Controlling *Haemonchus contortus* using bioactive compounds from plants with antifungal activity

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DECLARATION

I declare that the experimental work described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria, and has not been submitted in any other form to any University or academic institution. I, Bellonah M. Sakong, declare the above statement to be true.

Signed:

Dated:

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic aci
AF	*Aspergillus fumigatus*
AN	*Aspergillus niger*
Amp B	Amphotericin B
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
BEA	Benzene: ethyl acetate: ammonium hydroxide (90:10:1)
CA	*Candida albicans*
Ca	Calcium
CEF	Chloroform: ethyl acetate: formic acid (5:4:1)
CN	*Cryptococcus neoformans*
13CNMR	Carbon 13 Nuclear Magnetic Resonance
DMSO	Dimethylsulphoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC	*Escherichia coli*
EC50	Effective concentration 50%
EF	*Enterococcus faecalis*
EMW	Ethyl acetate: methanol: water (40:5.4:4)
EHA	Egg hatch assay
H NMR	Proton Nuclear Magnetic Resonance
IC_{50}	Inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
HC	*Haemonchus contortus*
Sba	*Schotia brachypetala* acetone
Spa	*Senna petersiana* acetone
Spw	*Senna petersiana* water
CGa	*Cassipourea gummiflua* acetone
CGw	*Cassipourea gummiflua* water
LC_{50}	Lethal concentration 50%
LDA	Larval development assay
µg	Microgram
Mg	Milligram
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

MS	Mass spectrometry
MH	Mueller-Hinton
Paa	*Peddiea africana* acetone
Paw	*Peddiea africana* water
Dwa	*Diospyros whyteana* acetone
Dww	*Diospyros whyteana* water
PA	*Pseudomonas aeruginosa*
EPG	Eggs per gram
Rf	Retardation factor
ROS	Reactive oxygen species
SA	*Staphylococcus aureus*
SD	Sabouraud dextrose
TA	Total activity
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
HWSETA	Health and Welfare Sector Education and Training Authority
UP	University of Pretoria
UPPGB	University of Pretoria Postgraduate Bursary
CSIR	Council of Scientific and Industrial Research
SABINA	Southern African Biochemistry and Informatics for Natural Products Network
TIA	Technology Innovation Agency
SI	Selectivity Index
Dox	Doxorubicin

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ABSTRACT

Infection of the gastrointestinal tract of livestock by the nematode parasite *Haemonchus contortus* is a serious challenge to livestock production. Infected animals, especially sheep and goats, frequently suffer from diarrhoea, anaemia, loss of appetite, reduced body weight and ultimately death if untreated. Anthelmintic drugs are widely used to control nematode infection, but problems of the development of resistance by parasites and unavailability or cost to rural smallholder farmers are serious problems. Their use has also resulted in the presence of residues in meat and milk, which affects food safety. Therefore, alternative control measures such as using plant extracts with putative anthelmintic properties can enhance parasite management.

The aim of this study was initially to investigate plant species used in ethnoveterinary medicine to control intestinal parasites for in vitro anthelmintic activity against the sheep nematode *Haemonchus contortus*. These extracts did not give promising results as the anthelmintic activities were generally low so further work on these plant species was discontinued.

The second part of the project was to test plant species with known antifungal activity for activity against *H. contortus* and a panel of fungal species, as some anthelmintic chemicals also have antifungal activity. For example the benzimidazole group of drugs has demonstrated good correlation between antifungal and anthelmintic activity. Antiparasitic activities of the acetone and water extracts of six plant species with known antifungal activity were determined using the egg hatch assay recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP). Cytotoxicity was determined by evaluating the viability of cells in the presence of the plant extracts using the tetrazolium-based colorimetric assay against Vero African Green monkey kidney cells. Efficacy of the extracts against various fungi was also tested using a serial microdilution method against three fungal pathogens (*Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*), as well as plant fungal pathogens. As parasitic anthelmintic assays are laborious and difficult, a major objective of this study was to investigate if a simple and rapid antifungal assay can be used as a model to isolate anthelmintic compounds from plant extracts.

The extracts of *Diospyros whyteana* and *Peddiea africana* had the strongest egg hatch inhibitory potential. The extracts had relatively low toxicity to normal mammalian Vero cells. Water extracts were slightly more toxic than acetone crude extracts. All the extracts had good antifungal activities with minimum inhibitory concentration (MIC) values as low as 0.04 mg/ml. *Peddiea africana*, followed by *Schotia brachypetala* extracts had the best antifungal activity. *Schotia brachypetala* inhibited the fungi at 0.04-0.08 mg/ml and the cytotoxicity was low,

leading to selectivity indices ranging from 0.59-13.43. The water extract of *Cassipourea gummiflua* was the most active of all the water extracts. The acetone extracts had a higher number of bioactive compounds than water extracts based on the number of fungal inhibition areas in bioautography.

In the anthelmintic egg hatch assay (EHA), the *Diospyros whyteana* acetone leaf extract had good activity. The extract was also active against *Candida albicans* with an MIC of 0.04 mg/ml. *Candida albicans* was therefore used as a model for bioassay-guided fractionation to isolate antifungal compounds from *D. whyteana*. Successive column chromatography resulted in isolation of three active compounds. Two compounds were not isolated in sufficient quantity to allow structural elucidation and identification but the third compound was identified as a mixture of α-amyrin and β-amyrin. This mixed compound had good antifungal (MIC values as low as 40 µg/ml) as well as anthelmintic activity (30-50% inhibition of egg hatch inhibition of *H. contortus*). This validates the use of the antifungal assay in bioassay-guided fractionation to isolate anthelmintic compounds in this case.

Additional useful activities for anthelmintic or antifungal plant-based remedies include antioxidant activity which may assist the patient in combating the disease. The total phenolic and flavonoid contents of the plant species were investigated, together with antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The total phenolic content of the extracts of *Diospyros whyteana* (water extract) was the highest followed by *Schotia brachypetala* (water extract). Generally, water extracts exhibited higher total phenolic contents than the acetone extracts. The plant extracts also generally had relatively high flavonoid contents. Phenolic and flavonoid compounds are often associated with antioxidant activity. The DPPH radical scavenging antioxidant activity of the extracts was less potent than those of the positive controls ascorbic acid and trolox. The acetone extracts of *Peddiea africana* and *Schotia brachypetala* had good DPPH radical scavenging ability.

This study showed that extracts of some plant species with good antifungal activities also have anthelmintic activity. This may indicate common biological pathways of inhibition or mechanisms of action worthy of further investigation. The use of the antifungal assay in this study assisted with the isolation of anthelmintic compounds. *Diospyros whyteana* had good anthelmintic and antifungal activity, and compounds active in both assays were isolated and identified from the acetone extract of this species. Further work on other plant species is necessary to confirm the correlation of antifungal and anthelmintic activity in plant extracts.

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CHAPTER 1: Introduction

1.1 Introduction

Economic losses from parasites can be significant and the issue of helminths, especially gastrointestinal nematodes, is a global health problem. Gillian et al. (2004) reported that nematode infections affect the health of millions of people and animals, causing huge economic losses in livestock farming in particular. Mulugeta et al. (1989) reported that infection is widespread in domesticated ruminants, causing major production losses. Nematode infection is rampant in most developing countries where poor pastures and the quantities of nutritious food consumed do not meet the nutritional requirements of animals (Leng, 1991). In addition, there is a lack of adequate veterinary care and the environment is favourable to nematode growth and transmission (Fikru et al., 2006). Nematode infections are also a serious animal health concern in South Africa (Van Wyk et al., 1999), manifesting especially in small ruminants (sheep and goats). The negative impact of nematode infection includes reduced immunity, lower fertility, a decrease in milk production and scours in animals, as well as a decrease in quantity of meat and wool (New Zealand Ltd., 2006), and even death in serious infections (Fikru et al., 2006; Hale, 2006).

The presence of nematode strains resistant to the available clinical drugs has escalated. Additionally, multiple drug-resistant populations of these parasites have also been detected (Mungube et al., 2012). Most parasite control programs are based upon a blend of chemotherapeutic control, grazing management, dietary management, biological control, vaccination and ethnoveterinary medicine (EVM) treatment (Waller, 1999; FAO, 2002). Problems have increased with parasites developing resistance to several families of chemical anthelmintics (Chandrathani et al., 2004). According to Leathwick et al. (2001) anthelmintic resistance in parasites is spreading at an alarming rate, most likely owing to intensive use of anthelmintics, and the increasing inefficacy of anthelmintic compounds is threatening stock health all over the world.

Many plants are cultivated and used as food, medicine and activators in farming. The majority may be are quite harmless, but in a small percentage of cases they are toxic and to some extent lethal (Munday, 1988). Most traditional medicine systems use a large number of medicinal plants and therefore, many traditional medicinal cases are linked to plant-based medicines. Animal medicines have also played a vital role in healing practices, rituals, and religions of many societies. It has been estimated that of 252 essential medicines selected by the WHO, 11.1% are derived from plants and 8.7% from animals (Alonso, 2001). Plants are a

potential source of antimicrobial compounds and hence they are used traditionally to combat many bacterial and fungal infections. The vast majority of antibiotics used in modern medicines are produced by microorganisms, yeasts or fungi. Higher plants mainly produce antimicrobials as a defence mechanism against infections or these substances may be constituents of cellular metabolism.

Ethnobotanical studies reveal that the indigenous knowledge of a community is a key player in the identification of medicinal plants, and such plants have been validated through routine use by generations of indigenous people (Cox, 2001). Many pharmaceutical companies have some form of research program which may incorporate investigating plants with the aim of isolating bioactive compounds and developing new marketable drugs. Researchers have screened many plants for anthelmintic activity in *in vivo* and *in vitro* studies (Githiori et al., 2004; Bizimenyera et al., 2006; Ademola and Eloff, 2011; Adamu et al., 2013). Results from such studies confirm the need to investigate natural medicines to control helminthosis. Nowadays, there is an increasing interest in the use of natural products as an alternative drug source among internal parasites, especially gastrointestinal nematodes in small ruminants (Min and Hart, 2003). Several researchers have started looking at bioactive compounds in certain plants as a way of controlling gastrointestinal tract (GIT) parasites in agricultural animals. Tannins are a group of bioactive compounds of interest being investigated as a natural way to control GIT nematodes (Min and Hart, 2003).

The azole class of drugs has diverse pharmacological activities including antifungal, anti-inflammatory, analgesic, anticancer, antiviral, pesticidal and antiarrhythmic (Ravindernath and Reddy, 2013). Albendazole, mebendazole, thiabendazole and triclabendazole are well-known anthelmintic drugs. Benzimidazoles have been used for decades as anthelmintic drugs in veterinary and human medicine and also as antifungal agents in agriculture (Katiyar et al., 1994). The correlation in anthelmintic and antifungal activities of this class of drugs provides a motivation for identifying similar possible associations in biologically active plant extracts.

Oxidation is a chemical reaction that involves the transfer of electrons. Oxidation processes including photo-oxidation may produce free radicals in food, chemicals and living systems. Antioxidants play a major role in neutralizing the effects of free radicals and are known to be effective in preventing free radical formation by scavenging or promotion of their decomposition (Maxwell, 1995). Antioxidants react *inter alia* by being reduced by free radicals and terminating the cascades of reactions thereby preventing damage to vital organs (Oroian and Escriche, 2015). Under the body's normal physiological conditions, the levels of free radicals which could be highly reactive and unstable are kept under control by a maintained balance between the generated free radicals and the endogenous antioxidant defence system

to avert their harmful effects (Dwyer et al., 2009, Ahmed et al., 2013). Therefore, antioxidant activity of plant components may be a useful adjunct of a plant-based remedy used in controlling helminth parasites.

1.2 Problem statement

Smallholder farmers in rural areas have challenges in accessing orthodox medicine to treat parasitic infections in animals. A major challenge in sheep production is the growing resistance of nematodes to currently used commercial anthelmintic drugs. Ethnoveterinary medicine may provide farmers with some promising remedies that can assist in combating the problems associated with nematode infestations in small ruminants. The efficacy, modes of action, dosage requirements and potential toxicity of these traditionally used medications need to be investigated. The anthelmintic benzimidazoles also have antifungal activity (Adamu et al., 2012; Eloff et al., 2016). As anthelmintic assays using parasitic nematodes are time-consuming and difficult to perform, it is worth investigating whether antifungal assays can be used to identify potential anthelmintic activity in plant extracts and to assist in bioassay-guided fractionation for purification of active compounds.

1.3 Justification of plant selection

Plant species were initially selected based on a survey of plants used in ethnoveterinary medicine against livestock nematode infections conducted by the Council for Scientific and Industrial Research (CSIR). A second set of plant species was selected from the screening of acetone extracts of more than 500 tree species against eight important bacterial and fungal pathogens (Pauw and Eloff, 2014). Plants with good *in vitro* antifungal activity (crude extracts with MIC < 0.1 mg/ml) were selected from the unpublished Tree Screening Database of the Phytomedicine Programme, University of Pretoria.

1.4 Aim

To determine the anthelmintic activity of extracts of plants selected on the basis of use against parasite infections in ethnoveterinary medicine and those with good antifungal activity to assess potential correlations in antifungal and anthelmintic activity.

To determine the best criterion to select plants with anthelmintic activity.

To isolate compounds with potential use in protecting animals against *Haemonchus contortus.*

1.5 Objectives

The objectives of the following study were:

1. To determine the *in vitro* anthelmintic activity of plant extracts against *Haemonchus contortus* based on their ethnoveterinary use and known antifungal activity

2. To determine cytotoxicity, antioxidant efficacy, phenolic and flavonoid content of active extracts.

3. To select the most promising plant species for isolation of anthelmintic compounds using bioassay-guided fractionation with antifungal activity as the test bioassay.

4. To investigate the antifungal, anthelmintic and cytotoxic activity of isolated compounds.

CHAPTER 2: Literature Review

2.1. Introduction

Small ruminant industries are found in almost all countries in the world and are important in mixed farming in both tropical and sub-tropical countries. The industry is suitable for resource-poor farmers simply because goats and sheep are more affordable than cattle. It has low input requirements such as small initial capital and maintenance costs, and is free of social, religious and cultural taboos (Terefe et al., 2012).

The industry is however faced with the great challenge of different diseases with helminthosis being the most common condition found in all countries with ruminants. The disease is caused by endoparasitic worms which reside within different parts of the hosts, including the gut, lungs, blood, gallbladder, tissues and internal cavities (Bashir, 2009).

2.2 Helminthosis

The prevalence of helminthosis worldwide is very high (Lone, 2012) and it depends on humidity, temperature, rainfall, vegetation and management practices (Degefu et al., 2011). The condition is severe in countries with little or no access to modern animal health care facilities (Farooq et al., 2012). Helminthosis in the livestock sector causes high economic losses through lowered fertility, reduction of food intake, reduced weight gain, lower milk production, treatment costs and mortality in severely parasitized animals (Degefu et al., 2011; Zeryehun, 2012). The condition affects all age groups, but its severity is highest in sheep and goats up to one year old. Malnourished animals are also highly susceptible (Lone et al*.,* 2012). Helminth parasites affecting ruminants are grouped into cestodes, trematodes and nematodes (Soulsby, 1982).

2.2.1 Cestodes

In ruminants, cestodes are acquired by consuming contaminated food or water (Joshi, 2001). The main genera of veterinary importance in this group infecting ruminants are *Moniezia* spp. and *Taenia* spp. (Joshi*,* 2001). In cattle, *Moniezia* is acquired through ingestion of herbage contaminated with mites carrying the infective stage of the parasite. If lambs are infected with *Moniezia,* diarrhoea is the main clinical manifestation (Bashir, 2009).

Taenia saginata is commonly known as the cattle tapeworm and has two hosts, namely humans and cattle. The definitive host is man and the intermediate host is cattle. The worms (segments) are passed out through the human faeces and later on, the cattle ingest the eggs. In the alimentary canal of the cattle the eggs hatch out as larvae (oncospheres), penetrating the gut wall and entering the mesenteric lymphatics before finally reaching the circulation. Later on, these larvae invade the muscular tissues to undergo further development to lead to the infective *Cysticercus bovis* cyst stage. Humans are infected when they eat undercooked beef containing the live cysts (Bashir, 2009).

2.2.2 Trematodes

Trematodes are commonly known as flukes and are found in the bile duct or small intestine, although sometimes they may affect the lungs. Trematodes of veterinary importance include *Fasciola* spp., *Schistosoma* spp. and *Paramphistomum* spp. (Bashir, 2009).

Fasciolosis causes great losses in the agricultural sector worldwide. It is estimated that the loss caused by the condition worldwide is about US$200 million per year and more than 600 million animals are affected annually (Bashir, 2009). Young animals are the most vulnerable and the infection may manifest itself as clinical or subclinical. The severity of the condition varies according to the number of parasites present and nutritional status of the animal (Soulsby, 1982).

Transmission of fasciolosis involves snails, with cattle acquiring the infection by ingesting moist and raw aquatic plants and grasses contaminated with infective metacercariae shed by the snails (Bashir, 2009). The metacercariae excyst into immature *Fasciola* in the abomasum and enter the peritoneum and the liver where they mature to become adult worms which lay eggs which are then passed through the faeces. When the eggs encounter water, they mature and invade the molluscan snail host. The mature cercariae emerge out of the snail and are encysted on aquatic grasses where they develop into metacercariae, the infective stage of the parasite (Bashir, 2009).

Schistosoma spp. are the only trematodes found in the bloodstream of warm-blooded hosts. The blood is a rich source of amino acids and glucose which are required by *Schistosoma* spp. for the egg laying process. The worms cause a condition known as schistosomiasis, commonly known as bilharziasis. Acute intestinal signs often manifest following infection; the mucosa of the intestine is severely damaged and so the animal develops profuse bloody diarrhoea, dehydration and loss of appetite. The condition is also zoonotic as it affects humans

(Joshi et al., 2001). The most prevalent *Schistosoma* spp. include *S. japonicum*, *S. bovis* and *S. spindale* (Joshi et al., 2001).

2.2.3 Nematodes

Nematodes are the most pathogenic helminths in both tropical and sub-tropical countries (Degefu et al., 2011). This group contains many helminths, but those of veterinary importance in small ruminants include *Haemonchus* spp., *Trichostrongylus* spp., *Nematodirus* spp. and *Cooperia* spp. (Degefu et al., 2011).

Nematodes produce microscopic eggs that are passed through the faeces and then within a few days the eggs hatch into larvae. The larvae typically develop into second and then third larval stages. The larvae infest pastures, so animals are infected upon grazing contaminated pastures. In the intestine of ruminants, the larvae mature into adults. The male and female adult worms (Figure 2.1) are required to mate to produce eggs (Soulsby, 1982). Adult roundworms cause anaemia, diarrhoea, poor growth and even death (Bashir, 2009).

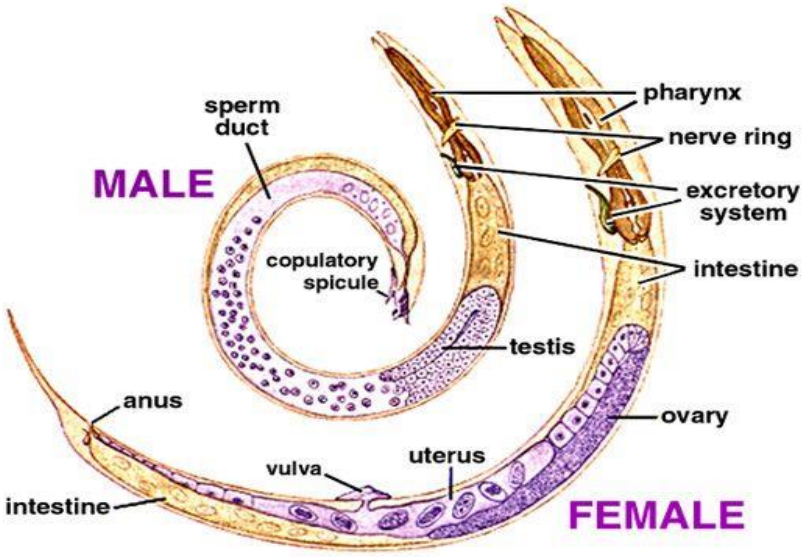

Figure 2.1. Structure of a nematode parasite of a male and female and the body parts by Travis Bauer (www.pharmawisdom.blogspot.in)

2.2.4. Life cycle of *Haemonchus contortus*

Gastrointestinal parasitism has become one of the most overwhelming animal health diseases in small stock animals (Min and Hart, 2003). The most relevant gastrointestinal parasites to

small ruminants belong to the Strongylida, superfamily Trichostrongyloidea (Zajac, 2006). The most important of these parasites is *Haemonchus contortus* because of its high reproduction rates and how it feeds on blood, causing the host animal to become severely anaemic following infection with large numbers of parasites (Zajac, 2006). The life cycle is generally the same in all species of the Trichostrongyloidea family (Figure 2.2). The adult female in the abomasum or small intestine produces eggs that are passed out in the faeces. The *H. contortus* life cycle takes 21 days to complete, beginning when a sheep on pasture ingests larvae in the infective (L3) stage. They then travel to the animal's abomasum, or fourth stomach. Development to the adult stage takes about three weeks in the gut when worms attach to the sheep's abomasal mucosa and feed on their blood.

The eggs produced during this stage are secreted in the animal's faeces, hatch if the conditions are right, and then developed to L1 and L2 stages. Once they reach the infective (L3) stage, they travel on to the pasture where they are re-ingested by sheep through the consumption of grasses (Machen et al*.*, 1998; Hepworth et al*.,* 2006; Besier, 2009). An adult female can lay up to 5 000 eggs daily and together the worms can consume up to 1/10th of an animal's blood in 24 hours (Hepworth et al*.,* 2006). The cuticle of *H. contortus* forms a small lancet in its oral opening and this is used for piercing the mucosa, thus causing capillary bleeding on which the worm feeds. Blood feeding begins at the fourth larval stage. *Haemonchus contortus* is also capable of undergoing a period of developmental arrest that is known as hypobiosis (O'Connor et al., 2006). During this period the larvae in the host do not develop directly into adults, instead they remain as L4 in the gastric glands of the abomasum for weeks or months.

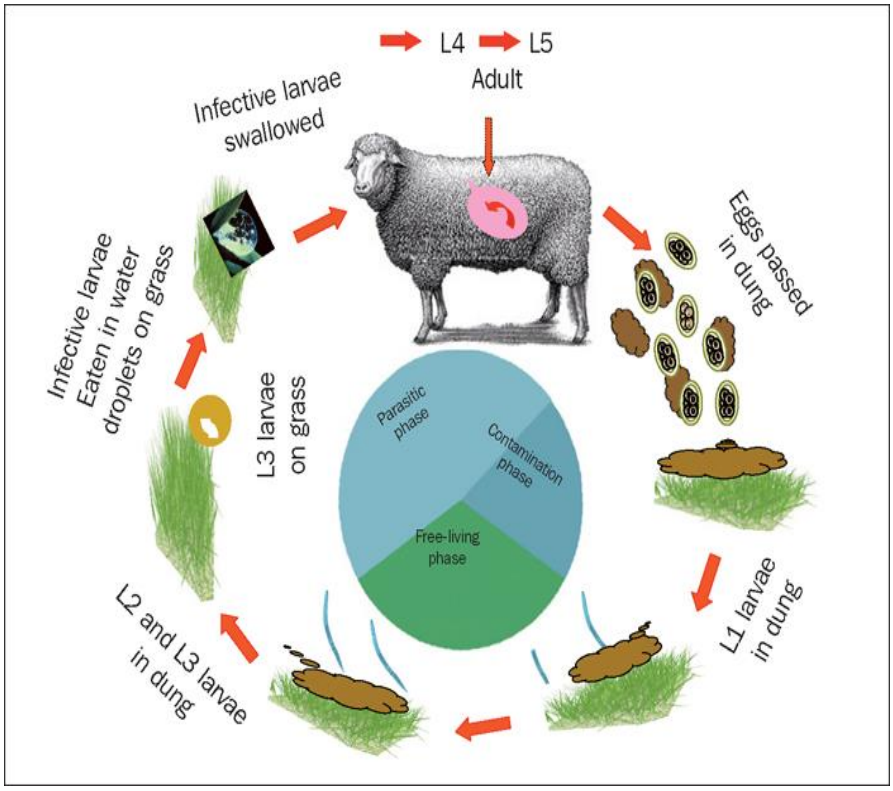

Figure 2.2. Life cycle of *Haemonchus contortus* from ingestion and infection to L5 stage (www.vettimes.co.uk)

2.3 Pathogenesis

Due to the worm's blood-consuming life style, the major clinical sign of haemonchosis is anaemia. Logically, higher worm burdens result in greater blood loss and an adult worm is capable of consuming 0.08 ml of blood per day (Attindehou et al. 2012). With low-level infections, blood loss would not be clinically significant. However, with favourable conditions, infections can easily increase to tens of thousands of worms, resulting in a rate of blood loss that exceeds the host's ability to regenerate red blood cells (Attindehou et al. 2012). Stress and poor nutrition worsen the effects of infection because they weaken the host's immune response to the parasite. Characteristic signs of severe *H. contortus* infection are pale mucous membranes and "bottle jaw", submandibular oedema due to hypoproteinaemia. Haemonchosis can result in reduced weight gain, weight loss, general unthriftiness, and death (Attindehou et al. 2012). Susceptibility varies from animal to animal due to individual genetic and environmental factors.

2.4 Control of helminth infections

2.4.1. Control measures

The infection of small ruminants by internal parasites (especially nematodes) is the most serious problem that challenges the small ruminant industry today (Waller and Prichard, 1985). Infections with these parasites can cause major economic losses to producers because of the cost of treatment, production loss, and death of heavily infected animals. Correct and effective management of internal parasites is critical for the survival of the small ruminant industry. The ability to detect the clinical signs of a major worm infection, to properly treat the infected animals, and to effectively reduce the flock's exposure to these parasites are all very important for effective internal parasite management (Waller and Prichard, 1985).

The disease haemonchosis is prevalent all over the world, especially in developing countries (Dhar et al., 1982) and is commonly associated with poor management practices and inadequate and inappropriate control strategies. An integrated approach is required for the effective control of helminths. This includes strategic and tactical use of anthelmintics in addition to careful management of grazing lands, including control of stocking rates and appropriate rotation strategies (Dhar et al., 1982). Vaccinations are also vital for the control of some parasitic diseases as in the case of lungworms. Attempts have been made to develop vaccines against several economically important gastrointestinal nematodes in small stock and cattle (Ekoja and Smith, 2010; Halliday and Smith 2011). Wirevax was launched first in South Africa by Afrivet, and was believed to provide stock owners with an affordable, cost effective product scientifically proven for South African conditions (Ekoja and Smith, 2010; Halliday and Smith, 2011). The vaccine acts against the blood-sucking *H. contortus* by stimulating antibodies that mix in the host's bloodstream so that the parasites drink antibodies specific for their own intestinal proteins. Wirevax contains tiny amounts of protein purified from the lining of the nematode's intestines. Like all vaccines, it works by stimulating the natural immune response in the animal after injection. These antibodies attach to the lining of the nematode, blocking digestion and starving the worm so that it produces far fewer eggs and eventually dies (Bassetto et al*.,* 2014).

Various problems have emerged with the use of anthelmintic drugs and among them resistance to different anthelmintic compounds and classes against various species of helminths is of utmost importance (Waller and Prichard, 1985). Also of importance is the resulting chemical residues and toxicity problems (Kaemmerer and Butenkotter, 1973).

2.4.2. Commercial anthelmintic drugs for the control of *H. contortus*

For more than half a century, the pharmaceutical industry has been distributing what are considered “modern” anthelmintic drugs aimed at a variety of animal parasites (Getachew et al., 2007). Some have been more successful than others. Ideal modern anthelmintic drugs have a wide margin of safety and spectrum of action, are easy to administer, have short residual periods, and are cost effective (Getachew et al., 2007). Prophylaxis for, or treatment of, nematode diseases can be achieved via the use of anthelmintic drugs. The anthelmintic drugs interfere with vital functions of the parasite, leading to starvation and/or paralysis followed by expulsion of the parasite (Getachew et al., 2007). There are various products in multiple drug classes available to combat parasitic nematodes. In 1961, thiabendazole, a benzimidazole, was the first successful product introduced with lower toxicity for treatment of nematodes (Kaplan, 2004). The group of benzimidazole anthelmintics represented the beginning of the modern chemical control of helminth parasites (Kaplan, 2004). This was followed by the main anthelmintic groups, levamisole and macrocyclic lactones. These anthelmintics act in different ways. The mechanisms of action of anthelmintic drugs are shown in Figure 2.3.

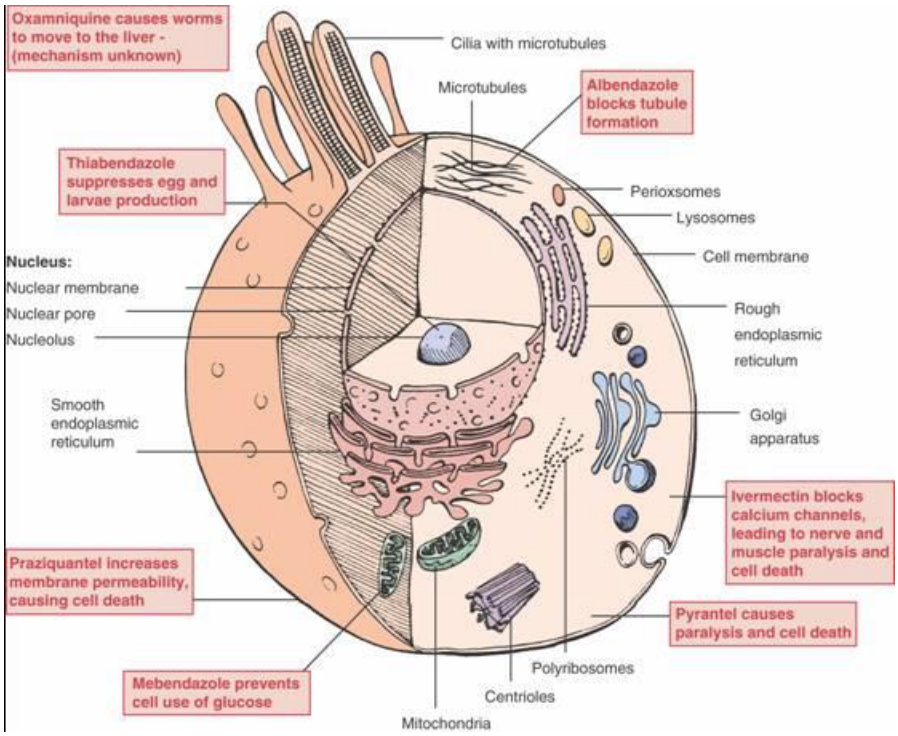

Figure 2.3. Mechanism of action of anthelmintic drugs (Veterinary Pharmacology-Chemotherapy, 2015)

2.4.2.1. Benzimidazoles

Benzimidazoles affect microtubules of the worm (Kohler, 2001) by binding to beta-tubulin, inhibiting the polymerization of tubulin and the formation of microtubules. The lack of microtubules inhibits many cellular functions such as transport, cell division, neural transmission, and cell differentiation, ultimately leading to cell death in the worm (Prichard, 2005).

The mode of action of the commonly used benzimidazole drug, mebendazole, is by binding to the cycloskeletal heterodimeric protein tubulin within the parasite (Figure 2.3), thus disrupting tubulin polymerization and leading to cell starvation (WHO, 2002). Benzimidazoles are used due to their high therapeutic index and absence of toxic residues in food animals (Townsend et al., 1990). They are available as oral pastes and as suspensions due to poor aqueous solubility. The slow transit through the rumen makes it ideal for use in ruminants (Harder et al., 2003).

The spectrum of activity of anthelmintics includes most nematodes such as *H. contortus*, *O. ostertagi, Nematodirus* spp. and *Dictyocaulus viviparous* (Taylor, 2000). In addition to that, albendazole is also effective against *Fasciola hepatica*. Albendazole and oxfendazole are not recommended to be used in pregnant animals due their teratogenic effects (Taylor, 2000).

2.4.2.2. Imidazothiazoles and tetrahydropyrimidines

Imidazothiazoles are nicotinic drugs, affecting the nervous system of the worm (Martin et al., 2005). Consequently, they cause sustained muscle contraction, leading to paralysis in nematodes and other parasites. These compounds have a broad-spectrum anthelmintic efficacy against nematodes but have no action against cestodes or trematodes (Kohler, 2001; Martin et al., 2005). Tetramisole is a racemic mixture of L and D isomers, while levamisole is the pure L-isomer. Pyrantel and morantel are structurally related to each other but different in structure from levamisole and are also effective anthelmintics (Martin et al., 2005). They act as nicotinic receptor agonists and elicit spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine receptors (Martin et al., 2005). Levamisole, tetrahydropyrimidines (e.g. pyrantel and morantel) and some other structurally related compounds are the main nicotinic drugs (Kohler, 2001).

2.4.2.3. Macrocyclic lactones

The avermectins/milbemycins bind to glutamate and gamma-amino butyric acid (GABA)-gates causing a hyper-polarization of nerve or muscle cells, leading to paralysis and killing of the parasite (Prichard, 2001).

2.4.2.4. Praziquantel

The action of praziquantel is linked to an induction of calcium flux across the integumental membrane (Prichard et al*.,* 1982) which increases tegumental calcium levels causing muscle contraction in the parasite body.

2.4.2.5. Closantel

Closantel has the ability to interfere with the proton gradient in the parasite's mitochondria that inhibits the generation of ATP by the parasite (Van den Bossche, 1985). Recent results, using nuclear magnetic resonance (31P-NMR) to measure effect of closantel on fluke intrategumental pH, suggest that closantel is a membrane-active molecule that is capable of affecting a number of helminth biochemical and physiological processes (Pax & Bennett*,* 1989).

2.4.2.6. Piperazine

Piperazine was first used as an anthelmintic in the 1950s and it is still the active constituent of over the counter remedies for thread worm infection (Miltsch et al., 2013). Piperazine acts to block neuromuscular transmission in the parasite by hyperpolarizing the nerve membrane, which leads to flaccid paralysis (Miltsch et al., 2013). It also blocks succinate production by the worm. The parasites, paralyzed and depleted of energy, are expelled by peristalsis (Miltsch et al., 2013).

2.4.2.7. Amino-acetonitrile derivative (AADs)

A new class of anthelmintics has recently been discovered. This class is known as amino-acetonitrile derivatives (AADs). AADs are thought to act on a nematode-specific group of acetylcholine receptor subunits and exhibit a broad spectrum of activity on parasites of sheep and goats. The prototype of AADs is monepantel (Kaminsky et al., 2008). It is effective against larval and adult stages of nematodes. Despite the availability of all these anthelmintics, control of the worms has become difficult due to development of resistance against all classes of available anthelmintics (Edward & Hoffman, 2008).

2.5. Anthelmintic drugs and resistance

Drug resistance is represented by the ability of worms in a population to survive drug treatment that earlier used to be effective against the same species and stage of infection at the same dose rate (Kaplan, 2004; Dobson, 2004). There are several aspects that affect the rate with which resistance develops. Some of these factors are related to the pharmacokinetics of the anthelmintic drugs, while others are related to the parasite biology, or environmental factors. Some drug-related factors contributing to resistance include the mode of action, frequency, timing, dose rate, method of delivery, persistence of the drugs, and specific rates of metabolism for different hosts (Dobson and Meagher, 1996).

The introduction of modern anthelmintic drugs was a great success, particularly for the small ruminant production industry. On one hand, anthelmintic drugs provided broad spectrum, safe, highly effective elimination of helminths that resulted in more productive and more profitable animals. On the other hand, the great dependability of these drugs to kill parasites within the host caused a shift in production practices such that producers relied on application of anthelmintic drugs alone and began to abandon other control methods to minimize parasite populations in the environment. Several years after the advent of modern dewormers, resistant strains of *H. contortus* were reported. The first broad-spectrum anthelmintic to become associated with resistant parasite strains in North America was thiabendazole, a benzimidazole, followed in the 1980's by levamisole and ivermectin (Craig, 2006). As early as 1997, Waller reported resistance to all three major drug classes in multiple countries in Africa.

The genetic diversity in different parasite isolates and the frequency of resistant alleles also contribute to resistance (Prichard, 2001). Several commercially available anthelmintic drugs being used all over the world are prone to the development of resistance. The frequent and incorrect use of three main anthelmintic classes, benzimidazoles, imidazothiazoles and macrocyclic lactones (ML), has escalated the rate of development and spreading of anthelmintic resistance (Watson et al., 1993). Currently benzimidazole-resistant nematodes of sheep have been reported and are a great threat to small ruminant production systems in many parts of the world (Perry et al., 2009). *Haemonchus contortus* has been considered as one of the leading parasites developing resistance against different anthelmintic compounds.

The mechanism of anthelmintic resistance is not completely understood. Drug resistance can develop in a limited number of ways (Wolstenholme et al*.,* 2004). There may be a change in the molecular target, so that the drug no longer recognizes the target, or a change in

metabolism that inactivates or removes the drug or that prevents its activation, or a change in the distribution of the drug in the target (Wolstenholme et al*.,* 2004).

2.6 Role of free radicals in infectious disease prevention and aetiology

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the mitochondria as a by-product of normal cellular metabolism forming part of the electron transport chain. ROS/RNS are involved in cellular activity regulation that includes defence against infectious pathogens and cellular signalling systems at a physiological level (Gulcin et al., 2003). In the presence of infections, burns, muscle damage or inflammation the enzyme phagocyte NADPH oxidase facilitates the production of ROS from leukocytes. The phagocyte NADPH oxidase is a multicomponent enzyme present in phagocytic leukocytes that consist of neutrophils, eosinophils, and monocytes/macrophages. This response triggers neutrophils, eosinophils, and macrophages/monocytes which are phagocytic cells to the site of infection. The phagocytic cells will then generate ROS, which will play a role in cell signalling, killing of the infectious organism, and stimulating the antioxidant repair process (Roos et al*.,* 2003). Defence against infectious pathogens occurs mainly by internalizing (phagocytosing) and exposing pathogens to reactive species (ROS/RNS) generated by the phagocyte NADPH oxidase (Roos et al., 2003).

Reactive oxygen species can be harmful if overproduced. Excessive generation of ROS/RNS without adequate neutralization mechanisms causes oxidative stress that involves the disturbance of endogenous antioxidant mechanisms in living organisms (Gulcin et al., 2003). Oxidative stress can lead to diseases due to cellular damage and a resultant loss of structural integrity and functionality. Oxidative stress can also cause damage of DNA and mitochondria and this is associated with diseases such as rheumatoid arthritis, cancer, Alzheimer's disease, Parkinson's disease, non-degenerative diseases and ischemic reperfusion (Droge, 2002). When considering rheumatoid arthritis for example it is believed that ROS such as NO together with superoxide (O_2^-) and their interaction products are responsible for tissue injury because of the disrupting oxidative reactions that they initiate. Reactive oxygen intermediates are thus mediators of inflammation and are responsible for the pathogenesis of tissue damage in rheumatoid arthritis (Valentao et al., 2002).

2.7. Alternative control strategies

Anthelmintics alone cannot combat ever-changing strains of *H. contortus*. The current state of crisis regarding widespread anthelmintic resistance calls for alternative control strategies. A

multifaceted approach that includes pasture management, careful planning and observation, and individualized treatment is required to control gastrointestinal helminths (van Zyl et al., 2017). The goal of alternative control is to develop methods or products that can be used in place of commercial dewormers, or as an adjunct to them, to reduce dependency on chemical control.

2.7.1 Plants used as anthelmintic drugs

A number of medicinal plants have been used to treat parasitic infections in man and animals (McGaw et al., 2000; McGaw et al., 2007; Nchu et al., 2011; Adamu et al., 2013, 2014). However, their scientific evaluation under controlled conditions compared to commercial anthelmintics is limited. There is some evidence to suggest that feeding forages containing tannins can significantly reduce nematode burdens (van Zyl et al., 2017). Tannins are secondary compounds of plants characterized by free phenolic groups. They are divided into two groups, namely hydrolyzable tannins and the more common proanthocyanidins or condensed tannins containing flavonoid units. Niezen et al. (1998b) concluded that feed containing condensed tannins leads to a reduction in worm burdens and faecal egg counts (FEC). In addition, Molan et al. (2002) reported that condensed tannins from dock (*Rumex obtusifolius*) had the greatest inhibitory effect on egg development, while Tzamaloukas et al. (2005) recommended the use of chicory (*Cichorium intybus*) as bioactive forage for the reduction of *Teladorsagia circumcincta*. *Zanthoxylum fagara* (Fagara) leaves had an inhibitory effect on nematode infection. Hounzangbe-Adote et al. (2005) reported that the administration of Fagara leaves to sheep was associated with a decrease in egg excretion and a significant reduction in the fertility of female worms, without changes in the worm number. Table 2.1 provides a summary of some plant species that have been tested for efficacy against internal parasites using *in vitro* and *in vivo* studies.

Table 2.1 Plants studied for *in vitro* and *in vivo* efficacy against internal parasites in animals

Plant species and family	Common name	Medicinal use	Plant part used	*In vitro* studies	*In vivo* studies	Parasite tested	Compound(s) isolated	LC_{50} (or % activity)	Reference
Aloe ferox Asphodelaceae	Bitter aloe	Wound healing, properties of healing arthritis	Leaves	N/A	*In vivo*	*Haemonchus contortus*	N/A	100%	Maphosa et al., 2010
Anogeissus leiocarpus Combretaceae	Chew stick	Livestock and humans' gastrointestinal worms	Bark and leaves	*In vitro*	N/A	*Haemonchus contortus*	N/A	0.36 mg/ml	Ademola and Eloff., 2011a
Anarcadium occidentale Anacardiaceae	Cashew	Tooth ache and gum problems	Leaves	*In vitro*	N/A	*Haemonchus contortus*	N/A	0.311 mg/ml	Ademola and Eloff., 2011b
Artemisia afra Asteraceae	African wormwood	Infusion for haemorrhoids and wounds	Leaves	*In vitro*	N/A	*Haemonchus contortus*	N/A	38.96%	Molefe et al., 2012
Cleome gynandra Capparidaceae	African cabbage	Colic pains	Leaves	*In vitro*	N/A	*Haemonchus contortus*	N/A	68%	Fouche et al., 2016
Curtisia dentata Cornaceae	Assegai	Stomach ache ailments and blood cleanser	Leaves	*In vitro*	N/A	*Haemonchus contorus*	Betulinic acid	40%	Shai et al., 2009
Elephantorrhiza elephantina Fabaceae	Elephant root	Remedy for dysentery, diarrhoea, treating intestinal disorders haemorrhoids, heart ailments and syphilis,	Root	*In vitro*	N/A	*Haemonchus contortus*	N/A	100%	Maphosa & Masika., 2011

Heteromorpha trifoliata Apiaceae	Parsley tree	Abdominal pains, gastrointestinal worms	Whole plant, leaves	*In vitro*	N/A	*Haemonchus contortus*	N/A	0.62 mg/ml	Adamu et al., 2013
Leucosidea sericea Rosaceae	Oldwood	Treatment of opthalmia and anthelmintic	Leaves	*In vitro*	In vivo	*Haemonchus contortus*	B-sitosterol, agrimol G (mixture of agrimol A and B)	0.72 mg/ml	Adamu et al., 2013
Monsonia angustifolia Geraniaceae	Crane's bill	Sexual stimulant and blood cleanser. Treatment of heartburn, anthrax, diarrhoea	Fruits	*In vitro*	N/A	*Haemonchus contortus*	N/A	56%	Fouche et al., 2016
Peltophorum africana Fabaceae	African wattle	Eye wash, stomach ailments	Leaves, stem bark and root bark	N/A	*In vivo*	*Haemochus contortus*	N/A	750MG/KG	Bizimenyera., 2008
Senna italica Fabaceae	Italian senna	Infusion is used to treat diarrhoea in infants	Leaves	*In vitro*	N/A	*Haemochus contortus*	N/A	25%	Mphahlele et al., 2016

2.7.2 Medicinal plants and toxicity

Parasites are eukaryotes and therefore share many molecular and biochemical properties with their eukaryotic hosts, making it often difficult to find antiparasitic drugs which are both effective and safe for animals. This limitation always has to be kept in mind when discussing the numerous findings that some compounds or extracts from a medicinal plant are active against parasites *in vitro*. In order to be medicinally useful, such a drug must have a good selectivity index, i.e. greater effectiveness against the parasite than toxicity against the host.

Toxic plants can pose a major health threat in small ruminants, especially during times of drought. Animals may consume more of these plants during drought because fewer alternative plants are available. Plants commonly used in traditional medicine are assumed to be safe. This presumed safety is based on their long use in the treatment of diseases according to knowledge accumulated over centuries (Schimmer et al., 1988). The dosage to be used can play an important role in efficacy or toxicity and is frequently not reported.

2.8 Conclusion

Helminthosis is a major constraint to production in small ruminant farming practices. Resistance of nematodes, particularly *Haemonchus contortus,* to all known synthetic anthelmintic drugs is a serious problem resulting in significant economic losses as well as animal welfare issues. Effective management of internal parasites is a critical issue and alternative and complementary therapies are being sought. Many studies have highlighted the potential efficacy of plant extracts in inhibiting egg hatching and growth of nematode parasites. More research is necessary to determine the potential of plants to assist in combating helminth infections. Anthelmintic assays are laborious and expensive, so it would be useful to determine an alternative, rapid and cheap bioassay to use as an alternative to identifying plants with activity against nematodes. Owing to the association between antifungal and anthelmintic activity in certain classes of drugs, this may be a route to investigate. Other useful biological activities of the plants could also contribute to their overall efficacy, and it is of course necessary to ensure that no potential toxicity exists in the plant extract.

CHAPTER 3: Anthelmintic activity of acetone extracts from South African plants plants used in ethnoveterinary medicine on egg hatching of *Haemonchus contortus*

Preface

In a joint project with the Phytomedicine Programme, the Council for Scientific and Industrial Research (CSIR, Biosciences Division, South Africa) compiled a list of South African plant species used traditionally for the treatment of internal and external parasites. The selection was based on different criteria such as interaction with traditional health practitioners, interaction with members of rural communities, databases of indigenous plants and their uses, information from medicinal plant books within the CSIR and literature searches. For this part of the study, I was provided with 15 plant species from the CSIR list. I was responsible for determining the biological activities of the extracts, analyzing results and assisting with drafting the manuscript. The results have been published in the following manuscript:

Fouché, G., Sakong, B.M. Adenubi, O.T., Pauw, E., Leboho, T., Wellington, K.W., Eloff J.N. 2016. Anthelmintic activity of acetone extracts from South African plants used on egg hatching of *Haemonchus contortus*. Onderstepoort Journal of Veterinary Research 83(1), 1165. http://dx.doi.org/10.4102/ojvr.v83i1.1164

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Anthelmintic activity of acetone extracts from South African plants used on egg hatching of *Haemonchus contortus*

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Abstract

The nematode, *Haemonchus contortus*, is responsible for major economic losses in the livestock industry. The management of parasites such as *H. contortus* has been with synthetic parasiticides. This has resulted in the formation of residues in meat and milk, which affects food safety. The development of resistance to available anthelmintics coupled with their high cost has further complicated matters. This has led to the investigation of alternative methods to manage nematodes including the use of plants and plant extracts as a potential source of novel anthelmintics. Acetone extracts were prepared from fifteen South African plant species and their anthelmintic activity determined using the egg hatch assay (EHA). The leaf extract of *Cleome gynandra* had the best inhibitory activity (68 ± 3%) at a concentration of 2.5 mg/ml followed by the stem extract of *Maerua angolensis* (65 ± 5%). The extracts had a relatively low toxicity on Vero cells determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) cellular assay.

Keywords: *Haemonchus contortus*, Anthelmintic activity, Egg hatch assay, Toxicity bioassay, Plant extracts, Acetone.

3.1. Introduction

Livestock production in tropical and developing countries is severely hampered by gastrointestinal parasites (Adejinmi and Harrison, 1997; Hounzangbe-Adote et al., 2005). The gastrointestinal parasitic nematode, *Haemonchus contortus*, also known as the barber pole worm, resides in the gut of sheep and other livestock. It accounts for about 80% of the global parasite afflictions of diseased animals (Arosemena et al., 1999) and is notorious for its high pathogenicity (Angulo-Cubillán et al., 2010).

In small ruminants gastrointestinal nematodes have traditionally been managed by the use of synthetic anthelmintic compounds (Mendonza de Gives et al., 1998). The systematic application of anthelmintic drugs in an effort to manage infections produced by *H. contortus* has led to the emergence of resistant strains (Prichard, 1994; Akhtar et al., 2000). There have been reports of parasite resistance to anthelmintic drugs in many countries (Schnyder et al., 2005; Melo et al., 2009) and multiple anthelmintic resistance has reached extreme levels (Torres-Acosta et al., 2012).

Certain drugs may also cause problems as food residues and environmental pollution (Hammond et al., 1997). This global problem has caused severe losses in productivity and is the main restricting factor for the livestock sector (Melo et al., 2009; Waller, 1994). Novel alternative methods are thus required. Research has been conducted on plant species as alternative anthelmintics to manage gastrointestinal infections in small ruminants (Adamu et al., 2013; Batista et al., 1999; Slomp et al., 2009). The use of anthelmintic plant extracts may be sustainable and environmentally acceptable and could provide an alternative to synthetic anthelmintics.

Furthermore, anthelmintic plant extracts have a mixture of active principles that could act in synergy yielding the anthelmintic effect and limit the development of resistance. This differs from commercial drugs that usually have only one molecule acting on the parasite when not administered as a combination formulation. Resistance is therefore likely to develop more slowly in the natural product. The aim of this study was to determine the *in vitro* anthelmintic action of acetone extracts from fifteen South African plant species used traditionally to control parasites of *H. contortus* using the egg-hatching assay (EHA). The toxicity of the acetone extracts was also determined against Vero cells.

3.2. Materials and methods

3.2.1. Plant material collection

Fifteen plants [*Aloe rupestris* Baker*, Antizoma angustifolia* (Burch.) Miers ex Harv., *Calpurnia aurea ssp. aurea* (Aiton) Benth.*, Senna italica* subsp *arachoides* (Burch.) Lock*, Cissus quadrangularis* L.*, Clematis brachiata* Thunb.*, Cleome gynandra* L.*, Ficus sycomorus* L.*, Hypoxis rigidula* Baker var rigidula*, Maerua angolensis* DC, *Monsonia angustifolia* E. Mey.ex A. Rich.*, Pelargonium luridium* (Andrews) Sweet, *Schkuhria pinnata* (Lam.) Kuntze ex Thell*, Sclerocarya birrea* (A.Rich) Hochst and *Tabernaemontana elegans* Stapf. were selected on the basis of available literature and ethnoveterinary usage over many years at the Council for Scientific and Industrial Research (CSIR) (unpublished data). These plants were collected from different locations in South Africa during the summer season.

3.2.2. Production of dried, ground plant material

Plant material was dried in an oven at 30-60°C followed by grinding to a fine powder using a hammer mill.

3.2.3. Preparation of the acetone extracts

The acetone extract was prepared by adding 200 ml of acetone to 20 g of each ground plant material, which was stirred for 1 h. The extract was decanted and filtered, the residue was re-extracted with the same volume of acetone once again for 1 h, and the third time the same volume of acetone was used but the mixture was stirred overnight. The extracts were combined and the acetone evaporated on a rotary evaporator.

3.2.4. Egg recovery and preparation

The method used was based on the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines described by Adamu et al. (2013). Briefly *H. contortus* eggs were collected from sheep that were housed indoors on a concrete floor. Approximately 10 to 15 g of sheep faecal pellets was crushed in water to form a slurry and cleared of organic debris by serially filtering it through sieves of pore sizes 150 and 63 and 20 µm. The eggs were collected on the 20 µm sieve and washed off with a 40% sugar solution (density 1.18) into 50 ml centrifuge tubes. The tubes were then centrifuged for 5 minutes at 1000 rpm to separate the floating eggs from other debris. The supernatant was decanted on a 20 µm sieve and the eggs were washed off with water and collected in a 500 ml container. The concentration of eggs in the egg suspension was determined by counting the eggs under a microscope (10x10 magnification) using a McMaster slide. The egg concentration was subsequently brought to a final concentration of 100 eggs per 200 µl. To avoid proliferation of fungi, 5 µg amphotericin B solution (Sigma, Germany) was added per millilitre of egg suspension.

3.2.5. Egg hatch assay (EHA)

The *in vitro* egg hatch assay was based on the procedure described by Adamu et al. (2013) which is based on the method described by Coles et al. (1992). The egg suspension (200 µl) containing approximately 100 fresh eggs were distributed into each well of a 24-well flat-bottomed microtitre plate. The same volume (200 µl) of the dried plant extract (5 mg/ml) dissolved in solvent (5% DMSO) was added to each well. Albendazole was used as positive control and 5% DMSO was used as the negative controls. Albendazole was dissolved in 5% DMSO in water and evaluated at various concentrations (0.008 to 25 µg/ml). The plates were incubated for 48 h at 27^{o}C at 70% relative humidity. The experiment was replicated three times for each extract on the same plate. After incubation, the hatched larvae and unhatched eggs

were counted using an inverted microscope under X20 magnification. The percentage inhibition of egg hatching was calculated using the formula of Bizimenyera et al. (2006):

Formula for calculation of results: Egg hatch inhibition (%) = 100 (1 - Number of larvae / Number of larvae and eggs in water control)

3.2.6. Determining the toxicity of the plant extracts

The toxicity of the plant extracts was determined by using the method employed by Adamu et al. (2013). Vero African Green monkey kidney cells were obtained from a confluent monolayer and then trypsinised and seeded (0.5×10^3 cells/ well) in a 96-well microtitre plate. This was followed by incubation overnight at 37°C in 200 µl of 5% minimal essential medium (MEM, Highveld Biological, South Africa) and supplemented with 0.1% gentamicin (Virbac R) and 5% foetal calf serum (Adcock-Ingram) (Adamu et al., 2013). The media were replaced with 200 µl of the extracts (1, 0.1, 0.01 and 0.001 mg/ml) after 24 h and incubated for another 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) as described by Mosmann (1983). Basically, the medium in each well was removed, replaced with fresh medium and 30 µl (5 mg/ml) MTT in phosphate-buffered saline (PBS) followed by incubation for 4 h. The medium was then removed before washing the cells with PBS and before the addition of DMSO (50 µl) to dissolve any formazan crystals (Adamu et al., 2013). A Versamax microplate reader at 570 nm (path length 1 cm) was used to measure the absorbance of the wells. Doxorubicin was used as a positive control and tested at different concentrations. The negative control was a well containing cells without extract. The percentage of cell viability was calculated relative to the pure growth. The LC_{50} value was calculated by determining the concentration of each plant extract resulting in 50% reduction of absorbance compared to untreated cells. Tests on the concentration of each extract were carried out in triplicate, and each experiment was repeated three times. The LC_{50} results are expressed as the mean ± standard deviation (s.d.) of the three replicates. A plant extract having an LC_{50} value > 20 µg/ml has an acceptable level of toxicity, whilst a value < 20 µg/ml is regarded as toxic (Kuete and Efferth, 2010).

Data analysis

Excel for Windows 7 was used to record the results produced in this study. Kinetica 5.0 (Thermo) using a sigmoid inhibitory model was used to calculate the LC_{50} values. The results are presented as the mean LC_{50} and the standard deviation of the mean.

3.3. Results

Acetone was selected as extractant because it has many advantages over other generally used extractants (Eloff, 1998a). The extraction yield was very similar for the different plant species and plant parts and varied from 5% to 6% (Table 3.1).

The yield that was obtained for each of the plant species is shown in Table 3.1.

Table 3.1. The plants and plant parts used for the solvent extraction, plant family, the mass and percentage of extract obtained

Entry	Plant and plant part used in extraction	Plant family	Solvent	Mass of extract % yield
1	*Aloe rupestris* (leaves)	Asphodelaceae	Acetone	1.0127 g (5%)
2	*Antizoma angustifolia* (roots)	Menispermaceae	Acetone	1.0619 g (5%)
3	*Calpurnia aurea* (leaves, flowers)	Fabaceae	Acetone	0.9409 g (5%)
	Calpurnia aurea (stems)	Fabaceae	Acetone	1.0491 g (5%)
4	*Senna italica* subsp. *arachoides* (roots, leaves, and fruit)	Leguminosae	Acetone	1.0920 g (5%)
5	*Cissus quadrangularis* (stems)	Vitaceae	Acetone	1.0063 g (5%)
6	*Clematis brachiata* (whole plant)	Ranunculaceae	Acetone	1.0430 g (5%)
7	*Cleome gynandra* (leaves)	Capparidaceae	Acetone	0.9699 g (5%)
8	*Ficus sycomorus* (bark and stems)	Moraceae	Acetone	1.0339 g (5%)
9	*Hypoxis rigidula* (bulb)	Hypoxidaceae	Acetone	1.1049 g (6%)
10	*Maerua angolensis* (leaves)	Capparaceae	Acetone	1.1714 g (6%)
	Maerua angolensis (stem)	Capparaceae	Acetone	1.0524 g (5%)
11	*Monsonia angustifolia* (whole plant)	Geraniaceae	Acetone	1.0013 g (5%)
12	*Pelargonium luridum* (whole plant)	Geraniaceae	Acetone	1.0100 g (5%)
13	*Schkuhria pinnata* (whole plant)	Asteraceae	Acetone	1.1115 g (5%)
14	*Sclerocarya birrea* (bark, root)	Anacardiaceae	Acetone	0.9109 g (5%)
	Sclerocarya birrea (fruit)	Anacardiaceae	Acetone	1.0142 g (5%)
15	*Tabernaemontana elegans* (leaves)	Apocynaceae	Acetone	1.0023 g (5%)

The dried extracts did not dissolve in water, and therefore DMSO was used to dissolve the extracts. The best results were obtained with 3.1 and 6.3% DMSO leading to 13.0% and 19.0% inhibition of egg hatching, respectively. The results for the negative control of 2.5% DMSO did not have a marked effect on the egg hatching. The lower degree of hatching with the negative

controls probably indicates damage to the eggs during processing. Consequently, the extracts were dissolved in 5.0% DMSO leading to a 2.5% final concentration after adding the same volume of egg suspension.

3.3.1. Determining the inhibitory activity of the plant extracts on *Haemonchus contortus* egg hatching

The two negative controls also had a degree of activity on the egg hatching. The results are presented in Table 3.2 as the mean egg hatch inhibition (%) and the standard deviation of the mean. At 2.5 mg/ml, extracts of *C. gynandra* (leaves), *M. angolensis* (stem), *M. angustifolia* (whole plant) and *S. italica* subsp. *arachoides* (roots, leaves and fruit) had a mean inhibition rate of between 55% and 68% which was much higher than the water and DMSO negative controls. Our choice of 2.5 mg/ml appeared to be a good concentration because only four species had what would have been an LC_{50} in the order of 2.5 mg/ml. Albendazole, the positive control, recorded 100% inhibition at the lowest concentration, 0.008 µg/ml. The extract of *C. brachiata* (whole plant) had the lowest activity (11%), which was even lower than the water control group (16%). The activity of the plant extracts was so much lower than that of albendazole that the feasibility of using plant extracts can be questioned. Plant extracts may be more active in the larval development assay (LDA).

Table 3.2. Mean inhibition percentages of the acetone extracts (2.5 mg/ml) on egg hatching of *H. contortus* from sheep and the toxicity values (LC_{50}) against Vero cells

Entry	Plant name and part used	Mean egg hatch inhibition (%) ± s.d.	Toxicity against Vero cells LC_{50} (µg/mL) ± s.d.
1	*Cleome gynandra* (leaves)	68 ± 3	553.61 ± 18.83
2	*Maerua angolensis (stem)*	65 ± 5	180.64 ± 3.4
3	*Monsonia angustifolia* (whole plant)	56 ± 6	120.37 ± 4.06
4	*Senna italica* subsp. *arachoides* (roots, leaves, fruit)	55 ± 13	46.31 ± 2.89
5	*Aloe rupestris* (leaves)	47 ± 7	63.46 ± 11.00
6	*Tabernaemontana elegans* (leaves)	47 ± 7	32.35 ± 0.88
7	*Schkuhria pinnata* (whole plant)	41 ± 14	39.93 ± 1.80
8	*Antizoma angustifolia* (roots)	37 ± 16	43.59 ± 6.28

9	*Calpurnia aurea (stems)*	32 ± 20	223.97 ± 5.3
10	*Sclerocarya birrea (fruit)*	28 ± 23	214.79 ± 14.0
11	*Calpurnia aurea* (leaves, flowers)	27 ± 16	166.63 ± 7.97
12	*Pelargonium luridum* (whole plant)	25 ± 10	30.58 ± 3.40
13	*Maerua angolensis* (leaves)	25 ± 6	73.76 ± 0.27
14	*Ficus sycomorus* (Bark & stems)	25 ± 5	172.94 ± 8.91
15	*Ficus sycomorus* (Stem)	21 ± 14	48.74 ± 1.32
16	*Hypoxis rigidula* (bulb)	17 ± 20	64.04 ± 2.53
17	*Clematis brachiata* (whole plant)	11 ± 15	117.00 ± 4.08
	2.5% DMSO	7 ± 17 14 ± 13	N.D.
	Doxorubicin	ND	2.97 ± 0.016

Note: Albendazole was the positive control and recorded 100% inhibition at all concentrations (0.008 to 25 µg/ml) used, while 2.5% DMSO recorded < 10 % inhibition.

3.3.2. Determining the cytotoxicity of the plant extracts

The tetrazolium-based (MTT) colorimetric assay (Mosman, 1983) was used to determine the viability of Vero African Green monkey kidney cells in the presence of each of the plant extracts and the results are shown in Table 3.2. From the results, it is apparent that none of the plant acetone extracts were as toxic as doxorubicin (2.97 ± 0.016 µg/ml = 5.12 µM ± 0.028 µM). The leaf extract of *C. gynandra* was the least toxic with (LC_{50} = 553.61 ± 18.83 µg/ml), followed by the stem extract of *C. aurea* (LC_{50} = 223.97 ± 5.4 µg/ml), the fruit extract of *S. birrea* (LC_{50} = 214.79 ± 14 µg/ml), the stem extract of *M. angolensis* (LC_{50} = 180.64 ± 3.5 µg/ml) and the bark and stem extract of *F. sycomorus* (LC_{50} = 172.94 ± 8.91 µg/ml). The whole plant extract of *P. luridum* (LC_{50} = 30.58 ± 3.40 µg/ml) was the most toxic of all the plants against Vero cells.

3.4. Discussion

The EHA is an *in vitro* assay used to evaluate the anthelmintic activities of natural products. The capacity to reduce egg hatching could help to modulate the risk of parasitism by limiting the infectivity of pastures grazed by ruminants (Max, 2010). This study determined the inhibitory activity of the acetone extracts of 15 plant species on egg hatching of *H. contortus*

in order to select the most promising plant species that could control the nematodes in the animal gut for further study. In previous studies, it was found that aqueous extracts contained few compounds, had very low biological activity (Eloff et al., 2005; Kotze and Eloff, 2002) and had low or negligible anthelmintic activity (Bizimenyera et al., 2006; Worku et al., 2009). Acetone was therefore selected as an appropriate extractant because it is miscible with organic and aqueous solvents, non-toxic to bacteria and fungi, and also has the capacity to extract a wide range of polar compounds (Eloff, 1998a). As shown in Table 3.2, it is evident that the extracts of four plant species had anthelmintic activity (inhibitory activity above 50%) at the concentration tested.

Cleome gynandra leaf extracts had the best anthelmintic activity with egg hatch inhibition of 68 ± 3% and low toxicity (LC_{50} = 553.61 ± 18.83 µg/ml) on Vero cells. Our results are in agreement with that of other researchers who have also reported on the anthelmintic activity of *C. gynandra*. Two authors (Jadhav et al., 2011; Thenmozhi et al., 2014) used the unverified assumption that the Indian adult earthworm (*Pheretima posthuma*) could be used as a model for the activity of *C. gynandra* (syn. *Gynandropsis pentaphylla*) extracts against intestinal roundworm parasites of human beings because of its anatomical and physiological resemblance. They also used a physiologically non-relevant high concentration of 25 mg/mL and concluded that these extracts had potent anthelmintic activity when it killed earthworms after 53 min without examining any helminths. Sowunmi and Afolayan (2015) also did a phytochemical analysis of the acetone extract of different parts of *C. gynandra.* The phenolic contents of the various parts of the plant were significantly high. Leaf acetone extracts of *C. gynandra* had the highest concentration of total phenolics (126.79 ± 0.55 mg/g), flavonoids (40.58 ± 0.06 mg/g) and flavanols (42.41 ± 0.05 mg/g), whilst the stem extract had the highest amount of proanthocyanidins (419.01 ± 0.67 mg/g) compared to the leaves (403.29 ± 0.89 mg/g) and fruits (107.18 ± 0.59 mg/g). The low concentration of saponins and alkaloids suggests that this plant may have low toxicity (Sowunmi and Afolayan, 2015). This suggestion is supported by the low toxicity we observed against Vero cells (LC_{50} = 553.61 ± 18.83 µg/ml). Alcohol and aqueous extracts from the leaves of *Cleome viscosa* Linn were also investigated for their anthelmintic activity against the adult Indian earthworm, *P. posthuma*, as well as *Ascaridia galli*. Three concentrations (50, 100 and 150 mg/mL) of each extract were studied, which entailed the determination of time of paralysis and time of death of the worm. Both the extracts had significant anthelmintic activity at the highest concentration of 150 mg/mL. The waterleaf extract had weaker activity than the methanolic leaf extract, and both extracts caused paralysis and death of worms. Phytochemical screening of the methanol extract showed that anthraquinone glycosides, phenolic compounds and steroids were present in *C. viscosa* Linn, whilst in the aqueous extract glycosides and phenolic compounds were present.

Flavonoids were identified as being one of the chemical constituents amongst the phenolic compounds in the crude extracts. Phenolic compounds are known for their anthelmintic activity (Kaushik et al., 1974; Lal et al., 1976; Szewezuk et al., 2003). Synthetic phenolic anthelmintics such as niclosamide, oxyclozanide and bithionol interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (Bate-Smith, 1962; Martin, 1997; Tandon et al., 1997). The phenolic content may therefore have produced similar activity in the extracts of *C. viscosa* and *C. gynandra*.

The second-best egg hatch inhibition of 65 ± 5% was by *M. angolensis* (stem) extract that had low toxicity (LC_{50} = 180.64 ± 3.5 µg/ml) on Vero cells. Phytochemical screening of the methanolic extract of the stem bark found that glycosides, tannins, saponins, terpenes, flavonoids, carbohydrates, proteins and alkaloids were present in *M. angolensis* (Ayo et al. 2013; Meda et al., 2013; Pl@ntUse). These compounds could also be present in the acetone stem extract tested in this study. It has been reported that *Maerua edulis* (Gilg and Gilg-Ben.) De Wolf and *Maerua subcordata* (Gilg) De Wolf have been used in traditional anthelmintic remedies in Kenya to treat sheep infected with *H. contortus* (Gakuya, 2001). In this study, aqueous extract from both unground and ground material of each plant material was prepared using boiling water. Twenty-one clinically healthy sheep of mixed breeds and sexes were randomly allocated to four treatment groups, four animals each. Faecal egg counts were performed for all the sheep. It was found that the crude extracts could control helminthoses to a reasonable extent and maintain the animal at a clinically healthy state. The brine shrimp assay was used to detect bioactivity in the water, chloroform and methanol extracts of *M. subcordata* and *M. edulis.* The chloroform extract was the most toxic to the brine shrimps compared to the water and methanol extracts (Gakuya, 2001).

The *M. angustifolia* (whole plant) extract had an egg hatch inhibition of 56% ± 6% and also had low toxicity on Vero cells (LC_{50} = 120.37 ± 4.06 µg/ml). Five compounds identified as aryl naphthalene lignans (5-methoxyjusticidin A, justicidin A, chinensinaphthol, retrochinensinaphthol methyl ether and suchilactone) were isolated during the fractionation of the organic (methanol-dichloromethane) extract of *M. angustifolia* (Khorombi, 2006). Lignans are a group of naturally occurring phenolic compounds. The drug podophyllum, a lignan, is obtained from the dried root and rhizomes of two species of *Podophyllum* (Berberidaceae), the American species *Podophyllum peltatum* and the Indian species *Podophyllum hexandrum* (*Podophyllum emodi*). The European settlers reported using the root extensively, particularly as a cathartic and anthelmintic (Konuklugil, 1995). Thus, the anthelmintic activity of *M. angustifolia* may be attributed to the lignans. *Senna italica* subsp. *arachoides* (roots, leaves, fruit) had an egg hatch inhibition of 55 ± 13% and had a higher toxicity on Vero cells (LC_{50} =

46.31 ± 2.89 µg/ml) than that of *C. gynandra* (leaves), *M. angolensis* (stem) and *M. angustifolia* (whole plant) which also had anthelmintic activity.

Aloe rupestris (leaves) only had an egg inhibition of 47 ± 7% against *H. contortus* and low toxicity (LC_{50} = 63.46 ± 11.00 µg/ml) against Vero cells. It has been reported that other *Aloe* species such as *Aloe ferox* can affect *H. contortus* of goats negatively (Maphosa et al. 2010). The amino acids, saponins and sterols in *A. ferox* can disturb protein structure and therefore affect the growth and repair of the nematode body (Mabusela, Stephen & Botha 1990). Additionally*, Tabernaemontana elegans* Stapf (leaves) also only had an egg inhibition of 47 ± 7% and low toxicity (LC_{50} = 32.35 ± 0.88 µg/ml) against Vero cells. In Guadeloupe (French West Indies), another species, *Tabernaemontana citrifolia,* has traditionally been used as an anthelmintic preparation for ruminants (Marie-Magdeleine et al., 2010). Marie-Magdeleine et al. (2010) prepared aqueous, methanolic and dichloromethane extracts from the fruit, leaves and roots of *T. citrifolia* for testing on four developmental stages of *H. contortus*. The EHA, the LDA, the L3 migration inhibition assay (LMI) and the adult worm motility assay (AWM) were employed in the testing. From the tests it was apparent that there were significant effects for the different parts of *T. citrifolia* when compared to the negative control and that the differences depended on the parasitic stage. The efficacies on the larval development of *H. contortus* ranged from 88.9 to 99.8% for fruit, from 72.1 to 83.8% for roots and from 33.5 to 85.0% for leaves. For the methanolic extract a dose-dependent effect was observed. Alkaloid compounds are present in the different parts of *T. citrifolia* and may be responsible for the observed anthelmintic activity against *H. contortus* (Marie-Magdeleine et al., 2010). Another species, *Tabernaemontana coronaria,* was also investigated for anthelmintic activity against the Indian adult earthworm *P. posthuma.* It was found that the ethanolic extract of the leaves had potent anthelmintic activity (Pushpa et al., 2011).

Anthelmintic activity has also been documented for other plant species where an organic extract was also used in the determination of the activity (Ademola and Eloff, 2010, Ademola and Eloff, 2011). Adamu et al. (2013) reported that *Heteromorpha trifoliata*, *Leucosidia sericea* and *Maesa lanceolata* had 100% inhibition, whilst *Clausenia anisata* had 80% inhibition at a concentration of 3.13 mg/ml in the EHA. Several other plant species have been documented as having anthelmintic activity. These are *Lantana camara* (Verbenaceae), *Tagetes minuta* (Asteraceae), *Mentha villosa* (Lamiaceae) (Albuquerque et al., 2007) and *Alpinia zerumbet* (Zingiberaceae) (Almeida, 1993).

In this study, extracts of *C. gynandra* (leaves), *M. angolensis* (stem), *M. angustifolia* (whole plant) and *S. italica* subsp. *arachoides* (roots, leaves and fruit) only had inhibitory activity

between 55 and 68% at 2.5 mg/ml. Higher inhibitory activity for these extracts may have been obtained if they had also been evaluated at 3.13 mg/ml.

The kidney is one of the main sites of excretion in animals, and therefore, renal cells in culture were used as an indicator of toxicity for this study. These cells were explicitly chosen because of the favoured blood supply of the kidney and their high metabolic capacity. The results of the toxicity study were encouraging because the extracts had low toxicity (LC_{50} > 20 µg/ml) against Vero cells. The determination of cellular toxicity is valuable because it will give a good indication of whether *in vitro* toxicity is also an indicator of *in vivo* toxicity.

The results of this study are important because egg hatch inhibition is an important method by which pasture contamination by animals can be reduced during grazing. Thus, there is the possibility that the plant material in this study can be administered as a feed additive to control helminths, although the concentrations required for efficacy were very high. Overall, the use of these botanicals could contribute to a helminth control programme.

3.5. Conclusions

The acetone extracts of *C. gynandra*, *M. angolensis*, *M. angustifolia* and *S. italica* subsp. *arachoides* have some anthelmintic activity against *H. contortus* egg hatching. The activity of each of these extracts was lower than that of the positive control, albendazole. The most promising plant species is *C. gynandra,* which may be further studied to identify the active constituents responsible for anthelmintic activity.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

G.F. conceptualised the study. G.F., K.W.W. and T.L. did the literature search and plant selection. T.L. prepared the plant extracts. J.N.E. and E.P. arranged for sheep to be infected, collected the eggs and guided the study. B.M.S. did the egg hatch assay on the extracts. O.T.A. screened the extracts for toxicity on Vero cells. K.W.W. wrote the first draft of the manuscript.

CHAPTER 4: Description, extraction and chemical composition of plants with antifungal activity

Preface

With the results of screening the plant species supplied by the CSIR used for ethnoveterinary purposes against parasites failing to demonstrate substantive *in vitro* anthelmintic activity a second group of plants needed to be evaluated. Because of previously reported good correlations between antifungal and anthelmintic activity of certain classes of compounds, plants with known promising antifungal activity were selected for further investigation of anthelmintic activity from the database of the Phytomedicine Programme, University of Pretoria. A first step in this part of the study was to describe what is known about the plant species and to investigate their chemical profile and complexity using thin layer chromatography.

4.1 Introduction

Nature has been a source of therapeutic agents for thousands of years and a good number of modern drugs have been isolated from natural resources (Prince and Prabakaran, 2011). The presence of different phytochemicals in different parts of plants supports the potential of plants as a source of natural medicines (Fayaz et al., 2017). Medicinal plants represent a rich source of secondary metabolites, many of which possess antimicrobial properties (Mahesh and Satish, 2008). They also represent a possible alternative treatment in non-severe cases of infectious diseases (Reddy, 2009).

Many studies have been carried out to screen medicinal plants for their antifungal activity. Masoko et al. (2007) screened hexane, dichloromethane, and methanol leaf extracts of 24 South African *Combretum* species against five fungal animal pathogens, namely *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenckii*. Minimum inhibitory concentration (MIC) values were in the range of 0.02 to 0.06 mg/mL (Masoko et al., 2007).

Adamu et al. (2013) reported good antifungal activities of 13 acetone leaf extracts with MIC values as low as 0.02 mg/ml for extracts of *Clausena anisata* against *A. fumigatus* and 0.04 mg/mL for extracts of *Zanthoxylum capense, Clerodendrum glabrum* and *Milletia grandis* against A. *fumigatus*. *Clausena anisata* extracts showed a reasonable selectivity index (2.65)

against *A. fumigatus*. They also had selective activity against *A. fumigatus*, an overall fungicidal activity of 98% and a total activity of 3395 mL/g against *A. fumigatus.*

Although many studies have been carried out on antifungal activity of plant extracts, relatively few reports are available of activity of plant species against parasitic nematodes of importance to human and animal health. This is most likely owing to the complexity of culturing the parasite and the labour-intensive and more difficult bioassays that are involved. Interestingly, some classes of compounds have shown both antifungal and anthelmintic (as well as other) activities.the azole class of drugs occupies a prominent place in medicinal chemistry because of its broad spectrum of pharmacological activities such as antifungal, anti-inflammatory, analgesic, anticancer, antiviral, pesticidal, cytotoxic and antiarrhythmic (Ravindernath and Reddy, 2013). Albendazole, thiabendazole, mebendazole and triclabendazole are well-known anthelmintic drugs which comprise attached imidazoles as the essential active moiety. Their anthelmintic efficacy is due to their ability to compromise the cytoskeleton through a selective interaction with β-tubulin (Borgers and De Nollin, 1975; Lacey, 1990) and interference with energy production. Benzimidazoles have been widely used since the 1960s as anthelmintic agents in veterinary and human medicine and as antifungal agents in agriculture (Katiyar et al., 1994). The targeting of parasitic β-tubulin is important as it is indispensable for the formation of the microtubular network, which is responsible for vital cell functions as motility, cellular shape, mitosis, coordination, transport and secretion in nematodes, cestodes and trematodes (Mavrova, 2006).

Little is known on the association between antifungal and anthelmintic activity in plants. In one study, Adamu et al. (2012) concluded that some southern African plant species used to treat helminth infections in ethnoveterinary medicine have excellent antifungal activities. In this part of the study, six plant species were selected for anthelmintic efficacy investigation based on known antifungal activity following a large scale screening of South African plant species for antimicrobial activity (Pauw and Eloff, 2014). The MIC values of acetone extracts of these species was below 0.1 mg/ml in all cases (Pauw and Eloff, 2014). Based on the known antifungal and anthelmintic activity of the benzimidazoles (Katiyar et al., 1994), the next step was to examine the possible anthelmintic activity and cytotoxicity of extracts of these antifungal plant species. The aim of the current chapter was to introduce the plant species selected for further study and to analyse extracts of the plants using thin layer chromatography with a variety of eluent systems of differing polarity to determine the complexity of the extracts. Acetone was used to prepare the extracts as Eloff (1998) concluded that it was the best extractant owing to its ability to extract compounds of a wide range of polarities, its nontoxicity to bioassay systems and because it is easy to remove when drying extracts.

4.2 Selected plant species based on their antifungal activity

A description of the evaluated plant species is provided below.

4.2.1 *Diospyros whyteana*

Diospyros whyteana (Hiern) P. White (Fig 4.1), a species from the Ebenaceae family, is an attractive tree for the garden with dark green, glossy leaves, fragrant flowers and intriguing balloon-like fruits. It is a shrub or small tree, branching low down and forming a dense canopy. In traditional medicine, the bark of this plant has been used for the treatment of menstrual pain, while the roots have anticancer properties (Fouche et al., 2006). The common names are:

English: Bladder-nut, Blackbark; Afrikaans: Swartbas, Bostolbos, Kraaibessie; IsiXhosa: umTenatane; IsiZulu: uManzimane, umKahze; Sesotho sa Leboa: Mohlatsane; Tshivenda: Munyavhili, Mwanda.

Figure 4.1. *Diospyros whyteana* (Bladdernut) [Left: Flowering plants of Africa (2009) by Cherise Viljoen, Kirstenbosch Botanical Garden; Right: Abu Shawka, Wikimedia Commons]

4.*2.2 Peddiea africana*

Peddiea africana Harv. (Fig 4.2) from the family Thymelaeaceae is a many-branched shrub or small tree at the fringes or in underbrush of evergreen forests. The glossy green, leathery leaves are simple, spirally arranged or alternate and have short petioles or are sessile. The

flowers grow in umbels and have no petals but have a tubular shape, with the tube being formed by the ribbed calyx ending in five rounded lobes. The flowers are greenish yellow, sometimes with a red-brown or maroon tint, and the berries are purple to black when ripe. Although the plant is poisonous, birds eat the fruits; the bark is used to make rope and to cure heartburn and other ailments (Coates Palgrave, 2002).

Figure 4.2. *Peddiea africana* (Flora of Zimbabwe by Bart Wursten Fen Gully)

4.2.3 *Cassipourea gummiflua*

Cassipourea gummiflua Tul. (Fig 4.3) from the Rhizophoraceae family is a variable plant. An evergreen shrub or tree, it can grow from 5 - 20 m tall, though occasionally it can be only 3 metres tall or up to 40 m. The bark has medicinal properties and is in great demand but there is not much information on how it is used traditionally (Pooley, 1993).

Figure 4.3. *Cassipourea gummiflua* (Photograph: J. Lewis)

4.2.4 *Schotia brachypetala*

Schotia brachypetala Sond. (Fig 4.4) is an evergreen tree that belongs to the Fabaceae family. It loses its leaves for a short period in the cooler areas. It has a wide-spreading, densely-branched, rounded crown and can grow from 3 to 22 m (Pooley, 1993). The bark and roots are used together to treat diarrhoea, heartburn, hangovers, to strengthen the body and purify the blood and to treat nervous heart conditions (Pooley, 1993; Coates Palgrave, 2002).

Figure 4.4. *Schotia brachypetala*. Picture by Tatters from Brisbane, Australia (Wikimedia Commons)

4.2.5 *Senna petersiana*

Senna petersiana (Bolle) Lock (Fig 4.5) of the Fabaceae family is a shrub or small deciduous tree up to 4 m in height (Pooley, 1993). The shiny, dark green, compound leaves have large, leaf-like stipules at the leaf stalk base and the yellow flowers occur in large terminal bunches (Pooley, 1993). The plant is used pharmaceutically in laxative preparations, and root and

leaf infusions are used as a purgative to treat stomach-ache and intestinal worms (Van Wyk et al., 1997).

Figure 4.5. *Senna petersiana*, Photograph by SANBI, PlantzAfrica

4.2.6 *Bowkeria citrina*

Bowkeria citrina Thode (Fig 4.6), a member of the Scrophulariaceae family, is a multi-stemmed shrub about 3 m tall, occasionally tree-like. The stems are greyish white and most of the plant is dotted with golden glands and covered with fine soft hairs. The leaves are narrowly eliptical, 70 X 10 mm, and have entire, finely scalloped margins and are borne in whorls of three to four. When they are crushed, they give a pleasant lemony aroma. It grows well under full sun in gardens with temperate summers and a good rainfall and it is frost tolerant but cannot withstand drought. Regarding medicinal uses, there is no record of the plant being used for traditional practices (Coates Palgrave, 2002).

Figure 4.6. *Bowkeria citrina* (courtesy of L. van Staden)

The plant species investigated, as well as their traditional uses where relevant, are listed in Table 4.1.

Table 4.1: List of plant species selected for anthelmintic testing and their traditional medicinal uses

Plant Names and family	**Common Name**	**Traditional uses**	**References**
Bowkeria citrina (Scrophulariaceae)	Yellow shell-flower bush	Bark is used to treat dysentery and diarrhoea, nervous heart conditions, flu symptoms, excessive beer drinking and as an emetic and to strengthen the body, while roots are also used to treat diarrhoea and heartburn. The seeds can be roasted and eaten	Kokwaro (1976)
Cassipourea gummiflua (Rhizophoraceae)	Large-leaved onionwood	Used for unspecified purposes	Pooley (1993)
Diospyros whyteana (Ebenaceae)	Bladder nut	Bark extracts are administered in the form of enemas to treat infertility, impotency and menstrual pain and leaf and root infusions are used to treat skin rashes	Malan et al. (2015)
Peddiea africana (Thymelaeaceae)	Green flower tree	Roots are toxic but have anti-cancer properties	Van Wyk et al. (1997)
Schotia brachypetala (Fabaceae)	Weeping boer-bean and African walnut	A decoction of the bark is taken to treat heartburn and hangovers. Bark and root mixtures are used to strengthen the body and purify the blood, to treat nervous heart conditions and diarrhoea, as well as for facial saunas	Germishuizen (2006)
Senna petersiana (Fabaceae)	Monkey pod	Constipation, stomach aches, and stomach worms are treated by drinking the infusion or decoction of the roots, or less often by using fresh leaves. In South Africa, this plant is used to treat malaria, schistosomiasis, gonorrhea and syphilis	Drummond (1972)

4.3. Materials and methods

4.3.1 Plant collection and preparation

The plant species were collected from the Manie van der Schijff Botanical Garden, Hatfield Campus,University of Pretoria). Voucher specimens were prepared, assigned accession numbers and deposited at the HGWJ Schweickerdt Herbarium of the Department of Plant and Soil Sciences at the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa.

Leaves were separated from the stems and air-dried at room temperature in shade conditions for two weeks (Eloff, 1998b). The dried leaves were then milled to a fine powder using a Macsalab mill (model 200 LAB, Eriez®, Bramley) and stored at room temperature in closed containers in the dark until needed.

4.3.2 Preparation of plant extracts

Separate aliquots of plant material were extracted with acetone (technical grade, Merck) at a ratio of 1:10 (m/v) for 6 h. Extraction bottles were vigorously shaken on a Labotec model 20.2 shaking machine. The supernatants were filtered through Whatman No 1 paper, using a Buchner funnel. The process was repeated three times on the powdered plant material to exhaustively extract it and all the volumes were combined. The extracts were concentrated under vacuum using a rotary evaporator (Labotec) and transferred into pre-weighed labelled containers and dried under a stream of air.

4.3.3 Analysis of extracts

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium–backed TLC plates (Merck, silica gel 60 F_{254}). An aliquot (10 µl) of each of the plant extracts dissolved to 10 mg/ml in acetone was loaded in a band 1 cm in length on TLC plates. Three TLC plates were developed under saturated conditions with each of the following eluents (Kotze and Eloff, 2002):

- Ethyl acetate, methanol, water [EMW] (40:6.5:5) (polar)
- Chloroform, ethyl acetate, formic acid [CEF] (50:40:10) (intermediately polar)
- Benzene, ethanol and ammonium hydroxide [BEA] (90:10:1) (non-polar)

To detect the separated compounds, the chromatograms were sprayed with vanillin-sulphuric acid solution in methanol (0.1 g vanillin (Sigma): 28 ml methanol: 1 ml sulphuric acid) and heated to 110ºC to optimal colour development (Stahl, 1969).

4.4 Results and discussion

Acetone extracts were prepared of the six plant species determined to have good antifungal activity against *Candida albicans* in the preliminary screening assay. The yields of extract from one gram of plant material of *Senna petersiana* and *Bowkeria citrina* were the highest at 350 mg and 260 mg, respectively (35 and 26%). *Diospyros whyteana* also had a good yield of extract with 13%, while *Cassipourea gummiflua* and *Schotia brachypetala* had relatively low yields of 7 and 1%, respectively.

The thin layer chromatographic fingerprints of the extracts covering a wide polarity range are presented in Fig. 4.7. The extracts of all plant species showed the presence of many different phytochemicals with distinct R_f values ranging from 0 to 0.95. The chromatogram of acetone and cold water extracts developed with benzene, ethanol and ammonium hydroxide (BEA) indicated the highest number of compounds. The purple colouration of some bands shown in Fig. 4.7 indicates the possible presence of terpenoids while the pink colours of other bands may indicate the presence of flavonols (Shahid, 2012).

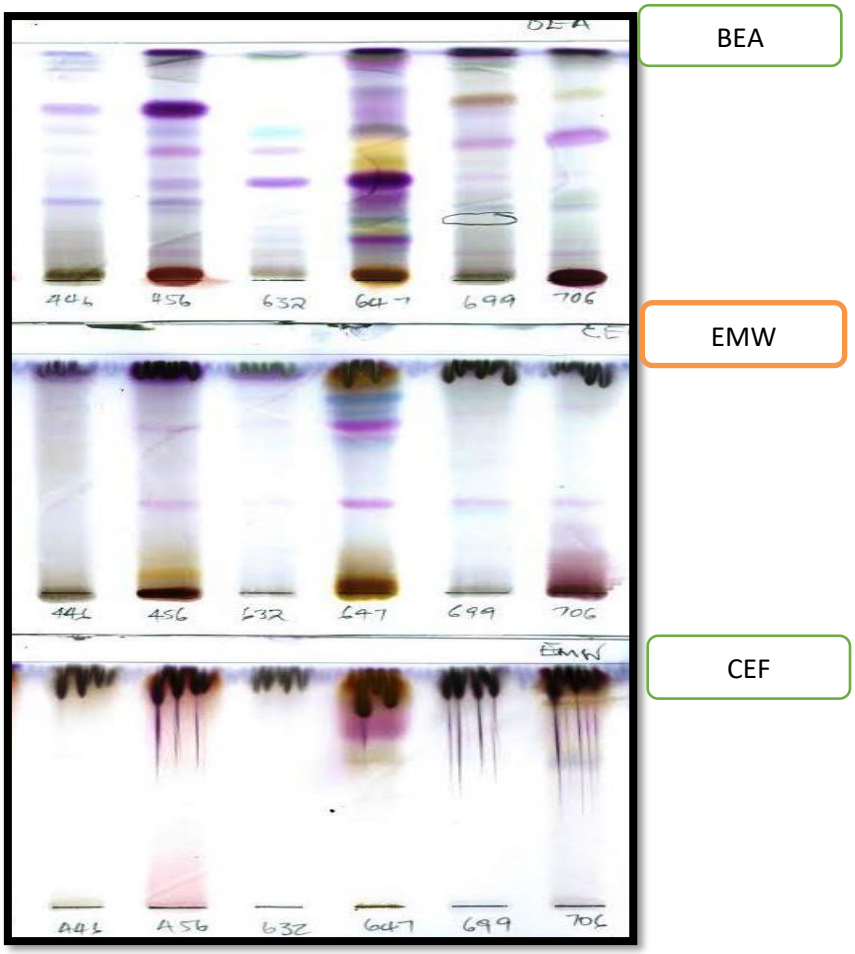


Figure 4.7. Chromatograms of extracts of ten (10) plant species developed in BEA, EMW and CEF sprayed with vanillin spray reagent

4.5 Conclusions

The greater the variety of the compounds that can be extracted by the extracting solvents used, the better the chance that biologically active compounds will be extracted. In the TLC analysis, of the three mobile systems used, BEA resulted in the best separation of compounds compared to CEF and EMW with all the extracts. This shows that most compounds in the prepared plant extracts are non-polar because BEA is non-polar in nature. The next step after identifying and selecting plant species with known antifungal activity was to screen antimicrobial and anthelmintic activity of the extracts to determine the presence of an association of activity in these different biological assays.

CHAPTER 5. The anthelmintic activity of extracts of six plant species with known antifungal activity against *Haemonchus contortus*

5.1 Introduction

Infections by helminths cause serious problems globally and are recognized as a major constraint to livestock production throughout the tropics and other parts of the world (Ibrahim et al., 1984; Waller, 1999; Githiori et al., 2004). Among the different types of helminths, nematodes are the most important based on prevalence and adverse effects they cause (Perry and Randolph, 1999; Perry et al., 2002). Nematode infections result in retarded growth, lowered productivity and high economic losses in diverse species of animals (Ashraf, 1985; Iqbal et al., 1993; Kochapakdee et al., 1995). *Haemonchus contortus,* also known as the barber's pole worm, twisted wireworm or large stomach worm, primarily infects sheep and goats. Haemonchosis affects millions of small ruminants worldwide, leading to estimated losses to the livestock industry of billions of dollars each year (Waller et al., 2005; Roeber et al., 2013).

Existing parasite control involves mainly the use of synthetic anthelmintics. For example benzimidazoles were initially developed as fungicides in plants but later they were developed as veterinary anthelmintics (Horton, 2000). These drugs are an important class of heterocyclic compounds for developing biologically active derivatives due to their various pharmacological activities (Gaballah et al., 2016). Other researchers documented biochemical and pharmacological studies, reporting their potential to inhibit the growth of fungal strains (Damu et al., 2013).

The continued and in some cases improper use of these has led to development of resistance to all the major classes of chemical anthelmintics (Chartier et al., 2001; Leathwick et al., 2001). There are additional challenges of chemical residues in the food chain and toxicity (Kaemmerer and Buttenkotter, 1973; Muhammad et al., 2004), as well as cost and non-availability of anthelmintics in remote areas. These challenges have compelled investigation of alternative control options such as grazing management, dietary management, natural control, vaccination and ethnoveterinary medicine (EVM) (Waller, 1999, Jabbar et al., 2006; Bizimenyera et al., 2006).

Historically, both human and animal medicine has relied on traditional treatments and plant materials. About 80 to 90% of the planet’s inhabitants still rely mainly on traditional treatments and practitioners (Plotkin, 1992). Plants contain a range of secondary metabolites, several of

which have activities against microorganisms associated with infections and against internal and external parasites. *In vitro* and *in vivo* studies have been carried out to study plant efficiency as alternative or complementary anthelmintics to control gastrointestinal infections in small ruminants (Slomp et al., 2009). Medicinal plants yield valued biological constituents that have possible worth in the cure of infections. It is thus necessary to study such plants to fully utilize their value (Abdul Ghani, 1990). Additionally, the rational use of drugs necessitates the standardization of basic methods, including preparation as well as investigation of their potential toxicity (Ibrahim et al., 1984). Researchers have screened plants for anthelmintic activity that may additionally have other biological activities using *in vivo* and *in vitro* studies (Aremu et al., 2012). Results from previous studies provide support for further investigation on using plant-derived products to control helminthosis (Githiori et al., 2004; Bizimenyera et al., 2006; Adamu et al., 2012; Adamu et al., 2013).

In this chapter, six plant species (*Peddiea africana, Diospyros whyteana, Cassipourea gummiflua*, *Schotia brachypetala*, *Bowkeria citrina* and *Senna petersiana* – described in Chapter 4) were selected based on their antifungal activity against *Candida albicans* from the Phytomedicine Plant Database (Pauw and Eloff, 2014). The aim of the current work was to evaluate the anthelmintic efficacy of these South African plant species with known antifungal activity and to determine whether antifungal activity may indicate anthelmintic activity in some cases. The antifungal activity of the extracts was also tested for confirmation of their effect against *C. albicans* as well as two other animal pathogenic fungi, *Cryptococcus neoformans* and *Aspergillus fumigatus.* The cytotoxicity of the extracts was also tested to ensure that the biological activity was not due to general toxicity.

5.2 Materials and methods

5.2.1 Plant collection

Six plant species (*Peddiea africana, Diospyros whyteana, Cassipourea gummiflua*, *Schotia brachypetala*, *Bowkeria citrina* and *Senna petersiana*) were collected from the Manie van der Schijff Botanical Gardens, Hatfield campus, University of Pretoria..

5.2.2 Extraction procedure

One gram of dried powdered leaves from each plant was extracted in centrifuge tubes (50 ml) with acetone (technical grade-Merck) and separately with distilled water, in a ratio of 1:10 (m/v). Extraction was by shaking on a Labotec model 20.2 shaking machine for 30 min. The supernatants were then filtered through Whatman No 1 filter paper, using a Buchner funnel.

The process was repeated three times to exhaustively extract the plant material and all the filtrates for each extractant were combined. The extracts were then concentrated under vacuum using a rotary evaporator (Labotec), transferred into pre-weighed labelled glass containers, and dried under a stream of air.

5.2.3 Antifungal assay

The two-fold serial microdilution assay (Eloff, 1998b) modified by Masoko and Eloff (2005) was used to confirm antifungal activity of the extracts and to determine their MIC values against three fungal isolates which were obtained from the Department of Veterinary Tropical Diseases, University of Pretoria. The fungal strains used were cultured from clinical cases of fungal infectious diseases in animals (before treatment). *Candida albicans* was isolated from a Gouldian finch, *Cryptococcus neoformans* from a cheetah, while *Aspergillus fumigatus* was isolated from a chicken which suffered from a systemic mycosis. Dried extracts were reconstituted in acetone or water to a concentration of 10 mg/ml. All the wells in sterile 96-well microplates were then filled with 100 µl of sterilized distilled water. Test plant extract samples (100 µl) were pipetted in the first well and serially diluted two-fold down until the last well from where 100 µl was discarded. Fungal cultures (containing approximately 10^9 cfu/ml) in fresh Potato Dextrose (PD) broth were pipetted into the wells (100 µl in each well). As an indicator of growth, 40 µl of 0.2 mg/ml of p-iodonitrotetrazolium violet (INT) dissolved in water was added to each microplate well (Eloff, 1998b) and incubated for 24 h at 37ºC at 100% relative humidity (RH). The MIC was recorded as the lowest concentration of the extracts that inhibited fungal growth after 24 to 48 h. All experiments were carried out in triplicate. Amphotericin B was used as a positive control, while acetone was used as the negative control.

5.2.4 Egg recovery and preparation

Faecal samples of *Haemonchus contortus* were collected from the rectum of monospecifically infected sheep that were housed indoors on a concrete floor. Three grams of faeces were weighed for the preparation of faecal egg count. Faecal samples were then crushed and washed with distilled water to make a slurry and filtered through different sieves of decreasing pore sizes, namely 250,150, 90, 63 and 38 µm. Eggs were trapped in the 38 µm sieve.

A 40% sodium chloride solution was used to float the eggs which were then centrifuged (1000 rpm) for 10 min. This procedure was done following the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines. The number of eggs was

determined by counting the eggs under a microscope at X10 magnification using a McMaster slide. The egg concentrate was subsequently brought to a final concentration of 100 per 200 µl. To avoid proliferation of fungi, 5 µg of amphotericin B at 2 mg/ml (Sigma, Germany) was added per millilitre of egg suspension. The hatching of the eggs was then determined.

5.2.5 Egg hatching assay

The *in vitro* egg hatch assay determines the ability of nematode eggs to hatch in different concentrations of test substance. The assay was performed according to the recommendations of the World Association for the Advancement of Veterinary Parasitology (WAAVP) with modifications (Coles *et al*., 1992). This test is recommended for the detection of anthelmintic resistance in nematodes.

Acetone and water extracts were dissolved in 100% DMSO and prepared to 10 mg/ml in phosphate buffered solution (PBS) as the stock solution. The final concentration of DMSO did not exceed 5%. Six concentrations of each extract (10, 5, 2.5, 1.25, 0.63 and 0.31 mg/ml) were used to ascertain the inhibition of egg hatching of *Haemonchus contortus*. Aliquots of the egg suspension (200 µl) were pipetted into each well of 24 well microtitre plates (Bizimenyera et al., 2006). In the test wells, 200 µl of the appropriate plant extract was added. Positive control wells contained 200 µl of the anthelmintic drug, albendazole (trade name Valbazen, at concentrations of 0.008 to 25 µg/ml) diluted in PBS, while the negative control was 200 µl of 5% DMSO and PBS together with 200 µl of the eggs in each well. Three replicates were analysed for each treatment.

The microtitre plates were then incubated at 27ºC for 48 hours. A drop of Lugol's iodine solution was added to each well to stop further hatching. All the unhatched eggs and L1 larvae in each well were counted. The percentage inhibition of hatching eggs was then calculated according to Coles et al. (1992) modified by Ademola and Eloff (2011). The equation is as follows:

$$\frac{\text{Number of larvae / Total number of larvae and eggs in wells with plant extracts}}{\text{Number of larvae / Total number of larvae and eggs in control well (no extract)}}$$

5.2.6 Cytotoxicity assay

The MTT (3-5 dimethyl-thiazol-2,5-diphenyl tetrazolium bromide) assay, as described by Mosmann (1983) was used to evaluate the toxicity of plant extracts against African green

monkey kidney (Vero) cells. The cells were obtained from the American Type Culture Collection (ATCC), number RL-10741 and grown in Minimum Essential Medium (MEM) supplemented with 5% foetal calf serum (WhiteSci). Cells were then seeded in 96-well flat bottomed culture plates at a cell density of 5 x 10^4 cell/ml and incubated overnight at 37ºC in a 5% CO_2 incubator to allow attachment of cells.

Thereafter, the cells were exposed to various concentrations of the extracts (1.0 - 0.0075 mg/ml) prepared in MEM in quadruplicate for each concentration and were then incubated again at 37ºC. At the end of the 48 h incubation period, the medium on the cells was removed and the cells rinsed with 150 µl phosphate buffered saline (PBS), whereafter fresh medium (200 µl) was pipetted onto the cells.

To each well, 30 µl of 5 mg/ml of MTT dissolved in PBS was added and the plates were incubated for another 4 h. The medium containing MTT was removed and 50 µl of dimethyl sulphoxide (DMSO) were then added to each well and the plates were gently swirled for 5 min at room temperature to dissolve the formazan crystals. The cytotoxic effects of the extracts on the cells were indicated by a clear appearance or lack of purple formazan colour in the wells. The absorbance of the plates was read at a wavelength of 570 nm using a Biotek Synergy microplate reader.

5.2.7 Selectivity Index

Selectivity index (SI) values of the extracts were calculated by dividing the cytotoxicity, LC_{50} (mg/ml), by MIC (mg/ml) for antifungal activity or EC_{50} (mg/ml) for anthelmintic activity. Where the cytotoxicity was >1 mg/ml, it was taken as being 1 mg/ml for the purposes of calculating the SI value. The higher the SI value, the safer the extract, so extracts with SI values below 1 are more toxic to the mammalian Vero cells than effective against the parasites.

5.3 Results and Discussion

5.3.1 Antifungal activity

Antifungal activity of the extracts of the six selected species was generally good to excellent with several MIC values below 0.1 mg/ml (Table 5.1). Both acetone and water extracts of *P. africana* had excellent MIC values of 0.04 mg/ml against *C. albicans*. The *S. brachypetala* acetone extract had MIC of 0.04 mg/ml and water extract had MIC = 0.08 mg/ml against the same fungal yeast. Furthermore, the *D. whyteana* water extract inhibited the growth of the

three test fungi with MIC values of 0.04 to 0.08 mg/ml while the acetone extract had MICs of 0.16 to 0.63 mg/ml. The lowest activity was shown with acetone and water extracts of *Bowkeria citrina* with MIC values of greater than 2.5 mg/ml against the filamentous fungal species, *Aspergillus fumigatus*. Extracts of this plant species had little activity against *Candida albicans* but much better activity against *Cryptococcus neoformans*. Amphotericin B was used as a positive control and it had MIC = 0.08 µg/ml against all three fungal organisms under the growth conditions used.

5.3.2 Egg hatch assay

Results of the egg hatch inhibition assay are presented in Table 5.2. The best activity was shown by the acetone extract of *D. whyteana* with EC_{50} of 0.738 mg/ml while the water extract inhibited the hatching of eggs with EC_{50} = 1.508 mg/ml. The acetone extract of *P*. *africana* had an EC_{50} of 1.088 mg/ml and the water extract had EC_{50} of 1.752 mg/ml. The next best activity was shown by *Senna petersiana* where the acetone extract inhibited hatching of eggs with EC_{50} = 1.176 mg/ml and the water extract with EC_{50} = 1.773 mg/ml respectively. *Bowkeria citrina*, *S. brachypetala* and *C. gummiflua* had lower activities in the egg hatch inhibition assay with EC_{50} values between 1.316 to 1.759 mg/ml with both acetone and water extracts. No reports of anthelmintic activity investigations could be found for these plant species. Adamu et al. (2013) proposed that extracts with EC_{50} greater than 6 mg/ml should be considered to have weak anthelmintic activity because of the difficulty to achieve such a high concentration in animals. Albendazole (the positive control) inhibited egg hatching with an EC_{50} of 0.037 mg/ml.

In other anthelmintic studies of acetone extracts of plants, similar EC_{50} values were obtained. Bizimenyera et al. (2006) reported that the leaf acetone extract of *Peltophorum africanum* had an EC_{50} of 0.62 mg/ml in the egg hatch assay. Similarly, Adamu et al. (2013) found that the EC_{50} of *Heteromorpha trifoliata* acetone leaf extract was 0.62 mg/ml which was lower than that of *Combretum molle* with EC_{50} of 0.87 mg/ml. Naturally, results that seem promising using *in vitro* testing need to be confirmed with *in vivo* studies as experiments in a laboratory do not always relate well to studies using an animal model, as various factors including metabolism need to be taken into account.

5.3.3 Cytotoxicity and selectivity index values

In the cytotoxicity assay, *Diospyros whyteana* acetone and water extracts both had LC_{50} values greater than 1 mg/ml, the highest concentration tested (Table 5.1). Additionally, water extracts of *B. citrina*, *P*. *africana*, *S. brachypetala* and *S. petersiana* had LC_{50} greater than 1 mg/ml, while acetone extracts showed little cytotoxicity at the highest concentration tested (1 mg/ml).

Regarding antifungal activity, the highest SI values were obtained with the *Peddiea africana* water extract with SI greater than 25 against *C. albicans* (Table 5.1). The antifungal activity of *D. whyteana* water extracts was better than that of the acetone extracts which is unusual, so it may be possible that compounds with non-specific antifungal activity such as tannins are extracted by water.

In the anthelmintic egg hatch study, the highest SI was recorded with the acetone extract of *D. whyteana* with SI value greater than 1.35, while the water extract had SI value of 0.66 (Table 5.2). This was followed by acetone extracts of *Cassipourea gummiflua* with SI value of 0.59 and the water extract with SI = 0.13. The lowest SI was recorded with the extract of *P. africana* with SI value of 0.03, while *B. citrina* had SI value of 0.04. Unfortunately there was insufficient plant extract to test cytotoxicity of the *Senna petersiana* extract but this extract had little antifungal or anthelmintic activity so this was not deemed important. Water extracts of most plant species had better SI values with respect to antifungal activity compared to the acetone extracts (Table 5.1). The average SI values reported in Table 5.2 reflected that the selectivity index for anthelmintic activity was very similar between extracts prepared using the two solvents, with acetone extracts having slightly better values. *Peddiea africana* and *D. whyteana* extracts had the best antifungal as well as anthelmintic activity, reflecting that antifungal activity of plant extracts may be a good lead to detect anthelmintic activity.

Not all recently published studies on *in vitro* anthelmintic activity of plant extracts against *H. contortus* using the egg hatch assay report toxicity of the extracts to normal human cells. It is is thus not possible to determine whether or not the extracts have promising anthelmintic efficacy. For example Ademola and Eloff (2011) recommended that the good activity of the acetone leaf extract of *Anogeissus leiocarpus* (LC_{50} = 0.36 mg/ml) could lead to its development as a useful anthellmintic therapy in veterinary practice. However, its potential toxicity and contribution of this effect to the activity was not considered.

Table 5.1. Antifungal activity of acetone and water extracts (MIC in mg/ml), cytotoxicity and selectivity index (SI) values

Samples	**Cytotoxicity (mg/ml)**													
			***C. albicans* MIC (mg/ml)**		**SI**		***C. neoformans* MIC**		**SI**		***A. fumigatus* MIC**		**SI**	
	A	W	A	W	A	W	A	W	A	W	A	W	A	W
Peddiea africana	0.039	>1	**0.04**	**0.04**	1.02	**>25**	**0.16**	**0.16**	0.24	**>6.25**	**0.04**	**0.08**	0.49	**>12.5**
Diospyrus whyteana	>1	>1	**0.16**	**0.08**	**>6.25**	**>12.5**	0.63	**0.04**	>1.59	**>25**	0.31	**0.08**	>3.23	**>12.5**
Cassipourea gummiflua	>1	0.182	1.25	0.63	>0.80	0.29	0.63	0.23	>1.59	0.79	>2.5	>2.5	0.4	0.073
Schotia brachypetala	0.106	>1	**0.04**	**0.08**	2.65	**>12.5**	**0.02**	**0.08**	5.30	**>12.5**	**0.04**	>2.5	2.65	0.4
Senna petersiana	0.251	ND	0.63	ND	0.40	ND	0.63	ND	0.40	ND	**0.08**	ND	3.14	ND
Bowkeria citrina	0.063	>1	2.5	>2.5	0.03	ND	**0.08**	0.04	0.79	**>25**	>2.5	>2.5	0.025	0.4
AVERAGE	0.410	0.836	0.770	0.666	1.858	**12.573**	0.358	0.110	1.692	**13.908**	0.912	1.532	1.656	**5.175**
Doxorubicin (µg/ml)	4.03													
Amphotericin B (µg/ml)				0.08				0.08				0.08		

*A = acetone; W = water; Selectivity index calculated by dividing LC_{50} / MIC. Values in bold indicate good antifungal activity and promising SI values (>5)

Table 5.2. Egg hatch assay (EHA, EC_{50}) cytotoxicity (LC_{50}) and selectivity index (SI) values of six South African plants

Plant extracts	**EHA (EC_{50} in mg/ml)**		**Cytotoxicity (LC_{50} in mg/ml)**		**SI**	
	Acetone	Water	Acetone	Water	Acetone	Water
Peddiea africana	**1.088**	1.752	0.039	> 1	0.03	>0.57
Diospyros whyteana	**0.738**	1.508	> 1	> 1	**>1.35**	>0.66
Cassipourea gummiflua	1.689	1.316	> 1	0.182	>0.59	0.13
Schotia brachypetala	1.413	1.672	0.106	> 1	0.07	>0.59
Senna petersiana	**1.176**	1.773	0.251	ND	0.21	ND
Bowkeria citrina	1.501	1.759	0.064	> 1	0.04	>0.56
AVERAGE	1.268	1.63	0.41	0.836	0.612	0.502
Albendazole	0.0366					

ND: Not Determined. Bold values highlight good activity in the egg hatch assay

5.4 Conclusions

The six plant species tested in this study had varying degrees of activity in the *H. contortus* egg hatch inhibition assay, and several had promising antifungal efficacy with little to no cytotoxicity. *Peddiea africana* and *Diospyros whyteana* had good antifungal activity as well as the highest inhibition of *H. contortus* egg hatching. In the case of these species it appears that high antifungal activity is associated with inhibition of egg hatching activity but a larger number of plant species need to be investigated for determining correlations in activity in the two bioassays and what classes of compounds may be responsible for such activities. Interestingly, water extracts had very good SI values for antifungal activity against the various fungal organisms tested, indicating that traditionally prepared water extracts may have useful therapeutic activities.

With several of the selected plant species being shown to have promising antifungal and anthelmintic activity, the next step was to determine whether these plants have additional useful bioactivities. Antioxidant activity is a useful property for medicinal plants as this may serve to protect animal and human tissues from damage caused by free radicals present as a result of infection by, for example, fungi and helminths. Phenolic compounds, including flavonoids, are important antioxidant compounds so the quantification of these compounds as well as detection of antioxidant activity was conducted on the selected plant extracts.

CHAPTER 6. Evaluation of flavonoid and phenolic content, and antioxidant activities of crude extracts of six plant species

6.1 Introduction

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. It is well known that plants produce these chemicals to protect themselves, but research demonstrates that they can also protect humans and animals against diseases (Hornok, 1992). Some of the well-known phytochemicals include lycopene in tomatoes, isoflavones in soy and flavonoids in fruits (Hornok, 1992). Since early ages, numerous plants have been used for different traditional purposes and are known to relieve and heal some ailments including cough, headache, fever and stomach cramps. Medicinal plants are used because of their biologically active constituents (Hornok, 1992). Different primary metabolites are responsible for cell division and growth of the cells, while secondary metabolites are most related to defence mechanisms by plants against variation to severe climatic situations, traumatic situations and protection against predation by snails, insects or even herbivores due to the fact that plants are sessile.

The use of plants with medicinal properties is an alternative or complementary mechanism of reducing the impact of growing resistance of infectious organisms and parasitic gastrointestinal nematodes, in addition to breeding resistant livestock, and improving livestock feed quality (Diehl et al., 2004). Medicinal plants can be cultivated, they are accessible and affordable, environmentally friendly and are also valued for their cultural importance (Pessoa et al., 2002; Umadevi et al., 2013). Bioactive compounds commonly found in plants have possible health benefits partly due to their antioxidative properties (Cao and Cao, 1999). It is well known that reactive oxygen species (ROS) are involved in a diversity of important processes in medicine, including among others inflammation, atherosclerosis, cancer and reperfusion injury (Kehrer, 1993). One of the major fundamental tissue destructive mechanisms is oxidative stress through an excessive release of reactive oxygen metabolites (ROM) (McCord, 2000). Antioxidants decrease inflammation and defend tissues from oxidative damage caused by free radicals, such as happens during helminth or fungal infections. Various phenolic and flavonoid compounds are known to be important antioxidants and may be efficient scavengers of free radicals (Iqbal et al., 2015).

Owing to the good antifungal and anthelmintic properties of the six plants chosen for investigation in this study, it was deemed necessary to further investigate the chemical composition and useful biological properties of these plants. Phenolics and flavonoids have a

multitude of different bioactivities so it was decided to determine the content of these phytochemicals in the plant extracts of interest. Additionally, antioxidant activity may be a useful adjunct to the other biological activities of the plant species investigated if they are to be developed further into botanical preventative or therapeutic medications. The antioxidant activity of the extracts via scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was therefore also determined.

6.2 Material and methods

6.2.1 Plant material

The leaves of the plants under investigation (*Peddiea africana, Diospyros whyteana, Cassipourea gummiflua*, *Schotia brachypetala*, *Bowkeria citrina* and *Senna petersiana*) were collected, dried and ground as described in Chapter 4, section 4.3.1). The dried plant material was stored at room temperature in closed glass jars in the dark until extraction.

6.2.2 Preparation of extracts

The powdered plant material (1 g) was individually extracted with 10 ml of acetone (technical grade, Merck) in polyester centrifuge tubes using a platform shaker (Labotec model 20.2 shaking machine) at room temperature for 30 min. The extracts were centrifuged, and the clear supernatant was filtered through Whatman No.1 filter paper using a glass funnel and gravity filtration. This procedure was repeated three times on the same plant material with fresh solvent to ensure exhaustive extraction. The filtered crude extracts of each plant sample were combined into pre-weighed glass vials and the solvent was left to evaporate at room temperature under a stream of air. Crude extracts obtained were used for the subsequent investigations.

6.2.3 Phytochemical screening

6.2.3.1 Determination of total flavonoid content

The total flavonoid content of the extracts was determined using the 96-well plate method adapted from Yadav and Agarwal et al. (2011). Extracts (100 µl) were dispensed into wells of a 96-well plate and 100 µl of 2% aluminium chloride was added. The final mixture was thoroughly shaken and incubated for 15 min at room temperature and absorbance was then read at a wavelength of 430 nm in a microplate reader (BioTek Synergy, South Africa). A

yellow colour indicated the presence of flavonoids. Total flavonoid determination was calculated from the linear regression curve of quercetin (as the positive reference compound) and outcomes were expressed as mg quercetin equivalent (QE) per g of crude extract.

6.2.3.2 Total phenolic content determination

Total phenolic content of crude extracts was determined according to the Folin Ciocalteu method (Folin and Ciocalteu, 1927). Extracts (20 µl) were aliquoted into the wells of a 96-well plate to which 100 µl of 50% Folin-Ciocalteu reagent and 80 µl of 7.5% sodium carbonate (Na_2CO_3) solution were added. The final mixture was shaken vigorously and incubated for 60 min in the dark and kept at room temperature and the absorbance was measured at 760 nm. The total phenolic content was calculated from the linear regression curve of gallic acid as the standard reference compound, and the results were expressed as mg gallic acid equivalent (GAE) per g of crude extract.

6.2.3.3 DPPH radical scavenging

To determine the radical scavenging ability of the extracts, the modified method using the 2,2-diphenyl picrylhydrazyl (DPPH) radical reported by Brand-Williams et al. (1995) was used. Acetone extracts (50 µl) and positive controls were prepared in serial dilutions of 8-250 µg/ml, whereafter 200 µl of DPPH in methanol (25 µg/ml) was added. The DPPH solution was prepared daily and stored in the dark. The change in absorbance at 517 nm was measured after 30 min incubation in the dark with a microplate reader. Ascorbic acid and Trolox were used as positive controls and extract without DPPH was used as a blank. Results were expressed as percentage reduction of the initial DPPH absorption. The concentration of extract leading to 50% reduction of DPPH (IC_{50}) was determined.

6.3 Results and Discussion

The total phenolic content, flavonoid content, and DPPH free radical scavenging activity of the acetone and water crude extracts of the six plant extracts are presented in Table 6.1. The total phenolic content of the extracts of *Diospyros whyteana* (water extract) was the highest (199.78 mg/g GAE), followed by *Schotia brachypetala* (water extract) with 183.80 mg/g GAE. Ondua et al (2019) reported the highest phenolic and flavonoid contents of fractions of the leaf extract of *Typha capensis* as 37.79 mg/g GAE and 134.63 mg/g RE (rutin equivalents), respectively.

The high phenolic content of the *D. whyteana* water extract may be responsible for the good antifungal and anthelmintic activity of this extract (Chapter 5). It would be useful to isolate and identify the phenolic compounds and test their antifungal and anthelmintic activity to confirm this supposition. Generally, water extracts of all species tested had higher total phenolic content compared to acetone extracts indicating that water was more efficient at extracting phenolic compounds.

Interestingly, the acetone extracts in several cases had higher flavonoid contents than the water extracts. *Bowkeria citrina* acetone extract had the highest flavonoid content of all the crude extracts with 62.69 QE/g, followed by *Diospyros whyteana* with 48.77 QE/g. The lowest flavonoid content was shown in the acetone extract of *Cassipourea gummiflua* with 5.16 QE/g, but water extracts had QE/g of 13.03.

The DPPH radical scavenging antioxidant activity of the acetone extracts was less potent than those of the positive control's ascorbic acid and trolox (Table 6.1). Among the six plants, extracts of *Schotia brachypetala* had strong DPPH radical scavenging activity with IC_{50} of 10.32 µg/ml. *Peddiea africana* had the next best DPPH radical scavenging ability with an IC_{50} of 14.35 µg/ml. The other four plant species had reasonable antioxidant activity in the DPPH assay with IC_{50} values between 27.94 and 35.71 µg/ml. The positive controls ascorbic acid and trolox vigorously scavenged the DPPH radical with IC_{50} values of 0.41 and 0.47 µg/ml respectively. A recent study (Ondua et al., 2019) reported that the IC_{50} of the acetone extract of *T. capensis in* the DPPH assay was as low as 7.11 µg/ml, which is close to that of *S. brachypetala* determined in this study. Phenolic compounds make an important contribution to the antioxidant activity of plant extracts and they act as radical scavengers, reducing agents, metal chelators, hydrogen donors and singlet oxygen quenchers (Khadri et al., 2010).

Table 6.1. Total phenolic content (GAE/g), total flavonoid content (QE/g), and DPPH scavenging activity (IC_{50} in µg/ml) of acetone and water extracts of six South African plant species

Samples	**Total phenolic content (GAE/g plant extract)**		**Flavonoid content (QE/g plant extract)**		**DPPH (IC_{50} in µg/ml)**
	Acetone	**Water**	**Acetone**	**Water**	
Peddiea africana	9.341±1.36	46.50±5.69	17.24±5.80	5.78±0.00	14.35±1.08
Diospyros whyteana	95.49±3.08	199.78±8.82	48.77±0.22	3.66±0.10	28.47±4.56
Cassipourea gummiflua	12.28±6.35	57.67±5.11	5.16±6.44	13.03±1.99	27.94±5.43
Schotia brachypetala	155.14±0.87	183.80±0.68	48.72±0.97	7.52±3.68	10.32±3.06
Bowkeria citrina	145.37±8.34	82.06±1.06	62.69±1.82	5.49±0.92	31.65±6.15
Senna petersiana	26.07±7.52	ND	44.42± 0.24	ND	35.71±1.79
Ascorbic acid					0.41±1.35
Trolox					0.47±1.38

6.4 Conclusions

This aspect of the study showed that the total phenolic content (TPC) of the six plant species tested was generally higher than the flavonoid content. Moreover, water extracts showed higher phenolic contents than acetone extracts so the polar extractant more efficiently extracted the polar flavonoids and phenolics. In the antioxidant DPPH assay, *Schotia brachypetala* (acetone) had very good activity, followed by *Peddiea africana*. Overall, there did not seem to be a relationship between the phytochemical content (total phenolics and flavonoids) of the plant species and antioxidant activity, although the phenolic and flavonoid content would most likely contribute to the overall antioxidant activity. *Diospyros whyteana*, which had good antifungal and anthelmintic activity in the previous chapters, had high levels of phenolics and flavonoids, but not extremely high activity in the DPPH assay. It would be recommended to conduct different antioxidant assays to confirm the antioxidant activity of the most active plant extracts via other mechanisms. As antioxidant activity in vitro is a chemical reaction, in vivo studies additionally need to be conducted to verify the therapeutic usefulness of antioxidant efficacty of the plant extracts.

Following this work it was decided to continue with *D. whyteana* for bioassay-guided fractionation of active compounds as it had both antifungal and anthelmintic activity as well as antioxidant activity, which may assist in reducing negative effects of infections.

CHAPTER 7. Bioassay-guided fractionation of active compound(s) from *Diospyros whyteana*

7.1 Introduction

Separation techniques leading to the isolation of bioactive compounds are important in the natural product isolation process. The complexity of a plant extract can be simplified through different separation techniques. One extraction technique aims to fractionate a plant extract by direct sequential extraction of phytochemicals from the same plant material with solvents of increasing polarities. The extraction process starts with the least polar solvent such as hexane and ethyl acetate to the most polar solvent such as water. Extracts of different polarities are obtained and each solvent extracts only the components that are soluble in it, therefore providing an optimum extraction (Wojcikowski et al., 2007). A conventional extraction method is maceration, which involves the use of extractants that can remove targeted phytochemicals maximally from the plant material (Wojcikowski et al., 2007).

Liquid-liquid fractionation is an effective separation technique to separate compounds in a complex mixture into fractions based on the polarity of the constituent compounds. This method is effective especially when the compounds within the mixture are coloured as this gives the scientist the ability to see the separation of the compounds (Jones, 2000). If the compounds are not visible, certain components can be observed by other visualization methods. This makes it relatively easy to collect fractions (Skoog et al., 2007). It is also a less expensive procedure than other methods of separation like high performance liquid chromatography (HPLC). This is because the most basic forms of liquid fractionation do not require expensive machines (Skoog et al., 2007). The addition of mobile phase, the detection of the success of each separation and collection of the fractions is done manually. Following each separation stage, bioassays may be conducted to detect the presence of the bioactivity to allow for bioassay-guided fractionation to isolate active components. When structure elucidation becomes more complicated, such as in the case of unusual or new compounds, other techniques such as mass spectrometry (MS), ultra-violet (UV), infra-red (IR) data and literature information are required to facilitate the structure determination (Jones, 2000).

The primary aim of this chapter was to isolate and elucidate the structure of antifungal compound(s) in the most active plant extract from the previous investigations, namely *Diospyros whyteana*. As it is relatively difficult and extremely time-consuming to use the parasitic anthelmintic assay for bioassay-guided fractionation of active extracts, the aim was to use a

simple antifungal activity assay to isolate active compounds and then to determine whether this could be a successful method to isolate anthelmintic compounds.

7.2. Materials and Methods

7.2.1. Bulk extraction and bioassay-guided fractionation of active compounds from *Diospyros whyteana*

A diagram of the isolation of active compounds from *D. whyteana* is shown in Figure 7.1. Powdered leaf material of *D. whyteana* (530 g) was extracted with 4L of acetone. Thereafter, two more successive extractions were performed with 2L of acetone each to exhaustively extract compounds from the plant material. The extract was concentrated using a Büchi Rotavapor R114 (Labotec) and dried under a stream of air at room temperature. Dried plant material was subjected to liquid-liquid fractionation following the method of Suffness and Douros (1979) using hexane, dichloromethane, ethyl acetate, butanol and water. At each fractionation step, the fractions were subjected to antifungal activity testing as described in Chapter 5, section 5.2.3 and fractionation was continued with the most active fraction.

The hexane fraction (with a mass of 240 g) was subjected to gravitational column chromatography on silica gel (column dimensions 2.5 cm×73 cm using 150 g silica, particle size 0.063–0.200 nm, 70–230 mesh, Merck). The eluting solvent mixture comprised hexane: ethyl acetate, starting with 100% hexane, and progressing to 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35 and finally 60:40 as mobile phase. Aliquots of the nine fractions were reconstituted in acetone (10 mg/ml) and 10 µl of the aliquot spotted on thin layer chromatography (TLC). Three mixtures of hexane: ethyl acetate (90:10, 70:30 and 60:40) as well as BEA (see Chapter 4, section 4.3.3) were used to develop separate plates. Fractions with similar chemical compositions as visualised on the TLC plates were combined. The purification of the compounds was achieved by bioassay-guided fractionation and repeated column chromatography until a single spot was obtained for each active compound using three different mobile phases (Chapter 4, section 4.3.3) to develop the TLC.

The hexane fraction (with a mass of 18.5 g) contained compounds with activity against *Cryptococcus neoformans* visualised in bioautography and was therefore subjected to further column chromatography with silica gel as stationary phase using a solvent gradient of 100:0, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30 and 60:40 (hexane:ethyl acetate). Twenty-five fractions (25) were collected from combinations of similar profiles in chromatograms and bioautograms of collected fractions. Fraction 10 (162 mg) contained anticandidal and anticryptococcal compounds on bioautograms and was subjected to further column

chromatography on silica gel using a gradient solvent system of 100:0, 98:2, 96:4, 94:6, 92:8 and 90:10 (hexane: ethyl acetate) to obtain purified compounds.

The structures of the compounds isolated in this study were determined by extensive NMR techniques and chemical methods, mainly by 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY) and by comparison with published literature data. This aspect of the work was done with the help of Dr Maurice Awouafack, a phytochemist.

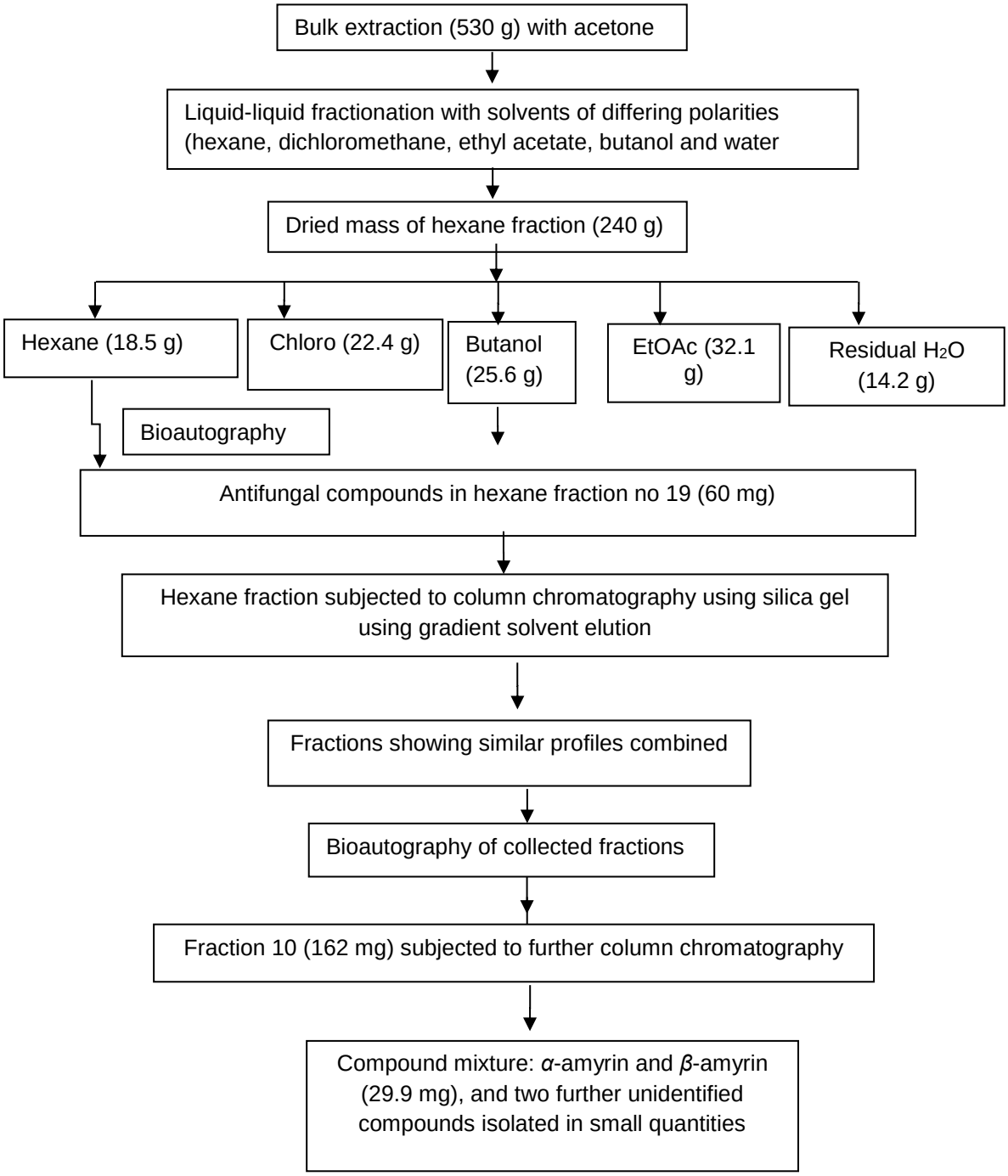


Figure 7.1. Diagram of procedure followed to isolate antifungal compounds from *D. whyteana*

7.2.2 TLC fingerprints

Aliquots of *D. whyteana* fractions were prepared on TLC plates (Chapter 4, section 4.3.3) by loading the extracts on 10 cm x 20 cm TLC plates and developing in hexane:ethyl acetate (80:20). The developed TLC plates were visualized under UV light at 254 nm and 365 nm to detect UV absorbing or fluorescing bands. The plates were then sprayed with vanillin spray reagent (0.1g vanillin dissolved in 28 ml methanol, with 1 ml sulphuric acid added) and heated at 110ºC to optimal colour development.

7.2.3 Bioautography

TLC plates of the fractions of *D. whyteana* were prepared using a hexane:ethyl acetate (80:20) mobile system and sprayed with actively growing cultures of *Candida albicans* and *C. neoformans* (Begue and Kline, 1972).

7.2.4 Antifungal activity, cytotoxicity and egg hatch inhibition of compounds

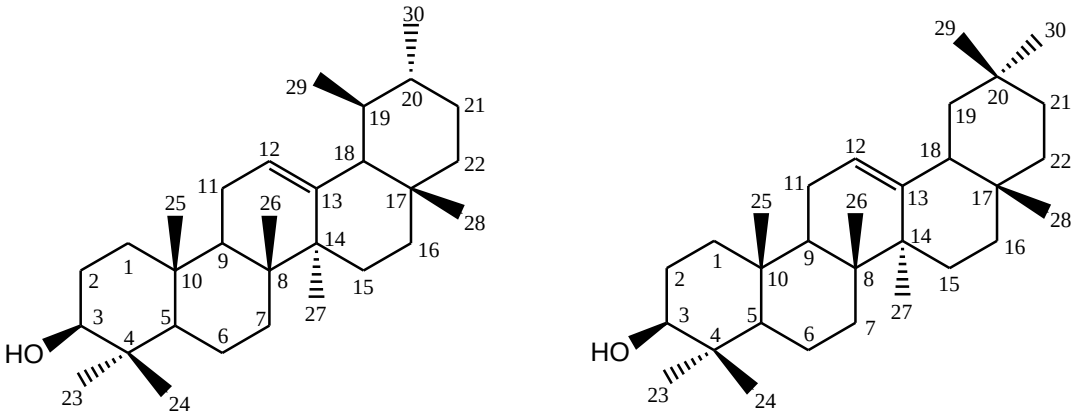
The purified compounds were tested against three animal pathogenic fungi (*Candida albicans, C. neoformans* and *Aspergillus fumigatus*) as well as four plant pathogenic fungi (*Rhizoctonia solani*, *Penicillium italicum*, *Aspergillus niger* and *Fusarium oxysporum*) for comparison purposes. The animal fungi were obtained from the culture collection of the Department of Veterinary Tropical Diseases, University of Pretoria. The plant pathogenic fungi were originally obtained from Prof Lise Korsten, Department of Plant and Soil Sciences, University of Pretoria. The method described in section 5.2.3 for MIC determination was used with amphotericin B as a positive control. Inhibition of the egg hatching of *Haemonchus contortus* eggs was carried out following the method described in Chapter 5, section 5.2.5 with Valbazen (albendazole) as positive control. Cytotoxicity was investigated according to the method in Chapter 5, section 5.2.6, and doxorubicin was included as the positive control.

7.3 Results and Discussion

7.3.1 Structure elucidation of compound mixture

Two compounds were isolated in very small quantities and were not able to be identified. Compound **2** was obtained as a white powder. Its ^{13}C NMR spectrum exhibited a total number of 30 carbons with 3 downfield displayed at δ 145.4, 121.9 and 79.3 ppm assignable to a triterpene skeleton with one ethylenic double bond (C=CH) and one oxymethine (HCOH) groups

characteristic for β-amyrin skeleton. The ^{1}H-NMR data of this compound had seven singlets assignable and data obtained matched those previously published (Bastert, 2001). A mixture of ***α***-amyrin and ***β***-amyrin was obtained (Figure 7.2) and it was not possible to separate these two compounds further. This appears to be the first time that amyrin has been isolated from *D. whyteana* although it is known to occur in other species of the genus (Rauf et al., 2017). Amyrins are known to have antibacterial and antifungal activity (Johann et al., 2007; Vázquez et al., 2012) although their activity against *H. contortus* egg hatching does not appear to have been published previously.



***α*-amyrin** ***β*-amyrin**

Figure 7.2. Structures of α-amyrin and β-amyrin

7.3.2 TLC analysis and bioautography

Thin layer chromatography (TLC) fingerprints of the crude extract and purified compounds are shown in Fig 7.3. Solvent systems using hexane:ethyl acetate in varying ratios were used to separate compounds based on polarity. Good separation was observed using the 4:1 system, and the separation of the three compounds from the crude acetone extract could be noted.

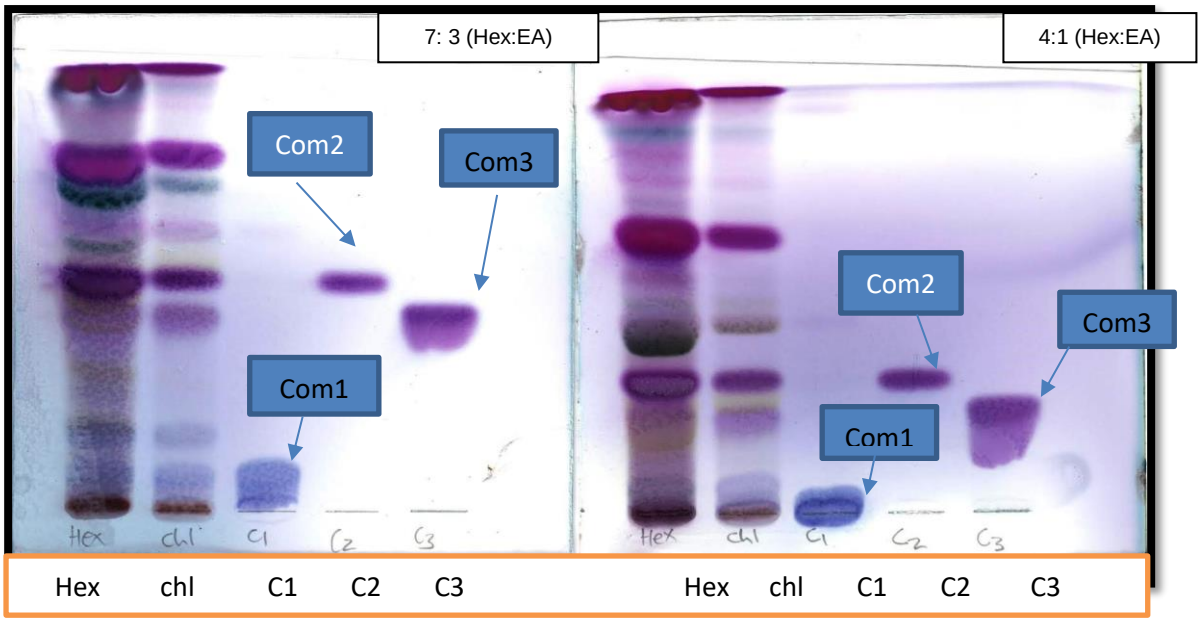


Figure 7.3. TLC plates of hexane and chloroform fractions, and isolated compounds from *Diospyros whyteana* developed in hexane:ethyl acetate (7:3 and 4:1) and sprayed with vanillin-sulphuric acid solution

A further TLC separation of the fractions of *D. whyteana* is shown in Figure 7.4. The hexane and chloroform fractions had similar chemicals present as the crude extracts. The more polar fractions including the ethyl acetate (EA) and butanol (but) fractions showed little separation of compounds as there was only a band on the origin. Bioautograms of replicas of the same TLC plate sprayed with *Cryptococcus neoformans* and *Candida albicans* are shown in Figures 7.5. The crude extract, hexane and chloroform fractions showed clear zones of inhibition when sprayed with *Cryptococcus neoformans*. Zones of fungal growth inhibition were not very clear when the chromatograms were sprayed with *C. albicans.*

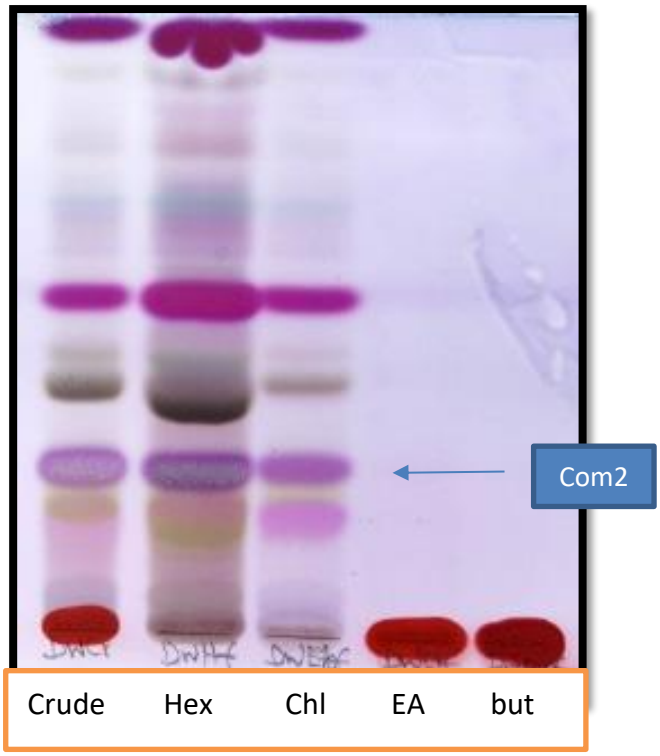


Figure 7.4. Chromatogram of *Diospyros whyteana* crude extracts and fractions sprayed with vanillin-sulphuric acid (elution system hexane:ethyl acetate = 4:1). Crude = crude acetone extract, Hex = hexane fraction, Chl = chloroform fraction, EA = ethyl acetatefraction, but = butanol fraction

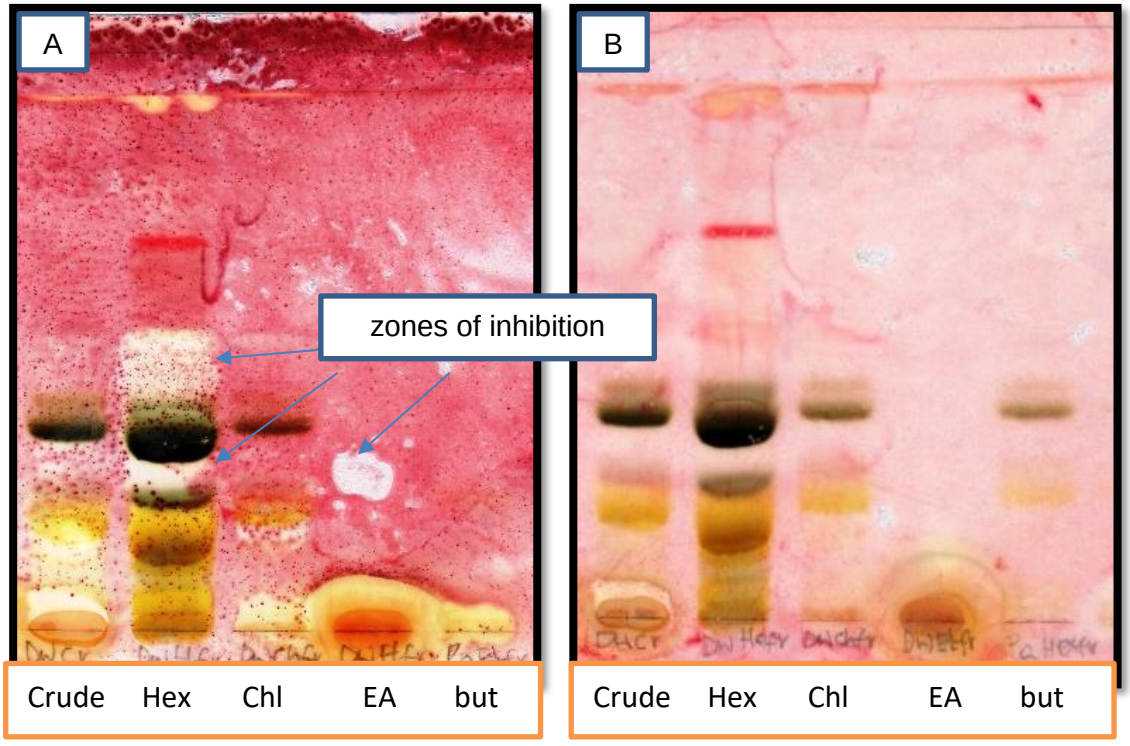


Figure 7.5. Bioautogram of *Diospyros whyteana* crude extracts and fractions sprayed with *Cryptococcus neoformans* (A) and *Candida albicans* (B) (elution system hexane:ethyl acetate = 4:1). Clear areas indicate zones of inhibition. Crude = crude acetone extract, Hex = hexane fraction, Chl = chloroform fraction, EA = ethyl acetatefraction, but = butanol fraction

7.3.3 Antifungal, anthelmintic activity and cytotoxicity of fractions and compounds

The antifungal activity of the *D. whyteana* crude extract and fractions against three animal and four plant pathogenic fungal species is presented in Table 7.1. The crude acetone extract and the ethyl acetate fraction had good activity against the animal pathogenic fungal species (*C. albicans* and *C. neoformans*) with the lowest MIC value of 40 µg/ml. Even lower MICs of 20 µg/ml and less were obtained against the plant pathogenic fungi, which is a promising result. The most susceptible fungal species was *P. italicum.* Activity of the purified compounds against the same fungal species, as well as their cytotoxicity against Vero cells and resulting selectivity index (SI) values are shown in Table 7.2. Varying degrees of antifungal activity were detected, with some extremely promising SI values. Again, the most susceptible fungal species was *P. italicum*, and compound 1 had extremely good activity against this species, with SI values as high as 61.25. This provides motivation for further studies targeted at isolating more of the compound and elucidating its structure. The other unidentified compound (3) did not have such good activity. The mixture of amyrins showed good activity with the highest SI value against *P. italicum.* It also had good activity against *C. albicans* with an SI of 11.25, but activity against the other fugal species was not noteworthy.

Table 7.1. Antifungal activity (MIC in µg/ml) of extracts and fractions tested against three animal and four plant fungal pathogens

Diosporus whyteana	*Candida albicans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*	*Aspergillus niger*	*Penicillium italicum*	*Rhizoctonia solani*	*Fusarium oxysporum*
Crude extract	40	40	20	<20	<20	40	<20
Hexane fr	40	160	40	<20	<20	40	<20
Chloroform fr	160	160	160	80	<20	160	80
Ethyl acetate fr	40	20	40	<20	<20	20	40
Amphotericin B	80	80	630	>250	<20	320	>250

Table 7.2 Antifungal activity (MIC in mg/ml) of isolated compound(s) tested against three laboratory isolates and four plant fungal pathogens together with cytotoxicity against Vero cells (LC_{50} in mg/ml) and SI values

Organisms	**Compound 1**			**Amyrin mixture**			**Compound 3**			**Amphotericin B**
	MIC	**LC_{50}**	**SI**	**MIC**	**LC_{50}**	**SI**	**MIC**	**MIC**	**SI**	
Candida albicans	0.16	2.45	15.31	0.16	1.8	11.25	0.16	1.00	6.25	0.08
Cryptococcus neoformans	>0.50	2.45	<4.9	>0.50	1.8	<3.6	>0.50	1.00	<2	0.04
Aspergillus fumigatus	0.32	2.45	7.66	0.32	1.8	5.63	0.32	1.00	3.13	0.08
Aspergillus niger	>0.50	2.45	<4.9	>0.50	1.8	<3.6	>0.50	1.00	<2	0.04
Penicillium italicum	0.04	2.45	61.25	0.04	1.8	45	0.04	1.00	25	0.02
Rhizoctonia solani	>0.50	2.45	<4.9	>0.50	1.8	<3.6	>0.50	1.00	<2	0.08
Fusarium oxysporum	0.63	2.45	3.89	0.63	1.8	2.86	0.63	1.00	1.59	Not active

Compound 2, a mixture of α- and β-amyrin isolated from *D. whyteana* had a percentage inhibition of helminth egg hatching of 30-50% compared to albendazole (trade name Valbazen), the standard drug, which had over 90% inhibition (Figure 7.6). It was interesting that this mixture of compounds which had good antifungal activity also had some anthelmintic activity. This lends support to the rationale to use a rapid and cheap antifungal assay to isolate compounds that may also have anthelmintic activity from plants. However, this would have to be tested on a large number of plant extracts and purified compounds. It was also noted that the butanol fraction (butFr) had a slight degree of inhibitory activity against helminth egg hatching. The crude extract (DWCRuA) showed similar activity to that of compound 2, the amyrin mixture. Therefore it is possible that the crude extract may be developed further as an anthelmintic preparation using the presence of the active amyrin compounds as a chemical marker in such a preparation.

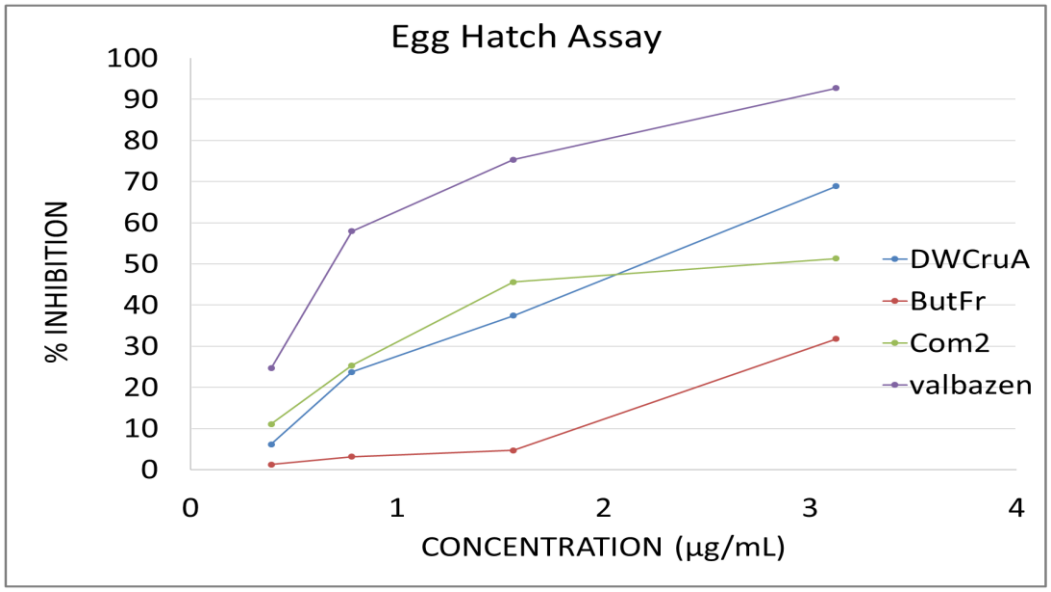


Figure 7.6. Inhibition of *Haemonchus contortus* egg hatching by *Diospyros whyteana* crude acetone extract, the butanol fraction, compound mixture of α- and β-amyrin and the positive control, Valbazen (albendazole)

7.4 Conclusions

Three compounds were isolated from the acetone extract of *D. whyteana*, using bioassay-guided fractionation with antifungal activity as a guide to isolate active compounds. One of the compounds proved to be a mixture of α- and β-amyrin, very closely related chemicals that could not be further separated. This is the first time that these compounds have been identified in *D. whyteana.* Two of the isolated compounds were not present in sufficient quantity to be structurally characterised. Activity of the isolated compounds indicated similar degrees of

microbial growth inhibition when tested against *C. albicans*. Isolated compounds showed relatively low toxicity to Vero cells. A dose dependent response of the fractions and compounds was noted in the nematode egg hatch inhibition assay. The antifungal amyrin mixture had anthelmintic activity, supporting the use of the antifungal assay as a guide for isolation of anthelmintic compounds from plants. As it is much easier to isolate antifungal compounds by bioassay-guided fractionation, this approach may facilitate the isolation of anthelmintic compounds. However, this supposition will have to be intensively investigated with other antifungal compounds isolated from a variety of plant extracts.

CHAPTER 8. General Discussion and Conclusions

8.1 General Discussion

Small ruminant gastrointestinal parasites result in great economic losses globally. Infection with helminth parasites such as *Haemonchus contortus* in sheep is a major problem. Inappropriate and extensive use of synthetic anthelmintic drugs against gastrointestinal nematodes in small ruminants has worsened the growing problem of anthelmintic resistance. Residues of these drugs in meat and milk are a further drawback, as are the negative environmental effects. Other measures are therefore necessary to control these parasites. Plant species have been investigated as alternative sources of anthelmintics to manage gastrointestinal parasites. They have been used in ethnoveterinary medicine for centuries to combat many diseases, and also those not used traditionally may contain interesting chemical compounds with useful biological activities. Anthelmintic plant extracts may be a sustainable and environmentally acceptable complement or alternative to synthetic anthelmintics. Furthermore, anthelmintic plant extracts have a mixture of active principles that could act in synergy, limiting the development of resistance. It is necessary to identify such compounds to use as biological markers if an active plant-based anthelmintic is to be developed. However, assays using parasitic helminths are inefficient and labour-intensive. It is often difficult to culture these organisms *in vitro* and usually they need to be cultivated in an animal host which leads to increased costs and ethical issues. Other means of detecting anthelmintic activity would be highly useful for alternative drug discovery platforms. As some classes of chemical compounds, such as the benzimidazole drugs, have antifungal as well as anthelmintic activity, it was proposed in this thesis that antifungal assays could be a mechanism to identify anthelmintic activity in plant extracts and be used to isolate anti-parasitic compounds. The aim of this study was therefore to investigate the efficacy of medicinal plant species used in ethnoveterinary medicine and those with known antifungal activity to control intestinal parasites and to determine the bioactive compound(s) responsible for such activities. It would be extremely useful to provide evidence to support the use of a simple, cost-effective antifungal bioassay in bioassay-guided fractionation to isolate anthelmintic compounds from plant extracts.

8.1.1 Objective 1

The first objective of the study was to determine the *in vitro* anthelmintic activity against parasitic *H. contortus* of extracts of plants selected firstly based on ethnoveterinary use and secondly on

known antifungal activity. An ethnopharmacological approach was initially used for the selection of the plant species investigated. The first part of the work was done in collaboration with the CSIR (Pretoria) where plants known to be used in ethnoveterinary medicine for parasite control were tested for anthelmintic efficacy. The acetone extracts of *Cleome gynandra*, *Maerua angolensis*, *M. angustifolia* and *Senna italica* subsp. *arachoides* had some anthelmintic activity against *H. contortus* egg hatching. In this assay, a concentration of 2.5 mg/ml of each extract was tested and a percentage inhibition of egg hatching was determined. The most active plant species was *C. gynandra*. Unfortunately results obtained were not very promising, so a further set of plants were selected based on their known antifungal activity for testing. The premise was that plants with antifungal activity may perhaps also have anthelmintic activity based on the antifungal and anthelmintic activities of compound classes such as the benzimidazoles.

These plant species (*Peddiea africana*, *Diospyros whyteana*, *Cassipourea gummiflua*, *Schotia brachypetala*, *Bowkeria citrina* and *Senna petersiana*) had activity against *Candida albicans* in a large-scale screening project and were selected for confirmation of their antifungal activity and testing of anthelmintic efficacy. Interestingly, a literature search revealed that many of these plant species had traditional medicinal uses although they were not selected on an ethnobotanical basis. Initially the extracts were analysed using thin layer chromatography with a variety of eluent systems of differing polarity to determine the complexity of the extracts. In this section of the work, a range of concentrations of the acetone and water extracts was tested to determine MIC values against fungal organisms and EC_{50} values for inhibition of nematode egg hatching. All plant crude extracts had activity in the nematode egg hatch inhibition assay, and several had very promising antifungal efficacy with little to no cytotoxicity. Acetone extracts had better activity against three fungal organisms associated with infections in animals and humans compared to water extracts. *Diospyros whyteana* had good antifungal and anthelmintic activity. Cytotoxicity analysis against normal mammalian cells (Vero) showed that the extracts had little to no cytotoxicity, leading to good selectivity index (SI, ratio of cytotoxicity to bioactivity) values.

8.1.2 Objective 2

The second objective of the study was to determine cytotoxicity and antioxidant efficacy of active extracts. The acetone and water extracts of leaves of the six plants had little to no cytotoxicity against Vero African green monkey kidney cells. Some SI values were very high (>25) when taking into account that an SI value greater than 10 is considered to be promising for further investigation. Surprisingly, the water extracts in general had extremely good SI values, better than those of the acetone extracts, with reasonable activity and low to no cytotoxicity indicating

that these should be further investigated for active compounds. However, there is a possibility that compounds such as tannins with non-specific biological activity may be present in these water extracts.

The plant extracts had relatively high levels of phenolics, more so than the flavonoid content. Water extracts, as expected owing to their higher polarity, generally contained higher contents of phenolics and flavonoids. Diverse structures of phenolics initiate from their character to protect plants alongside reactive oxygen, microorganisms and parasites, while their natural activities can offer active defence against some infections. *Schotia brachypetala* (acetone extract), had an extremely good IC_{50} value in the DPPH radical scavenging assay, demonstrating strong antioxidant activity. *Diospyros whyteana* had a high concentration of phenolics and flavonoids but lower antioxidant activity against the DPPH free radical than many of the other extracts.

8.1.3 Objective 3

The third objective of the study was to select the most promising plant species for isolation of anthelmintic compounds using bioassay-guided fractionation with antifungal activity as the test bioassay. *Diospyros whyteana* was selected for this purpose as it had good antifungal and anthelmintic activity and low cytotoxicity. Fractionation of the acetone leaf extract was conducted to isolate compounds. A dose dependent response of the fractions and compounds was noted in the egg hatch inhibition assay. The identified compound, which was a mixture of α-amyrin and β-amyrin, was effective in combating nematode egg hatching and also had moderate activity against *Candida albicans*. Amyrins are closely related compounds of the triterpene class and have been isolated from a number of plants as a mixture. This study revealed that there is a link between anthelmintic and antifungal activity in this case and the antifungal assay was a useful method to isolate anthelmintic compounds. This appears to be the first time that the antifungal and anthelmintic activity of *D. whyteana* crude extracts has been investigated and associated with amyrin derivatives.

8.1.4 Objective 4

The fourth objective of the study was to investigate the cytotoxicity and other potential uses of isolated compounds. Fractions and the isolated amyrin mixture as well as the two unidentified compounds showed relatively low toxicity to Vero cells. The compounds also had good activity against selected species of plant pathogenic fungi. There was unfortunately insufficient material

of two of the purified compounds to investigate more biological activities and confirm their chemical structures.

8.2 General conclusion and recommendations for future work

Helminth parasites are a significant health issue in both animals and humans, and lead to extensive production losses in livestock animals. Small ruminants are affected by the parasitic *H. contortus*, which has developed resistance to many anthelmintic drugs currently in use. The search for new anthelmintic lead compounds for development of new anthelmintics, or active plants which may be used in complementary therapy or prevention of infections, is urgent. Some classes of chemical compounds have antifungal as well as anthelmintic activity. As biological activity assays using parasitic nematodes are difficult, expensive and time-consuming, using a rapid and cost-effective antifungal assay to guide fractionation of plant extracts may be a potential mechanism of identifying anthelmintic compounds in plants. In this study several plants were screened for anthelmintic activity, and antifungal compounds were isolated from the most promising species, *D. whyteana*. The isolated antifungal compounds (one identified as a mixture of amyrins) had anthelmintic activity, supporting the use of the antifungal assay as a guide for isolation. As it is much easier to isolate antifungal compounds by bioassay-guided fractionation, this approach may facilitate the isolation of anthelmintic compounds.

Other extracts investigated in this project have potential for further investigation of active compounds, including *P. africana,* as this species had very good antifungal and antioxidant activity. More active compounds could be isolated from *D. whyteana* as two further compounds were isolated in insufficient quantity for elucidation of their chemical structures although preliminary bioassays showed that they had promising activity. Other active compounds may also be present, contributing to the activity of this species. Synergistic studies using these compounds in different combinations as well as in conjunction with currently used anthelmintic or antifungal drugs against which resistance is developing, could also yield interesting results. The activity of the extracts and purified compounds against plant pathogenic fungi is also worthy of further investigation to identify other uses for the plant.

It would also be of value to compare activity of the active extracts and compounds against other models of investigating anthelmintic activity such as free-living nematodes which are sometimes used in such investigations. These assays, as well as the culture of the organisms, are more complex and time-consuming than simple antifungal assays so it would not be as cost-effective to use these for bioassay-guided fractionation of active compounds.

A larger number of antioxidant assays could be used to elucidate the mechanism of antioxidant action and whether or not different types of antioxidant efficacy would be present in the plant extracts. Additionally, mechanisms of action of antifungal and anthelmintic activity could also be investigated to determine whether or not the extracts and purified compounds have a similar or different mode of action to currently used synthetic anthelmintic drugs. Combinations of plant-derived preparations and vaccines could perhaps also be tested to highlight potential adjuvant effects of plants with the vaccine. *In vivo* studies are ultimately the best manner in which anthelmintic and antifungal efficacy, as well as lack of toxicity, in an animal system can be determined and this is also an avenue of further research that could be followed.

In conclusion, this thesis has provided support for the presence of anthelmintic activity in antifungal plant extracts, as well as for the use of a simple and cost-effective antifungal assay using *C. albicans* as test organism for the bioassay-guided fractionation of anthelmintic compounds. The development and use of plant-derived antifungal and anthelmintic preparations is valuable in the search for complementary medications against such infections.

References

Adamu M., Naidoo V., and Eloff J.N., 2012. Some Southern African plant species used to treat helminth infections in ethnoveterinary medicine have excellent antifungal activities. BMC Complementary and Alternative Medicine 12: Article 213.

Adamu, M., Naidoo, V. and Eloff, J.N., 2013. 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*. BMC Veterinary Research 9: Article 38.

Adejinmi, J.O. and Harrison, L.J.S., 1997. Parasitic nematodes of domestic ruminants in Nigeria: Impact on ruminant production and control. Tropical Veterinarian 15: 137–151.

Ademola, I.O. and Eloff, J.N. 2010. *In vitro* anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae. Veterinary Parasitology 169: 198-203.

Ademola, I.O. and Eloff, J.N. 2011. Anthelmintic activity of acetone extract and fractions of *Vernonia amygdalina* against *Haemonchus contortus* eggs and larvae. Tropical Animal Health and Production 43: 521–527.

Ahmed, D., Zara, S., and Baig, H., 2013. In vitro analysis of antioxidant activities of *Oxalis corniculata* Linn. fractions in various solvents. African Journal of Traditional Complementary Alternative Medicine 10: 158-165.

Akhtar, M.S., Igbal, Z., Khan, M.N. and Lateef, M., 2000. Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo–Pakistan subcontinent. Small Ruminants Research 38: 99–107.

Albuquerque, U.P., Medeiros, P.M., Almeida, A.L., Monteiro, J.M., Neto, E.M.F.L., Melo, J.G., 2007. Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. Journal of Ethnopharmacology 114: 325–354.

Alonso, A., Sanchez, P. and Martinez, J.L., 2001. Environmental selection of antibiotic resistance genes. Environmental Microbiology, 3: 1-9.

Almeida, E.R., 1993. Plantas medicinais brasileiras – conhecimentos populares e científicos, Hemus, São Paulo, p. 341.

Angulo-Cubillán, F.J., García-Coiradas, L., Alunda, J.M., Cuquerella, M. and De La Fuente, C., 2010. Biological characterization and pathogenicity of three *Haemonchus contortus* isolates in primary infections in lambs. Veterinary Parasitology 171: 99–105.

Aremu, A.O., Finnie, J.F. and Van Staden, J., 2012. Potential of South African medicinal plants used as anthelmintics - Their efficacy, safety concerns and reappraisal of current screening methods. South African Journal of Botany 82: 134-150.

Arosemena, N.A.E., Bevilaqua, C.M.L., Melo, A.C.F.L. and Girão, M.D., 1999. Seasonal variations of gastrointestinal nematodes in sheep and goats from semi-arid area in Brazil. Revista Medicina Veterinária 150: 873–876.

Ashraf, M., 1985. Some pathological studies of lungs and regional lymph nodes in sheep and goats. http://agris.fao.org/agris-search/search.do?recordID=PK8700490 (Accessed 20 November 2019).

Attindehou, S., Salifou, S., Biaou, C.F., Gbati, O.B., Adamou-Nrsquo, M. and Pangui, L.J.2012. Epidemiology of haemonchosis in sheep and goats in Benin. Journal of Parasitology and Vector Biology, 4: 20-24.

Ayo, R.G., Audu, O.T., Amupitan, J.O. and Uwaiya, E., 2013. Phytochemical screening and antimicrobial activity of three plants used in traditional medicine in Northern Nigeria. Journal of Medicinal Plants Research 7: 191–197.

Barnes J., 2007. Pharmacovigilance of Herbal Medicines: A United Kingdom Perspective. In G. Bodeker and G. Burford (eds.), Traditional, Complementary and Alternative Medicine Policy and Public Health Perspectives, Imperial College Press, pp. 101-144.

Basch P.F. 1991. Schistosomes development, reproduction and host relation. Oxford University Press, New York.

Bashir, B.K., 2009. Seasonal prevalence of intestinal helminth parasites of goats (*Capra hircus*) of Khasibazar, Kalanki, Kathmandu. Master of Science Thesis, Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal.

Bastert, J., Schaller, M., Korting, H.C. and Evans, E.G.V., 2001. Current and future approaches to antimycotic treatment in the era of resistant fungi and immunocompromised hosts. International Journal of Antimicrobial Agents 17: 81-91.

Bate-Smith, E.C., 1962. The phenolic constituent of plants and their taxonomic significance, dicotyledons. Journal of the Linnean Society, Botany 58: 95–103.

Batista, L.M., Bevilaqua, C.M.L., Moraes, S.M. and Vieira, L.S., 1999. *In vitro* ovicidal and larvicidal effect of the plants *Spigelia anthelmia* and *Momordica charantia* against the nematode *Haemonchus contortus*. Animal Science 9: 67–73.

Begue, W.J. and Kline, R.M. 1972. The use of tetrazolium salts in bio-autographic procedures. Journal of Chromatography 64: 182–184.

Besier, B., 2009. Sheep worms – barbers pole worm. Note 476. Department of Agriculture and Food, Government of Western Australia, 1-4.

Biswas K., Chattopadhyay I., Banerjee R.K. and Bandyopadhyay U., 2002. Biological activities and medicinal properties of neem (*Azadirachta indica*). Current Science 82: 1336-1345.

Bizimenyera, E.S., Githiori, J.B., Eloff, J.N. and Swan, G.E. 2006. In vitro activity of *Peltophorum africanum* Sond. (Fabaceae) extracts on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*. Veterinary Parasitology 142: 336–343.

Brand-Williams, W., Cuvelier, M.E. and Berset, C.L.W.T., 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology 28: 25-30.

Cao, Y. and Cao, R., 1999. Angiogenesis inhibited by drinking tea. Nature 398: 381.

Craig, T.M., 2006. Anthelmintic resistance and alternative control methods. Veterinary Clinics: Food Animal Practice 22: 567-581.

Chandrawathani, P., Jamnah, O., Adnana, M., Waller, P.J., Larsen, T.M. and Gillespie, A.T., 2004. Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. Veterinary Parasitology 120: 177–187.

Chartier, C., Soubirac, F., Pors, I., Silvestre, A., Hubert, J., Couquet, C. and Cabaret, J. 2001. Prevalence of anthelmintic resistance in gastrointestinal nematodes of dairy goats under extensive management conditions in southwestern France. Journal of Helminthology 75: 325-330.

Coates Palgrave, K., 2002. Trees of Southern Africa 3rd edition. Struik, South Africa. pp. 458-459.

Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A. and Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 44: 35-44.

Cos, P., Vlietinck, A.J., Berghe, D.V. and Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology 106: 290-302.

Cox, F.E.G., 2001. Concomitant infections, parasites and immune responses. Parasitology 122: 23-38.

De Albuquerque, U.P., De Medeiros, P.M., De Almeida, A.L.S., Monteiro, J.M., Neto, E.M.D.F.L., de Melo, J.G. and Dos Santos, J.P., 2007. Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: a quantitative approach. Journal of Ethnopharmacology 114: 325-354.

De Roos, A.M., Persson, L. and McCauley, E., 2003. The influence of size-dependent life-history traits on the structure and dynamics of populations and communities. Ecology Letters 6: 473-487.

Degefu, H., Abera, C., Yohannes, M. and Tolosa, T., 2011. Gastrointestinal helminth infections in small-scale dairy cattle farms of Jimma town, Ethiopia. Ethiopian Journal of Science Technology 2: 31-37.

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

Diehl, M.S., Atindehou, K.K., Téré, H. and Betschart, B., 2004. Prospects for anthelminthic plants in the Ivory Coast using ethnobotanical criteria. Journal of Ethnopharmacology 95: 277-284.

Dobson, A., 2004. Population dynamics of pathogens with multiple host species. The American Naturalist 164: S64-S78.

Dobson, A. and Meagher, M., 1996. The population dynamics of brucellosis in the Yellowstone National Park. Ecology 77: 1026-1036.

Droge, W., 2002. Free radicals in the physiological control of cell function. Physiological Reviews 82: 47-95.

Drummond, R.B., 1972. A list of Rhodesian Legumes. Kirkia 8: 209-229.

Edward, C.L. and Hoffmann, A.A., 2008. Ivermectin resistance in a horse in Australia. Veterinary Record 162: 56-57.

Eloff, J.N., 1998a. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60: 1-8.

Eloff, J.N., 1998b. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64: 711-713.

Eloff, J.N., Famakin, J.O. and Katerere, D.R.P., 2005. *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. African Journal of Biotechnology 4: 1161–1166.

Eloff, J.N., Katerere, D.R. and McGaw, L.J., 2008. The biological activity and chemistry of the Southern African Combretaceae. Journal of Ethnopharmacology 119: 686-699.

Eloff J.N., Adamu M., van Zyl E., Botha F., McGaw L.J., and Naidoo V. 2016. Some success at last in the use of plants to combat helminth parasites in animal production. Planta Medica 81 (S01), 1002.

Farnsworth, N.R., 1994. The ethnobotanical approach to drug discovery: strengths and limitations. In: Prance G.T. Ethnobotany and the search for new drugs. In: Ciba Foundation Symposium, Chichester, 185: 42-59.

Farooq, Z., Mushtaq, S., Iqbal, Z. and Akhtar, S., 2012. Parasitic helminths of domesticated and wild ruminants in Cholistan desert of Pakistan. International Journal of Agricultural Biology 14: 63-68.

Fikru, R., Sori, T., Dhuguma, R. and Kiros, Y., 2006. Epidemiology of gastrointestinal parasites of ruminants in Western Oromia, Ethiopia. International Journal of Applied Research in Veterinary Medicine 4: 51-57.

Folin, O. and Ciocalteu, V., 1927. On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry 73: 627-650.

Fouché, G., Cragg, G.M., Pillay, P., Kolesnikova, N., Maharaj, V.J. and Senabe, J., 2008. In vitro anticancer screening of South African plants. Journal of Ethnopharmacology 119: 455-461.

Fyhrquist P., Mwasumbi L., Hæggström C.-A., Vuorela H., Hiltunen R. and Vuorela, P. 2002. Ethnobotanical and antimicrobial investigation on some species of *Terminalia* and *Combretum* (Combretaceae) growing in Tanzania*. Journal of Ethnopharmacology* 79: 169–177.

Gakuya, D.W., 2001. Pharmacological and clinical evaluation of anthelmintic activity of *Albizia anthelmintica* Brogn, *Maerua edulis* De Wolf and *Maerua subcordata* De Wolf plant extracts in sheep and mice. PhD thesis, University of Nairobi, Department of Veterinary Clinical Studies, Kenya.

Germishuizen, G., Meyer, N.L., Steenkamp, Y. and Keith, M., 2006. A Checklist of South African Plants. Southern African Botanical Diversity Network Report No. 41. Pretoria: SABONET.

Gillian, S., Behnke, J.M., Buttle, D.J. and Duce, L.R., 2004. Natural plant cysteine proteinases as anthelmintics? Trends in Parasitology 20: 322-327.

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

Grayer, R.J. and Harborne, J.B., 1994. A survey of antifungal compounds from higher plants, 1982–1993. Phytochemistry 37: 19-42.

Hale, M. 2006. Managing internal parasites in sheep and goats. www.attra.ncat.org. (Accessed 20 November 2019).

Hammond, J.A., Fielding, D. and Bishop, S.C., 1997. Prospects for plant anthelmintics in tropical veterinary medicine. Veterinary Research Communications 21: 213–228.

Harder, A., Schmitt-Wrede, H.P., Krücken, J., Marinovski, P., Wunderlich, F., Willson, J., Amliwala, K., Holden-Dye, L. and Walker, R., 2003. Cyclooctadepsipeptides—an anthelmintically active class of compounds exhibiting a novel mode of action. International Journal of Antimicrobial Agents 22: 318-331.

Hepworth, K., 2006. Managing internal parasitism in sheep and goats. https://www.extension.purdue.edu/extmedia/AS/AS-573-W.pdf. (Accessed 20 November 2019).

Hornok, L., 1992. Cultivation and processing of medicinal plants. John Wiley and Sons, Boston.

Hotez P.J., Molyneux D.H., Fenwick A., Ottesen E., Sachs S.E., Sachs J.D., 2007. Erratum: Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria (PLoS Medicine 2006) PLoS Medicine 4, 1553.

Hounzangbe-Adote, M.S., Paolini, V., Fouraste, I., Moutairou, K. and Hoste, H., 2005. In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode, *Haemonchus contortus.* Research in Veterinary Science 78: 155–160.

Ibrahim, M.A., Nwude, N., Ogunsusi, R.A. and Aliu, Y.O., 1984. Screening of West African plants for anthelmintic activity. https://cgspace.cgiar.org/handle/10568/4537. (Accessed 20 November 2019)

Iqbal, Z., Akhtar, M., Khan, M.N. and Riaz, M., 1993. Prevalence and economic significance of haemonchosis in sheep and goats slaughtered at Faisalabad abattoir. Pakistan Journal of Agriculture 30: 51-53.

Jabbar, A., Raza, M.A., Iqbal, Z. and Khan, M.N., 2006. An inventory of the ethnobotanicals used as anthelmintics in the southern Punjab (Pakistan). Journal of Ethnopharmacology 108: 152-154.

Jadhav, V.S., Ghawate, V.B. and Bhamber, R.S., 2011. Anthelmintic activity of ethanolic extract of seeds of *Cleome gynandra*. Journal of Pharmacy Research 4: 4479.

Johann, S., Soldi, C., Lyon, J.P., Pizzolatti, M.G. and Resende, M.A., 2007. Antifungal activity of the amyrin derivatives and in vitro inhibition of *Candida albicans* adhesion to human epithelial cells. Letters in Applied Microbiology 45: 148-153.

Jones, N.E., Davies, L.M., Brennan, J.S. and Bramble, S.K., 2000. Separation of visibly-excited fluorescent components in fingerprint residue by thin-layer chromatography. Journal of Forensic Science 45: 1286-1293.

Joshi, D.D., Karki, N.P.S. and Jimba, M., 2001. Status of meat slaughtering/inspection in Nepal. In Proceedings of the rabies slaughter workshop in Nepal to develop long-range solution NZFHRC. Tahachal, Kathmandu.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends in parasitology 20: 477-481.

Kaemmerer, K. and Buntenkötter, S., 1973. The problem of residues in meat of edible domestic animals after application or intake of organophosphate esters. In Residue Reviews: 1-240. Springer, New York, NY.

Kaminsky, R., Gauvry, N., Weber, S.S., Skripsky, T., Bouvier, J., Wenger, A., Schroeder, F., Desaules, Y., Hotz, R., Goebel, T. and Hosking, B.C., 2008. Identification of the amino-acetonitrile derivative monepantel (AAD 1566) as a new anthelmintic drug development candidate. Parasitology Research 103: 931-939.

Kaushik, R.K., Katiyar, J.C. and Sen, A.B., 1974. Studies on the mode of the action of anthelmintics with *Ascaridia galli* as a test parasite. Indian Journal of Medical Research 62: 1367–1375.

Kehrer, J.P. 1993. Free radicals as mediators of tissue injury and disease. Critical Reviews in Toxicology 23:21-48.

Khadri, A., Neffati, M., Smiti, S., Falé, P., Lino, A.R.L., Serralheiro, M.L.M. and Araújo, M.E.M. 2010. Antioxidant, antiacetylcholinesterase and antimicrobial activities of *Cymbopogon schoenanthus* L. Spreng (lemon grass) from Tunisia. LWT-Food Science and Technology 43: 331–336.

Khorombi, T.E. 2006. A chemical and pharmacological investigation of three South African plants. M.Sc dissertation, University of KwaZulu Natal South Africa, Pietermaritzburg, School of Chemistry.

Kivrak I., Duru M.E., Ozturk M., Mercan N., Harmandar M., Topcu G. 2010. Antioxidant, anticholinesterase and antimicrobial constituents from the essential oil and ethanol extract of *Salvia potentillifolia*. Food Chemistry. 116, 470-479.

Kochapakdee, S., Pralomkarn, W., Choldumrongku, S. and Saithanoo, S. 1995. Change in live-weight gain, blood constituents and worm egg counts in Thai native and crossbred goats raised in village environments in southern Thailand. Asian–Australasian Journal of Animal Science 78: 241-247.

Kokwaro, J.O., 1976. Medicinal plants of east Africa. University of Nairobi Press, Nairobi, Kenya.

Konuklugil, B., 1995. The importance of aryltetralin (podophyllum) lignans and their distribution in the plant kingdom. Journal of Faculty of Pharmacy of Ankara 24: 2.

Kosuth J., Koperdáková J., Tolonen A., Hohtola A. and Cellárová E., 2003. The content of hypericins and phloroglucinols in *Hypericum perforatum* L. seedlings at early stage of development. Plant Science 165: 515-521.

Kotze M., Eloff J.N. 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany 68: 62-67.

Kuete, V. and Efferth, T., 2010. Cameroonian medicinal plants: pharmacology and derived natural products. Frontiers in Pharmacology 1: Article 123.

Kunin, C.M., 1994. Urinary tract infections in females. Clinical Infectious Diseases 18: 1-10.

Lal, J., Chandra, S., Raviprakash, V. and Sabir, M., 1976. *In vitro* anthelmintic action of some indigenous medicinal plants on *Ascaridia galli* worms. Indian Journal of Physiology and Pharmacology 20: 64–68.

Leathwick, D.M., Pomroy, W.E. and Heath, A.C.G. 2001. Anthelmintic resistance in New Zealand. New Zealand Veterinary Journal 49: 227-235.

Leng, R.A., 1991. Application of biotechnology to nutrition of animals in developing countries. FAO Animal Production and Health Papers 90, pg 146.

Lone, B.A., Chrishti, M.Z., Ahmad, F. and Tak, H., 2012. Helminthic infestations in slaughtered cattle and goats of Ganderbal district, Kashmir. International Journal of Science 1: 65-67.

Mabusela, W.T., Stephen, A.M. and Botha, M.C., 1990. Carbohydrate polymers from *Aloe ferox* leaves. *Phytochemistry* 29, 3555–3558.

Machen, R.V., Craddock, F., Craig, T. and Fuchs, T.W., 1998. A *Haemonchus contortus* management plan for sheep and goats in Texas. Texas FARMER Collection. https://agrilifeextension.tamu.edu/library/ranching/a-haemonchus-contortus-management-plan-for-sheep-and-goats-in-texas/ (Accessed 21 December 2019)

Malan, D.F., Neuba, D.F. and Kouakou, K.L. 2015. Medicinal plants and traditional healing practices in Ehotile people, around the Aby lagoon (eastern littoral of Côte d'Ivoire). Journal of Ethnobiology and Ethnomedicine, 11: 21.

Maphosa, V., Masika, P.J., Bizimenyera, E.S. and Eloff J.N., 2010. In vitro anthelmintic activity of crude aqueous extracts of *Aloe ferox*, *Leonotis leonurus* and *Elephantorrhiza elephantina* against *Haemonchus contortus*. Tropical Animal Health and Production 42: 301–307.

Marie-Magdeleine, C., Mahieu,, M., D'Alexis, S., Philibert, L. and Archimede, H., 2010. In vitro effects of *Tabernaemontana citrifolia* extracts on *Haemonchus contortus*. Research in Veterinary Science 89: 88–92.

Martin, R.J., 1997. Mode of action of anthelmintic drugs. Veterinary Journal 154: 11–34.

Masoko, P., Picard, J. and Eloff, J.N., 2005. Antifungal activities of six South African *Terminalia* species (Combretaceae). Journal of Ethnopharmacology, 99: 301-308.

Max, R.A., 2010. Effect of repeated wattle tannin drenches on worm burdens, faecal egg counts and egg hatchability during naturally acquired nematode infections in sheep and goats. Veterinary Parasitology 169: 138–143.

Maxwell, S.R., 1995. Prospects for the use of antioxidant therapies. Drugs 49: 345-361.

McCord, J.M., 2000. The evolution of free radicals and oxidative stress. The American Journal of Medicine 108: 652-659.

McGaw, L.J., Jäger, A.K. and van Staden, J., 2004. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72: 247-263.

McGaw, L.J., van der Merwe, D. and Eloff, J.N., 2007. In vitro anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnveterinary medicine. Veterinary Journal 173: 366-372

Meda, N.T.R., Bangou, M.J., Bakasso, S., Millogo-Rasolodimby, J. and Nacoulma, O.G., 2013. Antioxidant activity of phenolic and flavonoid fractions of *Cleome gynandra* and *Maerua angolensis* of Burkina Faso. Journal of Applied Pharmaceutical Science 3: 036–042.

Melo, A.C.F.L., Bevilaqua, C.M.L. and Reis, I.F., 2009. Anthelmintic resistance to benzimidazole in gastrointestinal nematodes from small ruminants of semi-arid Brazilian North East. Animal Science 10: 294–300.

Mendoza de Gives, P., Crespo, J.F., Rodriguez, D.H., Prats, V.V., Hernandez, E.L. and Fernandez, G.E.O., 1998. Biological control of *Haemonchus contortus* infective larvae in

ovine faeces by administering an oral suspension of *Duddingtonia flagrans chlamydospores* to sheep. Journal of Helminthology 72: 343–347.

Mentz L.A. and Schenkel E.P., 1989. The consistency and reliability of therapeutic indications. Notebook Pharmacy 5: 93-119.

Miltsch, S.M., Krucken, J., Demeler, J., Ramunke, S., Harder, A. and Samson-Himmelstjerna, G., 2013. Interactions of anthelmintic drugs in *Caenorhabditis elegans* neuro-muscular ion channel mutants. Parasitology International 62: 591-598

Min, B.R. and Hart, S.P. 2003. Tannins for suppression of internal parasites. Journal of Animal Science. 81: 102-109.

Molan, A.L., Waghorn, G.C. and McNabb, W.C., 2002. The impact of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis in vitro.* Veterinary Record 150: 65–69.

Monteiro, M.V.B., Bevilaqua, C.M., Morais, S.M., Machado, L.K.A., Camurca-Vasconcelos, A.L.F., Campello, C.C., Ribeiro, W.L. and Mesquita, M.D.A., 2011. Anthelmintic activity of *Jatropha curcas* L. seeds on *Haemonchus contortus*. Veterinary Parasitology, 182: 259-263.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65: 55-63.

Muhammad, G., Abdul, J., Khan, M.Z. and Saqib, M., 2004. Use of neostigmine in massive ivermectin toxicity in cats. Veterinary and Human Toxicology 46: 28-29.

Muk, L., Naidoo, V. and Eloff, J.N., 2012. In vitro antibacterial activity of seven plants used traditionally to treat wound myiasis in animals in Southern Africa. Journal of Medicinal Plants Research 6: 4379-4388.

Mulugeta, H.S., Tafesse, M., Getachew, W., Kinfe, G., Getchew, T. and Teshome, Y., 1989. The significance of helminth parasites in livestock improvement production. Institute of Agricultural Research (IAR) proceedings of the 3rd National Livestock Conference, Addis Ababa, Ethiopia, 49- 53.

Munday, J., 1988. Poisonous Plants in South Africa Gardens and Parks, a field guide. Delta Books, Craighall.

Mungube, E.,O., Herve, S.V., Emmanuel, A.C., Diall, O., Boucom, A., Diarra, B., Sanogo, Y., Randolph, T., Bauer, B., Zessin, K.H. and Clause, P.H., 2012. Detection of multiple drug-resistant *Trypanosoma congolense* populations in village cattle of South-East Mali. Parasites and Vectors 5: 155.

Niezen, J.H., Robertson, H.A., Waghorn, G.C. and Charleston, W.A.G., 1998. Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. Veterinary Parasitology, 80: 15-27.

O'Connor, L.J., Walkden-Brown, S.W. and Kahn, L.P., 2006. Ecology of the free-living stages of major trichostrongylid parasites of sheep. Veterinary Parasitology 142: 1-15.

Ondua, M., Mfotie Njoya, E., Abdalla, M.A. and McGaw, L.J., 2019. Anti-inflammatory and antioxidant properties of leaf extracts of eleven South African medicinal plants used traditionally to treat inflammation. Journal of Ethnopharmacology 234: 27–35.

Oroian, M. and Escriche I., 2015. Antioxidants: Characterization, natural sources, extraction and analysis. Food Research International 74: 10-36.

Osbourne A.E., 1996. Preformed antimicrobial compounds and plant defense against fungal attack. The Plant Cell 8: 1821-1831.

Pauw, E. and Eloff, J.N. 2014. Which tree orders in southern Africa have the highest antimicrobial activity and selectivity against bacterial and fungal pathogens of animals? BMC Complementary and Alternative Medicine 14: Article 417.

Pax, R.A. and Bennett, J.L., 1989. Effect of closantel on intrategumental pH in *Schistosoma mansoni* and *Fasciola hepatica*. The Journal of Parasitology 75, 169-171.

Perry, B.D. and Randolph, T.F. 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Veterinary Parasitology 84: 145-168.

Perry, B.D. 2002. Investing in animal health research to alleviate poverty. ILRI (aka ILCA and ILRAD), Kenya.

Perry, J.A., Cvitkovitch, D.G. and Lévesque, C.M. 2009. Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA. FEMS Microbiology Letters 299, 261-266.

Pessoa, L.M., Morais, S.M., Bevilaqua, C.M.L. and Luciano, J.H.S. 2002. Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn and eugenol against *Haemonchus contortus*. Veterinary Parasitology 109: 59-63.

Plotkin, M.J. 1992. Ethnomedicine: past, present and future. Natural resources and human health: plants of medicinal and nutritional value. Proceedings, 79-86.

Pooley, E. 1993. Complete field guide to trees of Natal, Zululand and Transkei. Natal Flora Publications Trust, South Africa.

Prichard, R. 1994. Anthelmintic resistance. Veterinary Parasitology 54: 259–268.

Prichard, R.K., 1985. Interaction of host physiology and efficacy of antiparasitic drugs. Veterinary Parasitology 18: 103-110.

Prichard, R., 2001. Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends in Parasitology 17: 445-453.

Pushpa, B., Latha, K.P., Vaidya, V.P., Shruthi, A, and Shweath, C., 2011. In vitro anthelmintic activity of leaves extracts of *Tabernaemontana coronaria*. International Journal of Chemistry Technology Research 3: 1788–1790.

Rates, S.M.K., 2001. Plants as source of drugs. Toxicon, 39: 603-613.

Sacho H., Schoub D.B., 1993. Current perspectives on Nosocomial Infections. Natal Witness Printing and Publishing, Pietermaritzburg, South Africa.

Schimmer, O., Häfele, F. and Krüger, A., 1988. The mutagenic potencies of plant extracts containing quercetin in *Salmonella typhimurium* TA98 and TA100. Mutation Research/Genetic Toxicology 206: 201-208.

Schnyder, M., Torgerson, P.R., Schonmann, M., Kolera, L. and Hertzberg, H., 2005. Multiple anthelmentic resistance in *Haemonchus contortus* isolated from South African Boer goats in Switzerland. Veterinary Parasitology 128: 285–290.

Shahid, A.A., 2012. Biological activities of extracts and isolated compounds from *Bauhinia galpinii* (Fabaceae) and *Combretum vendae* (Combretaceae) as potential antidiarrheal agents. PhD Thesis, University of Pretoria, South Africa.

Skoog D.A., Holler F.J. and Crouch, S.R., 2007. Principles of Instrumental Analysis, Thomson Higher Education, USA.

Slomp, L., Pereira, P.S., Franca, S.D.C., Zingaretti, S. and Beleboni, R.O., 2009. *In vitro* nematocidal effects of medicinal plants from Sao Paulo state, Brazil. Pharmaceutical Biology, 47: 230-235.

Soulsby, E.J.L., 1982. Helminths, arthropods and protozoa of domesticated animals. Bailliere Tindall, London.

Sowunmi, L.I. and Afolayan, A.J., 2015. Phytochemical constituents and antioxidant properties of acetone extract of *Cleome gynandra* growing in the Eastern Cape, South Africa. Journal of Traditional Complementary and Alternative Medicines 12: 1–8.

Stahl E., 1969. Thin Layer Chromatography, Springer-Verlag, New York.

Steenkamp, V. and Gouws, M.C., 2006. Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. South African Journal of Botany, 72: 630-633.

Suffness, M. and Douros, J., 1979. Drugs of plant origin. Methods in Cancer Research 16: 73-126.

Sutherland I.A., and Leathwick, D.M., 2011. Anthelmintic resistance nematode parasites in cattle: A global issue? Trends in Parasitology 27: 176 – 181.

Szewezuk, V.D., Mongelli, E.R. and Pomilio, A.B., 2003. Antiparasitic activity of *Melia azadirach* growing in Argentina. Molecular Medicinal Chemistry 1: 54-7.

Tandon, V., Pal, P., Roy, B., Rao, H.S.P. and Reddy, K.S., 1997. In vitro anthelmintic activity of root-tuber extract of *Flemingia vestita*, an indigenous plant in Shillong, India. Parasitology Research 83: 492-498.

Taylor, M., 2000. Use of anthelmintics in cattle. In Practice London British Veterinary Association 22: 290-328.

Terefe, D., Demissie, D., Beyene, D. and Haile, S., 2012. A prevalence study of internal parasites infecting Boer goats at Adami Tulu Agricultural Research Center, Ethiopia. Journal of Veterinary Medicine and Animal Health, 4:12-16.

Thenmozhi, S., Varghese, N., Subasini, U., Dhanalakshmi, M., Manjuladevi, K. and Kameshwaran, S., 2014. Comparative study of anthelmintic activity of different extracts of *Gynandropsis pentaphyla.* International Journal of Pharmacy and Life Sciences 5: 3246–3248.

Torres-Acosta, J.F.J., Mendoza-de-Gives, P., Aguilar-Caballero, A.J. and Cuéllar-Ordaz, J.A., 2012. Anthelmintic resistance in sheep farms: update of the situation in the American continent. Veterinary Parasitology 189: 89-96.

Townsend, L.B. and Wise, D.S., 1990. The synthesis and chemistry of certain anthelmintic benzimidazoles. Parasitology Today, 6: 107-112.

Tzamaloukas, O., Athanasiadou, S., Kyriazakis, I., Jackson, F. and Coop, R.L., 2005. The consequences of short-term grazing of bioactive forages on established adult and incoming larvae populations of *Teladorsagia circumcincta* in lambs. International Journal for Parasitology 35: 329-335.

Umadevi, M., Kumar, K.S., Bhowmik, D. and Duraivel, S., 2013. Traditionally used anticancer herbs in India. Journal of Medicinal Plants Studies, 1: 56-74.

Van den Bossche, H.V. 1985. Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action. Current Topics in Medical Mycology 1: 313-351.

Valentao, P., Fernandes, E., Carvalho, F., Andrade, P.B., Seabra, R.M. and de Lourdes Bastos, M., 2002. Studies on the antioxidant activity of *Lippia citriodora* infusion: scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. Biological and Pharmaceutical Bulletin 25: 1324-1327.

Van Wyk, B.E., Oudtshoorn, B.V. and Gericke, N., 1997. Medicinal Plants of South Africa. Briza, Pretoria, South Africa.

Van Wyk, J.A., Stenson, M.O., Van der Merwe, J.S., Vorster, R.J. and Viljoen, P.G., 1999. Anthelmintic resistance in South Africa: surveys indicate an extremely serious situation in sheep and goat farming. Onderstepoort Journal of Veterinary Research 66: 273-284.

Van Zyl, E.A., Botha, F.S., Eloff, J.N., Msuntsha, P.P., Oosthuizen, P.A. and Stevens, C., 2017. The use of *Lespedeza cuneata* for natural control of gastrointestinal nematodes in Merino sheep. Onderstepoort Journal of Veterinary Research 84: 1-7.

Vázquez, L.H., Palazon, J. and Navarro-Ocaña, A., 2012. The Pentacyclic Triterpenes α,β-amyrins: A Review of Sources and Biological Activities. In: Phytochemicals - A global perspective of their role in nutrition and health. IntechOpen. https://www.intechopen.com/books/phytochemicals-a-global-perspective-of-their-role-in-

nutrition-and-health/the-pentacyclic-triterpenes-amyrins-a-review-of-sources-and-biological-activities (Accessed 20 November 2019).

Vicente M.F., Basilio A., Cabello A., Peláez F., 2003. Microbial natural products as a source of antifungals. Clinical Microbiology and Infections 9: 15–32.

Wadood, A., Ghufran, M., Jamal, S.B., Naeem, M., Khan, A. and Ghaffar, R., 2013. Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochemical Analytical Biochemistry, 2: 1-4.

Waller P.J., and Prichard, R.K., 1985. Drug resistance in nematodes. In: Campbell, W.C., Rew, R.S. (eds). Chemotherapy of Parasitic Infections. New York, USA: Phenum, pp. 339-62.

Waller, P.J., 1994. The development of anthelmintic resistance in ruminant livestock. Acta Tropica 56: 233–243.

Waller, P.J., 1999. International approaches to the concept of integrated control of nematode parasites of livestock. International Journal of Parasitology. 29: 155- 164.

Wangchuk, T., Inouye, D.W, Hare, M.P. 2008. The emergence of an endangered species; evolution and phylogeny of *Trachypithecus geei* of Bhutan. International Journal of Primatology 29: 565- 582.

Wangchuk P., Keller P.A., Pyne S.G., Taweechotipatr M., Tonsomboon A., Rattanajak R., Kamchonwongpaisan S., 2011. Evaluation of ethnopharmacologically selected Bhutanese medicinal plants for their major classes of phytochemicals and biological activities. Journal of Ethnopharmacology 137: 730-742.

Watson, R.R., 1993. Resistance to intestinal parasites during murine AIDS: role of alcohol and nutrition in immune dysfunction. Parasitology 107: S69-S74.

Waweru, D., 2001. Pharmacological and clinical evaluation of the anthelmintic activity of *Albizia anthelmintica, Maerua edulis* and *Maerua subcord* plant extracts in sheep and mice. Doctoral dissertation, University of Nairobi, Kenya.

WHO, 2002. World Health Organization Traditional Medicine Strategy 2002-2005. Geneva, Switzerland.

WHO, 1998. Quality Control Methods for Medicinal Plant Materials, World Health Organization. . https://apps.who.int/iris/handle/10665/41986. (Accessed 20 November 2019).

Williamson, E.M., Okpako, D.T. and Evans, F.J., 1996. Selection, preparation and pharmacological evaluation of plant material. Vol. 1. John Wiley & Sons, Boston.

Wojcikowski, K., Stevenson, L., Leach, D., Wohlmuth, H. and Gobe, G. 2007. Antioxidant capacity of 55 medicinal herbs traditionally used to treat the urinary system: A comparison using a sequential three-solvent extraction process. The Journal of Alternative and Complementary Medicine, 13: 103-110.

Wolstenholme, A.J., Fairweather, I., Prichard, R., Von Samson-Himmelstjerna, G. and Sangster, N.C., 2004. Drug resistance in veterinary helminths. Trends in Parasitology, 20: 469-476.

Worku, M., Franco, R. and Miller, J.H., 2009. Evaluation of the activity of plant extracts in Boer goats. American Journal of Animal and Veterinary Sciences 4: 72-79.

Yadav, R.N.S. and Agarwala, M., 2011. Phytochemical analysis of some medicinal plants. Journal of Phytology 3. https://updatepublishing.com/journal/index.php/jp/article/view/2737 (Accessed:19 November 2019).

Yue-Zhong S. 1998. Recent natural products based drug development: a pharmaceutical industry perspective. Journal of Natural Products 61: 1053-1071.

Zajac, A.M. 2006. Gastrointestinal nematodes of small ruminants: life cycle, anthelmintics, and diagnosis. Veterinary Clinics: Food Animal Practice, 22: 529-541.

Zeryehun, T. 2012. Helminthosis of sheep and goats in and around Haramaya, South-Eastern Ethiopia. Journal of Veterinary Medicine and Animal Health, 4: 48-55.

APPENDIX A

The interpretation of NMR data of one of the compounds allowed identification of it as a mixture of *α*-amyrin and *β*-amyrin in the proportions 4:1.

Table A1. NMR data of Compound 1

Position	***α*-amyrin**			***β*-amyrin**	
	δ_C	δ_H	**HMBC**	δ_C	δ_H
1	38.6			38.9	
2	27.2	1.44		27.2	
3	77.6	3.01 (m)	1, 23, 24	77.9	
4	38.7			38.5	
5	55.2			55.2	
6	18.2	1.23		18.2	
7	32.8	1.46		32.5	
8	39.9			39.7	
9	47.7	1.46		47.6	
10	36.7			36.8	
11	23.2	1.82		23.1	
12	124.6	5.07 (m)	9, 11, 14, 18	121.9	
13	139.5			144.9	
14	41.9			41.5	
15	26.4	1.74		26.0	
16	27.8	0.86		26.7	
17	33.6			33.6	
18	59.1	1.23		47.2	
19	39.55			46.7	
20	39.53			30.8	
21	31.0			34.4	
22	41.4	1.30 ; 1.18		36.9	
23	27.9	0.85 (s)	3, 5, 24	27.8	
24	15.5	0.66 (s)	3, 4, 5, 23	15.0	
25	15.2	0.86 (s)	1, 5, 9	15.5	
26	16.5	0.92 (s)	7, 8, 9, 14	16.4	
27	22.8	0.99 (s)	8, 13, 14, 15	25.5	
28	28.3	0. 70	18,17, 22	27.9	
29	17.0	0. 70	18, 19, 20	32.7	
30	20.8	0. 79	19, 20, 21	23.3	

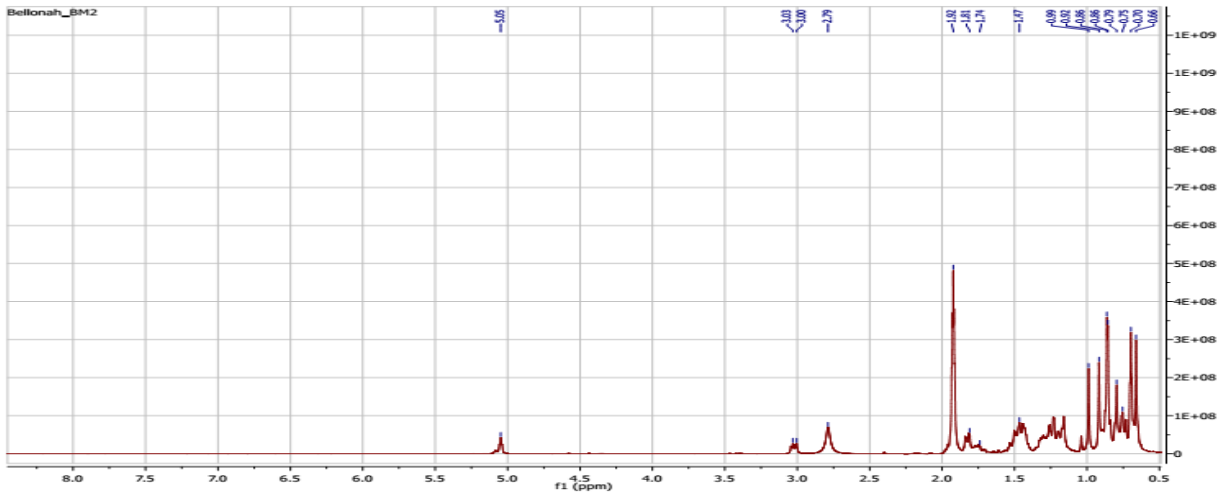


Figure A1. Spectra of RMN ^{1}H (500 MHz, acetone-d_6) of BM_2

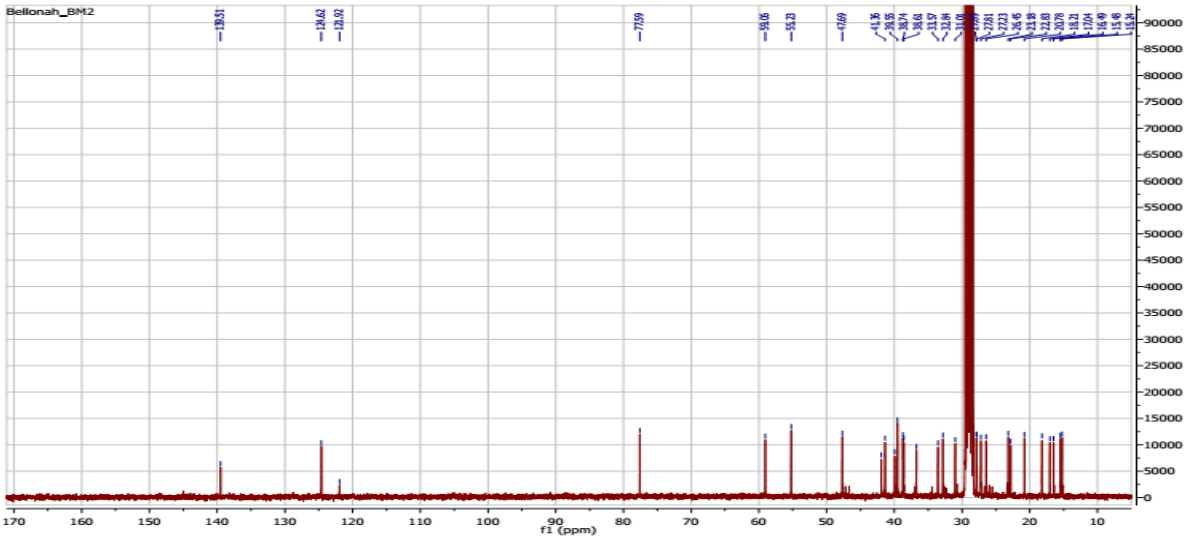


Figure A2. Spectra of RMN ^{13}C (125 MHz, acetone-d_6) of BM_2

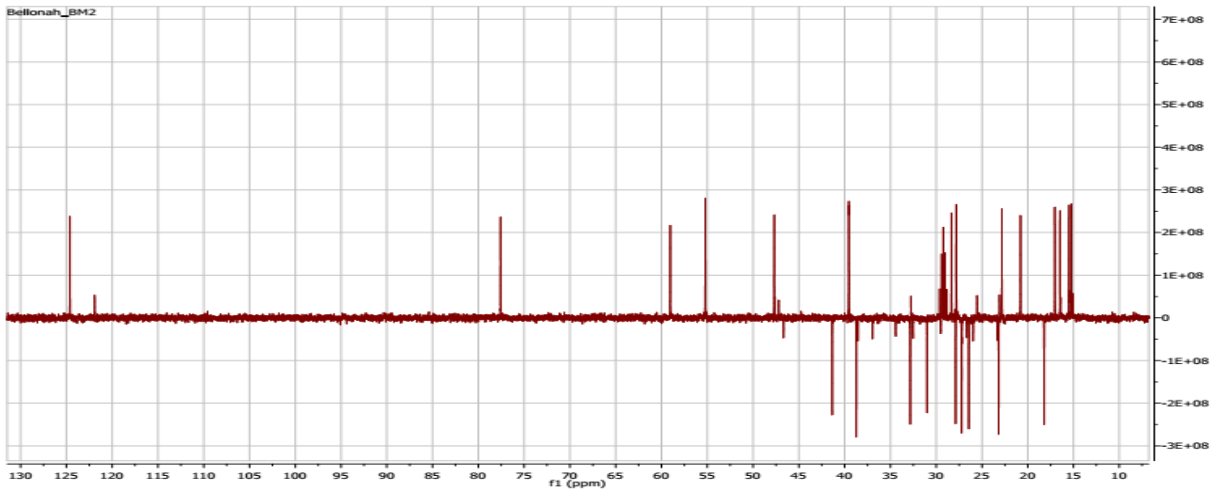


Figure A3. Spectra of DEPT (125 MHz, acetone-d_6) of BM_2

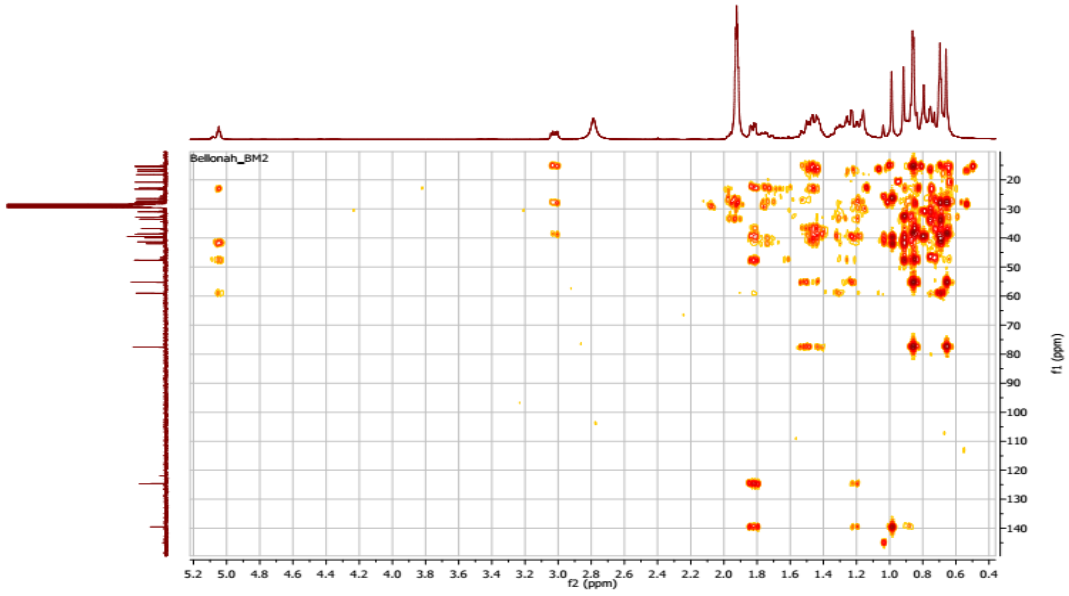


Figure A4. Spectra of HMBC (500 MHz: ^{1}H; 125 MHz: ^{13}C, acetone-d_6) of BM_2

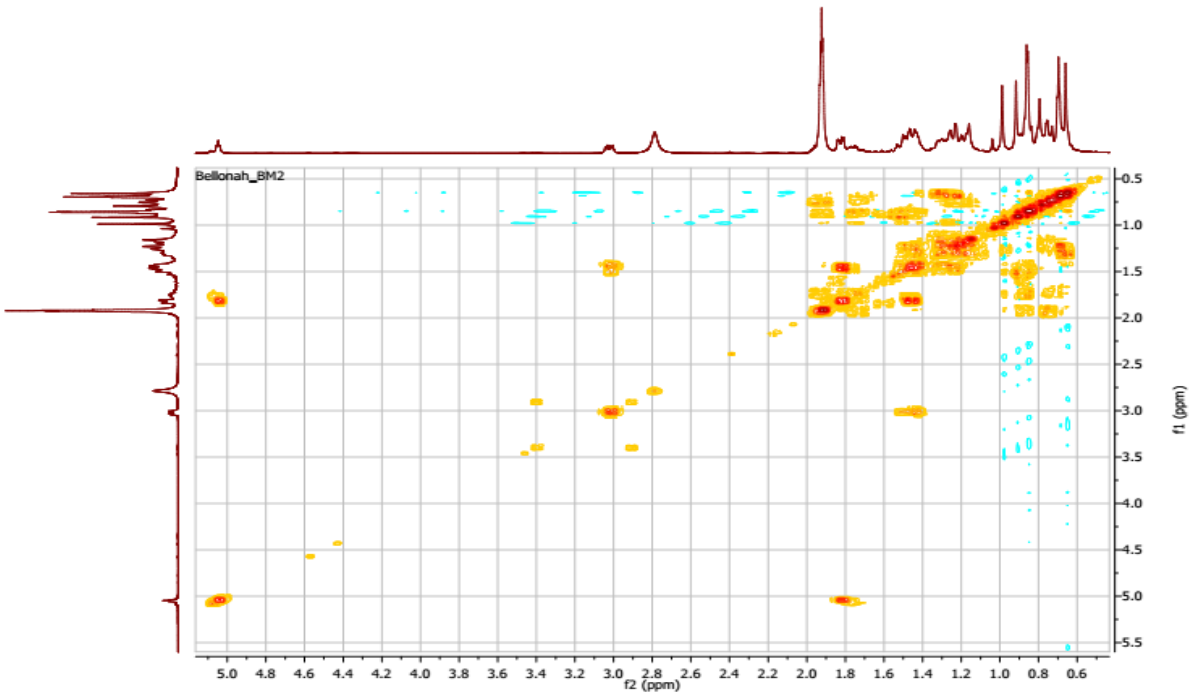


Figure A5. Spectra of COSY ^{1}H-^{1}H (500 MHz, acétone-d_6) of BM_2

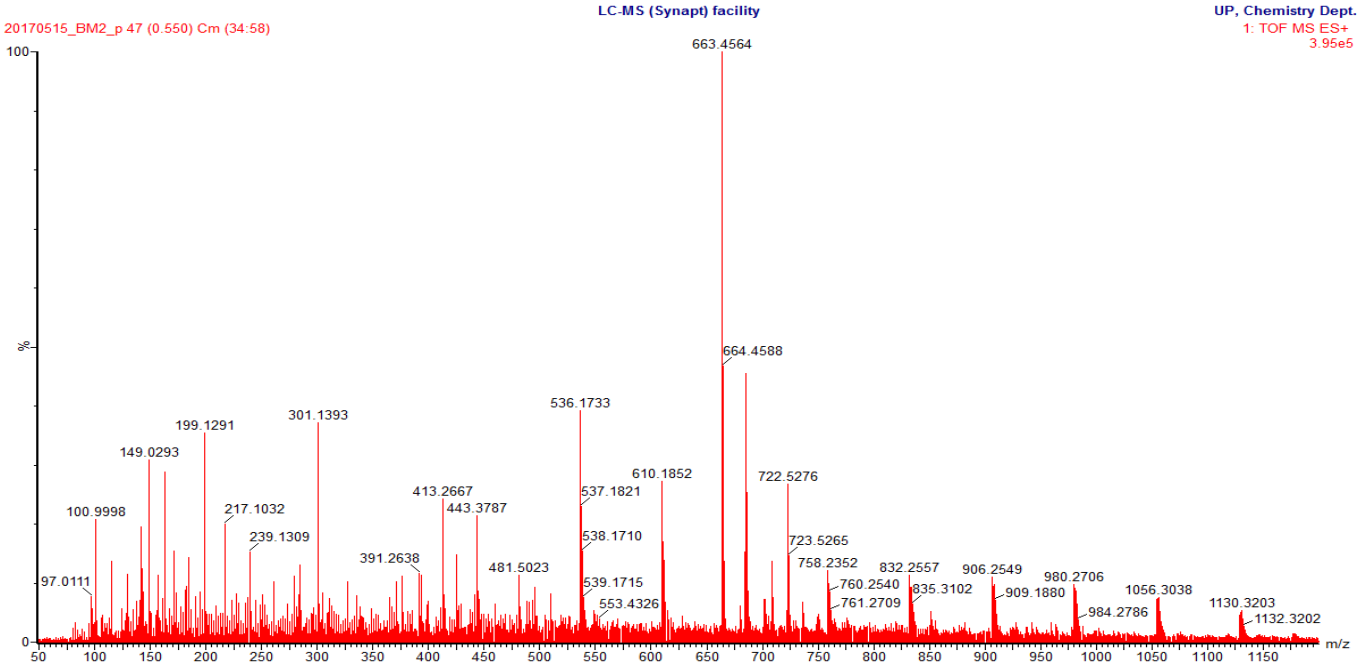


Figure A6. Liquid Chromatography and Mass spectrometry of BM2 Compound