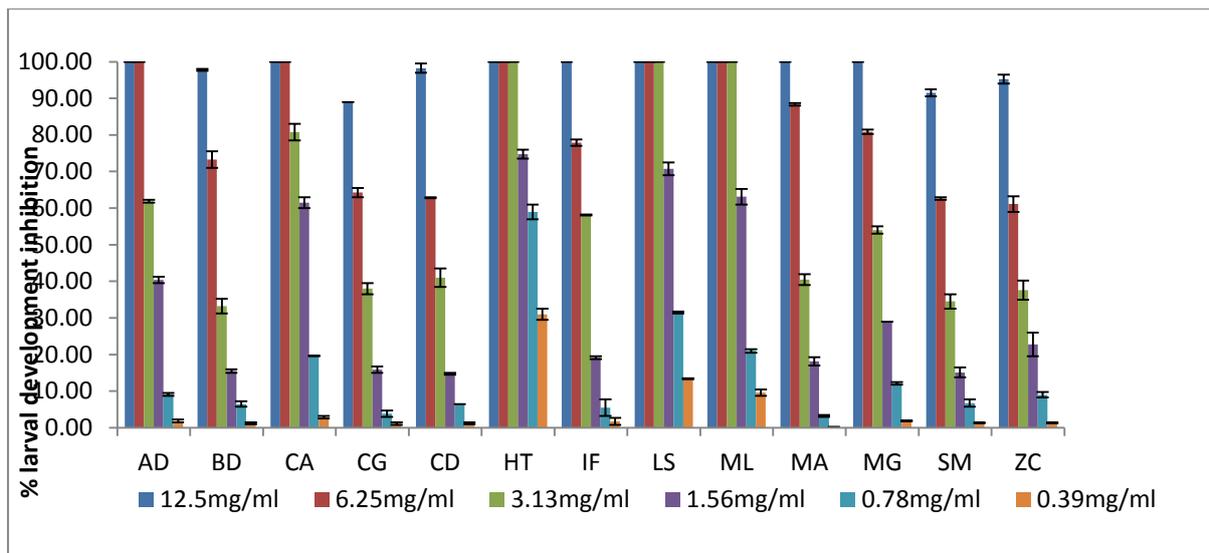
Figure 3.2: Percentage larval development inhibition (mean ±SE) of different concentration of acetone leaf extracts from 13 plant species (*AD; A. dimidiata; BD; B. discolor, CA; C. anisata, CG; C. glabrum, CD; C. dregei, HT; H. trifoliata, IF; I. frutescens, LS; L. sericea, ML; M. lanceolata, MA; M. azedarach, MG; M. grandis, SM; S. mitis, ZC; Z. capense).* Albendazole was positive control and recorded 100% inhibition at all concentrations (0.008 to 25 µg/ml) used.

Postscript

All the plant extracts possess varying degree of anthelmintic activity. We therefore went further to determine the antifungal activity of the same plant extracts. This is because some anthelmintic such as the benzimidazoles have known antifungal activity.

Chapter 4: Some southern African plant species used to treat helminth infections in ethnoveterinary medicine have excellent antifungal activities

Mathew Adamu, Vinasan Naidoo, Jacobus N Eloff

Preface

In addition to evaluating the antifungal activity against 3 fungal pathogens, bioautography was also done to determine the possible compounds responsible for the activity seen with the MIC's. Bioautography was an essential tool in selecting extracts that can be used for further study. The text in this chapter was submitted, accepted and published in BMC Complementary and Alternative Medicine 2012, **12**:213.

Abstract

Background: Diseases caused by microorganisms and parasites remain a major challenge globally and particularly in sub-Saharan Africa to man and livestock. Resistance to available antimicrobials and the high cost or unavailability of antimicrobials complicates matters. Many rural people use plants to treat these infections. Because some anthelmintics e.g. benzimidazoles also have good antifungal activity we examined the antifungal activity of extracts of 13 plant species used in southern Africa to treat gastrointestinal helminth infections in livestock and in man.

Methods: Antifungal activity of acetone leaf extracts was determined by serial microdilution with tetrazolium violet as growth indicator against *Aspergillus fumigatus, Cryptococcus neoformans* and *Candida albicans*. These pathogens play an important role in opportunistic infections of immune compromised patients. Cytotoxicity was determined by MTT cellular assay. Therapeutic indices were calculated and selectivity for different pathogens determined. We proposed a method to calculate the relation between microbicidal and microbistatic activities. Total activities for different plant species were calculated.

Results: On the whole, all 13 extracts had good antifungal activities with MIC values as low as 0.02 mg/mL for extracts of *Clausena anisata* against *Aspergillus fumigatus* and 0.04 mg/mL for extracts of *Zanthoxylum capense, Clerodendrum glabrum,* and *Milletia grandis*, against *A. fumigatus.*

Clausena anisata extracts had the lowest cytotoxicity (LC_{50}) of 0.17 mg/mL, a reasonable therapeutic index (2.65) against *A. fumigatus*. It also had selective activity against *A. fumigatus*, an overall fungicidal activity of 98% and a total activity of 3395 mL/g against *A. fumigatus*. This means that 1 g of acetone leaf extract can be diluted to 3.4 litres and it would still inhibit the growth. *Clerodendrum glabrum, Zanthoxylum capense* and *Milletia grandis* extracts also yielded promising results.

Conclusions: Some plant extracts used for treatment of parasitic infections also have good antifungal activity. Because it is much easier to isolate antifungal compounds by bioassay-guided fractionation, this approach may facilitate the isolation of anthelmintic compounds from active plant extracts. The viability of this approach can be tested by isolating the antifungal compounds and then determining its anthelmintic activity. Some of these plant extracts may also be useful in combating fungal infections.

Keywords:

Antifungal, *Candida, Cryptococcus, Aspergillus*, anthelmintic, therapeutic index, selectivity, plant extract, immune compromised patients.

4.1 Background

Apart from acting as a source of food, providing shelter and many other uses, plants have been a source of medicine for man and his animals. Infectious diseases caused by bacteria, fungi and viruses continue to be a major health concern especially in sub-Saharan Africa. These organisms cause untold hardship particularly in rural areas due to poor sanitary conditions, limited availability of potable drinking water and poverty. The situation is complicated with the HIV/AIDS pandemic that has ravaged a part of the world with the attendant problem of antimicrobial resistance and opportunistic infections. Even when antimicrobials are available, they remain expensive for the poorer communities and in some cases especially in the rural areas may be adulterated and of little value in the treatment of diseases caused by fungi in man and in animals [1]. Plants have consequently been widely used for the treatment of various ailments in animals. McGaw and Eloff [2] reported that of more than 200 plant species used in ethnoveterinary medicine (EVM) in South Africa, only 27 or 13% have been evaluated for any biological activity. More species need to be evaluated, and more in depth investigation of plants already tested needs to be carried out. Southern African plants are a potential source of undiscovered compounds with high biologically active extracts against a variety of disease-causing organisms [3], or disease-transmitting vectors [4].

The plant species used in this study were chosen based on documented traditional use for anthelmintic purposes. Because some commercial anthelmintics such as benzimidazoles have antifungal activity, we decided to investigate the possible antifungal activity and the cytotoxicity of extracts of these 13 plant species. Acetone was selected as the only solvent based on its ability to extract compounds with a wide range of polarities, its low toxicity in antimicrobial bioassays and because it is easily removed from the extract at low temperature [5, 6]. The fungal pathogens were selected based on their importance in opportunistic infections of immune compromised patients.

4.2 Methods

4.2.1 Plant collection

Leaves of thirteen plant species were collected in November 2009 at the National Botanical Garden in Pretoria, South Africa. The trees were identified and labelled and voucher specimens were kept in the Herbarium of the Garden (Table 1). Plant leaves were dried at room temperature in a well-ventilated room, milled to a fine powder in Macsalab Mill (Model 2000 LAB Eriez®) and stored in closed containers in the dark until use.

4.2.2 Plant extraction

Plant material (1 g) from each species investigated was extracted with 10 mL of acetone, (technical grade, Merck) in polyester centrifuge tubes [5]. The tube was vigorously shaken for 30 min on an orbital shaker, then centrifuged at 4000 x g for 10 min and the supernatant was filtered using Whatman No.1 filter paper before being transferred into pre-weighed glass containers. This was repeated twice and solvent was removed by evaporation under a stream of air in a fume hood at room temperature to produce the dried extract [7].

4.2.3 Chromatographic analysis

The extracted chemical components were made up to a concentration of 10 mg/mL and 10 µL was analysed by thin layer chromatography (TLC) separation using aluminium-backed TLC plates (Merck, Silica gel F254). The TLC plates were developed in saturated chambers using mobile phases of varying polarities, namely, ethyl acetate/methanol/water (40:5.4:5) [EMW] (polar/neutral), chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (intermediate polarity/acidic) and benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] (non-polar/basic) [8]. Separated components were examined under UV light at wavelengths of 254 and 365 nm after which TLC plates were sprayed with vanillin-sulphuric acid [9] and heated at 110 °C to optimal colour development.

4.2.4 Antifungal activity

The MIC values were determined using a serial microplate dilution method developed by Eloff [6] and modified by Masoko et al. [10]. Three fungal species, namely *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus* associated with opportunistic infections of immune compromised patients, were obtained from the fungal culture collection in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science at the University of Pretoria. *C. albicans* was isolated from a Goldian finch, *C. neoformans* from a cheetah and *A. fumigatus* from a chicken, all of which suffered from systemic mycosis. None of the animals had been treated prior to sampling. All fungi were maintained in Sabouraud dextrose agar (Oxoid, Basingstoke) until use. For growth inhibition, assays, fungal species were grown overnight in Sabouraud dextrose broth at 35 °C prior to the test. Fungi were cultured at 35°C in universal bottles as slants in Sabouraud dextrose agar (Oxoid,

Basingstoke) (65g dissolve in 1L distilled water and sterilized by autoclaving at 121°C for 30 min). Using sterile cotton swabs the collected conidia were inoculated into Sabouraud dextrose broth (Sigma, Germany) (30 g dissolve in 1L distilled water and sterilized by autoclaving at 121°C for 30 min) prior to bioactivity assays. Densities of fungal cultures used in bioautography and for MIC determinations were as follows: *C. albicans*, 2.5×10^6 cfu/mL; *C. neoformans*, 2.6×10^6 cfu/mL; *A. fumigatus*, 8.1×10^6 cfu/mL. *Candida albicans* was diluted to a density of about 2.5×10^4 cfu/mL, *C. neoformans*, 2.6×10^4 cfu/mL, and *A. fumigatus* 8.1×10^4 cfu/mL. Tetrazolium violet was used as an indicator of microbial growth [6, 10]. Growth of the microorganisms reduces the tetrazolium violet to a red formazan. Amphotericin B was used as a positive control in antifungal activity assays. MIC was registered as the lowest concentration of plant extract inhibiting microbial growth, indicated by a decrease in the intensity of the red colour of the formazan product. The total activity of the extracts was calculated as the total mass (mg) of the extract divided by the MIC value (mg/mL). Total activity value indicates the volume to which the extract derived from 1 g of plant material can be diluted and still inhibits the growth of the microorganism [11, 7].

4.2.5 Cytotoxicity assay using MTT

For this assay Vero monkey kidney cells obtained from a confluent monolayer cells were trypsinised and seeded (0.5×10^3 cells per well) in a 96 well microtitre plate and incubated overnight at 37°C in a 5% CO_2 atmosphere, in minimal essential medium 200 µl (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac^R) and 5% foetal calf serum (Adcock-Ingram). After 24 hours the media was replaced and 200 µl of the extracts (1, 0.1, 0.01, 0.001 mg/mL) were further incubated for 5 days. Viability of cells was determined using the tetrazolium-based colorimetric MTT assay (3-5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) described by [12]. In short the media in each well was removed and replaced with fresh media and 30 µl of 5 mg/mL MTT in PBS and subsequently incubated for four hours. Hereafter the medium was removed and cells washed with PBS, prior to the addition of dimethyl sulphoxide (50 µl) to dissolve any formazan crystals present. The absorbance of the wells was measured with a Versamax microplate reader at 570 nm. Different concentrations of berberine chloride (Sigma) were used as a positive control, while wells containing only cells without extracts were the negative control. The percentage cell viability relative to the pure growth was calculated. The LC$_{50}$ values was calculated by determining the concentration of plant extracts resulting in 50% reduction of absorbance compared to untreated cells. Tests were carried out in triplicate and each experiment was repeated three times.

4.2.6 Bioautographic investigations

For bioautography analysis, thin layer chromatography (TLC) plates were loaded with 10 µL of each extract of 10 mg/mL concentration and dried before developing in mobile phases of BEA, CEF and EMW. The solvent was evaporated from the plates in a stream of air for four days. Plates were then sprayed with concentrated cultures of fungi species in fresh growth medium until completely moist using a spraying gun. The moist plates were incubated at 37°C in an incubator for 24 h. The plates were then sprayed with 2 mg/mL of *p* iodonitrotetrazolium

violet (INT) (Sigma) and incubated for a further 12 h. The emergence of purple-red colour resulting from the reduction of INT into its respective formazan was a positive indicator of cell viability. Clear zones against the purple background were indicative of antifungal activity of compounds separated on TLC plates [13].

4.3 Results

4.3.1 Plant species yield

Different quantities were extracted with acetone from the ground dried leaves. *L. sericea* had the highest percentage yield of 6.27%, followed closely by *A. dimidiata* with 6.07%, and the lowest yield was obtained from *Z. capense* (0.81%). The percentage yields from other dried leaves were: *B. discolor* (3.30), *C. glabrum* (1.60), *H. trifoliata* (1.28), *S. mitis* (3.75), *M. lanceolata* (2.79), *I. frutescens* (2.05), *M. azedarach* (2.29), *C. anisata* (3.40), *C. dregei* (2.50) and *M. grandis* (1.24).

4.3.2 Phytochemical analysis

The compounds present in the different extract that react with the vanillin spray reagent were separated by thin layer chromatography using the CEF solvent system (Fig. 4.1). More than 13 compounds varying in polarity were separated.

4.3.3 Bioautography

With the three solvent systems used, 37 active bands were seen for the 3 fungal pathogens in the chromatograms of the different plants extracts. BEA system separated 17 (46%) of the active bands, followed by CEF 14(38%) and EMW 6 (16%) as the least. This indicates that most of the antifungal compounds are non-polar. In the antifungal bioautography, *A. dimidiata* (Rf; 019, 0.37, 0.63) and *L. sericea (*Rf; 0.18, 0.23, 0.28) had 3 active bands each against C. *albicans* in the BEA chromatogram (Figure 2). This was followed by *A. dimidiata* with two active bands in the BEA system against *A. fumigatus.* No antifungal compounds were seen in BEA chromatograms of *B. discolor* and *S. mitis* extracts (Figure 4. 2), CEF and EMW against the fungi organisms used in this study. An active compound with Rf value between 0.93-0.95 was present in *Z. capense* (0.95), *C. glabrum* (0.95)*, M. lanceolata* (0.95)*, I. frutescens* (0.94), *M. azedarach* (0.93) and *C. anisata* (0.93) extracts. This compound appears to be a common compound with zone of inhibition shown in all 6 plants listed above. *C. albicans* generally led to the most inhibition bands with the different plant extracts

4.3.4 Antifungal activity of extracts

MIC values: From the MIC values the average values for the different plant extracts against all three fungi were calculated (Table 4. 2). The average MIC values of all extracts against all three fungi varied between 0.09 and

0.63 mg/mL over the 12 and 24 hour incubation periods. The *Clausena anisata* extract had the best MIC (0.02 mg/mL) against *A. fumigatus.* Extracts of *Z. capense, C. glabrum,* and *M. grandis*, also had excellent antifungal activity (0.04 mg/mL MIC) against *A. fumigatus.* Extracts of *Z. capense, C. glabrum, L. sericea* and *B. discolor* had good activity (0.08 mg/mL MIC) against *C. neoformans* and also *I. frutescens* extracts against *A. fumigatus* (Table 4.2)*.*

Selective activity: If an extract has good activity against several microorganisms it may contain a general metabolic toxin that could also affect animal cells. The *C. anisata* and *M. grandis* extracts had good selective activity against *A. fumigatus*. In other cases some measure of selectivity was present. On average the most sensitive fungus to these extracts was *A. fumigatus* MIC (0.21 and 0.45 mg/mL after 12 and 24 h), followed by *C. neoformans* (0.26 and 0.41 mg/mL) and by *C. albicans* (0.35 and 0.48 mg/mL).

Fungicidal versus fungistatic activity: In an extract with fungistatic activity after a prolonged period growth would again take place. To measure the fungicidal activity we propose that the MIC of the extract after 12 h should be divided by the MIC after 24 h and multiplied by 100 to give the fungicidal activity. The time period would depend on the growth rate of the specific microorganism. The conidia collected and incubated at a high level led to a relatively high growth rate in our case. Extracts of three species *Clausena anisata, Milletia grandis* and *Cyathea dregei* had a fungicidal activity higher than 90% and the *L. sericea* extract had definite fungistatic activity.

The average total activity against the 3 fungal organisms varied from 1026 mL/g for *C. anisata* to 84 mL/g for the *H. trifoliata* extract (Table 4.3). The highest total activity value of 3395 mL/g against *A. fumigatus* was for the *C. anisata* extract and the lowest was 20mL/g for *H. trifoliata* against *A. fumigatus* (Table 4.3). Total activity values indicate which species could be the best source of an extract for use by poor communities or for organic production [11]. *Clausena anisata* with the highest total activity may be a good candidate for further study.

4.3.5 Cytotoxicity and therapeutic index

Clausena anisata had the highest LC_{50} (lowest toxicity) of 0.17 mg/mL, followed by *Melia azedarach* at 0.14 mg/mL. *C. dregei* on the other hand was highly toxic with an LC_{50} of 0.003 mg/mL (Table 4.4). The ratio between efficacy and safety of an extract is a very important parameter in developing any therapeutic product. The therapeutic index is an indication of the safety of the extract. If there is selective activity to the pathogen the therapeutic index will be higher than 1. Because both values are inversely related to activity the therapeutic index is calculated by dividing the cytotoxicity LC_{50} in mg/mL by the MIC in mg/mL. The higher the value of the therapeutic index the safer the extract. The *Clerodendrum glabrum* extract with a therapeutic index of 4.3 against *A. fumigatus* was the best. The *Clausena anisata* extract was the second best with a therapeutic index of 2.7 against *A. fumigatus* followed by the *Clerodendrum glabrum* extract (2.15) against *C. neoformans*. All other values were below 1 (Table 4.4).

4.4 Discussion

All 13 plant extracts had some degree of activity against the 3 fungal pathogens used. Many authors consider activity of a plant extract with MIC higher than 0.1 mg/mL as not significant [7, 14]. Extracts from 8 of the 13 species with the exception of *H. trifoliata, S. mitis, M. lanceolata, M. azedarach* and *C. dregei* extracts had MIC values lower than 0.1 mg/mL. It may be interesting to correlate the anthelmintic activity of the extracts with their antifungal activity.

Clausena anisata extracts had the best activity with an MIC of 0.02 mg/mL. *C. anisata* extracts also had the best antifungal activity when plant extracts of Tanzanian plant species were examined with an MIC value of 1 mg/mL against *C. albicans* [15] compared to 0.63 mg/mL against the same pathogen in the current study. It is satisfying that the results are close despite the use of different extractants, different origins of the plants and different *C. albicans* isolates. The MIC activity of *C. anisata* found here 0.04 mg/mL correlates very well with other published values of 0.05 mg/mL [3]. Bosman et al [16] reported that *L. sericea* is active against fungi using the non-quantitative disc diffusion method, but no activity was reported for acetone leaf extracts of *L. sericea.* The activity of *L. sericea* dichloromethane, ethanol and petroleum ether extracts against *C. albicans* was very poor with MICs ranging from 1.56-12.5 mg/mL [17] compared to our value of 0.31 mg/mL. The large difference obtained using similar methods may be ascribed to difference in activity of the plants examined.

We have frequently found that acetone is the best extractant for antibacterial and antifungal extracts [5, 8, 18]. In practically all cases antimicrobial compounds isolated were intermediate polarity compounds. The activity of this plant extract when compared to previous study by Suleiman et al., [3] who reported an MIC value of 0.05 mg/mL against *A. fumigatus* with a total activity value of 2740 g / mL with the acetone leaf extract of *Loxostylis alata* as against 0.04 mg/mL and 3395 mL total activity in this study remain one of the best activities recorded in our group so far.

The selectivity index of the extracts also helps to select plant extracts that will be useful for further study. In this study, *Clerodendrum glabrum* and *Clausena anisata* with selectivity index of 4.30 and 2.65 respectively, may have the potential for use in *in vivo* animal trials against *A. fumigatus.* The higher the selectivity index the higher the safety of the extract when used *in vivo*. Thus considering the results obtained in this study *C. anisata* and *C. glabrum* should be considered as candidate for further study especially in treatment of poultry infected with *A. fumigatus*.

4.4.1 Conclusions

After investigating the antifungal activity and cytotoxicity of the different plant extracts, the two most promising plant species for in depth analysis to treat fungal infections were *C. anisata* and *C. glabrum.* The potential use of

plant extracts in organic animal production instead of a commercial drug is not a pipe dream. Our group have shown that a *Loxostylis alata* extract was as effective as fluconazole in treating poultry infected with *Aspergillus fumigatus* [19]. *C. anisata* and *C. glabrum* extracts had a lower cellular toxicity than the *Loxostylis alata* extract and may deliver an even more useful product that could also be important in treating human patients.

There is a need to develop plant extracts to combat microbial and parasitic infections. Because the bioassays are simple and it is relatively easy to isolate antifungal compounds by bioassay-guided fractionation. In this study plants used traditionally to treat helminth infections generally had good antifungal and in some cases excellent antifungal activities. We are in the process of determining the anthelmintic activity of extracts of these traditionally used species. If a good correlation exists between antifungal and anthelmintic activity the search for new anthelmintic compounds or extracts with good anthelmintic activity may be accelerated. The Phytomedicine Programme (www.up.ac.za/phyto) has a database in which the antifungal activity of leaf extracts of more than 600 tree species has been determined. If there is indeed a reasonable correlation between antifungal and anthelmintic activities it means that antifungal assays amenable to robotic systems may be used to detect promising candidate plant species to discover anthelmintics.

Table 4.1: List of plant species used in the investigation, their traditional uses and references. (PRU voucher specimen numbers provided after family names)

Plant species	Family	Medicinal uses	Reference
Brachylaena discolor	Asteraceae (267)	Purgatives against intestinal parasites, anthelmintics for calves, sheep and goats	[20][21] [22]
Zanthoxylum capense	Rutaceae (96)	Gastric and intestinal disorders, anthelmintics, cough, bronchitis, pleurisy	[22] [20]
Clerodendrum glabrum	Lamiaceae (403)	Intestinal parasites, coughs, fever, and diabetes	[20] [21] [22]
Heteromorpha trifoliata	Apiaceae (491)	Intestinal worms, colic in horses and vermifuge, enemas for abdominal disorders	[20][21] [23]
Apodytes dimidiata	Icacinaceae (139)	Enemas for intestinal parasites, purgatives, inflammation of the ear	[20][21] [23]
Strychnos mitis	Strychnaceae (73)	Malaria, Fevers	[24]
Maesa lanceolata	Maesaceae (615)	Anthelmintics, treatment of wounds and infertility	[21]
Indigofera frutescens	Papilionaceae (675)	Anthelmintics	[21]
Leucosidea sericea	Rosaceae (288)	Treatment of opthalmia, anthelmintics, astringents and vermifuge	[21] [25]
Melia azedarach	Meliaceae (702)	Effective anthelmintics, emetic, cathartic and treatment of eczema	[21][26][22]
Clausena anisata	Rutaceae (317)	Anthelmintics, purgatives, rheumatism, fevers and myiasis	[27]
Cyathea dregei	Cyatheaceae (658)	Anthelmintics	[27]
Milletia grandis	Papilionaceae (704)	Anthelmintics and tranquilizers	[21] [28]

Table 4.2: MIC in mg/mL of the leaf extracts of 13 plant species against *A. fumigatus* (AF), *C. albicans* (CA) and *C. neoformans* (CN) incubated for 12h and 24 h. Values below 0.1 mg/mL in bold font. Degree of fungicidal activity (FCA) calculated by dividing average of 12 h MIC by average 24 h MIC and multiplying by 100.

Time and fungus	12h AF	24h AF	12h CA	24h CA	12h CN	24h CN	Average 12 h	Average 24h	Degree FCA
Brachylaena discolor	0.31	0.61	0.31	0.31	**0.08**	0.16	0.23	0.36	65
Zanthoxylum capense	**0.04**	**0.08**	0.16	0.31	**0.08**	**0.08**	**0.09**	0.16	60
Clerodendrum glabrum	**0.04**	**0.08**	0.31	0.63	**0.08**	0.16	0.14	0.29	49
Heteromorpha trifoliate	0.63	1.25	0.31	0.63	0.16	0.16	0.37	0.68	54
Apodytes dimidiata	0.31	0.31	0.31	0.63	**0.08**	0.16	0.23	0.37	64
Strychnos mitis	0.31	0.63	0.31	0.63	0.63	0.63	0.42	0.63	66
Maesa lanceolata	0.31	0.31	0.16	0.31	0.16	0.63	0.21	0.42	50
Indigofera frutescens	**0.08**	0.31	0.16	0.16	0.16	0.16	0.13	0.21	63
Leucosidea sericea	0.16	1.25	0.31	0.31	**0.08**	0.16	0.18	0.57	32
Melia azedarach	0.31	0.63	0.16	0.31	0.63	0.63	0.37	0.52	70
Clausena anisata	**0.02**	**0.04**	0.63	0.63	0.16	0.16	0.27	0.28	98
Cyathea dregei	0.16	0.31	1.25	1.25	0.16	0.16	0.52	0.57	91
Milletia grandis	**0.04**	**0.08**	0.16	0.16	0.63	0.63	0.28	0.29	95
Average	0.21	0.45	0.35	0.48	0.24	0.30	0.26	0.41	65.9

Table 4.3: Total activity of the leaf of 13 plant extract used as anthelmintic screened for antifungal activity using three fungal organisms.

	A. fumigatus		*C. albicans*		*C. neoformans*	
Incubation time	**12**	**24**	**12**	**24**	**12**	**24**
Brachylaena discolor	213	108	213	213	825	413
Zanthoxylum capense	405	203	101	52	203	203
Clerodendrum glabrum	798	399	103	51	199	51
Heteromorpha trifoliata	40	20	82	40	159	159
Apodytes dimidiata	392	392	392	193	1518	434
Strychnos mitis	242	119	242	119	119	144
Maesa lanceolata	180	349	349	180	349	89
Indigofera frutescens	513	132	256	256	256	256
Leucosidea sericea	783	100	404	404	1566	783
Melia azedarach	148	73	286	150	73	73
Clausena anisata	3395	1698	108	108	424	424
Cyathea dregei	313	161	40	40	313	313
Milletia grandis	618	309	154	154	39	39

Table 4.4: Cytotoxicity (LD$_{50}$ in mg/mL) of extract and selectivity index based on MIC of extract after 12 hours of extracts from thirteen plants examined. Therapeutic index calculated by dividing LD$_{50}$ by MIC

Plant species	Cytotoxicity	A. fumigatus	C. albicans	C. neoformans
Brachylaena discolor	0.004	0.01	0.01	0.05
Zanthoxylum capense	0.008	0.2	0.05	0.1
Clerodendrum glabrum	0.172	4.3	0.55	2.15
Heteromorpha trifoliata	0.043	0.27	0.55	0.27
Apodytes dimidiata	0.003	0.01	0.01	0.04
Strychnos mitis	0.043	0.14	0.14	0.07
Maesa lanceolata	0.104	0.34	0.65	0.65
Indigofera frutescens	0.052	0.65	0.33	0.33
Leucosidea sericea	0.016	0.1	0.05	0.2
Melia azedarach	0.145	0.47	0.91	0.23
Clausena anisata	0.053	2.65	0.08	0.33
Cyathea dregei	0.017	0.11	0.01	0.11
Milletia grandis	0.021	0.53	0.13	0.03

BD ZC CG HT AD SM ML IF LS MA CA CD MG
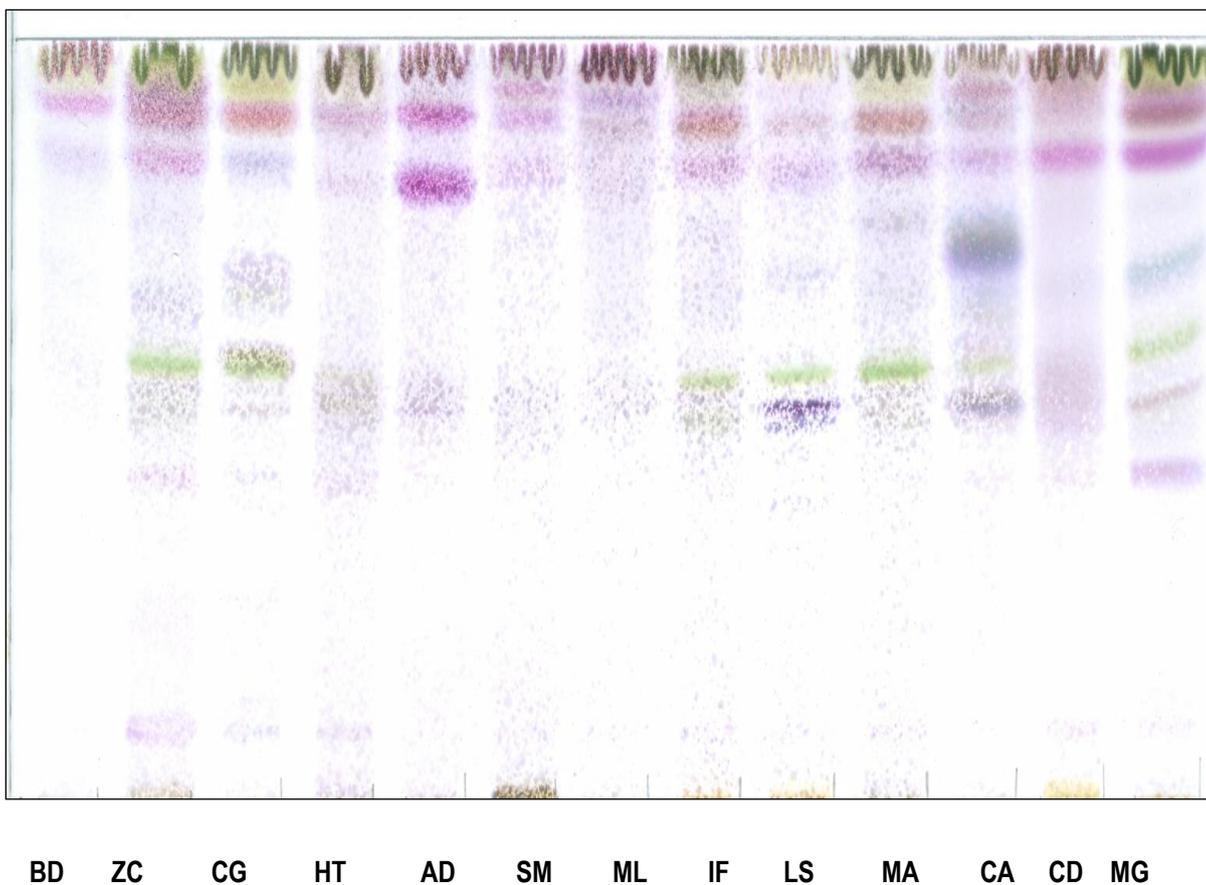
Figure 4.1: TLC plates developed in CEF system and sprayed with vanillin sulphuric acid showing varied chemical constituents of the thirteen acetone leaf extracts. KEY: BD, *B. discolor*; ZC, *Z. capense* ; CG, *C. glabrum*; HT, *H. trifoliata* ; AD, *A. dimidiata* ; SM, *S. mitis* ; ML, *M. lanceolata* ; IF, *I. frutescens* ; LS, *L. sericea* ; MA, *M. azedarach* ; CA, *C. anisata* ; CD, *C. dregei* ; MG, *M. grandis*

BEA (B)

| BD | ZC | CG | HT | AD | SM | ML | IF | LS | MA | CA | CD | MG |
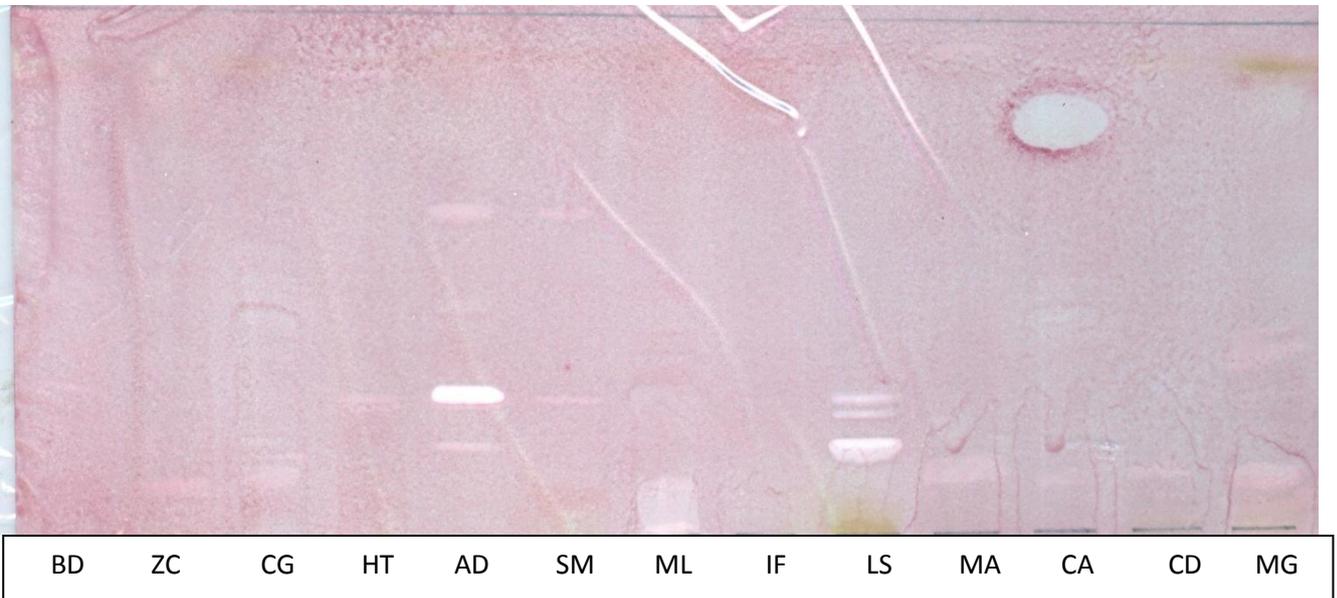
Figure 4.2: BEA Bioautogram of thirteen plant leaf acetone extracts against *C. albicans* showing antifungal bands. KEY: BD, *B. discolor*; ZC, *Z. capense* ; CG, *C. glabrum*; HT, *H. trifoliata* ; AD, *A. dimidiata* ; SM, *S. mitis* ; ML, *M. lanceolata* ; IF, *I. frutescens* ; LS, *L. sericea* ; MA, *M. azedarach* ; CA, *C. anisata* ; CD, *C. dregei* ; MG, *M. grandis*

Postscript: Since some of the extracts had activity against the fungal pathogens tested, we decided to test the activity against four bacteria organisms and their antioxidant activity. This will be worthwhile in the search for extracts with antibacterial activity and may possibly lead to extracts with wide therapeutic potential.

Chapter 5: The antibacterial and antioxidant activity of thirteen South African plant species used in ethnoveterinary medicine to treat helminth infections

Mathew Adamu, Vinny Naidoo and Jacobus N Eloff

Preface: This chapter evaluated the antibacterial activity of selected plant extracts against two Gram-positive and two Gram-negative bacteria to find out if the good antifungal activity was also associated with antibacterial activity. Because extracts with high antioxidant activity could be useful in treating inflammation caused by parasites the antioxidant activity of different extracts were also determined.

Abstract

Diseases caused by bacteria and fungi remain a major challenge globally and particularly in sub-Saharan Africa. The plants used in this study are primarily used in South Africa to treat helminth infections in livestock and humans. Their uses also extend to treatment of infection caused by bacteria. This study evaluated the antibacterial and antioxidant activity of the leaf acetone extracts of thirteen plant species. The antibacterial activity was determined by using a serial microdilution method. Bioautography was used to determine the number of antibacterial bands. The antioxidant activity was determined using the ABTS and DPPH methods. *Maesa lanceolata* and *Leucosidea sericea* with an MIC of 0.02 mg/ml had the highest antibacterial activity against *Enterococcus faecalis* and *Pseudomonas aeruginosa.* There was a poor correlation between antioxidant activity and antibacterial activity with $R^2=0.143$. *Maesa lanceolata* had a therapeutic index of 5.20, 2.60, 2.60 and 1.30 for *P. aeruginosa, E. faecalis, E. coli* and *S. aureus* respectively. *Strychnos mitis* had a therapeutic index of 1.08 for *E. coli*. This study shows that plant extracts used for ethnoveterinary medicine as an anthelmintic may also possess other therapeutic uses such as antibacterial activity.

5.1 Introduction

Resistance to available antibiotics is at a very alarming stage globally (Stuart and Bonnie, 2004). Efforts are urgently needed to replace current available antibiotics. This resistance is complex especially in Sub-Saharan Africa due to the menace of HIV/AIDS and associated secondary diseases such as tuberculosis. The situation is complicated with poor sanitary conditions and poor access to potable water and most importantly monetary inadequacies. In addition, antibiotics when available remain expensive for the poorer communities and in some cases especially in the rural areas are adulterated and of little value in the treatment of diseases caused by bacteria (Isturiz et al., 2000). Natural products from plant are a major source of chemical diversity and have provided therapeutic agents for numerous bacterial diseases (Payne et al., 2007). The antibacterial activity of plants is continuously attracting global attention (Rukayadi et al., 2009; Guzman et al., 2010). Tissue oxidation causes the release of free radicals with severe consequences as it has the capacity to amend cellular metabolism. Thus anti-oxidants have potential to prevent the activity of free radicals and reactive oxygen species thus helping in fighting diseases caused by bacteria and other pathogens.

South Africa is a potential source of undiscovered compounds with high activity extracts against a variety of bacterial and fungal pathogens (Shai et al., 2008). The plant species used in this study were chosen based on evidence of traditional use as anthelmintic, treatment of bacterial causing infections and availability. The aim of this study is to investigate the antibacterial and anti-oxidant activity of thirteen selected plant species against two Gram- positive and 2 Gram-negative organisms. Secondly, evaluate any possible correlation with anti-oxidant activity used for EVM in southern Africa. This investigation will assist in identifying plants for further study aimed at identifying compounds as potential source of new antibiotic for primary health care needs of man and his animals. If plant extracts that have anthelmintic activity are also useful in combating bacteria organisms it may also be useful.

5.2 Materials and Methods

5.2.1 Plant collection

Leaves of thirteen plant species were collected in November 2009 at the Pretoria National Botanical Garden in South Africa. The trees were identified and labelled and voucher specimens were kept in the Herbarium of the phytomedicine programme of the University of Pretoria with voucher numbers (Table 1). Plant leaves were dried at room temperature in a ventilated room, milled to a fine powder in Macsalab Mill (Model 2000 LAB Eriez®) and stored in closed containers in the dark until use.

5.2.2 Plant extraction

Plant material from each species investigated was extracted with acetone, (technical grade, Merck) in polyester centrifuge tubes at a ratio of 10 ml/g and repeated twice. Acetone was selected based on its superiority as extractant based on a number of parameters (Eloff, 1998a) especially because it extracts compounds with a wide range of polarities. The tubes were vigorously shaken for 30 min on an orbital shaker. Tubes were further centrifuged at 4000 x g for 10 min and the supernatant was filtered using Whatman No.1 filter paper before being transferred into pre-weighed glass containers. Solvent was evaporated under a stream of air in a fume hood at room temperature to produce the dried extract and for quantification (Eloff, 2004).

5.2.3 Chromatographic analysis

The extracted chemical components were analysed by separation with thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, Silica gel F254). The TLC plates were developed in saturated chambers using mobile phases of varying polarities, namely, ethyl acetate/methanol/water (40:5.4:5) [EMW] (polar/neutral), chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (intermediate polarity/acidic) and benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Separated components were visualised under UV light at wavelengths of 254 and 365 nm after which TLC plates were sprayed with vanillin-sulphuric acid (Stahl, 1969) and heated at 110 °C for optimal colour development.

5.2.4 Antioxidant activity

Anti-oxidant activity using qualitative and quantitative methods were employed. For the Qualitative anti-oxidant activity TLC plates were used to separate extracts and chromatograms sprayed with 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma) in methanol as an indicator (Deby and Margotteaux, 1970). The quantitative anti-oxidant activities were determined using the ABTS and DPPH methods (Re et al., 1999 and Brand-Williams et al., 1995).

5.2.5 Antibacterial activity

The MIC values were determined using a serial microplate dilution method (Eloff, 1998b). Bacterial organisms used were *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). These strains are recommended for antibacterial activity testing by the United States National Committee for clinical laboratory standards (NCCLS, 1990). Bacterial cells were inoculated into fresh Müller–Hinton (MH) broth (Fluka, Switzerland) and incubated at 37 °C overnight and density determined prior to the screening procedures. Densities of bacterial cultures after incubation overnight were as follows: *Staphylococcus aureus*, 2.6×10^{12} cfu/mL; *Enterococcus faecalis*, 1.5×10^{10}

cfu/mL; *Pseudomonas aeruginosa*, 5.2×10^{13} cfu/mL; *Escherichia coli*, 3.0×10^{11} cfu/mL. Acetone was used as solvent control, while gentamicin was the positive drug control.

5.2.6 Bioautographic investigations

Thin layer chromatography (TLC) plates were loaded with 10µL of 10 mg/ml of extract and dried before developing in mobile phases of BEA, CEF and EMW. The solvent were evaporated from the plates in a stream of air. Plates were then sprayed with concentrated cultures of bacteria species until completely moist using a spraying gun enhanced by a vacuum pump. The moist plates were incubated at 37 °C in an incubator for 12 h. The plates were then sprayed with 2 mg/ml of *p* iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 12 h (Begue and Kline, 1972). The emergence of purple-red colour resulting from the reduction of INT into its respective formazan was a positive indicator of cell viability (Begue and Kline, 1972). Clear zones against the purple background were indicative of antibacterial activity of compounds separated on TLC plates.

5.3 Results

5.3.1 Plant extracts yield

Leaves of the thirteen plant species were extracted using acetone as the extracting solvent, this gave varied percentage yield (Table 5.2), with *L. sericea* having the highest percentage yield of 6.27%, followed closely by *A. dimidiata* with 6.07%, and the lowest yield was 0.81 % by *Z. capense*.

5.3.2 Phytochemical profiling and Antioxidant activity

Extracts of plants were composed of various chemical constituents (Figure 5.1). The compounds varied from polar to non-polar and in some extracts compounds of intermediate polarity were observed.

M. lanceolata (ML) and *L. sericea* (LS) showed the highest number of anti-oxidant bands (Figure 5.2). This was consistent in all 3 solvent systems (BEA, EMW and CEF) and was supported by the ABTS and DPPH assays used for quantification of the antioxidant activity. *M. lanceolata* with a TEAC of 1.20 and *L. sericea* with an EC_{50} of 0.0051 as the best extracts using ABTS and DPPH respectively.

5.3.3 Bioautography

Three solvent systems were used. The plates elute by the BEA solvent system was the best with 56% of the total active bands against the four bacteria organisms (Figure 5.3). The highest number of active bands was in *L. sericea* BEA, with 6 active bands against *E. coli* (Rf values; 2.35, 2.67, 2.86, 3.20, 3.48 and 4.0) and *M. lanceolata* with 4 active bands (Rf values; 1.07, 2.29, 2.67 and 4.0). *S. mitis* had one active band (Rf 1.22) in BEA solvent against *S. aureus*. A compound with Rf value of 4.0 showed band of inhibition in *Z. capense* (Rf

2.11, 4.00), *C. glabrum* (4.00)*, H. trifoliata* (4.0), *M. lanceolata, I. frutescens* (2.00, 4.00), *L. sericea, M. azedarach* (2.00, 4.00), *C. anisata* (1.14, 4.00) and *M. grandis (*2.00, 3.48, 4.00)*. This band was common to nine extracts with zones of inhibition. The 13 plant extracts had better activity against *E. coli* than the other three tested organisms. In total, 75 (mean=18) active bands were seen on all the 3 solvent systems against the four bacteria organisms.

5.3.4 Minimal Inhibitory Concentrations and total activity against bacterial pathogens

The minimal inhibitory concentration (MIC) values for the antibacterial activity of the leaf extracts of the plant species are in Table 5.3. The results show 3 plants extracts; *L. sericea, M. lanceolata* and *I. frutescens* with average MIC of 0.16 mg/ml or less against the four bacteria organisms. *Clausena anisata* had a good MIC while the rest plant extracts had moderate MIC values with the exception of *Z. capense, A. dimidiata, H. trifoliata* and *C. glabrum* with poor MIC values. In our laboratory, MIC values of 0.16 mg/ml or less are considered excellent, while values above 0.16 mg/ml are good with MIC values of 1.25 and above as poor. *Pseudomonas aeruginosa* and *Enterococci faecalis* were the most susceptible bacteria organisms tested. The most effective plant extracts were *Leucosidea sericea* and *Maesa lanceolata* with average MIC of 0.04 mg/ml on all four bacteria organisms. The above two plants extracts had an MIC of 0.02 mg/ml against *P. aeruginosa* and *E. faecalis* as the best MIC. In contrast, *Z. capense* and *A. dimidiata* showed no activity against *E. coli* at 2.50 mg/ml the highest concentration used. The lowest average MIC of 1.02 mg/ml was recorded by *Z. capense*, followed by *A. dimidiata* with MIC of 0.95 mg/ml. Total activities value for the 13 plant extracts in the antibacterial assay showed *L. sericea* as the best with a total activity value of 6425.64 ml against *P. aeruginosa* and *E. faecalis* (Table 5.3)*. The total activity value is that volume to which 1 gram of the plant extract could be diluted and still retains activity against the microorganism. Average total activity showed *L. sericea* with 4216.83 ml as the best against all four bacterial organisms. The two plants extracts with the lowest total activity value were *Z. capense* and *H. trifoliata* with 32.44 ml and 35.70 ml respectively.

5.3.5 Therapeutic index

Based on the therapeutic index, that is the ratio of MIC to cytotoxicity value, *M. lanceolata* had the best therapeutic index of 5.20, 2.60 and 1.30 against *P. aeruginosa, E. coli* and *E. faecalis* and *S. aureus* respectively (Table 5.4). The results make such plant extracts a good candidate for possible isolation of compounds. The other extract with therapeutic index above 1 was *Strychnos mitis* on *E. coli* (1.08)*. All the other eleven extracts had values less than 1. The therapeutic values help to differentiate activity that is usually not due to toxicity but one that is probably mechanistic based.

5.4 Discussion

The results of this study shows extract with good antioxidant activity had a corresponding good antibacterial activity. *L. sericea* and *M. lanceolata* had good antioxidant activity when compared to the positive control Trolox. The two plant extracts had a corresponding average MIC value of 0.04 mg/ml for all four bacteria pathogen used. However, there was a poor correlation between antioxidant activity and antibacterial activity with $R^2=0.143$.The MIC results probably suggest that compounds in the two extracts (*L. sericea* and *M. lanceolata*) are probably acting in synergy, considering the good MIC. The results show plants extracts that were active against both Gram positive and Gram negative bacteria. However, the Gram positive bacteria were more susceptible to the extracts than the Gram negative bacteria (Table 5.3). This is because the cell wall of gram negative bacteria is less permeable to antimicrobial (Hodges, 2002).

Extracts of *L. sericea* and *M. lanceolata* had the best antibacterial activity with MIC of 0.02 mg/ml. A previous study (Bosman et al., 2004) reported *L. sericea* to have some activity against bacteria organisms using the disc diffusion method, but no activity for acetone leaf extracts of *L. sericea.* Our findings do not agree with theirs and this may likely be due to differences in methods used (microdilution method as against disc diffusion) for the antimicrobial assay. A study by Aremu et al., (2010) reported an MIC of 0.025 mg/ml using petroleum ether and dichloromethane leaf extracts of *L. sericea* against *Bacillus subtilis* and *Staphylococcus aureus,* which agrees with our results. The solvent used in our study differed to that used in that study. With solvent known to extracts compounds of different polarity from same plant, this may explain the different MIC values of 0.04mg/ml for S. *aureus* recorded in this study in comparison to the 0.025 mg/ml reported by Aremu et al., (2010). The activity of *L. sericea* may be due to the presence of active compounds such as aspindinol (Bosman et al., 2004), alkaloids, phenolics and saponins (Aremu et al., 2010) that had previously been reported in this plant. *Maesa lanceolata* extract with an antibacterial activity of 0.02 mg/ml is the first report of a good activity in this plant. Previous study (Sindambiwe et al., 1999) reported the extract lacks antibacterial activity at concentrations of 10 mg/ml. The results differ probably due to the method in use, as they used the agar dilution and broth dilution method for antibacterial and antifungal assays respectively. The poor antibacterial activity shown by *Z. capense* in this study agrees with earlier report (McGaw et al., 2000).

In this study, the plant species generally showed better activity against Gram-positive bacteria than against Gram-negative bacteria. This is usually the norm as Gram-positive bacteria are more susceptible to antimicrobials than are Gram- negative bacteria (Vlietinck et al., 1995) due to differences in their cell wall composition.

5.4.1 Conclusions

The plants extract tested had varying degree of antibacterial and anti-oxidant activity. *L. sericea* and *M. lanceolata* extracts are the two most effective plant species in the antibacterial assay. The results of this study report a poor relation between antioxidant and antibacterial activity. The study confirms that extracts with good anthelmintic activity such as *M. lanceolata* and *L. sericea* do have a corresponding good antibacterial activity. Thus, activity guided fractionation and isolation of compounds of *L. sericea* and *M. lanceolata* the two most promising plant extracts should be carried out.

Table 5.1: List of plant species used in the investigation, their traditional uses and references. Only the leaves of these plants species were used in the study.

Plant species	Family	Medicinal uses	Reference
Brachylaena discolor	Asteraceae(267)	Purgatives against intestinal parasites, anthelmintics for calves, sheep and goats	Bryant,1966; Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Zanthoxylum capense	Rutaceae(96)	Gastric and intestinal disorders, anthelmintics, cough, bronchitis, pleurisy	Hutchings, 1996; Bryant, 1966.
Clerodendrum glabrum	Lamiaceae(403)	Intestinal parasites, coughs, fever, and diabetes	Bryant, 1966; Watt and Breyer-Brandwijk, 1962, Hutchings, 1996.
Heteromorpha trifoliata	Apiaceae(491)	Intestinal worms, colic in horses and vermifuge, enemas for abdominal disorders	Bryant, 1966; Watt and Breyer-Brandwijk, 1962, Gerstner, 1938.
Apodytes dimidiata	Icacinaceae(139)	Enemas for intestinal parasites, purgatives, inflammation of the ear	Bryant, 1966; Watt and Breyer-Brandwijk, 1962, Gerstner, 1938.
Strychnos mitis	Strychnaceae(73)	Malaria, Fevers	Biset, 1974
Maesa lanceolata	Maesaceae(615)	Anthelmintics, treatment of wounds and infertility	Watt and Breyer-Brandwijk, 1962

Table 5.1 continue: List of plant species used in the investigation, their traditional uses and references. Only the leaves of these plants species were used in the study.

Plant species	Family	Medicinal uses	Reference
Indigofera frutescens	Papilionaceae(675)	Anthelmintics	Watt and Breyer-Brandwijk, 1962.
Leucosidea sericea	Rosaceae(288)	Treatment of opthalmia, anthelmintics, astringents and vermifuge	Watt and Breyer-Brandwijk, 1962 and Jacot Guillarmod, 1971.
Melia azedarach	Meliaceae(702)	Effective anthelmintics, emetic, cathartic and treatment of eczema	Watt and Breyer-Brandwijk, 1962;Oliver-Bever 1986;Hutchings, 1996
Clausena anisata	Rutaceae(317)	Anthelmintics, purgatives, rheumatism, fevers and myiasis	Doke and Vilakazi, 1972.
Cyathea dregei	Cyatheaceae(658)	Anthelmintics	Doke and Vilakazi, 1972.
Milletia grandis	Papilionaceae(704)	Anthelmintics and tranquilizers	Watt and Breyer-Brandwijk, 1962 ; Palmer and Pitman 1972

Figures in brackets are voucher specimen numbers and have a prefix PRU.

Table 5.2: Percentage yield and antioxidant activity of thirteen South Africa plant extracts used for antibacterial activity

Plant species	% Yield	EC_{50} DPPH	TEAC
Heteromorpha trifoliata	3.30	4.36	0.17
Indigofera frutescens	0.81	0.01	0.45
Zanthoxylum capense	1.60	4.02	0.44
Milletia grandis	1.28	4.62	0.63
Brachylaena discolor	6.07	2.61	0.22
Clerodendrum glabrum	3.75	3.52	0.45
Strychnos mitis	2.79	3.50	0.30
Cyathea dregei	2.05	2.98	0.41
Apodytes dimidiata	6.27	3.50	0.26
Melia azedarach	2.29	3.34	0.78
Clausena anisata	3.40	2.47	0.18
Maesa lanceolata	2.50	1.43	1.20
Leucosidea sericea	1.24	0.01	0.69

Table 5.3: Minimal inhibitory concentrations (MIC) of the leaf of 13 plant species evaluated for antibacterial activity using four bacteria organisms.

Plant species	*S. aureus*	*E. coli*	*E. faecalis*	*P. aeruginosa*
Brachylaena discolor	1.25	0.63	0.16	0.31
Zanthoxylum capense	1.25	2.50	0.31	0.31
Clerodendrum glabrum	1.25	0.31	1.25	0.63
Heteromorpha trifoliate	1.25	0.63	1.25	0.63
Apodytes dimidiata	0.31	2.50	1.25	0.31
Strychnos mitis	0.31	0.04	0.63	0.16
Maesa lanceolata	0.08	0.04	0.04	0.02
Indigofera frutescens	0.16	0.16	0.08	0.31
Leucosidea sericea	0.08	0.08	0.02	0.02
Melia azedarach	0.63	0.31	0.16	0.63
Clausena anisata	0.16	0.31	0.31	0.31
Cyathea dregei	0.31	0.31	1.25	0.31
Milletia grandis	0.31	0.31	0.31	0.31
Gentamicin	<0.02	<0.02	<0.02	<0.02
Acetone	>2.50	>2.50	>2.50	>2.50
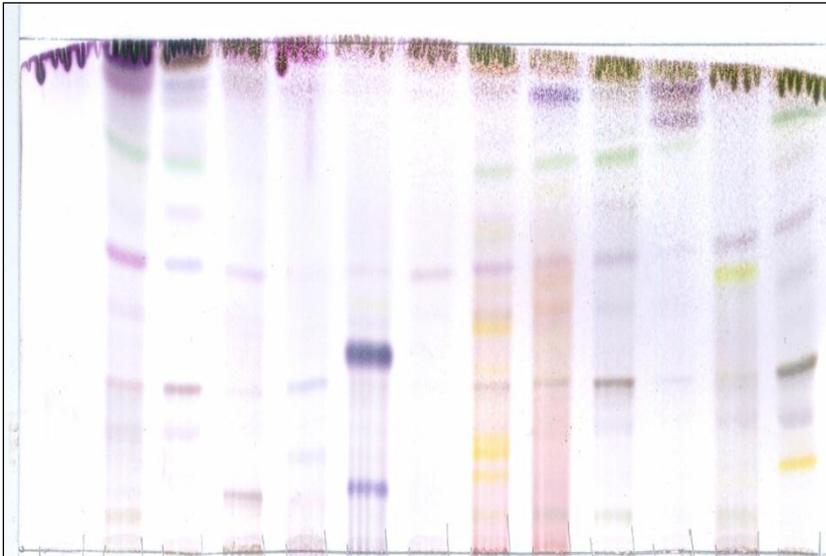
BD   ZC   CG   HT   AD   SM ML IF   LS MA   CA   CD   MG

Figure 5.1: TLC plate of 13 plant species sprayed with vanillin-sulphuric acid

KEY: **BD**, *B. discolor*; **ZC**, *Z. capense* ; **CG**, *C. glabrum*; **HT**, *H. trifoliata* ; **AD**, *A. dimidiata* ; **SM**, *S. mitis* ;

ML, *M. lanceolata* ; **IF**, *I. frutescens* ; **LS**, *L. sericea* ; **MA**, *M. azedarach* ; **CA**, *C. anisata* ; **CD**, *C. dregei* ;

MG, *M. grandis*
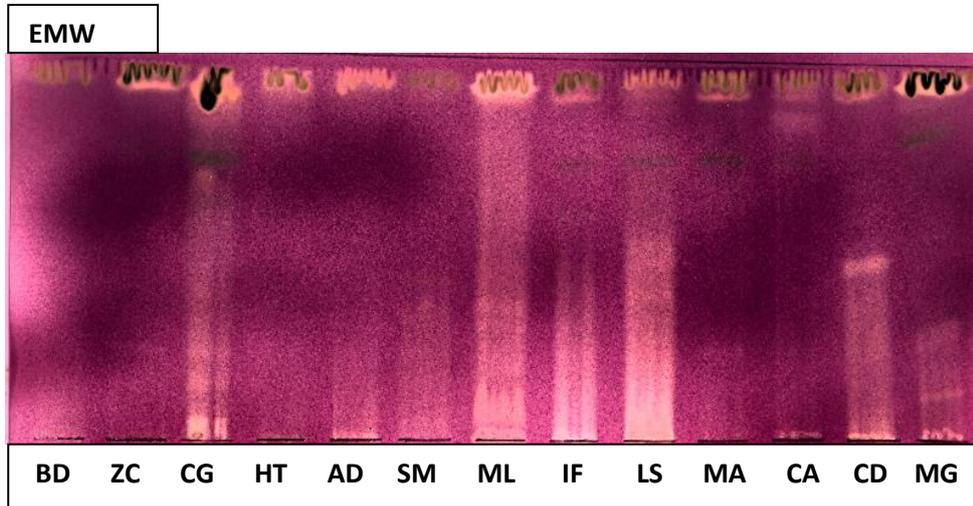
Figure 5.2: TLC plates showing antioxidant activity of 13 plant species.

KEY**:** BD, *B. discolor*; ZC, *Z. capense* ; CG, *C. glabrum*; HT, *H. trifoliata* ; AD, *A. dimidiata* ; SM, *S. mitis* ; ML, *M. lanceolata* ; IF, *I. frutescens* ; LS, *L. sericea* ; MA, *M. azedarach* ; CA, *C. anisata* ; CD, *C. dregei* ; MG, *M. grandis*.
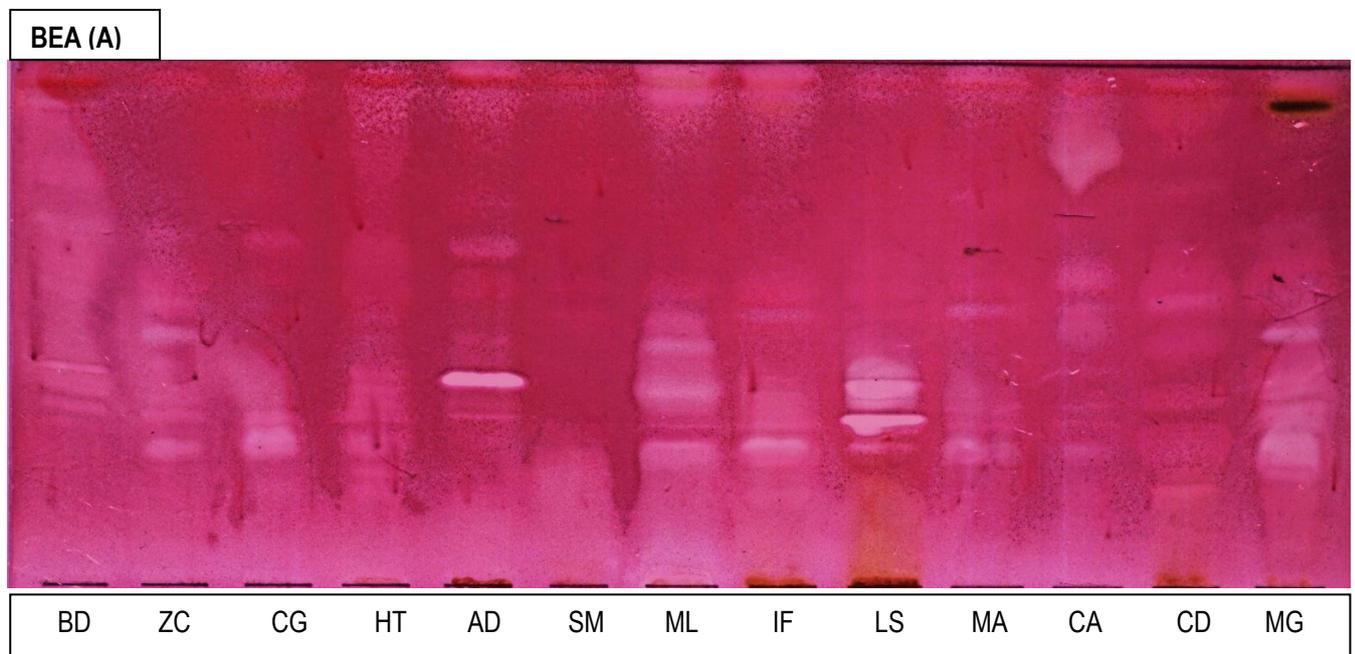
Figure 5.3: BEA (A), Bioautogram of different plant leaf acetone extracts against *E.coli* showing antibacterial bands.

 KEY: BD, *B. discolor*; ZC, *Z. capense* ; CG, *C. glabrum*; HT, *H. trifoliata* ; AD, *A. dimidiata* ; SM, *S. mitis* ; ML, *M. lanceolata* ; IF, *I. frutescens* ; LS, *L. sericea* ; MA, *M. azedarach* ; CA, *C. anisata* ; CD, *C. dregei* ; MG, *M. grand*

Postscript: Based on the anthelmintic, antifungal, antibacterial, antioxidant and cytotoxicity of the extracts of the thirteen selected plant species. *Leucosidea sericea* was selected for further in depth study to evaluate the fractions of the acetone extracts against the egg and larvae of *Haemonchus contortus*, cytotoxicity and to isolate compounds from the most active extracts and fractions. Isolated and characterized compounds will be tested for anthelmintic activity using the EHA and the LDT assays.

Chapter 6: Anthelmintic phloroglucinol derivatives and antifungal activity of fractions from *Leucosidea sericea* (Rosaceae)

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Preface

To simplify the isolation of the anthelmintic compounds from the leaf extract of *L. sericea*, the extracts were fractionated into four different fractions using solvent-solvent fractionation and tested for activity against the eggs and larvae of *Haemonchus contortus.* The major compounds present in active extracts and fractions will be isolated characterized chemically and biologically. The result in this chapter is being prepared for submission to Journal of Ethnopharmacology.

Abstract

Ethnopharmacological relevance: The leaves of *Leucosidea sericea* are used traditionally as anthelmintic and to treat opthalmia in South Africa.

Aim of the study: Anthelmintic activity had been reported from this plant, the aim of this study was to identify and characterized the compounds responsible for the anthelmintic activity using bio-assay guided fractionation.

Materials and methods: Bioassay-guided isolation of the ethyl acetate fraction of the acetone crude extract of *L. sericea* was achieved using repeated silica gel column chromatography techniques. The structures of isolated compounds were identified by interpretation of their NMR, MS and IR data and by comparison with reported values. The anthelmintic assay was conducted using guidelines approved by the World Association for Advancement for Veterinary parasitology. The antifungal activity was determined using a microplate dilution method, while cytotoxicity on Vero monkey cells was tested using the MTT assay.

Results: The isolated compounds were identified from their NMR data as β-sitosterol, agrimol G and a mixture of agrimols G and A. The Egg hatch assay EC_{50s} for agrimol G was 0.52 mg/ml, while that of the mixtures of agrimols G and A was 0.28 mg/ml. The larval development test EC_{50s} for agrimol G was 0.08 mg/ml and 0.11 mg/ml for mixture of agrimols G and A.

Conclusion: This study is the second report of compound been isolated from this plant species. The compounds isolated have *in vitro* anthelmintic activity*.*

Keywords: *Leucosidea sericea*; Rosaceae; Phloroglucinol derivatives; Anthelmintic; Antifungal; Cytotoxicity.

6.1. Introduction

Helminths remain the most important cause of production losses in livestock in Africa (Anon, 1991). Whilst a number of different parasites occur throughout the continent, *Haemonchus contortus* remains the singular most pathogenic nematode of small ruminants (Krecek, 2006). This blood-sucking parasite colonises the abomasum in the thousands with resultant severe anaemia, hypoproteinaemia and ultimately a drop in production and even death (Taylor et al. 2007). Current treatment options centre on the use of anthelmintics (Kaminsky et al., 2008), selected farming practice such as rotational grazing (Waller, 2006) and most recently vaccinations (LeJambre et al., 2008). Of these rotational grazing is a mitigating tool to lower total levels of infection by relying on different species being run on properly maintained pastures, with the premise being that non-susceptible species clear out the worms before susceptible species start grazing. Unfortunately due to the level of management required for a rotational grazing system, it is not practically implemented among subsistence farmers. The use of vaccines, while effective, is still a developing technology and only provides short-term protection due to antibody weaning in addition to being cost prohibitive to the subsistence farmer.

As a result chemical anthelmintic is the most widely recognised method of worm control. Unfortunately through years of use and abuse, the development of severe drug resistance has had a major impact on animal production. At present, *H. contortus* is resistant to all but the newest anthelmintics which are also too costly for small scale farmers to use. A cheaper means of helminth treatment in animals is required. It has been suggested that herbal remedies, may prove to be a viable alternative as several plants species are being tested for activity (Githiori et al., 2006). *Leucosidea sericea* (Eckl. & Zeyh) (Watt and Breyer-Brandwijk, 1962, Pooley, 1993) a member of the Rosaceae family consist of approximately 3000 species and 107 genera with nine indigenous to South Africa (Jordan, 2000). It is a 7 m tall small tree of great character which grows in the eastern parts of South Africa (Watt and Breyer-Brandwijk, 1962, Coates-Palgrave, 2002). The leaf of this plant is used mainly for anthelmintic purpose, while the Zulu people use a paste made from it for treating opthalmia (Hutchings, 1996). The *in vitro* anthelmintic activity of this species have been reported (Bosman et al., 2004, Aremu et al., 2010) and previous phytochemical investigation of the leaves led to the isolation of Aspidinol as the only compound so far (Bosman et al., 2004). The anthelminthic activity motivated further investigation on this species as well as 12 others (Adamu *et al*., 2012). Several plant species with anthelmintic activity has been reported in literature, some with good activity (Hoste et al., 2012). The compounds responsible for this activity are not been reported partly because of lack of a standard bio-guided assay for their isolation. In a preliminary screening acetone extracts from this plant showed promising efficacy against *H. contortus* egg and larvae. For this study the effect of four

fractions obtained by liquid-liquid fractionation from the acetone crude extract of the leaves of *L. sericea* were evaluated against the egg and larva of *H. contortus* in order to potencies the plant extract as well as to isolate bioactive compounds.

6.2. Materials and Methods

Plant materials were milled to a powder using a Macsalab Mill (Model 2000 LAB Eriez). Whatman paper No 1 was used for filtration. Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh. Percolated plates of TLC silica gel 60 F_{254} (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with vanillin-sulfuric acid spray reagent followed by heating at 110 °C for 3 min. IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Optik GmbH, Germany). [1] H- and 13C-NMR spectrum was recorded with a Bruker spectrometer at 500 MHz, and chemical shifts (δ) are quoted in ppm with TMS as internal standard.

6.2.2. Plant material

The leaves of *L. sericea* were collected in March 2011, from the National Botanical Garden in Pretoria, South Africa. After identification, voucher specimens were kept in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria with voucher number PRU 288.

6.2.3. Extraction and bio-guided isolation

The dried powdered leaves (500 g) of *L. sericea* were extracted with acetone (5 L) for 24 h to give crude extract (68.52 g) after filtration and removal of solvent using rotary evaporator. Part of this extract (30 g) was dissolved in a mixture of chloroform and water and fractionated by solvent-solvent extraction to yield hexane (15.21 g), chloroform (5.37 g), ethyl acetate (10.37 g) and n-butanol (13.52 g) (Suffness and Douros, 1979; Eloff, 1998a). The chloroform, hexane and n-butanol fractions had lower anthelmintic activity and were not further investigated. The ethyl acetate fraction (3 g) was subjected to silica gel open column chromatography (2.5 cm 73 cm, 0.0630.200 nm, 100 g) using hexane with increasing amount of ethyl acetate to yield 99 fractions of 500 ml each that were combined to 15 fractions after monitoring with comparative TLC. Fraction 5 (500 mg) obtained from hexane: ethyl acetate (95:5) was re-chromatographing on silica CC using isocratic mixture of hexane: ethyl acetate (96:4) to afford compound 1 (5 mg). Fraction 9 (342 mg) obtained from hexane: ethyl acetate (93:7) was similarly subjected to a purification silica gel CC as described for fraction 5 to give 2 (10 mg) while fraction 10 (1301 mg) obtained from hexane: ethyl acetate (90:10) was re-chromatograph on silica gel CC using isocratic mixture of hexane: ethyl acetate (92:8) to afford compounds 2 and 3 (7 mg).

6.2.3.1. Agrimol G (2)

Amorphous yellowish powder; IR ν_{max}: 3400-3100, 2934, 1610, 1269, 1154, 1106, 984, 652 cm^{-1}; ^{1}H-NMR (CDCl$_3$, 500 MHz) δ 1.15 (4H, d, J = 8.5 Hz, H-11/H-12/H-11'/H-12'), 1.19 (2H, d, J = 8.4 Hz, H-9"/H-10"), 2.12 (2H, s, H-8/H-8'), 3.70 (6H, s, 4-OMe/4'-OMe), 3.82 (4H, s, H-7/H-7'), 3.86 (2H, m, H-10/H-10'), 4.02 (1H, m, H-8"), 9.22 (br s, 2"-OH), 9.60 (br s, 2'-OH), 9.70 (br s, 6-OH), 10.70 (br s, 2-OH), 15.50 (br s, 6"-OH), 15.87 (br s, 6'-OH), 16.07 (br s, 4"-OH); ^{13}C-NMR data (CDCl$_3$, 125 MHz) δ: 212.5 (C-9/C-9'), 211.8 (C-7"), 160.0 (C-2"/C-4/C-4'/C-6/C-6'), 157.9 (C-2/C-2'/C-4"/C-6"), 106.6 (C-1/C-1'/C-1"/C-3"), 105.8 (C-3/C-3'), 104.8 (C-5/C-5'/C-5"), 62.1 (4'-OCH$_3$), 39.3 (C-8"), 38.3 (C-10/C-10'), 19.8 (C-9"/C-10"), 19.3 (C-11/C-11'/C-12/C-12'), 16.9 (C-7'), 16.5 (C-7), 9.2 (C-8/C-8').

6.2.3.2. Agrimol A (3)

Amorphous yellow powder; IR ν_{max}: 3400-3150, 2914, 1610, 1269, 1150, 1100, 984, 652 cm^{-1}; ^{13}C-NMR data (CDCl$_3$, 125 MHz) δ: 212.5 (C-9/C-9'), 204.2 (C-7"), 160.4 (C-2"/C-4/C-4'/C-6/C-6'), 159.7 (C-2/C-2'/C-4"/C-6"), 109.4 (C-1/C-1'/C-1"/C-3"), 108.0 (C-3/C-3'), 105.7 (C-5/C-5'/C-5"), 61.4 (4-OCH$_3$/4'-OCH$_3$), 52.9 (C-9"), 46.0 (C-8"), 38.3 (C-10/C-10'), 36.6 (C-10"), 24.9 (C-13"), 22.7 (C-11/C-11'/C-12/C-12'), 19.2 (C-11"/C-12"), 16.6 (C-7'), 16.4 (C-7), 9.1 (C-8/C-8').

6.2.4. Anthelmintic assay

6.2.4.1. Recovery and preparation of eggs

The helminth eggs were prepared according to the method of the World Association for the Advancement of Veterinary Parasitology (WAAVP) with modification (Coles et al., 1992). Eggs were donated by BosVet from their donor sheep with a monospecific infection of non-resistant *H. contortus*. BosVet housedthe sheep indoors on concrete floor, fed hay, commercial concentrate pellets and had free access to potable water. The faecal pellets were mashed in an ordinary blender to make a relatively liquid suspension (slurry), and serially filtered through a sieve of sizes 400, 250, 150, 90, 63 µm and 38 µm to trap the eggs on the latter. The material on the 38 µm mesh was washed into 50 ml centrifuge tubes, resuspended in a magnesium sulphate solution (SG of 1.10) and centrifuged at 1000 g for 10 minutes to separate the eggs from other debris. The resultant supernatant was again passed through a 38 µm sieve to finally harvest the eggs into 1l of distilled water. The egg concentration was subsequently brought to a final concentration of 100 eggs per 0.2 ml.

6.2.4.2. Egg hatch assay (EHA)

The EHA was conducted according to WAAVP guidelines, (Coles et al., 1992). Briefly 0.2 ml of the egg suspension was distributed into a 48-flat-bottomed microtitre plate and mixed with 0.2 ml of each plant fraction (4, 2, 1, 0.5, 0.25 and 0.13 mg/ ml), albendazole (0.008 to 25 µg/ml dissolved in 5% dimethyl sulfoxide (DMSO)

as the positive control or water as the negative control. After incubation at 27°C for 48 h, a drop of Lugol's iodine solution was added to each well and the number of motile larvae and unhatched eggs counted. The percentage inhibition of egg hatching was calculated using the formula below. All experiments were undertaken in triplicate on three separate occasions (n=9).

$$Egg\ hatch\ assay = \left(\frac{a}{b} \div \frac{a}{c}\right) \times 100\%$$

Where a= Number of larvae, b= Total number of larvae and eggs in wells with plant extract and c= Total number of larvae and eggs in control well which contained ⟨water ⟩

6.2.4.3. Larval development test (LDT)

The egg suspension (0.2 µl diluted to 150 µl) was placed into 48-well plates, with 20 µl of a suspension of lyophilised *Escherichia coli* (ATCC 9637) (Hubert and Kerboeuf, 1992), 10 µl of amphotericin B (Sigma), 20 µl of nutritive media (comprising of 0.1 g yeast extract in 0.9 ml of normal saline and 0.1 ml of Earle's balanced salt solution) and incubated as above for 48 h. After incubation, 200 µl of the test extracts (4, 2, 1, 0.5, 0.25 and 0.13 mg/ ml) and albendazole (0.008 to 25 µg/ml) were added to the wells (n=3) and further incubated for 5 days with a negative control. Hereafter, the assay was stopped by addition of one drop of Lugol's iodine solution and the L_1, L_2 and L_3 larvae in each well were counted. A percentage inhibition of development to L_3 was calculated using the formula adopted from Coles et al., (1992) as modified by Ademola and Eloff, (2011c).

$$Larval\ development\ test = \left(\frac{a}{b} \div \frac{a}{c}\right) \times 100\%$$

Where a= Number of third stage larvae, b= Total number of larvae in wells with plant extract and c= Total number of larvae in control well

6.2.5. Antifungal assay

The MIC values were determined using a serial microplate dilution method developed by Eloff (1998a) with slight modifications by Masoko et al (2005) for the fungal assay. Three fungal species, namely *Candida albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus,* were maintained in Sabouraud dextrose agar (Oxoid, Basingstoke) until used. For growth inhibition, tests fungal species were grown overnight in Sabouraud dextrose broth at 35 °C prior to the screening test. Densities of fungal cultures used in bioautography and for MIC determinations were as follows: *C. albicans*, 2.5×10^6 cfu/ml; *C. neoformans*, 2.6×10^6 cfu/ml; *A. fumigatus*, 8.1×10^6 cfu/ml. *Candida albicans* was diluted to a density of about 2.5×10^4 cfu/ml, *C. neoformans*, 2.6×10^4 cfu/ml, and *A. fumigatus* 8.1×10^4 cfu/ml. Tetrazolium violet was used as an indicator of microbial growth and was

added 1 h before the MIC readings were taken. Growth of the microorganism reduces the tetrazolium violet to a red formazan. Amphotericin B was used as a positive control. MIC at 24 and 48 h was determined as the lowest concentration of plant extract inhibiting microbial growth, indicated by a decrease in the intensity of the red colour of the formazan product. Acetone was used to dissolve the various plants extracts (Eloff, 1998b).

6.2.6. Cytotoxicity assay using MTT

For the assay confluent Vero monkey kidney cells were trypsinised and seeded (0.5 x 10^3 cells per well) in a 96 well microtitre plate and incubated overnight at 37°C in 5% 200μl minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac, South Africa) and 5% foetal calf serum (Adcock-Ingram). After 24 hours the media was replaced with 200 μl of the extracts (1, 0.1, 0.01, 0.001 mg/ml) and further incubated for 5 days. Viability of cells was determined using the tetrazolium-based colorimetric MTT assay (3-5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) described by Mosmann, (1983). In short the media in each well was removed and replaced with fresh media and 30 μl of 5 mg/ml MTT in PBS and subsequently incubated for 4 h. Hereafter the medium was removed and cells washed with PBS, prior to the addition of DMSO (50 μl) to dissolve any formazan crystals present. The absorbance of the wells was measured with a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control, while wells containing only cells without extracts were the negative control. The percentage cell viability was calculated relative to the pure growth with the LC_{50} being the concentration that resulted in 50% reduction of absorbance compared to untreated cells. Tests were carried out in triplicate and each experiment was repeated three times. Selectivity index (LC_{50}/EC_{50} or MIC) were calculated using values from the cytotoxicity.

6.2.7. Statistical analysis

The results generated were analysed using the pharmacology software Kinetica 5.2 (Thermo) using the Hills pre-programmed model to obtain the concentration required to kill 50% of the organisms (EC_{50}). Results were subjected to descriptive statistics.

6.3. Results

The crude acetone extract of the leaves of *Leucosidea sericea* was subjected to solvent-solvent fractionation to give hexane, chloroform, ethyl acetate and n-butanol fractions with a yields of 28.69%, 2.90%, 18.46% and 49.94%, respectively. The hexane soluble portion was subjected to repeated silica gel CC to afford three compounds (**1**-**3**). The structures of the isolated compounds were identified by interpretation of their NMR and IR data and by comparison with reported data. The compounds were identified as *β*-sitosterol (**1**) (Al-Oqail *et al*., 2012), agrimol G (**2**) (Yamaki et al., 1989; Lounamaa et al., 1973) and a mixture of agrimols G and A (**2** and **3**) (Singh and Bharate, 2006; Yamaki et al., 1989; Lounamaa et al., 1973). The isolation of these compounds (**1** - **3**) from this plant is reported here for the first time.

The *in vitro* anthelmintic activity of the fractions from the crude extract and compounds (**2** and **3**) was determined against the egg and larvae of *Haemonchus contortus* (Tables 6.1, 6. 2 and 6.4, Figs.6.1 and 6. 2). The cytotoxicity of the compounds (**2** and **3**) was also evaluated against Vero cell lines (Table 6.4).

The egg hatch results are presented in Table 6.1. The hexane fraction of *L. sericea* was most active with EC_{50} of 0.69 mg/ml, followed by 0.92 mg/ml for the ethyl acetate fraction. Chloroform and butanol fractions recorded an EC_{50} of 1.39 and 1.68 mg/ml respectively. Albendazole induced a 100% inhibition at all tested concentrations. The larval development assays are presented in Table 6.1. The ethyl acetate fraction of *L. sericea* was the most effective with an EC_{50} of 0.79 mg/ml, followed by 1.14 mg/ml for the hexane fraction. An EC_{50} of 1.70 and 2.28 mg/ml for the chloroform and butanol fractions. Albendazole had a 100% inhibition at all tested concentrations. The selectivity index of the various fractions was determined for both assays. The ethyl acetate fraction had the best with values of 308.15 and 358.86 for the EHA and the LDT respectively. Based on the above results the ethyl acetate fraction was selected for isolation of bioactive compounds with anthelmintic activity. This method was used as no method exist for a throughput bioassay-guided test for anthelmintic unlike bacteria and fungi were bioautography gives zones of inhibition as leads for isolation of compounds.

The MICs were generally weak to poor (Table 6.2). Chloroform fraction of *L. sericea* had MIC of 0.31 mg/ml against *C. albicans* and 0.63 mg/ml against *A. fumigatus* for hexane, butanol and ethyl acetate fractions. The chloroform fraction had an MIC of 0.73 mg/ml. The fractions of this plant had poor activity on the fungal pathogens. This fraction therefore had good selectivity as it targeted bacteria and fungi, indicating that the extract was activity via a specific mechanism and not as a general metabolic toxin.

Cytotoxicity and selectivity index of fractions of *L. sericea* on the fungal pathogens are presented in Table 6.2. Based on the LC_{50} the butanol fraction was the least toxic with values of 330.20 mg/ml and 283.50 mg/ml for the ethyl acetate fraction. The chloroform and hexane fractions had LC_{50} of 4.18 and 2.42 mg/ml respectively. The selectivity index of the fractions had the butanol and ethyl acetate fractions with 175.95 and 1056. The chloroform and hexane fractions had selectivity index below 5.

The EC_{50} of agrimol G against the egg and larvae of *H. contortus* are 0.52 and 0.08 mg/ml while that of agrimols G and A are 0.28 and 0.11 mg/ml. The cytotoxicity of the compounds against Vero cells showed both compounds to be non-toxic with LC_{50} of 6780 mg/ml for agrimol G and 4030 mg/ml for agrimols G and A, and a very high selectivity index (Table 6.3). Figures 6. 2 and 6.3 represent the dose response activity for the egg hatch and larval development for both compounds. The peak of activity for both compounds was attained at a concentration of 0.25 mg/ml for the larval development test. Agrimol G had a better activity for the LDT than agrimols G and A. The egg hatch assay had agrimols G and A with better activity than agrimol G. At concentration of 1 mg/ml a 100% inhibition was achieved for both compounds for the EHA.

6.4. Discussion

The activity of four fractions of *L. sericea* was evaluated against the development of egg and larva of *Haemonchus contortus in vitro*. The cytotoxic activity of the fractions was determined on fungal pathogens and Vero cells. Bioassay-guided fractionation using the egg and larvae of *Haemonchus contortus* was used for the isolation of compounds from the most effective fraction. Two phloroglucinol derivatives were isolated from the ethyl acetate fraction of *L. sericea* leaf and β sitosterol. The activity of the two phloroglucinol compounds on the egg and larvae of *H. contortus* was determined and their toxicity using the MTT assay. The quantity of β sitosterol was not enough to allow for further assay.

The aim of fractionation was to potentiate the activity of the acetone leaf extract of *L. sericea.* The acetone crude extracts had an EC_{50} of 1.08±0.11 and 1.27±0.07 mg/ml for the egg hatch and larval development study for *L. sericea* (Adamu et al., 2012). The results for the extracts and various fractions of *L. sericea* were not different statistically. This shows that fractionation did not potentiate the activity of the fractions. The result of this study was within the range of values recorded by Ademola and Eloff (2010; 2011a) using the acetone extracts and fractions of *Combretum molle* and *Cassia alata*. The activity demonstrated by the extracts and fraction suggests the possibility of synergism of action between the secondary metabolites involved in the anthelmintic activity. The differences in the chemical constituents of the fractions could lead to multiple mechanisms of action. Thus synergism of action will help to delay the onset of resistance which may be useful for anthelmintic efficacy and the delay of anthelmintic resistance (Ademola and Eloff, 2011c). The hexane and ethyl acetate fractions of *L. sericea* have higher possibilities of containing compounds with anthelmintic activity. The butanol and chloroform fractions had poor EC_{50} compared to the crude acetone extract. The activity in the fractions was evident in the polar solvent. The bioactive compounds may likely be a polar compound, thus isolation process targeted the ethyl acetate component of our fractions.

For safety testing the activity of the fractions were evaluated on fungi and mammalian cells. The poor effect of our fractions in inhibiting fungal growth gave an insight to the selective activity of the fractions. The premise for the use of this assay as an indicator of general toxicity was to determine if non-specific inhibitory activity was present. An example of this can be seen with the condensed tannins. The protein precipitating activity of condensed tannins, lead to an excellent inhibitory activity *in vitro* which do not correspond in *in vivo* activity. In addition the fractions were relatively non-toxic to the Vero cell line with all LC_{50} being above 1.40 mg/ml. Fractionation led to fractions becoming less toxic when compared to the crude acetone extracts with LC_{50} of 0.016 mg/ml (Adamu et al., 2012). Based on the overall safety and efficacy of the different fractions, the ethyl acetate fraction was used for isolation of bioactive compound(s).

The compounds isolated were agrimol G, mixture of agrimols G and A, and β sitosterol. These compounds are been isolated for the first time in this plant and as far as authors know this the second report of compound been

isolated from this plant after Bosman et al., (2004) isolated Aspindinol. Agrimols G and A are phloroglucinol derivatives and include compounds such as kosotoxin and protokosin. The phloroglucinol derivatives are known anthelmintic compounds that have been isolated from *Hagenia abyssinica* and *Dryopteris ferns* (Lounasmaa *et al*., 1973). The activity of the two phloroglucinol derivatives further confirms this group of compounds have anthelmintic potential that can be explore for commercial purpose by synthetic production. Attempt at synthesising this compound has been unsuccessful. The phloroglucinol derivatives was not toxic at 200 mg/kg but was toxic at 50 mg/kg intraperitoneally in mice (Tibebe et al, 1992). Establishing that anthelmintic compounds such as agrimol G exist in the leaf of *L. sericea* and with high safety when administered orally gives a good indication for the use of this species. Even if this safety is due to poor availability from the gastrointestinal tract, it may still have merit as an anthelminthic as efficacy may be enhanced as poor absorption could result in high concentration within the abomasum. Unfortunately, there is also the possibility that it is been metabolised to inactive products by the bacteria in the gut lumen. The use of the leaf as a forage in sheep to help combat helminth infection may be a possible option in the continue search for cheap, renewable and eco-friendly alternative to chemical anthelmintic especially to poor farmers. Plants with phloroglucinol derivatives such as *Hagenia abyssinica* has been in use in Ethiopia and was part of many European pharmacopoeias as an anthelmintic (Lounasmaa *et al*., 1973).

6.5. Conclusions

This study report activity against fungi and Vero cells as a tool for accessing the toxicity of plant extracts. A general non-selective activity against micro-organisms and mammalian cells will probably imply a general metabolic toxicity and such extracts should be tested further for toxicity or be used with caution. The fractions had a selective activity due to the poor effect on fungi but very good anthelmintic activity. Fractionation of the acetone extracts did not lead to potentiation as no fraction showed better activity than the extract. Two phloroglucinol derivatives, agrimols G and A were isolated and have proven activity against egg and larvae of *H. contortus in vitro*. The leaf of *Leucosidea sericea* could be used to combat helminth infection in sheep. Further study will involve infecting sheep with *Haemonchus contortus* and treating with the leaf extract of *Leucosidea sericea*.

Table 6.1: Effect of fractions from acetone extract of *L. sericea* on the egg and larvae of *Haemonchus contortus* as well as their cytotoxicity and selectivity index

Fractions	Assay	EC$_{50}$ (mg/ml)	Cytotoxicity LC$_{50}$ (mg/ml)	Selectivity index
Hexane	EHA*	0.69	2.42	3.51
n-Butanol	EHA	1.68	330.20	196.55
Chloroform	EHA	1.39	4.18	3.01
Ethyl acetate	EHA	0.92	283.50	308.15
Hexane	LDT**	1.14	2.42	2.12
n-Butanol	LDT	2.28	330.20	144.82
Chloroform	LDT	1.70	4.18	2.46
Ethyl acetate	LDT	0.79	283.50	358.86

*EHA: egg hatch assay, **LDT: larval development test.

Table 6.2: Minimum inhibitory concentration (mg/ml) of fractions of the acetone extract of *Leucosidea sericea* against fungi pathogens as well as their selectivity index.

Fraction	Duration (h)	Fungi (MIC values in mg/ml)				Selectivity index
		C. neoformans	*C. albicans*	*A. fumigatus*	*Average*	
Hexane	24	2.50	2.50	0.63	1.88	1.29
	48	2.50	2.50	1.25	2.08	1.16
Ethyl acetate	24	2.50	2.50	0.63	1.88	151.07
	48	2.50	2.50	1.25	2.08	136.08
n-Butanol	24	2.50	2.50	0.63	1.88	175.95
	48	2.50	2.50	1.25	2.08	158.50
Chloroform	24	0.63	0.31	1.25	0.73	5.73
	48	1.25	0.31	1.25	1.35	3.09

Table 6.3: Anthelminthic activity of compounds (**2** and **2/3**) from *L. sericea* on the egg and larva of *H. contortus* as well as their cytotoxicity and selectivity index

Compounds	Anthelmintic activity EC$_{50}$ (mg/ml)		Cytotoxicity	Selectivity index	
	EHA*	LDT**	LC$_{50}$ (mg/ml)	EHA	LDT
Agrimol G (**2**)	0.52	0.08	6780	13038.46	84750
Agrimols G and A (**2/3**)	0.28	0.11	4030	14393	36636

*EHA: egg hatch assay, **LDT: larval development test
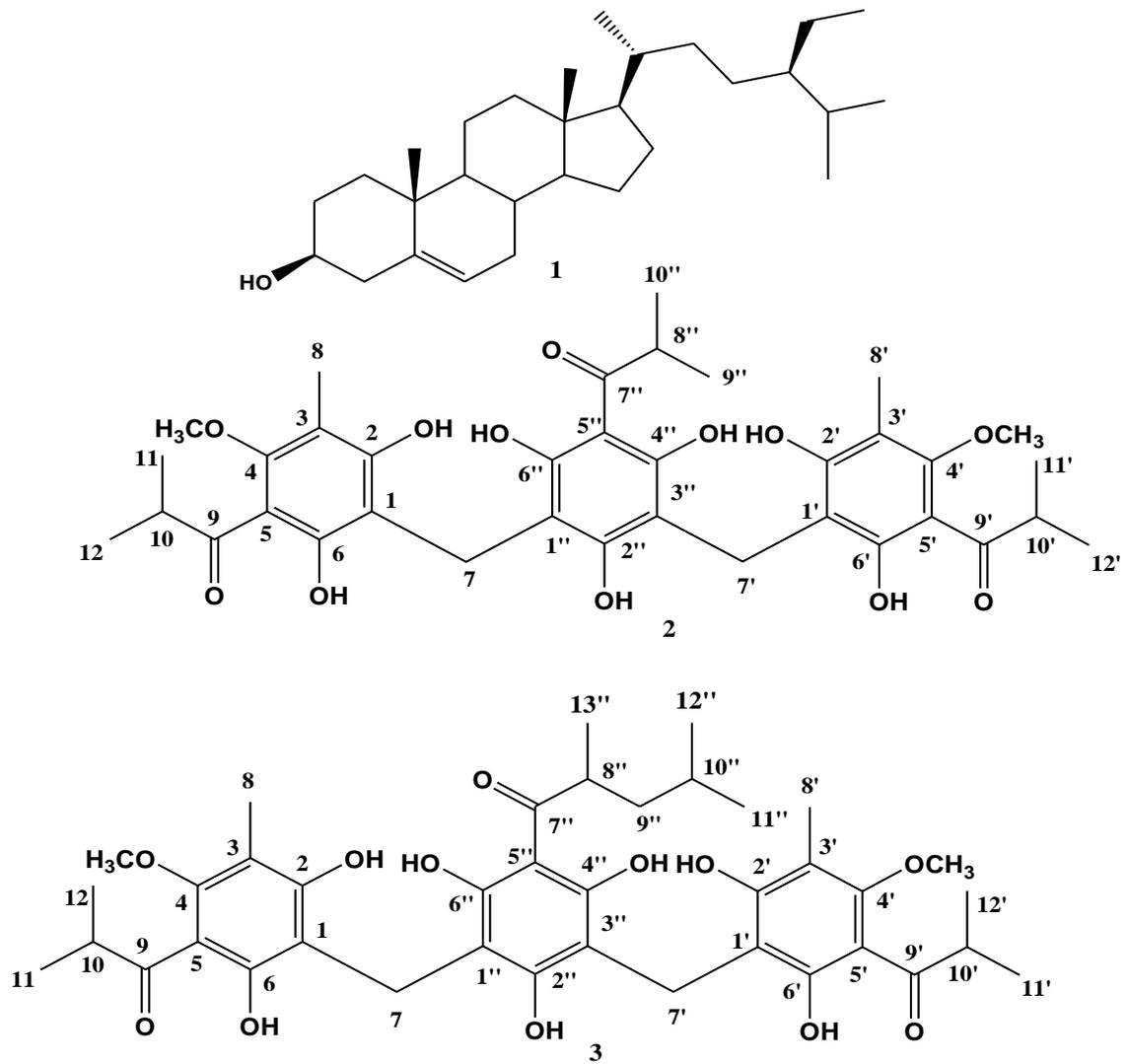
Figure 6.1: Structures of compounds (1 - 3) from *Leucosidea sericea*
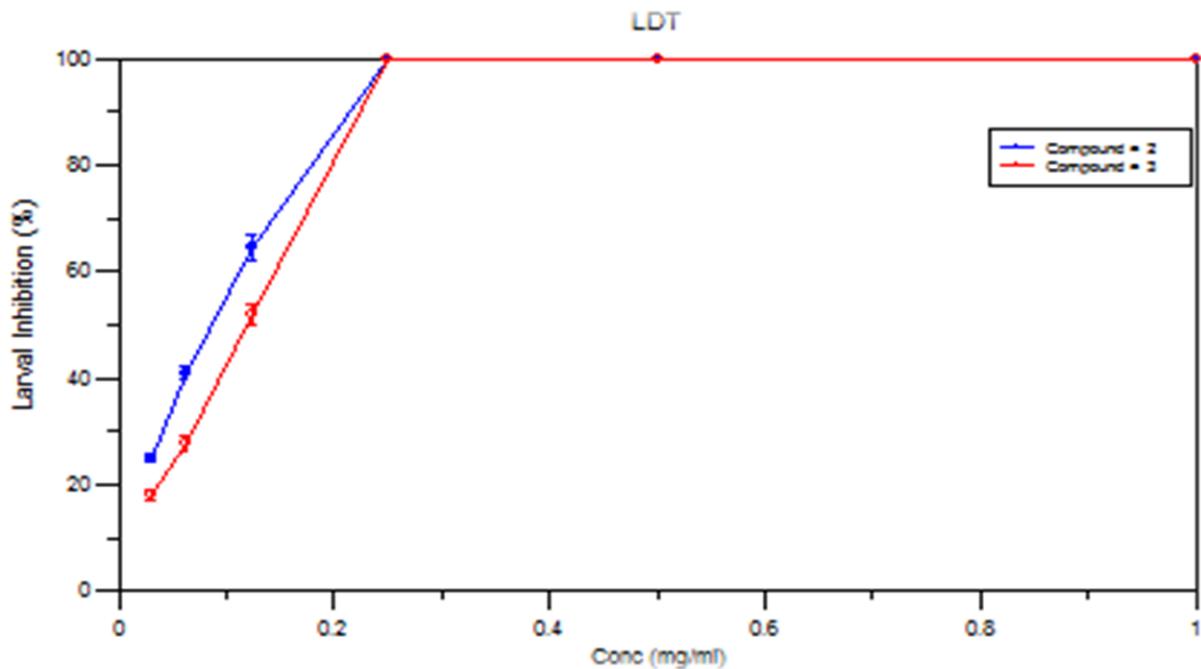
Figure 6.2: Larval development inhibition test of agrimol G and agrimol G and A
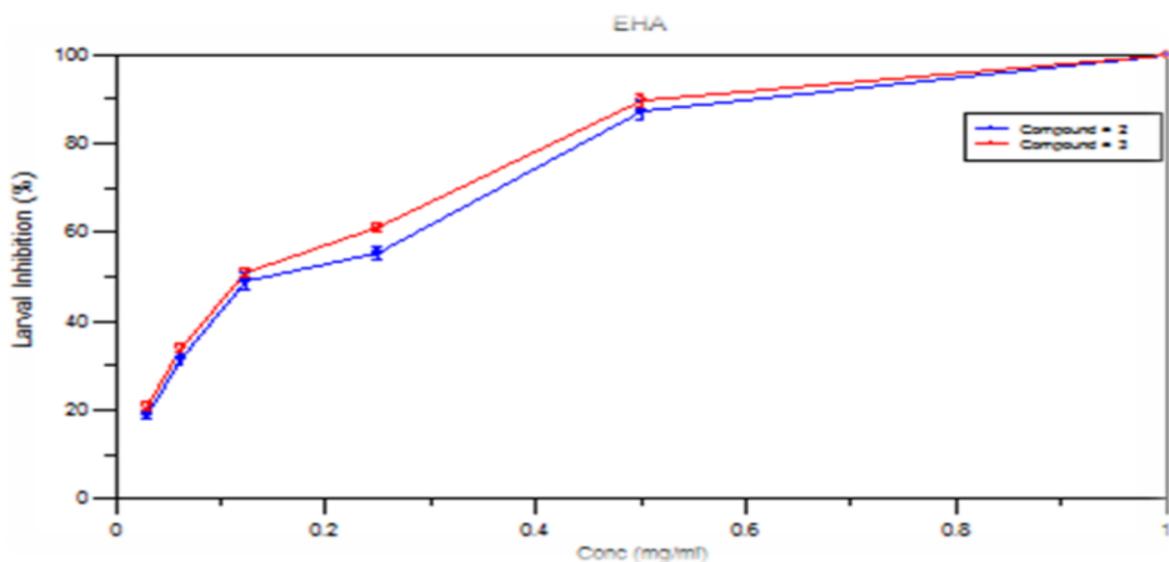
Figure 6.3: Egg hatch inhibition of agrimol G and agrimol G and A

Postscript: The two anthelmintics compounds isolated from *L.sericea* were isolated for the first time from this plant. There was need to evaluate the effect of this compound and the extract on the ultrastructure of adult *Haemonchus Contortus.* This may give a better insight on the possible mechanism of action of these compounds and our extracts.

Chapter 7: Establishing the effects of phloroglucinol derivatives isolated from *Leucosidea sericea* on the ultrastructure of *Haemonchus contortus*, using electron microscopy

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Preface: The activity of extracts and compounds isolated need to be further evaluated to help in understanding the possible mechanism of action. The effects of extracts, compounds and anthelmintic drugs ivermectin and albendazole with PBS control on adult *Haemonchus* ultrastructure may help to understand the possible mechanism of action.

Abstract

Plant extracts used for the treatment of helminth infections in sheep are an alternative to chemical anthelmintics. Previous studies have reported the anthelmintic activity of acetone leaf extracts of *Leucosidea sericea*. However the mechanisms of action remain unknown. The aim of this study was to determine through scanning and transmission electron microscopy the effect of the acetone leaf extract of *L. sericea* and two phloroglucinol derivatives on the ultrastructure of *Haemonchus contortus*. A positive drug control treatment using albendazole and ivermectin was included. The main changes identified were the alteration and destruction of the cuticle, changes to the hypodermis, vacuoles within the cytoplasm, cytoplasmic degradation, cellular swelling and in a few cases, some abnormality within the mitochondria. The control for albendazole and ivermectin had the expected internal changes of destruction of internal architecture and paralysis respectively. The lesions seen were probably due to bioactive plant metabolites and the route of entry. The possible mode of action is direct as it depends on contact between the bioactive plant metabolite and *Haemonchus contortus.*

7.1 Introduction

Haemonchus contortus is an abomasa nematode parasite of production animals. While the parasite can occur in any of the domestic ruminant species, sheep are the major susceptible species. Once infected, Haemonchus can induce substantial production losses which can only be ameliorated through the use of relatively costly anthelmintics. Unfortunately, through years of use, Haemonchus has developed severe resistance to the major class of anthelmintics with the result that sheep farming in certain areas of the world are facing major constraints. Not surprisingly, the demand for new anthelmintic has increased research in the development of new agents. In addition, with new focus being given to environmental toxicity, phytochemicals are commonly being screened for their effect and mechanism of effect (Waller, 2004, Hoste et al., 2006). *Leucosidea sericea* leaf extracts, of the Rosaceae family, has been reported to have good anthelmintic activity *in vitro* (Bosman et al., 2004, Aremu et al., 2010. Adamu et al., 2012). After extensive literature review, the possible mechanism by which the extract has its anthelmintic effect remains unknown.

While the anthelmintics have different mechanisms of action, they tend to function within two broad categories. The first being parasitic paralysis and expulsion due to an inability of the parasite to maintain its position within the gastrointestinal tract against peristalsis. The drugs that promote parasitic paralysis function by targeting the parasite neuromuscular control pathways via the GABA, nicotinic, potassium channels or acetyl choline receptor systems. The agents which function via this mechanism are the macrocyclic lactones such as abamectin, doramectin, eprinomectin, ivermectin, moxidectin, the amino acetonitrile derivatives (AADs) with Monepantel as the sole drug in this group and the imidazothiazoles examples are levamisole, tetramisole. On electron-microscopy, the visible effects are minor as the parasites are usually alive. A major effect seen is muscle contraction which is evident as a contracture of the parasite.

The second mechanism is the interference with energy production and subsequent parasite starvation and lysis. One way of achieving this mechanism is by inhibition of the Fumarate Reductase system and subsequent inhibition of glucose transport leading to starvation and includes the benzimidazoles such as albendazole. On electron microscopy, the commonly seen signs are loss of micro-villi and general vacuolisation. Another way is by the uncoupling of oxidative phosphorylation leading to a failure to produce energy with subsequent depletion of energy reserves and eventual death of the parasite and includes the salicylanilides like closantel, niclosamide-bases, oxyclozanide and rafoxanide or the diphenylsulphides, such as bithionol. On electron microscopy, the commonly seen signs are mitochondrial death and vacuolisation.

The mechanisms of actions for extracts with anthelmintic activity are usually not reported. The aim of this study is therefore to investigate through scanning and transmission electron microscopy the general class of effect of the extract of *L. sericea* and the two phloroglucinol derivatives have on the ultrastructure of *Haemonchus contortus* through the use of electron microscopy.

7.2 Material and Methods

7.2.1 Study compounds

Plant material (1 g) was extracted with 10 ml of acetone, (technical grade, Merck) in a polyester centrifuge tube as previously undertaken with studies demonstrating efficacy (Adamu et al., 2012). The tube was vigorously shaken for 30 min on an orbital shaker, centrifuged at 4000 g for 10 min, filtered through Whatman No.1 filter paper and finally dried under a stream of air in a fume hood at room temperature. The extract was reconstituted in 2.5% DMSO and tested in the assays. The two phloroglucinol derivatives, agrimol G and a mixture of agrimols G and A, previously isolated from the leaves of *L. sericea*, were also evaluated. These pure compounds were made up to a concentration of 1200 µg/ml in 5 % DMSO.

7.2.2 Parasite

Adult *H. contortus* parasites were obtained from the abomasum of infected sheep on the day of slaughter of sheep following an *in vivo* study (Study was approved by the University of Pretoria animal use and care committee). The abomasum was tied up from both ends and washed into a bucket, and the motile adult parasite removed by manual hand picking with the help of a needle and parasite identity was confirmed using MAFF (1986) guidelines. A minimum of 10 adult parasites were then put into tubes and incubated for 3 h at 20 °C in an incubator with the extract of *L. sericea*, the 2 phloroglucinol derivatives, albendazole or ivermectin, all at a concentration of 1200 µg/ml. A PBS negative control was also included. After incubation, the parasites were washed three times in PBS and then transferred into 2% glutaraldehyde solution for further processing.

7.2.3 Transmission Electron Microscopy

Samples for electron microscopy were fixed in 2.5 % glutaraldehyde. Thereafter, the fixative was carefully decanted into appropriate waste bottles by means of a pipette. After post-fixation in osmium tetroxide for 1 hour, the tissue blocks were rinsed in Millonigs buffer for 10 min, distilled water for a further 20 min. Thereafter, the specimens were dehydrated using a graded series of ethanol (50%, 70%, 80%, 96%, and 100%) for 10 min each. The sections were then infiltrated with Mollifex twice for 10 min and propylene oxide twice for 10 min each. The sections were then infiltrated with propylene: Epoxy resin mixture at a ratio of 2:1 for 30 min to 1 hour, and then embedded in a 100% epoxy resin overnight. The specimens were cured in embedding oven at 65 degrees Celsius overnight. Polymerised resin blocks were now ready for ultramicrotomy. Ultra-thin resin sections were contrasted with uranyl acetate and lead citrate before being examined in a Philips CM10 Transmission Electron Microscope (TEM) operated at 80 kV.

7.2.4 Scanning Electron Microscopy

Samples for electron microscopy were fixed in 2.5 % glutaraldehyde. Thereafter, the fixative was carefully decanted into appropriate waste bottles by means of a pipette. After post-fixation in osmium tetroxide for 1 hour, the tissue blocks were rinsed in Millonigs buffer for 10 min, distilled water for a further 20 min. Thereafter, the specimens were dehydrated using a graded series of ethanol (50%, 70%, 80%, 96%, and 100%) for 10 min each. The ethanol was discarded and replaced with equal volume of HMDS in a fume cupboard. Specimens were incubated for 30 minutes at room temperature and then resuspended in a small volume of HMDS. A drop of this suspension was placed on a coverslip and air dried. After drying the specimen were coated with gold and viewed at different magnifications using a Zeiss Scanning Electron Microscope.

7.3. Results

A total of ninety four SEM micrographs and fifty one cross sections for TEM were recorded for adult *Haemonchus contortus* following treatment with acetone extract of *Leucosidea sericea,* albendazole, agrimol G, agrimol A, Ivermectin and PBS control. Several ultra-structural internal changes were seen in the treated parasites. The changes seen were cuticular damages, general vacuolization and loss of mitochondria cristae, marked intracellular disorganisation (Fig 7.2). The agrimol G and a mixture of agrimols G and A treatments led to some degenerative changes (Figs 7.3 and 7.4) albeit not as pronounced as the extract. The lesions seen were damages to the cuticle and slight disorganisation of the internal structures such as vesicle formation (Figs 7.3 and 7.4).

The parasites incubated in PBS negative control had a clear intact cuticle and hypodermis (Fig. 7.1). The internal morphology was well preserved when compared to the treated specimens. The parenchyma was without vacuoles and the general architecture of the parasite was well preserved. The microvilli retained its tubular shape characteristic of a digestive cell. The parasites incubated with ivermectin also showed no evidence of damage to the external and internal structures (Fig 7.5), except that the muscular layer showed clear evidence of contraction which was not evident in the negative control specimens. In contrast albendazole treated parasites had a normal cuticle (c) and underlying hypodermal tissues (Fig 7.6), with an overall increase in electron lucency of the parenchyma, and loss in the microvilli lost its architecture in the presence of normal musculature.
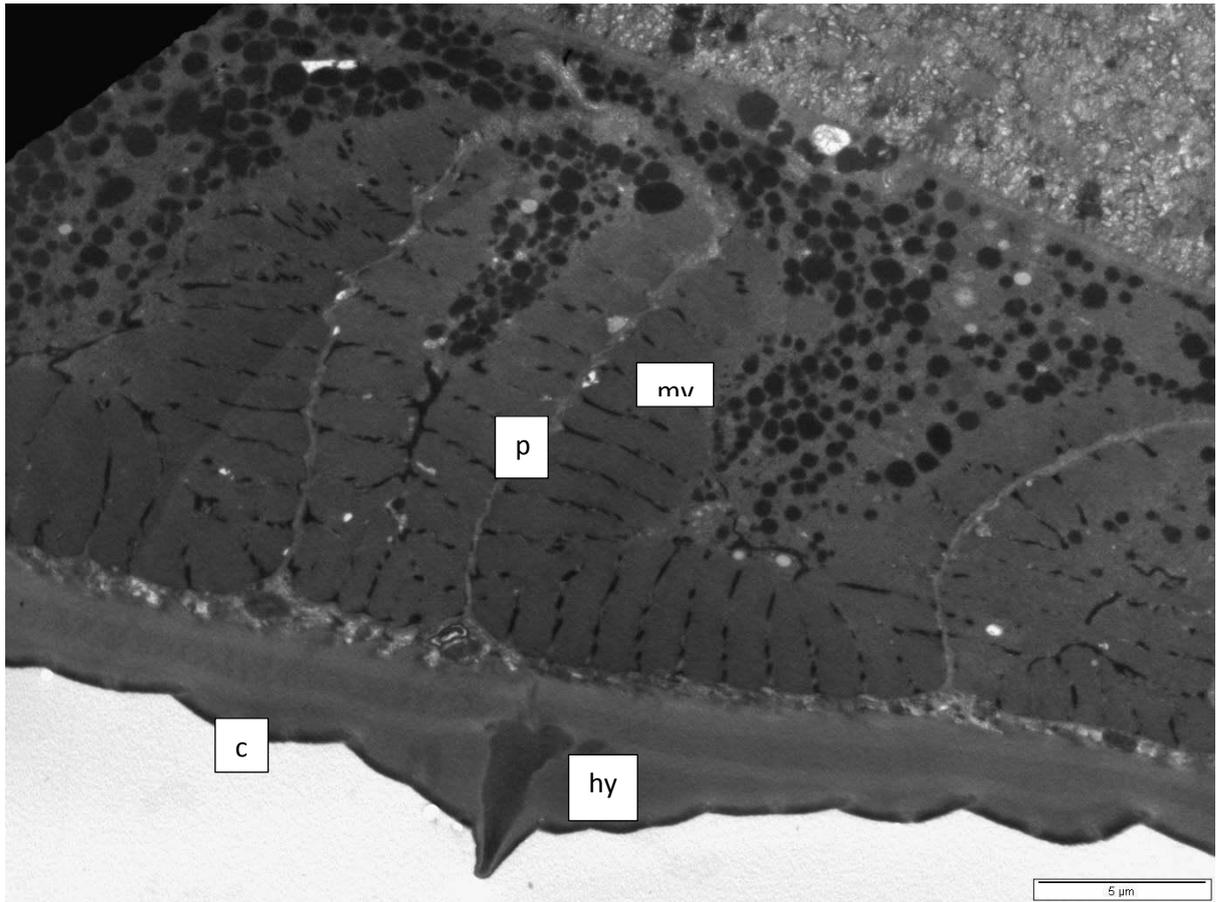
Figure 7.1: TEM micrograph of adult *Haemonchus contortus* after incubation for 3 h in Phosphate buffer saline (PBS), showing intact cuticle (c), hypodermis (hy), parenchyma (p) and microvilli (mv).
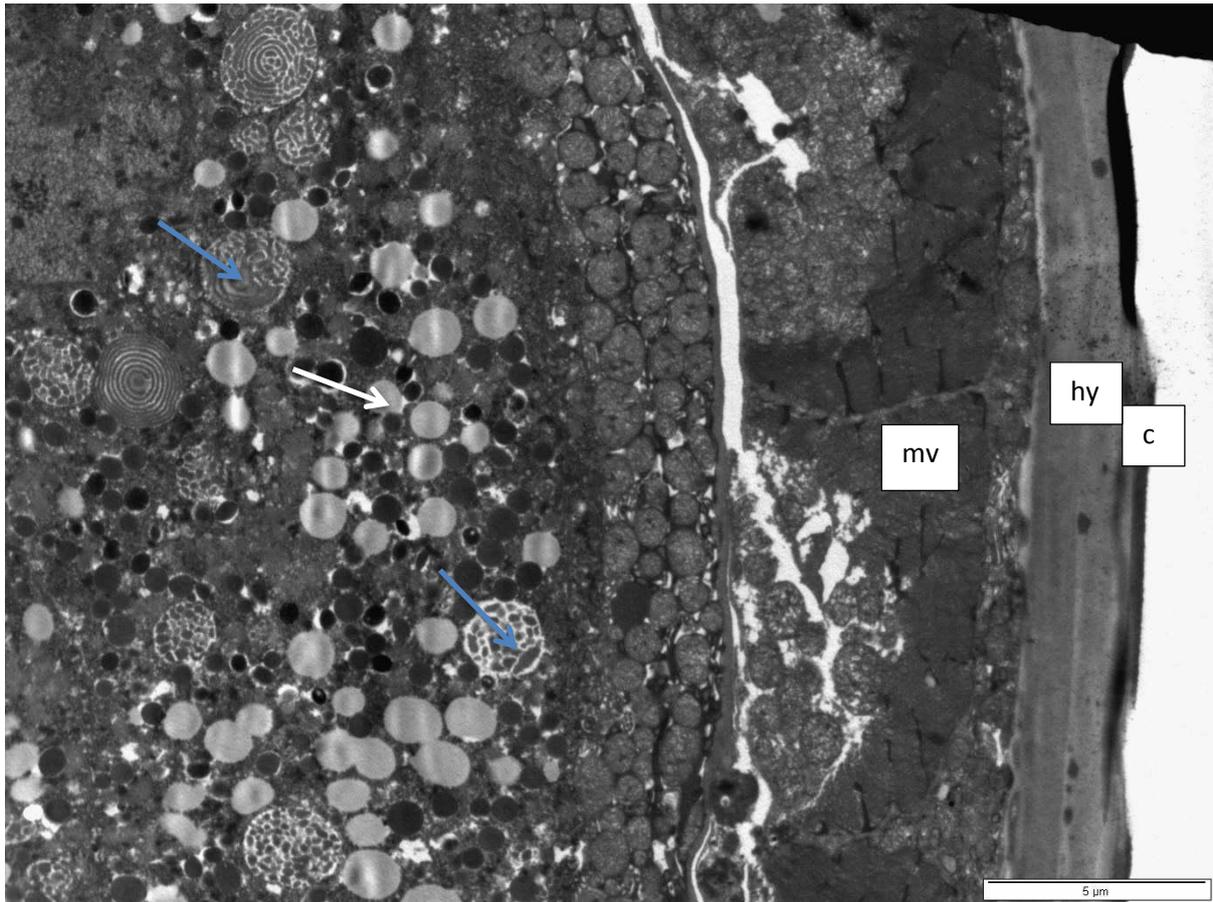
Figure 7.2: TEM micrograph of *Haemonchus contortus* after 3 h incubation with 1200 µg/ml acetone leaf extract of *Leucosidea sericea* showing cuticular damages and vacuolization of the parenchyma (White arrow) and a loss or complete lack of cristae within the mitochondria (Blue arrow) and marked intracellular disorganisation. Microvilli (mv) showing loss of architecture.
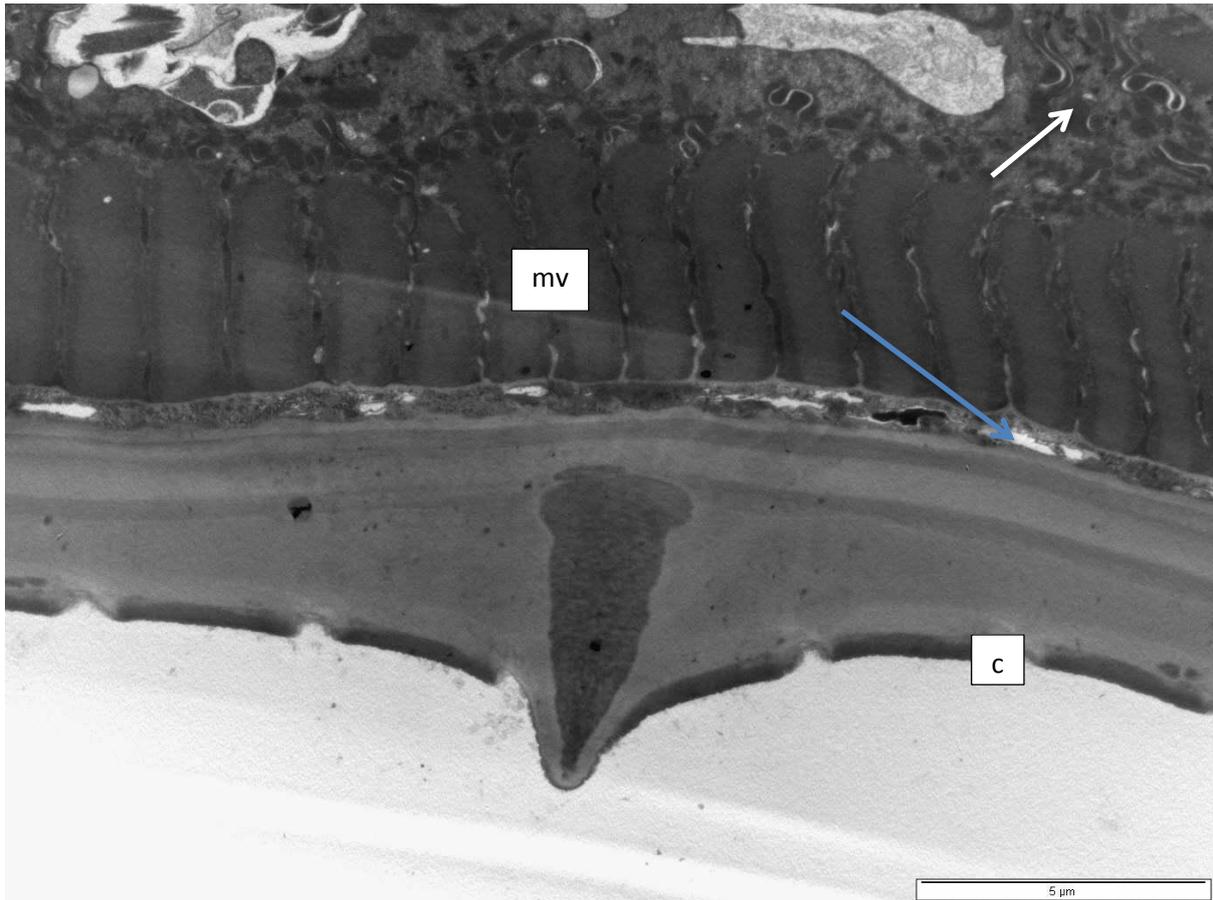
Figure 7.3: TEM micrograph of *Haemonchus contortus* cross section following treatment with agrimol G at 1200 µg/ ml isolated from the leaf extract of *Leucosidea sericea* showing cuticular damage, breakup of tegumental cells (Blue arrow) and slight parenchyma disorganisation and accumulation of vesicles (white arrow). Microvilli lost its normal architecture and look dense and compact.

Figure 7.4: TEM micrograph showing cross section of *Haemonchus contortus* following treatment with 1200 µg/ml of agrimols A&G showing slight cuticular damages and parenchyma damages (white arrow) and vesicle formation (Blue arrow).
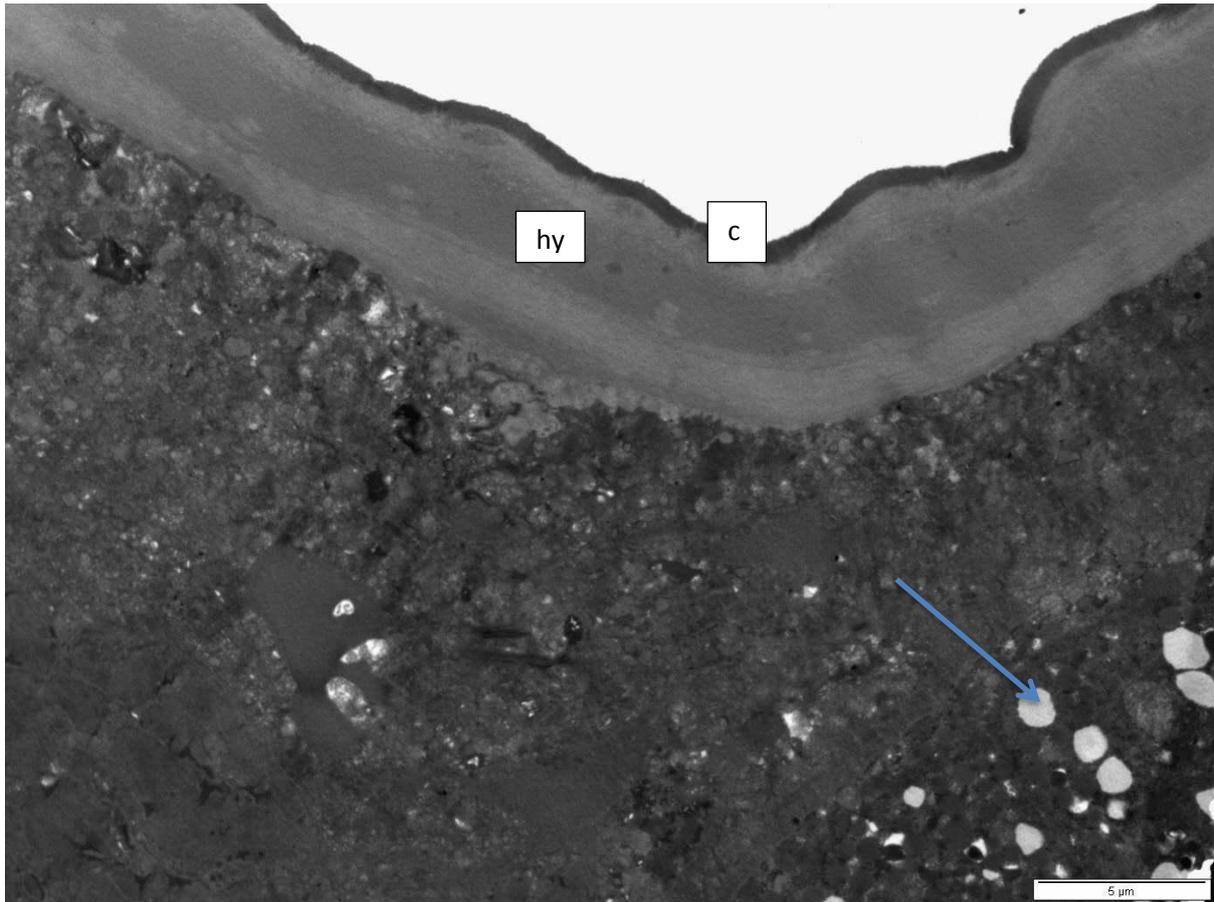
Figure 7.5: TEM micrograph showing cross sections of *Haemonchus contortus* following treatment with ivermectin at 1200 µg/ml showing the cuticle(c) and hypodermal muscles (hy) in a contracted form, possibly due to paralysis of the muscular layer, vesicle formation and overall increase in electron lucency.
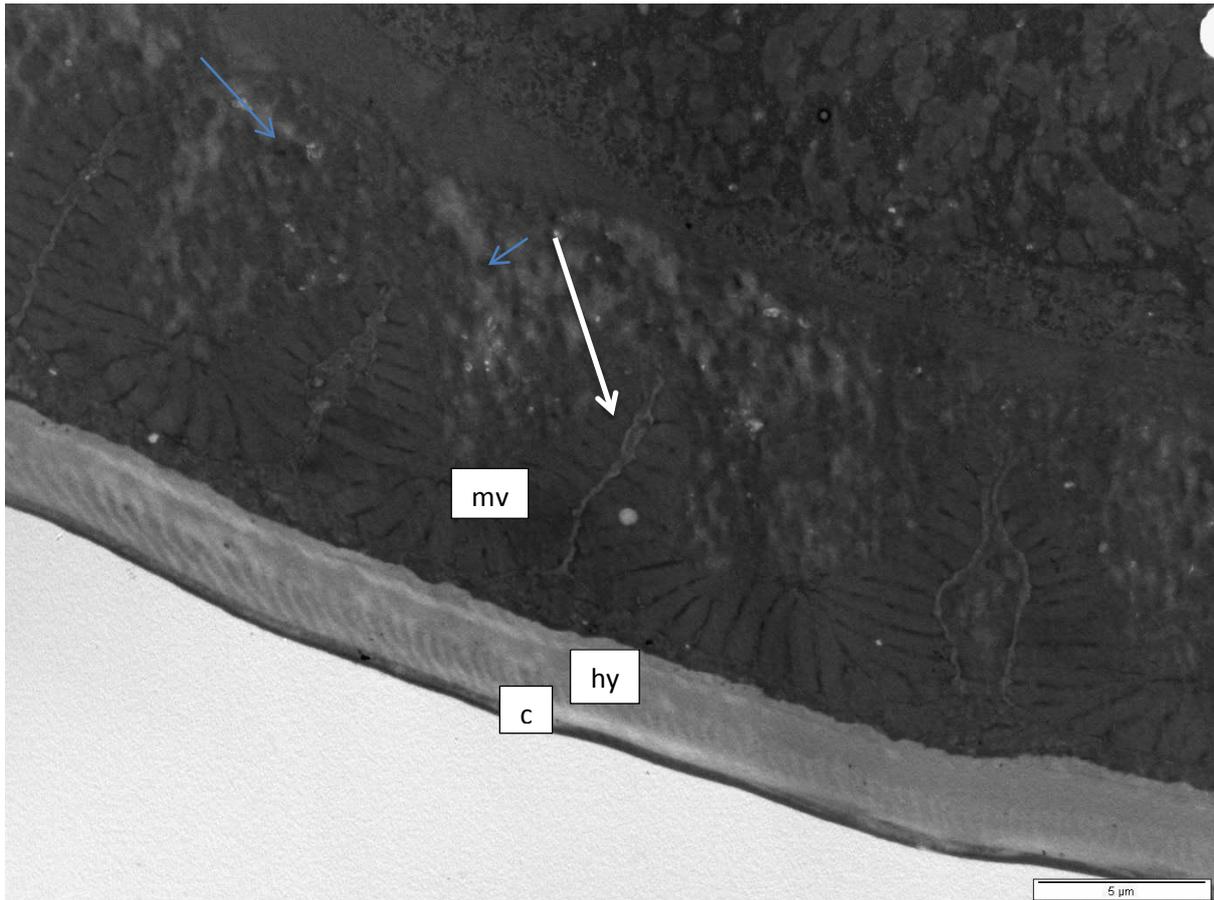
Figure 7.6: TEM micrograph showing cross section of *Haemonchus contortus* following treatment with albendazole at 1200 µg/ml showing normal cuticle (c) and underlying hypodermal tissues . See overall increase in electron lucency of the parenchyma (white arrow) and loss of digestive cell architecture seen with the microvilli. Blue arrow showing secretory granules.

The SEM was used to demonstrate the effects of treatments on the outer surface of the parasites. The albendazole treated (Fig 7.7A) and the *L. sericea* acetone treated parasites (Fig 7.7B) both showed similar external surface disruption, albeit with the albendazole effect being more marked. This was characterised by distorted circular and longitudinal annulations of the parasites. Severe damage to the parasites were also seen in Figs 8C and 8E for parasites treated with agrimol G and agrimols A & G, evident as the complete destruction of the circular and longitudinal annulations of parasites (Fig 7.8D). The vulval flap seen in Fig 7.8D was also damaged. The parasite treated with ivermectin (Fig 7.8E) showed less damage to the parasite morphology in comparison to those treated with extract, compounds and albendazole, with the circular and longitudinal annulations being preserved. The parasite in PBS control (Fig7. 9) showed clear and intact outer surface morphology, with the longitudinal annulations and the entire cuticular surface well preserved.
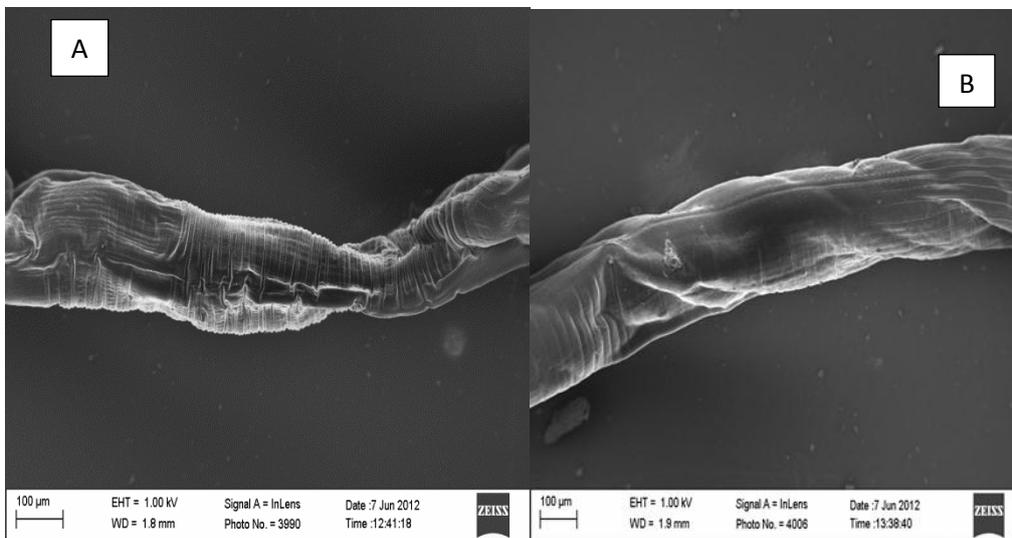
Figure 7.7: Scanning electron micrograph showing *Haemonchus contortus* following treatment with albendazole (A) and acetone leaf extract of *L.sericea* (B) with marked damage to the cuticular surface.
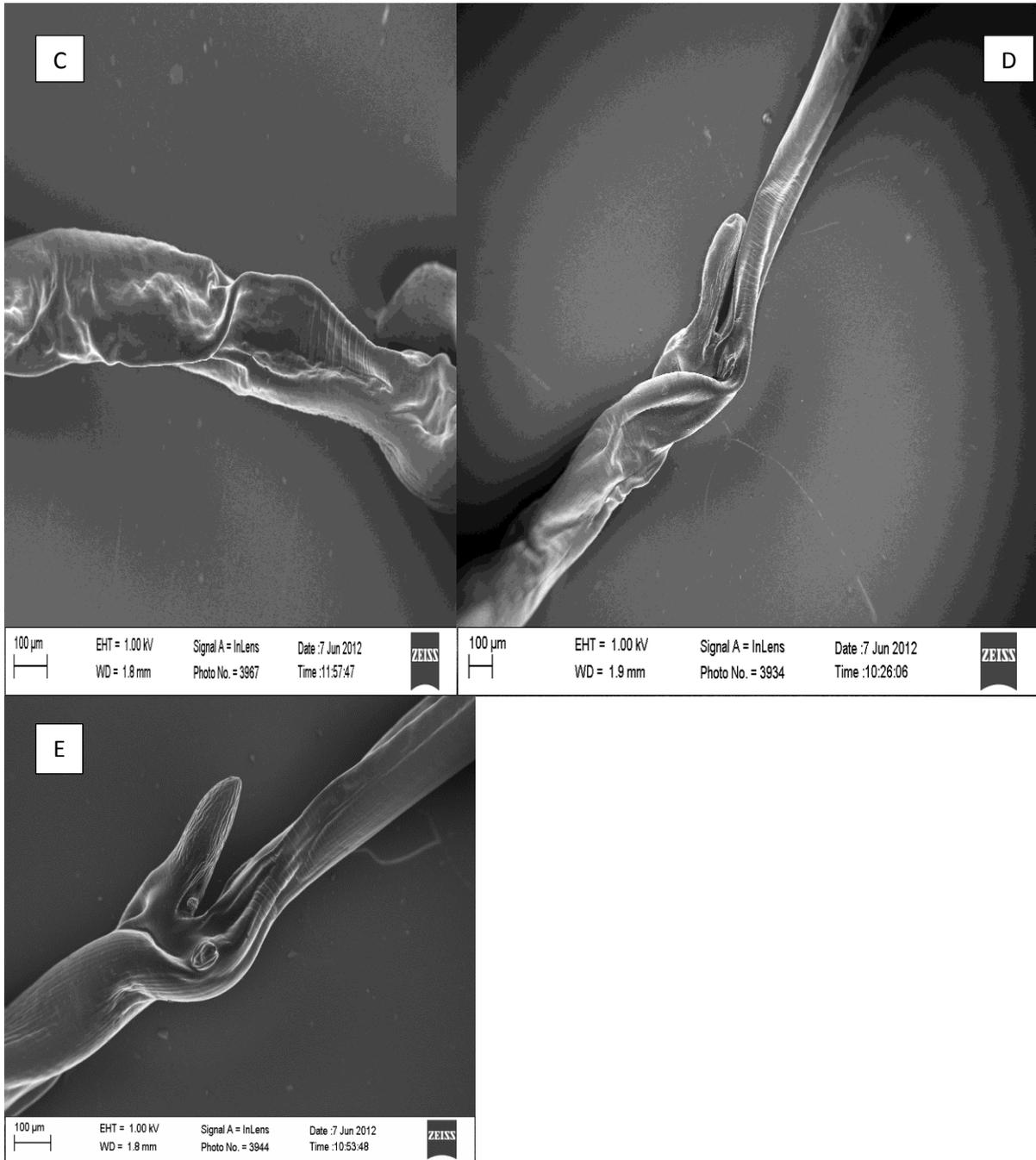
Figure 7.8: Scanning electron micrograph showing *Haemonchus contortus* after treatment with agrimol G(C), agrimol A (D) and Ivermectin showing cuticular damage.
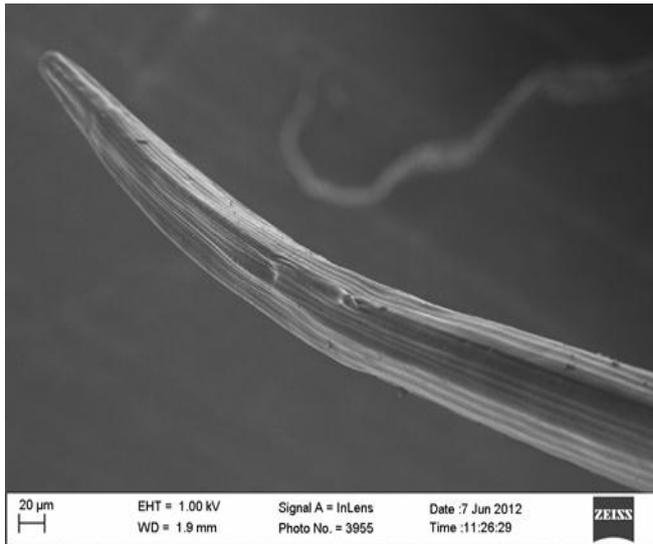
Figure 7.9: Scanning electron micrograph showing *Haemonchus contortus* following incubation for 3 h in PBS control with smooth cuticular surface and clear longitudinal annulations

7.4. Discussion

The main objective of this study was to understand the possible mode of action of *Leucosidea sericea* extract and their phloroglucinol compounds against adult *H. contortus*. This is the first report describing harvesting and incubation of adult H. *contortus* to determine the effect of extract or compounds on their ultrastructure. Three hours was used to incubate the parasites, to allow sufficient time for the penetration of the drugs or extracts into the parasites. This time period was in accordance with earlier studies using third stage larvae of this parasite by Brunet et al., (2011). Two major changes were observed following exposure of *H. contortus* to the extract and compounds of *L. sericea*, ivermectin and albendazole. These changes were on the cuticle and on the internal structures. The changes observed can be related to the biology of the parasite, its susceptibility and the way the anthelmintic penetrates the cell. The PBS control worms showed no abnormalities with every aspect of parasite morphology being intact.

For the ivermectin treated group, the parasite were contracted which is consistent with spastic paralysis as a known mechanism of action for ivermectin, levamisole or pyrantel. In all three cases these substance induce contraction by interfering with neurotransmitter activity. For ivermectin, this action is via the stimulation of GABA receptor while for the levamisole/pyrantel it involved inhibition of acetylcholine esterase. Based on the absence of contractile activity in the extract and pure compound treated worms, it appears that the mechanism of activity is not related to change in muscular activity or parasite motility.

In the albendazole treated adult parasites, changes seen were disruption of the intestinal microvilli and increased electron lucency, in agreement with findings of Beugnet et al., (1996). From a mechanistic standpoint, albendazole acts by interference with energy production by inhibiting Fumarate Reductase and blocking tubulin synthesis. It also induces mitochondria damage through the direct effect of uncoupling oxidative phosphorylation (McCracken and Sillwell, 1991). While the damage to the mitochondria was not evident for this study, there was evidence of altered energy utilisation as evident by the lucency, which would indicate the parasite changing energy utilisation to stored depots. We can only speculate that if the parasites were exposed for longer period, more signs of parasitic death such as mitochondrial damage would have been present as seen for the 6-48 h incubation of Beugnet (1996).

For this study the extract showed signs of microvilli damage, integument damage, damage to the mitochondria, and vacuolisation. From a mechanistic point, all the effects except the integument damage can be explained by interference with energy metabolism. From literature, the two major classes which induce this effect are the benzimidazoles and the niclosamides. For the former the effect is described above and the latter the effect is due to inhibition of mitochondrial electron-chain transport. In comparison to the albendazole, the plant extract induced more severe changes making it unlikely that the effect is due to a benzimidazole like effect. In contrast the effect is almost identical to that published for closantel in effect and time (Rothwell and Sangster 1996).

Therefore it is likely that the effect is in part due to interference with mitochondrial function, probably at the level of the electron-chain transport. This disruption of the cuticle by the extract is not however explained by the disruption of the electron transport chain. This leads to the possibility that the extract is effective due to the presence of two of more compounds with difference mechanisms of action.

This is clearly evident by the effects produced by the isolated compounds. Both compounds interfered with the cuticle with the absence of major internal disruption. As a result these compounds would be responsible for the cuticular effect on the parasite while yet unidentified compounds are responsible for interfering with energy generation. The effect on the cuticle has a serious implication on the survival of the parasite since it is the outer layer that provides protection between the environment and the internal structures.

This was the first attempt at using SEM to study the effect of a plant extract on this parasite. The results of the SEM did not provide any useful information on the effect of the various treatments on the parasites. This may be due to the fact that the SEM did not expose the effect of the various treatments as was the case with the TEM. Thus TEM appears to be a better tool for studying effect of extracts or drugs on nematodes of animals.

This study has reported on the effect of extracts and compounds on adult *H. contortus* as a means of understanding how extracts acts on the parasite. Future study should look at molecular changes that may be associated with treatment of parasites with extracts.

From our results it does appear that the effect of the extract of *L sericea* is probably due to two different points of action, energy metabolism and cuticle damage. The latter effect appears to be due to the presence of agrimol G and a mixture of agrimols G and A. The former effect may be due to an unknown compound that exist in the extract but has not been isolated. Future study should target isolation of more compounds and evaluating their effect on the parasite.

Postscript: The activity of the acetone extracts of *L. sericea* showed more damaging effects on the parasites than the compounds isolated. Considering the small quantity of compounds we had and the efficacy when compared to the extract. We decided to use the acetone leaf extract of *L. sericea* to evaluate its effect in sheep infected with *H. contortus* on faecal egg count reduction.

Chapter 8: The anthelmintic effect of acetone leaf extract of *Leucosidea sericea* in sheep artificially infected with *Haemonchus contortus*

M. Adamu, J.N. Eloff, V. Naidoo

Abstract

A two phase independent study was carried out to evaluate the effect of an acetone leaf extract of *L. sericea* with *in vitro* activity to the parasite in sheep artificially infected with *H. contortus.* Following infection and treatment at 109 mg/kg body weight a 73.1 % reduction in faecal egg counts was achieved. At a dosage of 500 mg/kg body weight we achieved 83.5 % reduction in faecal egg counts. Albendazole had a 100 % reduction following a single treatment. The results of the liver enzyme assay also support our clinical observation as ALT levels were within normal range during this study. Following infection, all animals had a lowered haematocrit at the time of the treatment but the differences were not statistically significant and all values were within the normal range. Eosinophil's values also increased following infection in phase 2 of this study. The extract may have some immunomodulatory effect there by slowing down cell mediated immunity and supporting humoral immunity. This extract had no toxicity at the highest dosage used. The use of this plant extract had a beneficial effect on the treatment and control of *Haemonchus* infection in sheep. Higher dosages may lead to a high enough concentration within the abomasum to lead to complete control.

8.1 Introduction

The nematode *Haemonchus contortus* is the single most economically important sheep parasite in the world (LeJambre et al., 2008). Currently, anthelmintic therapy is the main means of combating infection. However, resistance development by *H. contortus* to all major anthelmintic groups has been reported except for the new anthelmintic monepantel and derquantel (Kaminsky et al., 2009, Little et al., 2010). Resistance development to available anthelmintic leads to increase cost of production, loss in production in terms of meat and milk, infertility and mortality. As a result newer treatment remedies need to be found. For this reason various control options are being investigated, one of which is the use of plant and plant derived products. Currently, few veterinarians in clinical practice have accepted the use of plants and plant derived products. This may be due to lack of scientific validation of most plant anthelmintic, lack of standardization of dosages and toxicity profiles of most products.

Leucosidea sericea, a tree belonging to the family Rosaceae, has been used as a deworming agent in South Africa (Watt and Breyer-Brandwijk, 1962). This tree is known as umTshitshi by the Zulu's, isiDwadwa by the Xhosa and Ouhout in Afrikaans. It is often a straggly shrub or a dense, small, evergreen tree, which grows up to 7 m in height (Watt and Breyer-Brandwijk, 1962, Coates-Palgrave, 2002). Studies on the *in vitro* anthelmintic activity have previously been reported using *Caenorhabditis elegans and H. contortus* (Bosman et al., 2005; Aremu et al., 2010, Adamu et al., 2012). The *in vitro* results indicated a minimum lethal concentration of 0.26 mg/ml for the ethanol and dichloromethane leaf extracts against *C. elegans* (Aremu et al., 2010) and an EC_{50} of 1.08 mg/ml and 1.27 mg/ml against the egg and larvae of *H. contortus* (Adamu et al., 2012). Anthelmintic compounds have also been isolated from leaf extracts of this plant. Aspindinol (Bosman et al., 2005) and two phloroglucinol derivatives agrimol G and agrimol G and A (Adamu et al., 2012) were isolated. The possible mechanism of action of phloroglucinol derivatives may be via degenerative changes within the parenchyma and vacuolization of adult *Haemonchus* parasite (Adamu et al., 2012).

Despite the promising *in vitro* activity no study has yet reported on the anthelmintic activity of this plant in animal experiment. The aim of this study was to evaluate the anthelmintic activity of the acetone leaf extracts of *L. sericea* in a controlled dose finding study in a sheep artificial model of haemonchosis.

8.2 Materials and methods

The procedures used in this study were approved by the Animal Use and Care Committee (AUCC) at the Faculty of Veterinary Science University of Pretoria (Ref V035-11) in accordance with the international guidelines for the use of animals in experimentation.

8.2.1 Plant material and extract preparation

The leaves of *L. sericea* were collected at the National Botanical Garden in Pretoria, South Africa. After identification, voucher specimens were kept in the Department of Paraclinical Sciences, Faculty of Veterinary Science University of Pretoria (voucher number PRU 288). The dried leaves were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eriez®, Bramley) and stored at room temperature in closed containers in the dark until used. Extraction was carried out on finally ground plant material with a 10:1 ratio of acetone to plant material using standard procedures (Eloff, 2004).

8.2.2 Infective larvae

Third-stage larvae were obtained from a donor sheep infected with a pure strain of *H. contortus* (Bosvet, Pretoria). Once the infection became patent (presence of nematode eggs in faeces) the faecas was collected, and cultured at 28 °C for 7 days. The resulting third stage larvae (L3) were harvested using a modified Baermann technique (Hansen and Perry, 1993).

8.2.3 Animals and experimental design

Experimental design was according to the method recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Wood et al., 1995) with slight modification as the number of animals per group was reduced from 15 to 3 as reported by Lone et al., (2012) for discovery studies. Dorper sheep were bought from reputable breeders for this study. The animals were housed indoors at the University of Pretoria Biomedical Research Centre (UPBRC), Onderstepoort. The animals were fed an *ad lib* standard diet of lucerne and teff hay, with sheep pellets offered in limited quantities in the evenings. The animals were kept for 4 weeks for conditioning and acclimatization. All sheep were vaccinated with pulpy kidney vaccine from Onderstepoort biological products before they were infected with the parasites. The faecal egg counts and other blood parameters were evaluated and all animals were found to have haematology values within normal range. The animal's temperature and general health conditions were observed and recorded daily. A cut-off point of salvage treatment for anaemia was a packed cell volume (PCV) value of 15%.

The phase one of the study had a four way parallel study design with animals randomly assigned to positive control, negative control or tests group with the help of table of random numbers using weight of animals. Animals were hereafter infested with 3500 Larvae of *H. contortus* and allowed 6 weeks for parasite to develop. Mono-specific environmental strains of larval suspensions of *H. contortus* were obtained from Bosvet (Pretoria). A total of 3500 infective larvae were administered orally with a syringe in three divided doses over a 3 days period. The animals were monitored for presence of eggs in faeces 3 weeks post infection. The faecal egg count was monitored three times a week for duration of the experiment. Treatment with extracts and control drug were

instituted upon attainment of a faecal egg count of 1000 and above. Faecal sample were monitored for eggs and counted using the McMaster method (Soulsby, 1982).

For the second phase of an independent study 8 male Dorper sheep were used. This second phase made use of a two way parallel study with animals randomly assigned to control and test group. This phase followed the same procedure as phase 1.

8.2.4.1 Treatment procedure

Each animal was weighed before the study and every week. Blood was collected for full blood count and liver enzyme analysis a day before infecting the animals with the larvae (pre-infection), day of commencement of treatment (post-infection) and a day before necropsy (post treatment). Following confirmation of *H. contortus* infestation animals were subjected to 5 days of treatment and 7days of post-treatment monitoring prior to sacrifice. For phase 1 animal were treated with extract (189 mg/kg), albendazole (1 ml / 10 kg body weight) or left untreated. One group was used as the uninfected untreated control. Phase 2 made use of only two groups, extract (500 mg/kg) or untreated as the effect of albendazole on the strain was established in the previous strain.

All animals were observed for signs of any adverse reaction as a result of treatment, including effect on feed intake. At the end of this period, the animals were sacrificed and the abomasum tied and freed of excess fat and mesenteric attachments. Each abomasum was opened and its contents thoroughly washed into a graduated bucket and the volume of the wash made to 2-3 L. After thorough mixing, 10% aliquots of the contents were mixed with 10% formalin. The nematodes in the 10% aliquots were stained with Lugol's iodine for ease of counting under a stereomicroscope. The group treated with albendazole were administered at manufacturers recommended dosage orally once.

8.2.6 Faecal egg counts (FECs)

Faeces were collected from the rectum and stored in a clearly labelled container with animal identification and date. Faecal egg count was done using the McMaster technique (Soulsby, 1986). Briefly, 4 gram of faecal material was ground using a pestle and mortar, 56 ml of saturated sodium chloride was added and thoroughly stirred. This was then poured through a tea strainer and collected in another container. An aliquot was then taken from the surface of the suspension using a pasture pipette and slowly dispensed into the two chambers of the McMaster slide. The slide was then placed on the microscope stage and allowed 5 minutes to settle. The eggs were then counted using X10 objective and the total count from both chambers added and multiplied by 50 to give the faecal egg count or egg per gram (epg).

8.2.7 Clinical pathology

Blood was collected aseptically from the jugular vein using a syringe. This was transferred into 5 ml tubes with and without an anticoagulant. The blood specimens were analysed for complete blood count (CBC) using ADVIA 2120 Haematology System from Siemens. The serum was analysed for ALT (alanine amino transferase) and AST (aspartate amino tranferase) using the Cobas Integra 400 plus from Roche. Analysis was undertaken by the clinical pathology unit of the Onderstepoort Academic Hospital Faculty of Veterinary Science University of Pretoria

8.2.9 Statistical analysis

All results generated were entered in Excel spread sheet and statistical analysis done using 1BM SPSS STATS Version 20 (2011). The mean, standard deviation and difference within and between group at p=0.05 were determined. Comparisons between groups were not undertaken as the sample size was small to accommodate this discovery study.

8.3 Results

8.3.1. General clinical observation

The animals remained apparently healthy throughout the duration of the experiment except for one sheep that died due to bloat and pulpy kidney. No adverse reactions were generally observed except for a single incidence of mild diarrhoea on day 4 of treatment with the extract at 500 mg/kg.

8.3.2 Faecal egg count reduction test (FECRT) and abomasal worm counts

The mean faecal egg counts for sheep treated with *L. sericea* and their controls for study phase 1 and 2 are presented in Tables 8.1 and 8.2 respectively. In phase 1The mean pre-treatment faecal egg counts for group treated with extract, albendazole and untreated control were 6133, 16266.7and 7750 respectively. Following treatment with extract and albendazole, the group treated with extract had a reduction in faecal egg count to 3617 on day 2 of treatment and a further decline to 1300 on day 3 of treatment. Albendazole treated sheep had a rapid reduction in faecal egg counts which reached zero as early as day 3 post-treatment. The untreated control had FEC fluctuating from 7275 to 4150 and to 2975. After 5 days of continued treatment with extract FEC reduced to 1033 and untreated control FEC was 1850.

In phase 2 the pre-treatment FEC was 2333 for treatment and 1217 for control group. Following treatment the FEC of treatment reduced to 1783 on day 1 of treatment and to 2833 after last day of treatment. FEC for control group was 1417 on the last day of sample collection. The results for the FECRT are in Table 8.2 for phase 2 of this study. Five days of continued treatment with extract of *L. sericea* at a dosage of 500 mg/kg body weight resulted in a reduction to only 16.5 % of pre-treatment FEC left. The control FEC increased by 148 % from the initial value. The FECRT for phase 1 of this study was not calculated due to lack of a definite trend and differences between treated and control group.

The difference in FECRT between treated and control group was statistically significant with p=0.561 for phase 2 of our study in contrast to phase 1 where there were no statistically significant differences.

The abomasal worm counts for both phase 1 and 2 of this study are presented in Tables 8.1 and 8.2. The mean worm count for extract treated sheep were 1075, control 2000, albendazole treated were 325 and 600 for uninfected sheep. For phase 2 mean worm count for treated sheep were 397and 623 for the negative control. The presence of abomasal worms in the uninfected negative control may be due to cross contamination from infected group. However, there were no eggs excreted from the negative control group throughout the duration of the study, probably because infection was at the late stage of the experiment and parasites had not started producing eggs.

8.3.3 Effect on haematology and liver enzymes following treatment with *L. sericea* and their controls

The blood count and liver enzymes (ALT and AST) activities are presented in Tables 8.3 and 8.4. In phase 1, all the animals were healthy at the start of the study. Following the induction of disease, all animals showed a lower haematocrit at the time of the treatment. Following treatment, the albendazole group had an increase in haematocrit compared to the pre-infection concentrations. Both the extract treated and untreated groups had a marginal increase. As a result it would appear that the extract did not have an effect on the blood loss experienced by the treated animals. The only other visible change was an increase in the eosinophil count from pre-infection to post-infection. None of the other haematology parameters showed any signs of change. No change in the liver enzymes activities was observed.

The blood and liver enzymes (ALT and AST) values are presented in Tables 8.3 and 8.4. In phase 2, all animals were healthy at the start of the study. Following infection, all animals had a lowered haematocrit at the time of the treatment. In both studies there was no statistically significant difference and all values were within the normal range 0.22-0.44. The eosinophil values also increased following infection in phase 2 of this study. The pre-infection values for eosinophil were 2.00 and 3.00 for treatment and control group, this value increased to 6.75

post-treatment for both treated and control group and the difference was statistically significant. Following treatment the eosinophil values for treated group dropped to 4.50, while the control group increased to 7.00.

Liver enzyme alanine aminotransferase (ALT) were within the normal range of values of 9-45 for all groups throughout the duration of this study. The AST values were above the normal range even at pre-infection for the albendazole treated group.

Table 8.1: Mean faecal egg counts for *L. sericea* extracts compared with untreated controls for phase 1 of study.

Group		Pre-infection	Pre-infection	Pre-infection	Post-treatment	Post-treatment	Post-treatment	Post-treatment	Post-treatment	Post-treatment	Worm count
Extract	Mean	6716.67	11083.33	6133.33	7416.67	3616.67	1300.00	3916.67	5050.00	1033.33	1075.00
	SD	5191.90	7517.70	2980.91	3752.78	1373.26	100.00	2750.15	1381.12	246.64	125.00
Albendazole	Mean	2850.00	14716.67	16266.67	3083.33	33.33	0.00	0.00	0.00	0.00	325.00
	SD	1475.64	1814.75	5372.46	453.69	57.74	0.00	0.00	0.00	0.00	62.36
Untreated	Mean	3900.00	3775.00	7750.00	7275.00	4150.00	2975.00	12950.00	5725.00	1850.00	2000.00
	SD	141.42	35.36	989.95	1025.30	424.26	1025.30	212.13	742.46	141.42	100.00
Uninfected	Mean	0	0	0	0	0	0	0	0	0	600.00
	SD	0	0	0	0	0	0	0	0	0	163.30

SD=standard deviation, bw = body weight

Table 8. 2: Mean faecal egg counts for *L. sericea* extracts treated sheep at 500 mg/kg bw compared with untreated controls for phase 2 of study.

ANIMAL ID	Rx	Days after treatment						Worm Count	FECRT	FECRT
		0	1	3	4	5	6		C	T
1	T	3100.00	1800.00	1150.00	1100.00	800.00	650.00	130.00	100	100
5	T	3200.00	2950.00	1350.00	2500.00	700.00	0.00	460.00	77.51	78.66
7	T	700.00	600.00	250.00	1050.00	850.00	200.00	600.00	111.99	38.33
Mean		2333.33	1783.33	916.67	1550.00	783.33	283.33	396.67	100.85	87.87
SD		1415.39	1175.09	585.95	823.10	76.38	332.92	241.32	141.41	56.37
2	C	250.00	250.00	250.00	300.00	550.00	600.00	220.00	147.99	16.51
4	C	700.00	150.00	900.00	500.00	600.00	650.00	240.00		
9	C	2700.00	3000.00	2900.00	3000.00	3200.00	3000.00	1410.00		
Mean		1216.67	1133.33	1350.00	1266.67	1450.00	1416.67	623.33		
SD		1304.16	1617.35	1381.12	1504.44	1515.75	1371.43	681.35		

T= Treated, C= untreated control, SD= standard deviation, R_X= Treatment, bw= body weight, FECRT= faecal egg count reduction test

Table 8.3: Mean haematology and liver enzyme of sheep infected with *Haemonchus contortus* and their controls phase 1

Parameters	Units	Pre-infection Extract	Post-infection Extract	Post-treatment Extract	Pre-infection Albendazole	Post-infection Albendazole	Post-treatment Albendazole	Pre-infection Untreated	Post-infection Untreated	Post-treatment Untreated	Pre-infection Uninfected	Post-infection Uninfected	Post-treatment Uninfected
Haemoglobin	g/dl	119.67	96.33	96.67	122.33	101.67	123.33	120.00	94.50	99.00	134.33	131.33	127.33
Red Cell Count	$\times 10^{12}$/L	13.64	9.61	9.30	13.63	10.03	11.70	13.86	9.34	9.59	14.89	13.67	13.26
Haematocrit	l/l	0.33	0.26	0.28	0.34	0.29	0.35	0.33	0.26	0.29	0.38	0.36	0.35
MCV	Fl	23.90	27.37	29.47	24.83	28.43	29.87	23.65	28.25	29.80	25.37	26.00	26.40
MCH	Pg	8.77	10.13	10.43	9.00	10.20	10.53	8.70	10.10	10.30	9.00	9.70	9.63
MCHC	g/dl	36.83	37.00	35.43	36.33	35.80	35.27	36.70	35.70	34.70	35.60	36.97	36.53
RDW		26.73	26.10	24.03	20.87	27.30	24.90	21.55	29.45	24.30	21.83	21.00	20.60
WCC	$\times 10^{9}$/L	6.26	5.55	5.52	6.79	5.08	6.31	6.80	5.19	5.73	8.52	7.13	6.88
Neutrophil(mat)		34.33	44.00	38.67	59.00	37.33	39.33	50.50	46.00	50.00	62.67	51.67	45.00
Neutrophil(immat)		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lymphocytes		61.33	51.33	55.00	35.67	51.33	55.00	46.50	50.00	42.50	35.67	43.33	51.00
Monocytes		4.00	2.33	2.33	3.67	6.67	3.67	1.00	2.00	3.50	0.33	2.67	4.67
Eosinophil		0.33	1.67	3.33	1.33	4.00	1.33	2.00	2.00	2.50	1.33	2.33	4.33
Basophil	$\times 10^{9}$/L	0.00	0.67	0.67	0.33	0.67	0.67	0.00	0.00	1.50	0.00	0.00	1.67
N (mat) abs	$\times 10^{9}$/L	2.15	2.44	2.14	4.00	1.89	2.50	3.44	2.40	2.88	5.34	3.66	3.09
N (immat) abs	$\times 10^{9}$/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lymphocyte abs	$\times 10^{9}$/L	3.84	2.85	3.03	2.43	2.61	3.46	3.16	2.58	2.42	3.04	3.12	3.55
Monocyte abs	$\times 10^{9}$/L	0.25	0.13	0.13	0.25	0.34	0.23	0.07	0.10	0.20	0.03	0.19	0.32
Eosinophil abs	$\times 10^{9}$/L	0.02	0.10	0.18	0.09	0.21	0.08	0.14	0.10	0.14	0.11	0.16	0.30
Basophil abs		0.00	0.04	0.04	0.02	0.03	0.04	0.00	0.00	0.08	0.00	0.00	0.11
Platelets C		366.00	471.00	534.33	352.67	315.00	530.33	410.50	436.00	500.00	470.33	402.33	413.67
MPV	u/l	5.50	5.67	5.97	5.37	5.47	5.17	4.75	5.40	5.05	4.87	4.97	5.53
ALT	u/l	23.00	19.00	24.00	14.67	13.00	14.33	25.50	27.00	25.50	24.00	25.33	25.33
AST		237.33	179.33	191.33	373.00	350.33	443.33	272.00	245.50	259.00	292.67	336.33	360.00

Table 8.4: Mean haematology and liver enzyme of sheep infected with *Haemonchus contortus* and their controls phase 2

Parameters	Units	Pre-infection Treated	Pre-infection Untreated	Post-infection Treated	Post-infection untreated	Post-treatment treated	Post-treatment untreated
Haemoglobin	g/dl	101.75	101.75	107.75	106.00	124.75	120.50
Red Cell Count	x10^{12}/L	11.14	12.12	11.56	10.94	12.45	11.63
Haematocrit	l/l	0.29	0.29	0.31	0.29	0.34	0.32
MCV	Fl	26.48	23.60	26.70	27.15	27.65	27.88
MCH	Pg	9.23	8.40	9.40	9.78	10.10	10.45
MCHC	g/dl	34.78	35.65	35.18	36.00	36.55	37.55
RDW		19.30	18.20	17.78	18.98	17.45	17.95
WCC	x10^9/L	8.69	11.30	10.92	9.79	10.63	10.41
Neutrophils(mat)		24.25	43.00	27.50	33.00	28.00	34.25
Neutrophil(immat)		0.00	0.00	0.00	0.00	0.00	1.25
Lymphocyte		68.75	48.25	57.75	56.50	63.50	54.25
Monocyte		4.25	5.25	6.25	3.25	3.50	3.00
Eosinophil		2.00	3.00	6.75	6.75	4.50	7.00
Basophil		0.50	0.50	1.75	0.50	0.50	0.25
N (mat) abs	x10^9/L	2.20	4.94	2.90	3.13	2.99	3.55
N (immat) abs	x10^9/L	0.00	0.00	0.00	0.00	0.00	0.14
Lymphocyte abs	x10^9/L	5.88	5.37	6.41	5.64	6.75	5.65
Monocyte abs	x10^9/L	0.39	0.61	0.69	0.31	0.39	0.32
Eosinophil abs	x10^9/L	0.16	0.33	0.73	0.67	0.45	0.73
Basophil abs	x10^9/L	0.05	0.05	0.19	0.04	0.06	0.03
Platelets C		462.00	526.25	306.00	409.50	604.00	265.00
MPV		5.65	5.48	5.73	5.53	3.63	5.30
ALT	u/l	36.75	10.25	11.00	13.75	14.25	14.00
AST	u/l	253.00	126.50	242.50	201.75	196.00	292.50

8.4 Discussion

The aim of this study was to evaluate the anthelmintic effect of the acetone leaf extracts of *Leucosidea sericea* in sheep artificially infected with *H. contortus.* The acetone leaf extract of *L. sericea* at a dosage of 189 mg/kg body weight had a 73.18% reduction in FEC and at 500 mg/kg body weight a 83.49% reduction in FEC. In contrast albendazole reduced the FEC by 100% after only one treatment. Failure of the product to achieve a 100% reduction in FEC may be due to a number of factors, such as pH changes, activity of rumen micro flora or extract not attaining effective concentration within the abomasum. A methanolic extract of *Euphorbia helioscopia* led to a 86.1% reduction in FEC (Lone et al., 2012). Simon et al., (2012) reported a 63 % reduction in FEC at a dosage of 200 mg/kg for extract of *Combretum molle*, while at 2000 mg/kg the FEC reduction was 96.23 %. The effect of our extract was better with 83.5 % reduction at 500 mg/kg.

The activity of the acetone leaf extracts may be due to compounds that have been isolated from the leaf of this plant. Compounds such as aspindinol (Bosman et al., 2005), agrimol G and mixture of agrimol G and A and β-sitosterol (Adamu et al., 2012) have been isolated from the leaf of *L .sericea*. These compounds have anthelmintic activity *in vitro* against the egg and larvae of *Haemonchus contortus* (Adamu et al., 2012). The probable mechanism of action of the phloroglucinol derivatives was earlier reported (Adamu et al., 2012), as they cause degenerative changes within the parenchyma and vacuolization of the internal structure of adult *Haemonchus* parasite. These compounds have not been isolated previously from this plant and no anthelmintic activity of these compounds has been reported. The concentration of these compounds in the leaf of *L. sericea* was actually low with about 10 mg isolated from 500 g of plant material. The compounds isolated were in small quantity from the extract administered to give an effective concentration, as only about 9 g of the extract was administered at the dosage of 500 mg/kg body weight. This may be a reason why extracts and compounds have good activity *in vitro* but lower activity *in vivo.* In future increasing the dosage of this extract or synthesizing compounds and using for animal trial will be required. If there is direct dose related response a dose of 1125 mg/kg body weight would be required to reduce the FEC by 100%. This dosage is achievable and realistic considering higher dosages of extracts that are been administered in animal experiments if there are no toxic effects.

The administration of this extract did not show any adverse clinical effect due to toxicity. The results of the liver enzyme assay also support our clinical observation as ALT levels were within normal range during this study. Assessing liver enzymes may give an indication to toxicity as the liver is the main organ for metabolism. Increase in the level of these enzymes especially ALT may indicate probable toxicity due to damage to the liver parenchyma. This study attests to the safety of this extract at the highest dosage used as clinical toxicity was not observed and ALT levels were within the normal range of 9-45.

The extracts had no negative effect on haematology indices measured, only eosinophil counts were higher than normal. This may likely be due to the parasite infection as pre-infection levels were normal. A rise in eosinophil is normal especially with haemonchosis. Following, treatment with extract the eosinophil level dropped, while that of the control was on the increase. The extract may have some immunomodulatory effect there by slowing down cell mediated immunity such as eosinophil production.

This study has failed to demonstrate an anthelmintic effect of the leaf of *Leucosidea sericea* as the adult worms were not killed. The extract did however, have a biological effect as it reduced the fecundity of the adult parasite, thereby reducing the egg output of the parasites. This therefore suggests that a higher dose or further potentization of the extract may be needed to demonstrate better efficacy.

8.5 Conclusions

Based on the results of this study, it could be proposed that this plant extract can be examined as alternative to commercially chemical anthelmintic drugs especially in rural areas. The study also confirms that the leaf of this plant is useful for the treatment of helminth infection in animals. The plant extracts should undergo more detail studies for efficacy and safety by synthesizing or extracting enough of the isolated compounds and evaluating their efficacy and safety *in vivo.*

Chapter 9: General Discussions and Recommendations

9.1 General Discussions

The main aim of this study was to scientifically validate the anthelmintic activity of traditionally used anthelmintic plants with the intent of isolating anthelmintic compound(s) or plant extracts that could be used for treatment of *H. contortus* in small ruminants especially sheep. To achieve this aim, the following objectives were set out:

❖ Preliminary *in vitro* screening of acetone extracts of thirteen plant species using the egg hatch assay and the larval development test against *H. contortus* eggs and larvae.

❖ Evaluation of the plant extracts for activity against 4 bacteria and 3 fungi pathogens and relationship with anthelmintic activity.

❖ Evaluation of the *in vitro* toxic effect of the acetone extracts of the thirteen plant species using Vero cells

❖ Isolation of anthelmintic compounds from the most active plant extract and fraction using bioassay-guided fractionation.

❖ Establishing the effects of compound(s) isolated from *Leucosidea sericea* on the ultrastructure of *H. contortus*, using electron microscopy.

❖ Evaluate the activity of the acetone leaf extracts of *L. sericea* in sheep infected with *H. contortus* on ability to reduce faecal egg count and total worm counts.

The extent to which these objectives were attained is discussed herein.

The first objective was to screen acetone leaf extracts of thirteen selected South African plant species for activity against the egg and larvae of *H. contortus.* Using a bench-top screening assay, the results for the activity of the thirteen plant extracts showed varied efficacy that did not support the anthelmintic effect and the folkloric use of these plant extracts. Only three plant extracts, *Leucosidea sericea, Heteromorpha trifoliata* and *Maesa lanceolata* were defined by sufficiently efficacious $EC_{50s.}$

Our second objective was to screen the activity of these extracts for activity against three fungal pathogens and four bacteria organisms. The antifungal activity was based on the premise that some anthelmintic such as the benzimidazoles have antifungal properties, making it was beneficial to evaluate the antifungal activity of these extracts. The results of this study demonstrated that some traditionally used anthelmintic plant species had excellent anti-fungal activity that was comparable to other known anti-fungal plant extracts. For example, the antifungal activity of C*lausena anisata* against *A. fumigatus (*Adamu et al., 2012) was better than that of *Loxostylis alata* (Suleiman et al., 2010) with MIC of 0.02 mg/ml and 0.05 mg/ml respectively. Another sub-objective was to determine the antibacterial activity of the selected plant extracts, against four bacteria pathogens partly because some of these plant species had documented use against bacterial infections. The results indicated that several plant extracts were characterised by MIC values below 0.1 mg/ml.

The third objective was to evaluate the cytotoxic activity of these extracts against Vero cells. Most of the plant extracts were toxic to the Vero cells, with the LC_{50} of 0.17 mg/ml for *C. anisata* being least toxic while *C. dregei* was the most toxic with LC_{50} of 0.003 mg/ml. Based on the efficacy against the egg and larvae of *H. contortus,* cytotoxicity value and selectivity index and considering past *in vitro* and *in vivo* anthelmintic activity reported. We selected *Leucosidea sericea* for further study. The fractions and compounds isolated from *L. sericea* were also evaluated for toxicity. The cytotoxicity of the acetone extracts of *L. sericea* was 0.05 mg/ml, while that of the ethyl acetate fraction was 283.50 mg/ml. Fractionation showed the various components to be less toxic to the parent acetone extracts. The two phloroglucinol compounds isolated were also not toxic with LC_{50} values in the thousands mg/ml.

The fractions of the acetone leaf extracts of *Leucosidea sericea* was also evaluated against the egg and larvae of *H. contortus*. Based on the selectivity index of the four fractions the ethyl acetate fractions with its best selectivity index (308 and 359) against egg and larvae and was selected for isolation. β-sitosterol, and 2 phloroglucinol derivatives; agrimol G and a mixture of agrimols G and A were isolated from the fraction. The activity of the phloroglucinol derivatives were tested for activity against egg and larvae development and had an EC_{50} of 0.52 mg/ml for agrimol G, while that of the mixtures of agrimols G and A was 0.28 mg/ml. The larval development test EC_{50s} for agrimol G was 0.08 mg/ml and 0.11 mg/ml for mixture of agrimols G and A. The amount of β-sitosterol was not sufficient for further testing. While the compounds isolated were known, this is the first record of their occurrence in this plant. Of interest are the phloroglucinol derivatives as another compound kosidin, within this family is a known anthelmintic.

In an attempt to characterise the mechanism of action of these extract and isolated compounds. The effect of the extracts and two compounds on the ultrastructure of adult *H. contortus* was evaluated. The main changes identified were destruction of the cuticle, changes to the hypodermis, vacuolization of the cytoplasm, cytoplasmic degradation, cellular swelling and abnormality within the mitochondria.

Our final objective was to evaluate the effect of the acetone leaf extract of *L. sericea* in sheep artificially infected with *H. contortus* and their effect on faecal egg count reduction. A two phased closed study was conducted with a dose of 109 mg/kg body weight and 500 mg/kg body weight. Both phases demonstrated the potential towards a reduction in faecal egg count of 73.1% for the lower dose and a reduction of 83.5 % for the higher dose. No clinical signs of toxicity, or unscheduled deaths were observed during the study. In both cases since these phases were designed to be pilot studies for ethical reasons, statistical significance could not be established. With the leaf acetone extract of *L. sericea* demonstrating benefit in reducing sheep haemonchosis egg production, a larger efficacy study could be of value.

9.2 Recommendations

The leaf of *L. sericea* has beneficial effect in the treatment of *H. contortus* infection in sheep from the results of this study. The plants also had no toxicity at the maximum dosage used for our study. We therefore recommend that this plant can be used for treatment of helminth infection in sheep. We also recommend that in future high dosage or possibly the compounds should be evaluated for activity and safety.

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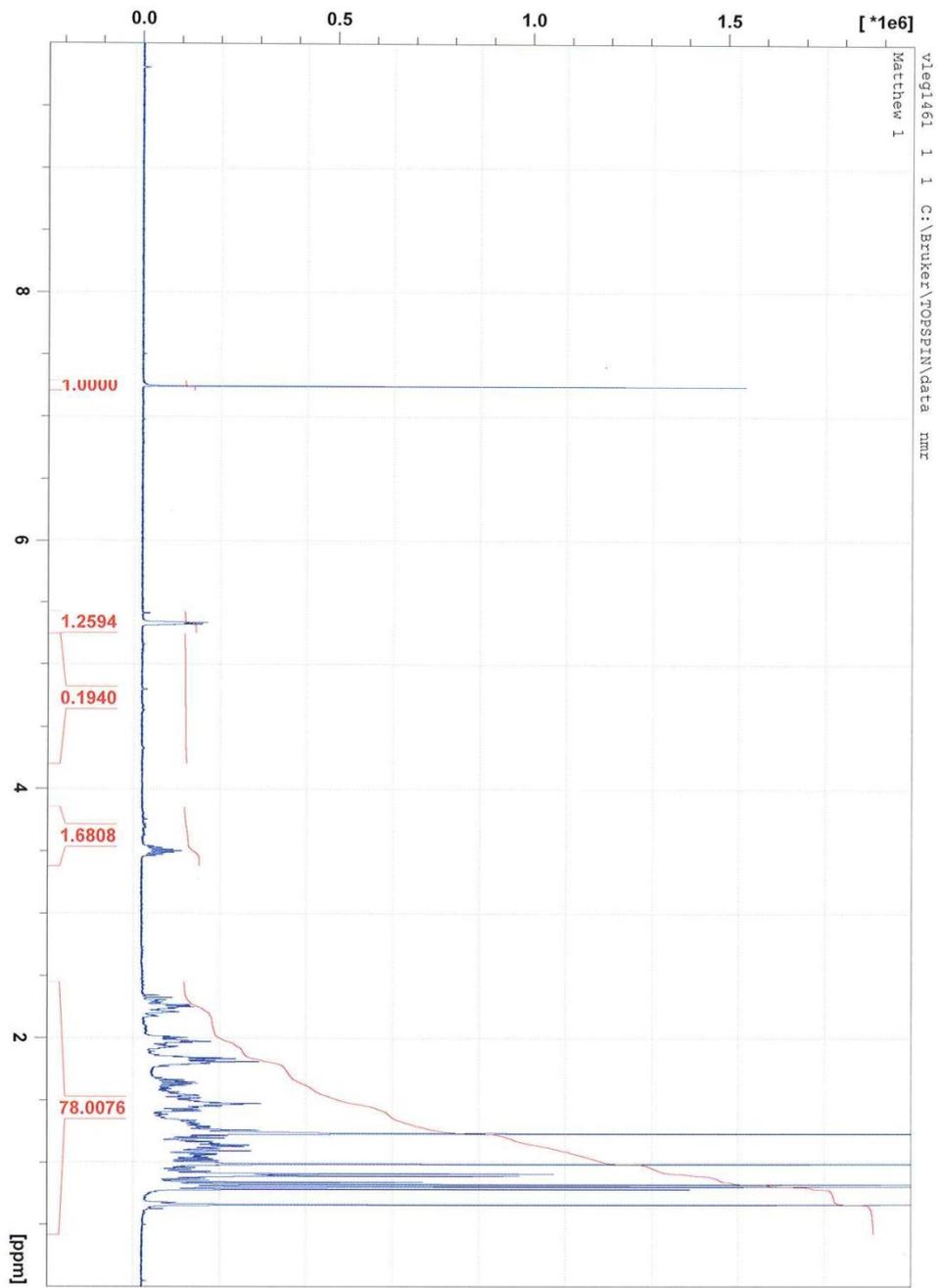
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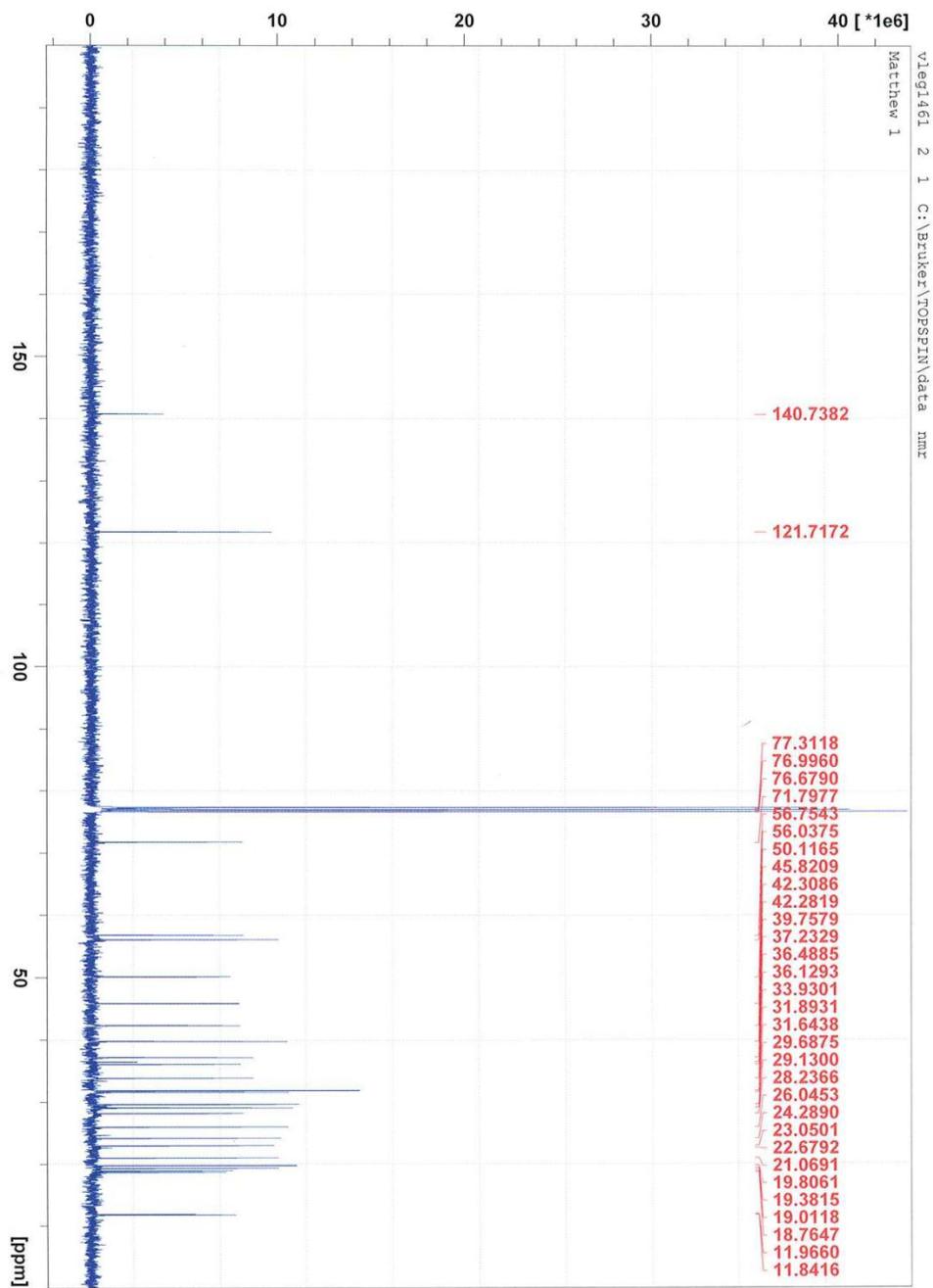
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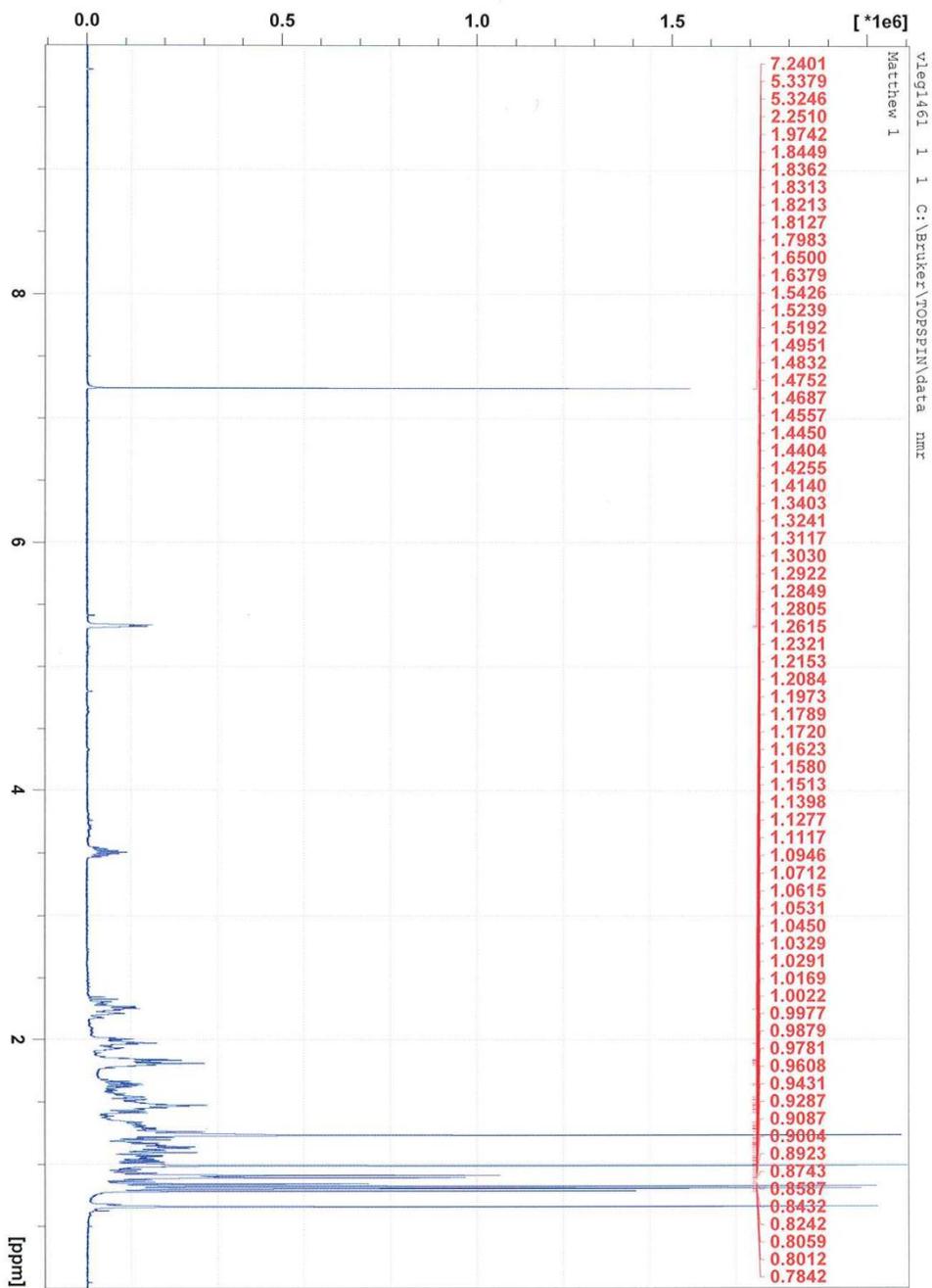
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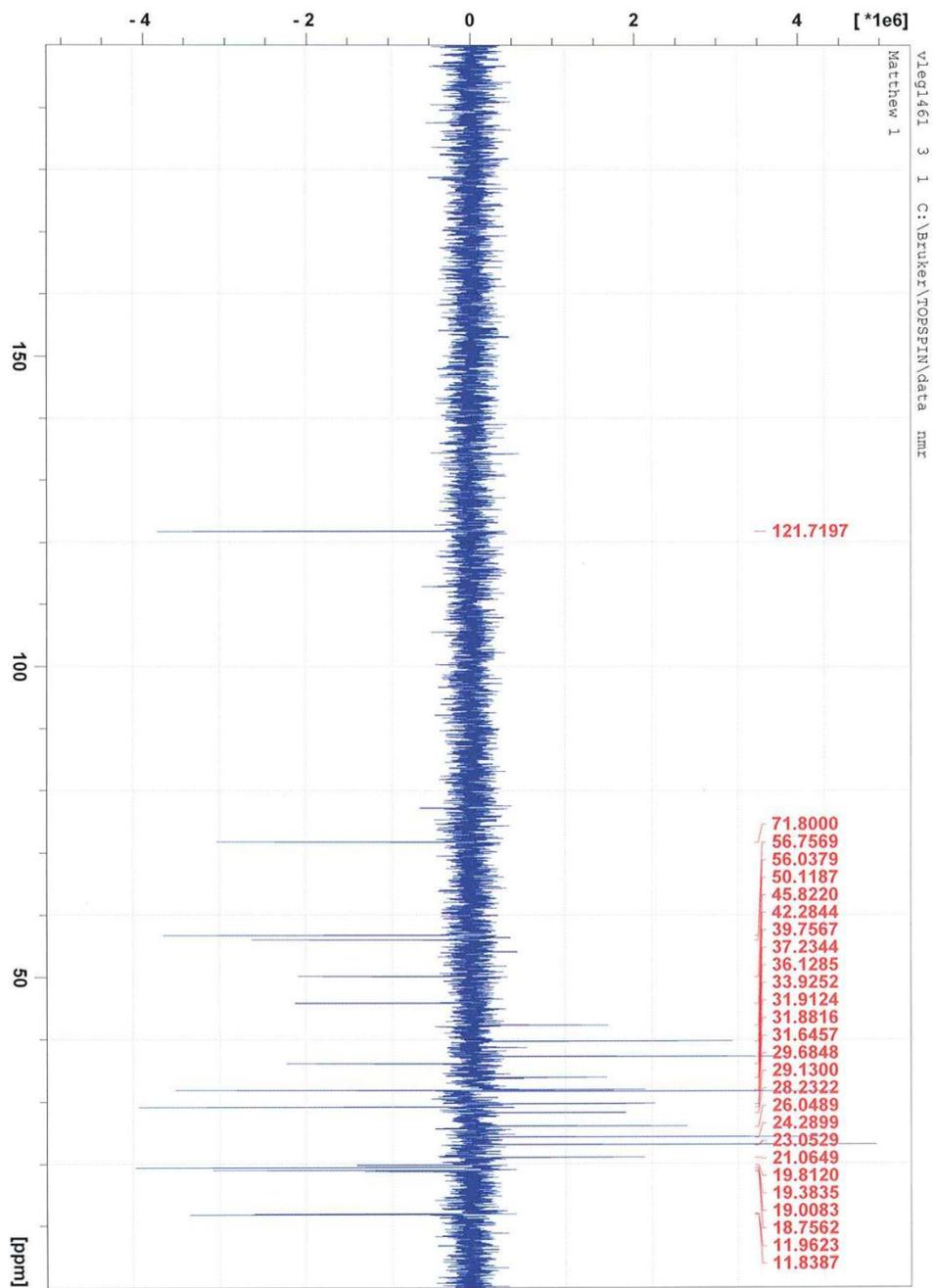
Appendix

The following are NMR Spectra for compounds isolated from the leaf of *L. sericea* Mathew 1 denote β sitosterol, Mathew 2 denote agrimol G and Mathew 3 denote mixture of agrimol A and G.

vleg1461 2 1 C:\Bruker\TOPSPIN\data nmr

Matthew 1

140.7382

121.7172

77.3118
76.9960
76.6790
71.7977
56.7543
56.0375
50.1165
45.8209
42.3086
42.2819
39.7579
37.2329
36.4885
36.1293
33.9301
31.8931
31.6438
29.6875
29.1300
28.2366
26.0453
24.2890
23.0501
22.6792
21.0691
19.8061
19.3815
19.0118
18.7647
11.9660
11.8416

vleg1461 3 1 C:\Bruker\TOPSPIN\data nmr

Matthew 1

[*1e6]

- 4   - 2   0   2   4

150   100   50   [ppm]

— 121.7197

71.8000
56.7569
56.0379
50.1187
45.8220
42.2844
39.7567
37.2344
36.1285
33.9252
31.9124
31.8816
31.6457
29.6848
29.1300
28.2322
26.0489
24.2899
23.0529
21.0649
19.8120
19.3835
19.0083
18.7562
11.9623
11.8387

151

vleg1462 3 1 C:\Bruker\TOPSPIN\data nmr

Mathew 2

- 77.2003

62.1031

- 52.9036

39.3041
38.3113

24.9939
22.8171
19.7624
19.2460
16.4884
11.9296
9.2052