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The potential use of the invasive species *Cereus jamacaru* (Cactaceae) to control nematode infections in sheep

by

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Fig.1.Cereus jamacaru. Source: www. *Cereus jamacaru* on flickr-photosharing

DECLARATION

This research was carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria and at Onderstepoort Veterinary Institute (OVI), under the supervision of Prof J.N. Eloff and Dr A.F. Vatta and Dr I.O. Ademola.

This dissertation represents work done by Clément Kandu-Lelo, except where the work of others is acknowledged and the results have not been submitted anywhere else before.

Clément Kandu-Lelo

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LIST OF ABBREVIATIONS USED

AAEAC	ascorbic acid equivalent antioxidant capacity
Ace	acetone
A.f.	*Aspergillus fumigatus*
BEA	benzene:ethanol:ammonia (90:10:1 v/v/v)
BuOH	butanol
CEF	chloroform:ethyl acetate:formic acid (5:4:1 v/v/v)
C.a.	*Candida albicans*
C.n.	*Cryptoccocus neoformans*
COWP	copper oxide wire particles
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picryl-hydrazyl
E.c.	*Escherichia coli*
E.f.	*Enteroccocus faecalis*
EMW	ethyl acetate:methanol:water (40:5:4 v/v/v)
Epg	eggs per gram
FEC	faecal egg count
Hex	hexane
INT	p-iodonitrotetrazolium violet
L_1, L_3	first, third stage larvae
LDVA	larval development and viability assay
LWT	live weight
MeOH	methanol
MHB	mueller Hinton Broth
MIC	minimum inhibitory concentration
OVI	Onderstepoort Veterinary Institute
P.a.	*Pseudomonas aeruginosa*
PCV	packed red cell volume
S.a.	*Staphyloccocus aureus*
SOD	super oxide dismutase
TEAC	trolox equivalent antioxidant capacity
TLC	thin layer chromatography
UV	ultraviolet light

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SUMMARY

This study was stimulated by a publication of Mr Mike Bosch (Bosch 2007) that *Cereus jamacaru* DC (Cactaceae) used by him on his farm is effective for gastrointestinal nematode control in livestock. This plant, widely known as Queen of the night, is a serious invasive weed. We evaluated this claim in *in vitro* assays and *in vivo* experiments in sheep experimentally infected with *Haemonchus contortus* and *Trichostrongylus colubriformis*. This is the first report of such an anthelmintic trial in livestock involving *C. jamacaru*.

The first study was to repeat the farmer's work under strictly controlled conditions. *In vivo* studies were conducted to determine the possible direct anthelmintic effects of *C. jamacaru* on ovine gastrointestinal nematodes. Eighteen sheep were allocated to three groups and were infected with 4000 *H. contortus* and 6000 *T. colubriformis* infective larvae given in 3 divided doses over a period of three days. From day 0 until day 49 of the experiment, sheep were drenched once a week with fresh blended *C. jamacaru* plants with the core removed at the same (32.33 g/sheep) or double the dose (64.66 g/sheep) used by Mr Bosch. No negative effects of the double dose were observed during the period of the experiment. Faeces were collected twice a week for faecal egg count. Based on the *in vivo* experiments, *C. jamacaru* was not effective in reducing *H. contortus* and *T. colubriformis* in sheep to the 70% reduction levels of the control treatments considered to be a useful reduction in FEC. Nevertheless, its *in vivo* activity was substantial at the higher dose and reduced the FECs by 65%.

To investigate the matter further some additional experiments were carried out. Specimens of *C. jamacaru* were collected and dried in the shade. For the phytochemical analysis and *in vitro* experiments, the dried material was milled to a fine powder, it was extracted with acetone and five fractions (hexane, butanol, water, chloroform and 35% water in methanol) were obtained by solvent-solvent fractionation. The chemical composition of the fractions and crude extract was analysed by thin layer chromatography using three solvent systems of varying polarity and pH. To detect the separated compounds, vanillin-sulphuric acid-

methanol was sprayed on the chromatograms and heated at 110°C for optimal colour development.

The antioxidant activity in plant extracts may influence the immune systems of animals and have an indirect effect. The antioxidant activity was therefore determined. For qualitative analysis of antioxidant activity, the 2,2, diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a test for the radical scavenging ability of the compounds present in the different extracts. In the DPPH qualitative analysis of antioxidant activity there were a number of antioxidant compounds present in some of the extracts and fractions but the activity appeared to be low. This observation was confirmed in the TEAC quantitative analysis of antioxidant activity. Even the fraction with the highest activity was about 8 times less active than trolox or ascorbic acid. It therefore appears that stimulation of the immune system by antioxidant activity does not explain the results found on the farm.

The antibacterial and antifungal activities were determined against 4 bacteria (*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa*) and 3 fungi (*Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans*). The minimum inhibitory concentration (MIC) values for plant extracts varied from 0.04 to 2.5 mg/ml. *E. coli* was the most sensitive and the fungi were in general resistant to the extract and fractions. In general the activity was not very good, especially if the dosage in an aqueous system on the farm is considered. Antimicrobial activity also does not explain the results obtained.

The nematocidal activity was evaluated by an egg hatch assay and larval development viability assay (LDVA) *in vitro*. The acetone extract inhibited egg hatchability and killed infective larvae of *H. contortus* nematodes of sheep in a concentration-dependent manner. The percentage inhibition of egg hatching of the acetone extract and the butanol, chloroform, 35% water in methanol, hexane and water solvent-solvent fractions were 100%, 100%, 94%, 91%, 9% and 16%, respectively. The percentage inhibition of larval development of the acetone extract and chloroform, 35% water in methanol, hexane and water fractions were

93%, 84%, 49%, 85%, 31%, respectively. The chloroform fraction was significantly more active than all the other fractions ($p<0.05$). The extracts had *in vitro* activity against the nematodes. The activity was however much lower than the positive control, albendazole.

It is disappointing that our results do not provide an explanation for the success obtained by Mr Bosch on his farm. Even if we do not understand how it works it may be useful to prepare suitable dosages (using low level technologies adaptable to rural conditions) for use by resource-poor rural communities where *C. jamacaru* occurs as an invasive weed.

PAPERS PREPARED FROM THIS DISSERTATION

Kandu-Lelo,[1] C., Vatta[2], A.F., Ademola,[1] I.O. and J.N. Eloff.[1] Direct anthelmintic effects of *Cereus jamacaru* (Cactaceae) on trichostrongylid nematodes of sheep: *in vivo* studies

Kandu-Lelo,[1] C., Vatta[2], A.F., Ademola,[1] I.O. and J.N. Eloff.[1] Evaluation of ovicidal and larvicidal activity of *Cereus jamacaru* acetone extract and fractions on *Haemonchus contortus*

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CHAPTER 1

INTRODUCTION

1.1. Background

Infestations with helminth parasites of livestock are among the most common and economically important diseases of grazing livestock (Perry & Randolph, 1999). These are serious problems of the developing world particularly where nutrition and sanitation are poor (Sharkhuu 2001; Faye, Leak, Nouala, Fall, Losson & Geerts 2003). These infections are responsible for lower outputs of animal products (meat, milk, hides, skins and manure) and traction, which all impact on the livelihoods of smallholder farmers (Perry & Randolph 1999). The greatest losses associated with nematode parasite infestations are sub-clinical, and economic assessments show that the financial costs of uncontrolled intestinal parasitism are enormous (Preston & Allonby 1979).

Livestock producers have derived substantial benefits from the use of anthelmintics in controlling livestock parasitoses. Synthetic anthelmintics are the main way of controlling nematode parasites of livestock today. However, these drugs may not be readily available to smallholder farmers, or to remote pastoralist communities.).

In Africa, synthetic drugs are sometimes unavailable or expensive and unaffordable by resource-poor farmers. These constraints lead to the use of poor quality or altered products (Monteiro, Wanyangu, Kariuki, Bain, Jackson & Mckellar 1998). The use of plants as medicines provides a low-cost alternative but requires much work to produce high-quality preparations that are safe and efficacious.. The misuse of these synthetic anthelmintics has led to the development of anthelmintic resistance (Lans & Brown 1998). Scientific validation of the anti-parasitic effects and possible side-effects of plant products in ruminants is necessary prior to their adoption as novel methods for parasite control (Githiori, Athanasiadou & Thamsborg 2006). Because of

the rapid escalation of anthelmintic resistance worldwide, other approaches to nematode control in developing countries are urgently required (Jackson & Coop 2000). This work will focus on studying the effectiveness of *Cereus jamacaru*, “Queen of the Night” cactus, a declared weed in South Africa, but reportedly used as a deworming agent by a farmer close to Pretoria.

1.2. Statement of the problem

i. Parasitic gastroenteritis is one of the major constraints to ruminant production in Africa.
ii. Synthetic anthelmintics are often not available or are unaffordable to small-scale and smallholder farmers in the developing world.
iii. In commercial farming systems, anthelmintic resistance has emerged as a serious threat to sustainable livestock production.

1.3. Justification of the study

Plants offer a vast, virtually untapped reservoir of chemical compounds with many potential uses. One of these uses is in agriculture to manage pests with less risk than with synthetic compounds that are often toxicologically and environmentally undesirable.

1.4. Aims and objectives

1.4.1. Hypothesis

It is hypothesized that *C. jamacaru* has an anthelmintic effect against *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep based on its use by a local farmer.

In addition, it is conjectured that *C. jamacaru* will also possess antimicrobial, antifungal and antioxidant properties because many plant extracts have these activities.

1.4.2. Aims

To evaluate the use of *C. jamacaru* in protecting sheep against helminth infections in order to validate the apparent success obtained by a commercial farmer using this product.

1.4.3. Objectives

- To evaluate possible toxic effects of feeding animals with a higher dose of *C. jamacaru* than that used by Mr Mike Bosch.
- To evaluate the *in vitro* efficacy of *C. jamacaru* as an anthelmintic
- To evaluate the *in vivo* efficacy of *C. jamacaru* against *H. contortus* and *T. colubriformis* at different dosage levels.
- To evaluate the antibacterial, antifungal and antioxidant properties of *C. jamacaru* extracts *in vitro* as an alternative explanation to the success of the commercial farmer.
- To make recommendations on using this procedure based on the results obtained.

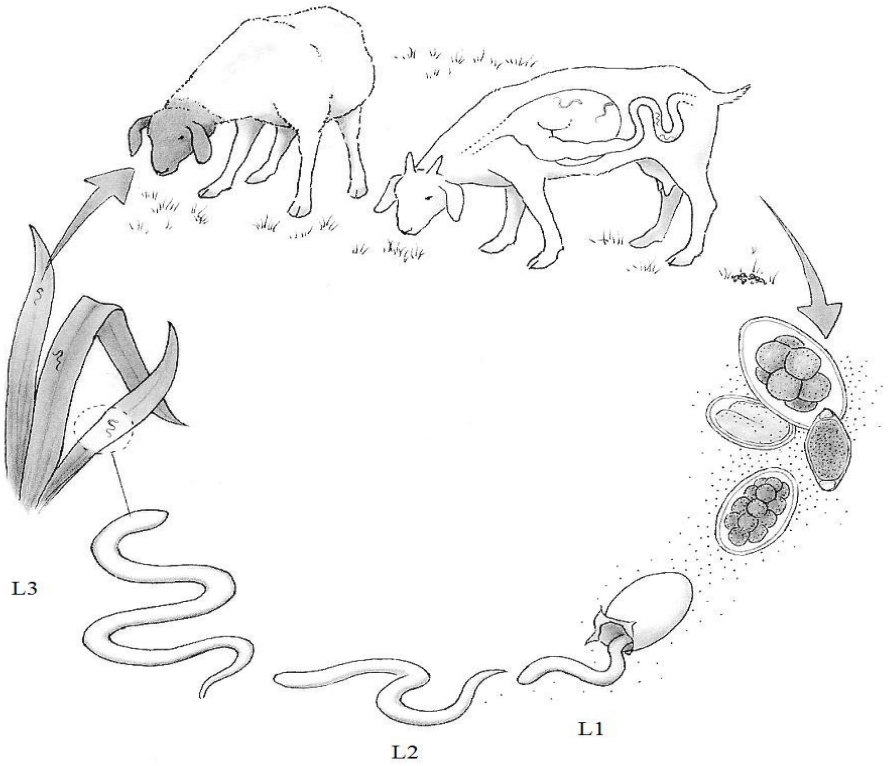
CHAPTER 2

LITERATURE REVIEW

2.1. Nematodes of sheep

Haemonchus contortus and *Trichostrongylus colubriformis* are among the top ten most common nematodes hampering production of sheep and goats in the tropics (Horak, Ursula & Purnell 2004; Almalaik, Bashar & Abakar 2008). The life cycle is direct (Fig. 2) and typical for the strongyle nematodes. Adult *H. contortus* are 10 to 30 mm long and those of *T. colubriformis* are 5.5 to 8 mm long. The females of *H. contortus* can shed 5000 to 15000 eggs per day in the host's faeces (Hansen & Perry 1994), whereas the females of *T. colubriformis* are less prolific.

The hatched larva undergoes five stages of development separated by four moults (Reinecke 1983). First-stage larvae (L_1) develop and hatch in a day or two and feed on microorganisms. After a moult, the resulting second-stage larvae (L_2) also feed on microorganisms. The second moult is started but not completed in the external environment so that the infective third-stage larvae (L_3) remain encased in the cuticle of the second stage until they are ingested by an animal. The sheath is cast off in the abomasum and the now parasitic third stage larvae undergo a moult to the fourth stage (L_4). The fourth stage sooner or later moults to the fifth or adult stage (Bowman 1995).



A: Eggs passed onto pasture in manure; B: Eggs hatch, and larvae develop from first larval stage (L_1) to infective third stage (L_3); C: Infective larvae are ingested by grazing sheep.

Fig. 2. Principal life-cycle of gastrointestinal nematodes. (Illustration: Katarina Näslund).

In arid areas, *H. contortus* survives the dry season as inhibited fourth-stage larvae within the abomasal mucosa of the host (Gatongi, Prichard, Ranjans, Gathuma, Munyua, Cheruiyot & Scott 1998). The inhibited larvae resume their development a few weeks before the onset of the new rainy season. This phenomenon is accompanied by a drastic increase of the egg output of infected animals before the wet season starts ("spring rise").

The mature *H. contortus* worms are voracious blood-suckers within the abomasum. Consequently, anaemia, submandibular oedema ("bottle jaw"), rough coat, and weight loss are the main symptoms of haemonchosis. *Trichostrongylus colubriformis* tunnels through the villi of the small intestine and causes seepage of serum proteins into the lumen. As a result, infection caused by *T. colubriformis* is characterized by protracted diarrhoea with a dark stool, weakness, loss of production and death (Urquhart, Armour,

Duncan, Dunn & Jennings 1996). Since control programs for *Haemonchus* spp usually result in the control of intestinal worms (Table 2-1), they will not be considered separately in this study.

Table 2-1. Families, examples and predilection sites of strongyles of sheep and goats common in the tropics (Soulsby 1982)

Family	Parasites	Predilection Site
Trichostrongylidae	*Trichostrongylus axei*	Abomasum
	Haemonchus contortus	"
	Ostertagia ostertagi	Small intestine
	Teladorsagia circumcinta	"
	Trichostrongylus vitrinus	"
	Trichostrongylus colubriformis	"
	Cooperia curticei	"
	Cooperia oncophora	"
	Nematodirus spathiger	"
Strongylidae	*Oesophagostomum venulosum*	Large intestine
	Oesophagostomum columbianum	Large intestine
	Syngamus nasicola	Trachea
Ancylostomatidae	*Bunostomum trigonocephalum*	Small intestine
	Gaigeria pachyscelis	"
Metastrongylidae	*Dictyocaulus filaria*	Lungs

2.2. Control of helminth infections

Some types of worm infection control are: anthelmintics, grazing management, targeted drenching, biological control, copper oxide wire particles, strategic nutritional supplementation, use of plants with antiparasitic properties, and breeding and use of resistant and resilient breeds.

2.2.1. Anthelmintics

Anthelmintics are drugs used to treat infections with parasitic worms (flukes, tapeworms and round worms). They are of huge importance for human tropical medicine and for veterinary medicine. Parasitic worms also infect livestock and crops, affecting food production with a resultant economic impact. Synthetic anthelmintics are separated into classes on the basis of similar chemical structure and mode of action (Holden-Dye & Walker 2007). In South Africa, three broad-spectrum classes of synthetic anthelmintics are used for the control of gastrointestinal nematode parasites of ruminant livestock; they have high levels of efficacy against all stages of the parasites:

- The benzimidazole/probenzimidazole group interferes with the polymerization of microtubules (Harder 2002). The drugs bind to the protein tubulin of the parasite, therefore causing death by starvation (Roos 1997).
- The tetrahydropyrimidine/imidazothiazole group affects acetylcholine neuro-transmission by interfering with nicotinic acetylcholine receptors (Roos 1997; Harder 2002).
- The macrocyclic lactones (MLS) or avermectin/milbemycin group work through an interaction with choline channels on helminth gamma aminobutyric acid (GABA) receptor complexes, and also inhibit pharyngeal pumping (and hence feeding), motility and fecundity in susceptible nematodes, resulting in paralysis and ultimately elimination from the host (Harder 2002).

The production of sheep and goats in South Africa is being threatened by the widespread and increasing problem of resistance to all anthelmintic groups by

nematode parasites (Van Wyk, Stenson, Van der Merwe, Vorster, & Viljoen 1999; Bakunzi 2003). Considerable research effort has been, and is currently being, expended on alternative approaches to the control of internal parasites of livestock. Occurrence of resistance to anthelmintics and increased awareness concerning drug residues entering the food chain and harming the environment, have implied a reduction in the regular, preventive use of anthelmintics and an increase in the use of non-chemical means to control parasitic infections in small ruminants (Athanasiadou, Houdijk & Kyriazakis 2008).

2.2.2. Anthelmintic resistance

In South Africa, it appears that the level of anthelmintic resistance may be the highest that has so far been recorded in the world and that resistant strains of gastrointestinal nematodes of sheep and goats are emerging that may soon not be controllable by treatment with any of the existing anthelmintics (Van Wyk *et al*. 1999). Farmers in the summer rainfall region, if not the whole country, must be alerted to the immediate need for testing the gastro-intestinal parasites of their sheep for susceptibility to preparations in at least all three groups of broad-spectrum anthelmintic compounds currently available. Alternative methods of integrated worm control must be sought and implemented with urgency, to reduce further selection for resistance. The ways to induce reversion of the resistance that have already developed, should be examined (Van Wyk *et al*. 1999; Bakunzi 2003).

2.2.3. Targeted drenching

Wilson & Grenfell (1997) have shown that in a flock or herd, only a few individuals are infected with the majority of the parasites and many animals have few or no parasites. Targeted drenching is based on the selective treatment of those individual animals that are diagnosed to be infected, and having clinical symptoms of the disease. The chemical control measures include the targeted, selective use of anthelmintics, which is achieved either by monitoring and treating individual animals, or by targeting specific nematode species and consequently minimising the use of anthelmintics (Athanasiadou *et al*. 2008).

The FAMACHA© system developed in South Africa .was founded on this concept The system enables anaemic sheep and goats infected with *H. contortus* to be identified clinically (Kaplan, Burke, Terrill, Miller, Getz, Mobini, Valencia, Williams, Williamson, Larsen & Vatta 2004). The system consists of a chart which has five eye scores (1-5), which have been correlated with packed cell volumes. Animals in categories 1 or 2 (red or red-pink) do not require treatment whereas animals in categories 4 and 5 (pink-white and white) do. Animals in category 3 may or may not require treatment depending on various factors, such as the time of year, levels of helminth infection in the animals and build-up of larvae on pasture. Thus, instead of treating the whole flock, only those animals with infection-inducing anaemia are treated with an effective anthelmintic (Van Wyk, Bath & Malan 1998; Vatta, Letty, Van der Linde, Van Wijk, Hansen & Krecek 2001).

Bath & Van Wyk (2001) reported that the system has worked well on both intensive and extensive farms in the summer rainfall area of South Africa where farmers reported a significant drop in treatment costs, averaging a 58.4% reduction. According to Mahieu, Arquet, Kandassamy, Mandonnet & Hoste (2007), the use of the FAMACHA© method allowed a dramatic decrease in anthelmintic use during the periparturient period in a Creole goat flock grazing under an oceanic–tropical climate.

2.2.4. Grazing management

Pasture management may be planned so that the levels of infective larvae on pastures are unlikely to cause severe infections with internal parasites in grazing ruminants. Grazing management consists of two main types: continuous and rotational grazing. In continuous grazing programs, animals are placed in a camp when the forage is first ready to graze and left in that camp for the entire grazing period of each year. This principle has long been condemned by grassland management advisors in South Africa (Tainton 1999). Controlled mating, weaning and parasite control are at best difficult to achieve and animals do not easily or regularly come under the herdsman's eye (Tainton 1999).

Rotational grazing is a type of management which requires that the grazing allotted to a group or groups of animals for the entire grazing period be subdivided into at least one enclosure more than the number of animal groups. It involves the successive grazing of these enclosures in rotation so that not all the veld is grazed simultaneously.

One approach to control parasites is to move animals from a contaminated pasture to a parasite-free or nearly parasite-free pasture. Such a pasture is one that has been tilled or given a prolonged rest of 6 months or more during the cool season and 3 months or more during the warm season or where tall forages are planted for animals to graze at shoulder or higher height. Another approach is to move animals to the clean pasture only days after they been dosed (Scarfe 1993). However, this may select strongly for anthelmintic resistance.

Resistance has developed because past recommendations did not consider “refugia”, which is the proportion of a population of worms that is not exposed to the anthelmintic treatment. When treating all the animals in a flock/herd, as has been practiced in the past, only resistant worms survive. If these animals are then moved to a “clean” pasture, only resistant worms can develop on that pasture.

2.2.5. Biological control

Due to rapidly developing anthelmintic resistance in parasitic helminths and increasing concern about chemical residues in livestock products and the environment (Sanyal 2001) alternative strategies have to be investigated. Biological control methods using nematophagous fungi could be an alternative approach for the control of nematodes and trematodes in ruminants (Sanyal 2005). The thought behind biological control is to make use of one or more of the natural enemies of the nematodes, making it possible to reduce the infection on pasture to a level where grazing animals can avoid both clinical and subclinical effects of the parasitic nematodes. The presence of the fungi in the faecal pats where the development of the pre-parasitic larvae takes

place is a prerequisite. Therefore, to be effective, the fungi should pass through the gastrointestinal tract of the host without loss of viability (De & Sanyal 2009).

Biological control by means of the nematode-destroying microfungus *Duddingtonia flagrans* has been extensively researched. This nematophagous fungus forms sticky traps that hold infective larvae found in the faeces, avoiding the third-stage larvae from migrating onto the pasture and potentially infecting future hosts. When resting spores (chlamydospores) of this fungus are fed daily to grazing animals for a period of time, the pasture infectivity and thus the worm burdens of grazing animals are reduced, especially in young lambs. However, the practical use of this fungus within production systems has been difficult to achieve and there is currently little hope that commercial application will be found (Larsen 2006).

The use of vaccines may also be a potentially useful control method applied to gastrointestinal infections in small ruminants. Although the possibility of control by vaccination is currently being investigated, in practice wide-scale vaccine production has not yet proved to be successful for gastrointestinal nematodes (Walkden-Brown & Eady 2003).

2.2.6. Copper oxide wire particles (COWP)

Besides being an efficient and effective means of treating copper deficiency in livestock, copper oxide wire particles can also be potentially useful as an anthelmintic against abomasal parasites (Knox 2002). The mechanism of action is assumed to be based on the lethal effects on the parasite of ionic copper liberated from the copper oxide in the acid environment of the abomasum. The concentration for an anthelmintic effect and the potential for toxicity in copper-sufficient animals or those exposed to copper-accumulating plants, are still need to be established. However, Burke and Miller (2006) showed that a dose of 2 g of COWP was effective against gastrointestinal nematode burdens in sheep. In subsequent work the same authos did not recommended the use of copper wire particles (Burke & Miller 2008).

Copper boluses (Copasure©, Animax Ltd, England) are available for use for copper deficiency in cattle. The minimum dose that has been shown to be effective in some studies is 0.5 g, but as much as 2-4 g may be required. If necessary, treatment can be repeated after 4-6 weeks. When 0.5 or 1 g is used animals should not receive more than two to four COWP boluses containing 2 or 4 g of copper in a worm season. COWP has been found to be effective in reducing only abomasal (*H. contortus*) and not intestinal worms. Most of the time it has also been found to be effective only against *H. contortus* in mature goats. Recently, Vatta, Waller, Githiori & Medley (2009) found that COWP boluses have the potential to be used in the place of conventional anthelmintics for the control of established *H. contortus* infections in indigenous South African goats.

2.2.7. Strategic nutritional supplementation

Gastrointestinal nematode infections impair animal productivity by reducing the voluntary feed intake and/or reduction in the efficiency of feed used, especially through the inefficient use of absorbed nutrients (Coop & Kyriazakis 2001). Disturbances in protein metabolism and reduced absorption and/or retention of minerals are significant during parasite infection (Coop & Kyriazakis 2001). The importance of these effects is influenced by the size of the larval challenge and the number and species of parasites present.

A common feature of gastrointestinal infection is the increased loss of endogenous protein into the gastrointestinal tract (Coop & Kyriazakis 2001). It was shown that improved dietary protein supply may reduce production losses and mortality from gastrointestinal nematode parasites (Coop & Kyriazakis 2001). According to Svensson, Hessle & Hoglund (2000), parasite control methods involving grazing management combined with nutritional supplementation with concentrates and/or forages is the most frequently reported anti-parasite strategy in Sweden.

2.2.8. Resistant and resilient breeds

Resistance is the ability of the host to prevent or limit the establishment or development of infection (Van Houtert & Sykes 1996). Resistant animals may be identified by examining faecal egg counts (FEC). Selection for resistance can be combined with conventional nematode control methods, as the animals can be evaluated during a test period sufficiently short so that their production is not greatly compromised (Kisielewicz, Fraser & Eady, 1995). The Red Masai sheep breed of Kenya is more resistant against *H. contortus* than the Merino, Dorper and Hampshire breeds (Preston & Allonby 1979). The Canaria Hair Breed sheep, of the Canary Islands, has a greater resistance to *H. contortus* infection than Canaria sheep (González, Herna'andez, Molina, Ferna'ndez, Raadsma, Els Meeusen & Piedrafita 2008).

Resilience is the ability of an animal to maintain reasonable levels of production when subjected to parasitic challenge (Van Houtert & Sykes 1996). According to Woolaston & Baker (1996), the identification of resilience may be done by means of a simple method in which selection pressure to a disease trait is applied, allowing the disease to take its course, and then animals that perform well under those conditions are selected.

2.2.9. Ethnoveterinary medicine

Ethnoveterinary medicine is the use of people's beliefs, knowledge and skills in the practice of healing animals (Mc Corkle 1986). The use of herbal remedies is empirical, and it is usually transferred directly by oral teaching from generation to generation (Masika, Van Averbeke & Sonandi 2000). Preparations derived from plants were the original therapeutic interventions used by man to control diseases and parasites, both within humans and livestock. Developed and developing countries show a great interest in indigenous medicine, and many developing countries use traditional medicines at the primary health care level (McGaw, Jager & Van Staden, 2000).

McGaw & Eloff (2008) reported that most non-commercial, rural South African livestock owners use traditional remedies to treat both themselves and their

animals for common diseases. The use of ethnoveterinary medicine is limited by several conditions such as the seasonal availability of certain plants, the scarcity of treatment against infectious disease, inadequate ethno-diagnosis and existing harmful practices. In South Africa, the use of herbal remedies is widespread because these remedies are cheap, locally available and convenient to administer. Farmers also use herbal remedies because they do not have knowledge of conventional remedies (Masika *et al*. 2000). According to Luseba & Van der Merwe (2006), some plants are used not only as alternatives to expensive pharmaceutical products but also because in certain diseases or chronic cases, they are thought to be more efficacious.

About 25% of currently available therapeutic compounds are plant-derived and or synthetic analogues derived from those compounds. Many plants are recorded and used as having curative activity but few have been subjected to scientific validation. In South Africa, Van der Merwe, Swan & Botha (2001) identified eight plants that were used as dewormers of cattle in the Madikwe area, although cattle owners in that area had a poor understanding of helminth infections of cattle. According to Luseba & Van der Merwe (2006), diagnosis from traditional farmers is not always clearly defined; priority is given to physico-biological elements. Internal parasite infections, for instance, are strongly linked to diarrhoea and lack of appetite.

Plant species that are purportedly used by traditional healers are listed, often without supplying a proper description of the method of preparation (Guarrera 1999). However, some *in vitro* studies have been used to evaluate plant preparations for efficacy against nematode parasites. Leaf, bark and root extracts of *Peltophorum africanum* have been demonstrated, *in vitro*, to be active against *H. contortus* (Bizimenyera, Githiori, Swan & Eloff 2006). Roots of *Adhatoda vesica* were shown to have a toxic effect against adults of *H. contortus* (Lateef, Iqbal, Khan, Akhtar & Jabbar 2003). Leaves of *Vernonia amygdalina* were toxic against eggs of *H. contortus* (Alawa, Adamu, Gefu, Ajanusi, Abdu, Chiezey, Alawa & Bowman 2003). Hoskin, Wilson, Barry, Charleston & Waghorn (2000) reported that tannin-rich forages, such as lucerne (*Medicago sativa*), birds' foot trefoil (*Lotus corniculatus*) and sulla

(*Hedysarum coronarium*) lowered faecal egg counts in sheep grazing these pastures.

Some plants do not only affect the nutrition of animals, but also have antiparasitic effects. Certain plants contain condensed tannins which are effective antimetabolites, and in amounts exceeding 5%, which is common in tropical forages, can impair rumen microbial functioning in ruminants (Singh & Bhat 2003). *Sericea lespedeza*, *Hedysarum cornarium* and *Leucaena diversifolia* are some of these tannin-containing plants with antiparasitic properties (Min & Hart 2003; Shaik, Miller, Kouakou, Kannan & Kallu 2004).

Tannins and flavonoids have therapeutic uses due to their anti-inflammatory, anti-fungal, antioxidant, anthelmintic and other biological properties (Athanasiadou, Kyriazakis, Jackson & Coop 2000). Ruminants can therefore benefit from the presence of condensed tannins in their diets; the consumption of average concentrations of condensed tannins can result in increased weight gain, milk secretion and wool growth (Barry & McNabb 1999) and decrease the detrimental effects of gastrointestinal parasitism (Aerts, Barry & McNabb 1999).

2.3. *Cereus jamacaru* DC (A.P. de Candolle, 1828)

Cereus jamacaru (Cactaceae) is commonly known as “Queen of the night” cactus because it blooms at night with a beautiful flower. This tree-like cactus originated from Brazil, grows up to 18 m tall, and has segmented stems with a main trunk that can be over 60 cm thick. It is easily propagated by cuttings, but is also easy to grow from seeds, although the cactus is then slow to grow (Gimre 2001).

In Brazil, people in the interior of the country sometimes utilize the plant as a living fence, planting it around the house as a kind of hedge. It is also utilized as a source of food for livestock in drought periods. In times of great drought, farmers cut off young branches of the plant to feed livestock. The outermost part of the ribs is cut off in order to remove the spines, and then the branches are chopped up before the cactus is given to cattle (Gimre 2001). *C. jamacaru*

contains phenolic compounds (Sousa, Alencar, De Amorim & Albuquerque 2008). Phenolic compounds, which include tannins and flavonoids, may act as feeding attractants, feeding deterrents, floral pigments, and structural components of plants (Harborne 1982, 1998). According to Sousa (2001), a spoonful of mashed stem pulp with sugar is used against stomach ulcers, while roots are used against respiratory and renal diseases in humans. *C. jamacaru* is recorded among ten native plants examined by Albuquerque, Muniz de Medeiros, De Almeida, Monteiro, Machado de Freitas Lins Neto, Gomes de Melo & Dos Santos (2007) in Brazil, which they qualified as "potential targets for future phytochemical and pharmacological studies." They indicated that the plant may be used therapeutically against renal problems, hepatic problems, respiratory problems, influenza, cough, bronchitis, ulcers, constipation, hypertension, rheumatism, enteritis, and fever, and as an expectorant, as an anti-emetic, for syphilis, spinal problems, urethral problems and injury and as a diuretic.

In Cuba the juice of the stems is used to expel parasitic worms from the human body; an infusion of the stems and flowers is used as a cardiac tonic and to combat rheumatism (Socha 2003). In Europe, an alkaloid compound called "cactine" is extracted from the plant and used to treat irregular heartbeat, angina pectoris, and cardiac neuralgia (Socha 2003). It has been found to have a spasmolytic effect on the coronary arteries that promotes blood circulation. Prostate diseases, bladder irritation, congested kidneys, and nervous headaches are also treated with infused stems (Socha 2003). In Egypt, recent studies on native and cultivated plants have shown that *C. jamacaru* has reproducible *in vitro* antischistosomal activity (Yousif, Hifnawy, Soliman, Boulos, Labib, Mahmoud, Ramzy, Yousif, Hassan, Mahmoud, El-Hallouty, El-Gendy, Gohar, El-Manawaty, Fayyad & El-Menshawi 2007). *C. jamacaru* is not indigenous in South Africa, where it is a declared weed and is classified in a group of plants that are very harmful to the country. It is an invasive alien plant, forming impenetrable thickets or barriers that prevent access to streams, pastures, shade trees or plantations (Henderson 2001). According to Klein (2006), in South Africa, Queen of the Night infestations are limited mainly to the warmer parts of Gauteng, North West, Mpumalanga and Limpopo Provinces, where they especially occur in the bushveld to the north

of the Magalies Mountains. One of the worst infestations is at Kameelpoort, north-east of Pretoria, where Queen of the Night has infested some 3 000 ha of land. Smaller infestations occur in the vicinity of Soutpan, Warmbaths, Pienaars River, Hammanskraal, Pretoria North, Rust de Winter, Groblersdal, Thabazimbi, Rustenburg, Brits, Krugersdorp and the Cradle of Humankind area. It is, however, found as a cultivated ornamental in many South African gardens where, especially in the warmer areas, it could be a potential source of infestation. It is sometimes planted as security hedges.

However, a commercial farmer between Pretoria and Mantsole apparently derives benefits from this weed by using it for nematode control in his livestock, after he had observed that kudu apparently auto-medicate themselves (Bosch 2007).

2.4. Antimicrobial resistance

According to the International Food Safety Authorities Network (INFOSAN 2008), antimicrobial agents are drugs of great concern to human and animal clinical medicine as well as for animal welfare. To a large extent, the same classes of antimicrobials are used in animals and humans. When added to feed and water, antimicrobials can promote growth and increase feed efficiency. This long-term prophylactic use at a low-dose has resulted in the development of antimicrobial resistance in animal production.

The evolution of resistance to microbes is one of the most significant problems in modern medicine, posing serious threats to human and animal health. Since their discovery during the 20th century, antibiotics and related medicinal drugs have substantially reduced the threat posed by infectious diseases. Over the years, antimicrobials have helped to bring many serious infectious diseases under control. These drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century. These gains are now seriously in danger by another recent development: the emergence and spread of microbes that are resistant to cheap and effective first-choice, or "first-line" drugs. The bacterial infections which contribute most to human disease are also those in which emerging

anti-microbial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections, and hospital-acquired infections.

Some important examples include penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, multi-resistant salmonellae, and multi-resistant *Mycobacterium tuberculosis*. Resistance development is not confined to bacteria and fungi but occurs in viruses, protozoa and helminths (Soulsby 2008). In many of these, the mechanism of resistance is unknown, and hence their control is still in question. The development of resistance to drugs commonly used to treat malaria is of particular concern, as is the emerging resistance to anti-HIV drugs. When antimicrobials are used incorrectly (for too short a time, at too low a dose, at inadequate potency or for the wrong disease) the likelihood that bacteria and other microbes will adapt and replicate rather than be killed is greatly enhanced (WHO 2002).

2.5. Antifungal agents

Fungi are eukaryotic microorganisms, which are heterotrophic and essentially aerobic with limited anaerobic abilities. They possess chitinous cell walls, plasma membranes containing ergosterol, 80SrRNA and microtubules composed of tubulin. Fungi can grow as yeasts (*Candida albicans, Cryptococcus neoformans*), moulds or filamentous fungi (*Aspergillus fumigatus*) or a combination of both. Persons suffering from AIDS, cancer and those receiving organ transplants are particularly vulnerable to aspergillosis. Among the most dangerous fungal pathogens are *Mucor* and *Aspergillus* species (Charvalos, Tzatzarakis, Van Bambeke, Tulkens, Tsatsakis, Tzanakakis & Mingeot-Leclercq 2006). Immunoincompetence is the primary predisposing factor in *Aspergillus* infections in humans.

Aspergillus infections present as pulmonary complications in immunocompromised patients, often resulting in a necrotizing pneumonia, with widespread dissemination to other organs. Moulds (filamentous fungi) can infect dairy cattle causing a disease referred to as mycosis mostly during

stressful conditions. Mycotic infections in dairy cattle are mainly caused by *A. fumigatus* and the clinical signs correlate closely with Hemorrhagic Bowel Syndrome (HBS) which impairs milk production, infects the ruminant gut at various sites and causes enteric haemorrhage (Wang & Forsberg 2003).

2.6. Antioxidants

The term "antioxidant" refers to the activity demonstrated by numerous vitamins, minerals, and other bioactive chemicals found in plants to provide protection against the damaging effects of highly reactive molecules known as free radicals. During healthy metabolism, carbohydrates and sugars are metabolized in the presence of oxygen to supply the animal with energy. Most of the raw materials involved in metabolism are converted to energy. In all energy reactions in the body, however, there are some molecular fragments that aren't totally used up. Some of these chemically active fragments have an electrical charge due to an abnormal or deficient amount of electrons. These charged molecules are named free radicals. Free radicals are highly unstable because they have one or more unpaired electrons. They scavenge the animal's body to grab or donate electrons, thereby damaging cells, proteins, and DNA itself. Under normal circumstances, reactive oxygen species (ROS) and free radicals are seriously eliminated by antioxidant protection systems such as antioxidant enzymes and non-enzymatic factors. Nevertheless, under pathological conditions, the balance between production and elimination of ROS is split apart. As a result, biomacromolecules including DNA, membrane lipids and proteins are damaged by ROS-mediated oxidative stress (Je, Park, Yoon, Kim & Ahn 2009). Free radical reactions and oxidative damage have been associated with many of the diseases of aging such as arthritis and cancer. This same oxidative process causes oils to become rancid, peeled fruits to brown, and iron to rust.

Animals who are already stressed, aging animals with weak organ systems, and sick animals may need some help to eliminate free radicals. External sources of antioxidants are thought to be a useful antidote for an excess of free radicals.

According to Arizona (2006) and Hollman (2001), phenolic compounds are to a great extent distributed in the plant kingdom and plants produce a great variety of organic compounds that are not directly included in the primary metabolic processes of growth and development. They produce a wide range of differing biological effects, such as antioxidant, anti-inflammatory, anti-allergic and anti-carcinogenic activities. The observed result of Wang, Zhang, Duan & Li (2009) suggest that phenolic compounds might be major contributors to the antioxidative activities of plant compounds.

Although there are negatives surrounding Queen of the Night cactus, there are many positives, such as its ability to be utilized as a source of food for livestock (Gimre 2001) and its therapeutic attributes (Sousa 2001; Albuquerque *et al*. 2007; Socha 2003). The aim of this work is the evaluation of the anthelmintic, antimicrobial, antifungal and antioxidant properties of C. *jamacaru*, and the chemical investigation of the plant material. The *in vitro* and *in vivo* effects will be tested to determine its effectiveness as an anthelmintic against *H. contortus* and *T. colubriformis*. Hence, the current study aims at using *C. jamacaru* as a possible control measure against worm infection in livestock.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant collection, processing and chemical extraction

3.1.1. Plant collection and processing

Voucher specimens were identified and deposited in the H.G.W.J. Schweickerdt Herbarium (PRU number: 096502) in Pretoria. The specimen used for the fingerprint was collected in the Johannesburg area. Thorns and ribs were removed from the stem of the cactus. Then the plant material was sliced, dried at 24ºC in a drying machine (locally constructed by Eloff; Fig.3-1.) for three days before being ground in a Macsalab mill (Model 200 Lab, Eriez®, Bramley, RSA). The *C. jamacaru* powder was stored in the dark in closed glass bottles and extracts thereof were analyzed for anti-oxidant, antibacterial, antifungal and anthelmintic activities. For the anthelmintic efficacy tests, plant material (ribs) was collected in the morning between September and November 2008 from Boschveld Farm located in the Pienaars River area in Limpopo Province.

Fig. 3.1. Drying machine drying plant material in an air stream with temperature control (locally constructed by Eloff).

3.1.2. Plant material extraction

The dried powder of *C. jamacaru* (368.91 g) was dissolved in 4 l of acetone and placed in a shaking machine (Labotec®, Model 202, South Africa) for 30 minutes. The solution was left to settle and the supernatant was filtered out with a Whatman® (# 1) filter paper into a clean marked container. This process was repeated three times and the filtered solution was combined. The solvent was removed under a stream of air in a fume cupboard at room temperature overnight. The mass of the plant material extract was 22.13 g.

The solvent-solvent extraction method developed by the National Cancer Institute (Suffness & Douros 1979) was used for partial purification of the crude plant material according to polarity. An amount of 4.91 g of crude acetone extract was used for the solvent-solvent extraction.

- Equal parts of water and chloroform (50 ml of each) were added to the acetone extract in a round bottomed flask and after mixing well, the

contents were poured into a separation funnel. The components were separated with the water fraction on top and the chloroform fraction at the bottom.

- The water fraction was then separated further by adding an equal volume of butanol to the water fraction in a separation funnel to yield the water and butanol fractions.
- The chloroform fraction was dried in a rotary evaporator below 40˚C with a pre-weighed round bottom flask under reduced pressure and increased temperature and then dissolved in a 1:1 mixture of hexane and 10% water in methanol. The hexane fraction and the 10% water and methanol fractions were collected.
- The 10% water in methanol fraction was further diluted to a 35% water in methanol solution. Chloroform was added to the 35% water in methanol solution. This was partitioned in a separation funnel to obtain chloroform and 35% water in methanol fractions.
- The five fractions (hexane, chloroform, butanol, water and 35% water in methanol) were individually collected in pre-weighed containers and dried under a fan overnight. The masses of the respective fractions were determined (Fig. 3-2).

The carbon tetrachloride step was skipped because of toxicity concerns. There was insufficient material removed in the butanol extract and it was therefore excluded for the larval development and viability assay.

Fig. 3-2. Illustration of fractionation. Solvent-solvent extraction of plant extracts developed by the National Cancer Institute (Suffness & Douros 1979).

Step skipped because of toxicity concerns.

3.2. Anthelmintic experiments

3.2.1. In vitro experiments

3.2.1.1. Preparation of donor sheep

The study was conducted with the permission of the Onderstepoort Veterinary Institute (OVI) Animal Ethics Committee (Project: C-Kandu-UP) and the

University of Pretoria Animal Research Committee (Protocol V054/08) and in line with the guidelines of these committees. The on-station study itself was conducted at OVI. Twenty two adult castrated male sheep were used. Fourteen of these sheep were used to titrate the correct dose of *Ehrlichia ruminantium* for a vaccine challenge experiment in June 2008, but had fully recovered before use in the present experiment. The remaining 8 animals had not been used in any animal experiments. Eighteen animals were used for the trial while 4 served as donors.

On arrival at OVI approximately 12 months before the start of the present study, the animals were dewormed twice with 200 µg/kg moxidectin (Cydectin Injectable, Fort Dodge, South Africa) at an interval of 24-hours. The sheep were individually ear-tagged and were maintained in an insect-free facility on concrete floors that were swept clean daily, until the animals were transferred to the facilities for the present experiment. All the sheep were housed in a common pen with concrete floors which were swept clean of faeces on a daily basis to prevent any accidental nematode infection. A commercial pelleted feed, lucerne hay and free access to water were provided.

At the time of infection, the donor sheep were transferred to their own separate walled pens, one pen for the two *Haemonchus* donors and a separate pen for the two *Trichostrongylus* donors. The sheep were checked for nematode infection by means of the McMaster technique and a sensitive egg flotation technique (see section 3.2.3 Parasitological and other experimental procedures) and were found to be negative on both tests.

3.2.1.2. Infection of animals

Mono-specific larval suspensions of *H. contortus* and *T. colubriformis* were obtained from two different sources:

H. contortus:

A freshly harvested batch of larvae were obtained from Biozetica Agri-Source (Pty) Ltd.

References: *H. contortus* ex F. van Schalkwyk.
Biozetica Agri-source (Pty) Ltd. *Haemonchus contortus* (OP)
Strain no: BZ/OP/2/P2/HcOP
Donor no: 229

T. colubriformis:

The larvae were obtained from cryopreserved infective larval (L_3) stocks of OVI and defrosted according to standard procedures.
References: Trich. 6686/4. 36000L3. 89/11/22

For both species, the concentration of larvae was estimated by counting aliquots of 1 ml of larval suspension under a stereomicroscope. The concentration was adjusted by adding clean tap water to a large aliquot of the original concentrated larval suspension and checking aliquots of the diluted suspension. Larvae remaining from the day's infection were retained, their motility checked on the following morning and additional larvae added from the original concentrated larval suspension to make up the required numbers.

A total dose of 4000 infective larvae of *H. contortus* was given in 3 divided doses over a period of three days. On the first day 3 ml of larval suspension containing 1395 motile L_3 larvae was administered at the back of the animal's tongue. The larval suspension was well stirred by swirling. An amount of water equivalent to the amount of larval suspension was drawn up in the same syringe and administered immediately thereafter (Vatta *et al*. 2009). This was followed by the administration of 1233 and 1239 motile L_3 on the second and the third day respectively. The motility of larvae was checked after administration and estimated to be >90%, 96% and 97% on the three days, respectively.

For *T. colubriformis*, a total dose of 5968 larvae was administered to the sheep in 3 divided doses over a period of 3 days. The larvae were administered as for *H. contortus*. The motility of the larvae was checked after

administration and estimated to be >90%, 82% and 77% on the three days, respectively.

3.2.1.3. Egg recovery and preparation

Procedures for the recovery and preparation of strongyle nematode eggs for use in the egg hatch assay were based on the methods for the detection of anthelmintic resistance in nematodes of veterinary importance (Hubert & Kerbeuf 1992). When the infections had become patent, fresh faecal samples were collected from the sheep that had previously been infected with *H. contortus*. The faecal pellets were mashed and the slurry was then washed through a series of sieves with meshes of decreasing aperture size (150 µm, 63 µm and 25 µm).

Using a wash bottle a magnesium sulphate (density 1:10) solution was used to backwash the material of the sieve for transfer into centrifuge tubes. The tubes were centrifuged at 1 000 rpm for 5 minutes. The supernatant was decanted on to a 25 µm sieve. The eggs retained on the sieve were transferred to a 50 ml tube. The concentration of eggs in the suspension was determined by counting the number of eggs in 200 µl of egg suspension and the concentration was adjusted to give a final concentration of 500 to 600 eggs per ml.

3.2.1.4. Egg hatch assay

The *in vitro* egg hatching test was based on the method described by Coles, Bauer, Borgsteede, Geerts, Klei, Taylor & Waller (1992). An amount of 0.2 ml of egg suspension was distributed in a 48-well-flat-bottomed microtitre plate (Cellstar, Germany) to give approximately 100 to 120 fresh eggs per well and mixed
with the same volume of plant extract at concentrations of 10 mg/ml in 8 serial dilutions for the acetone extract and 1 mg/ml in 8 serial dilutions for the 5 fractions.

Albendazole (99.8% pure reference standard, Sigma, USA) was used as a positive control. Albendazole was dissolved in 0.1% dimethyl-sulphoxide

(DMSO) and diluted at concentrations of between 5 µg/ml and 0.04 µg/ml. Preliminary investigations into the maximum concentration of DMSO (0.1%) used to dilute the albendazole and the acetone used to dilute the plant extracts indicated that these solvents had no significant effect on the hatching of eggs of *H. contortus*.

The eggs were incubated in this mixture for 48 h at 27^{o}C and 70% humidity. After this time had lapsed, a drop of Lugol's iodine was added to each well. The hatched larvae and unhatched eggs were then counted using an inverted microscope under 40x magnification. The experiment was replicated three times for each concentration on the same plate.

3.2.1.5. Larval development and viability assay

The nutritive medium was as described by Hubert & Kerbeuf (1992) and composed of Earle's balanced salt solution plus yeast extract (Sigma-Aldrich, USA) diluted in saline solution (1 g of yeast extract per 90 ml of saline solution). The test was carried out in a 48-well microtitre plate (Cellstar, Germany). Thirty microlitres of nutritive medium was added to 150 µl of nematode egg suspension containing approximately 100 eggs per well. Amphotericin B (Sigma–Aldrich, Germany) at a concentration of 5 µg/ml was included in the egg suspension to inhibit fungal growth. Twenty microlitres of faecal filtrate obtained from the first filtration of the faeces with the 25 µm sieve while recovering the nematode eggs was added to provide bacteria for the larvae.

The plates were covered and put in an incubator at 27^{0}C and 70% relative humidity for 48 hours. By then the parasites would have developed to 1st stage larvae. A stock solution of the plant extract (10 mg/ml for the acetone extract and 1 mg/ml for the five fractions) were reconstituted with the acetone and serially diluted 1:2 with water to give 8 concentrations. A stock solution of albendazole (5 µg/ml) was also prepared and serially diluted with 0.1% (DMSO) to give 8 concentrations. Aliquots (200 µl) of each extract concentration, albendazole, DMSO or acetone (controls) were transferred into the wells. The third stage larvae were obtained seven days later. At this time

the parasites were counted with an inverted microscope (X40) by separating the larvae into two classes, living third stage larvae and dead larvae.

3.2.1.6. Determination of the relative bioactivity of fractions

The egg hatch and larval development test activities were determined from the percentage of egg hatch and larval development. The relative bioactivities of the fractions were further assessed by comparing the percentage values of the various fractions by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests, which was performed using GraphPad Prism version 4.01 for Windows, San Diego, USA.

3.2.2. *In vivo* experiments

3.2.2.1. Preparation of sheep

The experimental animals were adapted to the housing conditions beginning 8 weeks before the first treatment (from week -8). All the sheep were observed for any signs of ill health at least twice daily, in the morning and the afternoon. During this adaptation period, one animal developed urolithiasis and was managed surgically through partial penile amputation. At that stage, the animal was maintained in a separate pen but was fed the same diet as the main group of sheep.

The faecal nematode egg counts (FECs) of the animals were checked during week -8 and 1 sheep had an FEC of 33 eggs per gram of faeces (EPG) while the remainder had negative counts. Three of the sheep were positive on the egg flotation technique, but were negative on FEC, indicating a very low grade infection. Given the low FECs of these animals, no further anthelmintic treatments were administered. The sheep were artificially infected with larvae of *H. contortus* and *T. colubriformis* at the same time and in the same manner as the donor animals (week -6). During weeks -5 to week -1, the FECs of the animals were monitored twice a week and the infections were allowed to develop to patency.

When the infections were fully patent, the animals were grouped into control (C), single dose (S) and double dose (D) groups. The groups were balanced for FECs and live weights based on their FECs and live weights for the faecal samples examined in week -1. This was done as follows. Sheep with similar FECs and live weights were grouped into clusters of three. Thereafter, the three sheep in each cluster were randomly allocated to an experimental group: control, single dose or double dose.

3.2.2.2. Plant collection and preparation

On the day before administration of the plant extract, fresh material was harvested from the farm of the commercial farmer that claimed efficacy of the plant. Thorns were removed from the plant material and the material was kept at room temperature. From the material collected at the farm, a 25 cm piece was cut off. The hard centre core was removed from the cactus before it was weighed. The first 25-cm piece of cactus that was administered weighed 808.19 g so that the weight of each subsequent piece of cactus used was adjusted to make it exactly equal to 808.19 g. The 808.19 g of cactus were blended with a small volume of water to make up the volume of cactus-water suspension to an amount that was easy to work with.

The dosage used in the study was in line with that used by the farmer. One dose of the cactus was, therefore, the final volume of this suspension divided by 25. This is explained as follows. The farmer claimed fed a length of 1 m of cactus per 100 sheep. He would remove the thorns and shred the pulp with a penknife into a trough for the sheep to consume. By extrapolation, therefore, one dose corresponded to 1 cm of the plant stem and a double dose corresponded to 2 cm. The total quantity required should have been 18 cm of cactus (1 cm x 6 for single dose, plus 2 cm x 6 for double dose) but we used more to prevent any shortage. A 1-cm piece weighed 32.33 g (808.19 g ÷ 25 = 32.33 g) after the centre core was removed.

3.2.2.3. Treatment and follow up procedures

The blended cactus was given to the animals using a 50 ml syringe. The pasty material was deposited at the back of the tongue of the sheep. The animals of

groups S and D were treated with a single dose (32;33 g) or double dose (64;66 g), respectively, of the test substance. After dosing, a volume of 50 ml of water was carefully administered by syringe into the month of the sheep to ensure swallowing of the full dose. The first treatment was given on day 0 in week 1 and repeated on days 7, 14, 21, 28, 35 and 42 after the first treatment. Animals in the control group received a volume of water equivalent to 1 dose of the test substance. Observations of the sheep for any adverse reactions to the cactus were made at hourly intervals for 4 hours after each administration.

The live weights of each animal were taken with a scale (Ruddweigh 500, Ruddweigh, Australia) once a week at the same time of the day, on the day that the plant extract was administered. The body condition score, packed cell volume and FAMACHA© score was also taken for each sheep once a week on the day of treatment. The FAMACHA© score was determined by the same technician. Faecal egg counts were carried out on the day of treatment and three days thereafter using the Visser Filter Method, from day 0 to day 49. Faecal samples (10–15g per sheep) were collected directly from the rectum of each animal with a lubricated gloved hand, placed in a cooler box with freezer packs and stored in a refrigerator until processed later in the day. To reduce the variation inherent in egg counts, samples were collected at the same time each morning. The faeces remaining after the egg counts had been done were pooled by experimental groups and faecal cultures for recovery of third-stage nematode larvae were prepared, from day 15 onwards.

3.2.3. Parasitological and other experimental procedures

3.2.3.1 Faecal egg counts

Faecal egg counts were carried out according to the Visser Filter Method (Van Schalkwyk, Schröder, Malan & Van Wyk 1995). Briefly, 4g of faeces were washed through a series of three sieves (110 µm, 70 µm and 25 µm) where nematode eggs were retained in the 25 µm sieve. The eggs suspended in *c*. 15 ml water were then drained into calibrated containers. Granulated sugar (15-20 g) was added to the egg suspension and this is made up to a volume

of 60 ml with water. Nematode eggs are counted in the 3 chambers of a 3-chambered McMaster slide. The FEC is then calculated according to the following formula:

$$\text{EPG} = \frac{\text{Number of eggs counted } x\ 100}{3}$$

3.2.3.2. Egg flotation technique

An egg flotation technique modified from Reinecke (1983) was used. Five grams of faeces were sieved through the Visser Filter Method sieves described above. The egg suspension in the outer sieve was released into a 150 ml flat-sided glass medicine bottle, which was then filled with a 40% saturated sucrose solution to form a positive meniscus on the top of the bottle. Care was taken not to allow any air into the bottle when the cap was screwed on. The bottle was left lying on its side for 20 minutes to allow any nematode eggs in the sample to attach to the uppermost side of the bottle. The sucrose solution was then carefully drained. A small amount of water was added to the bottle using a wash bottle, taking care not to splash water against the uppermost surface of the bottle but simply to suspend sediment remaining on the bottom of the bottle. This was drained off as well.

About 5 ml of 40% saturated sugar solution was then added to the bottle, the bottle cap was screwed on and the bottle was shaken well. The solution was poured into a 15 ml test tube. The test tube was filled with a 40% saturated sucrose solution until a positive meniscus was formed. A glass microscope slide was placed over the top of the tube test, and this was left to stand for 20 minutes. The slide was then removed, turned so that the wet side was uppermost and a cover slip was placed on the slide. The slide was examined for the presence of strongyle eggs with the aid of a compound microscope. Where more than one sample was processed at the same time, the tip of the

suction pump was cleaned between samples by sucking up a small amount of clean water out of a jar.

3.2.3.3. Larval cultures

Larval cultures were prepared according to Reinecke (1983). Faeces were collected by hanging a faecal collection bag on the appropriate donor animal over-night. The faecal pellets were then broken up in a large plastic tray and mixed with a similar volume of vermiculite, to improve the aeration of the culture and to facilitate maximum hatching of the eggs. A 2 cm thick wooden stick was placed upright in the centre of a 1 ℓ glass fruit jar and faeces were slowly added to the jar and pressed down with a second stick until the layer of faeces was 5 to 7 cm thick. The centre hole is used to improve aeration. The outside of the jar was thoroughly cleaned to avoid contamination.

By using a water bottle, the inside of the jar as well as the compacted faecal mixture were moistened, without soaking the contents. The lid was then lightly screwed on to the bottle and incubated at 27°C for 5 to 8 days. The culture was harvested by rinsing the sides of the culture jar into a 100 ml glass bottle, while holding the bottle at a slant. The larvae were identified according to the key of Van Wyk, Cabaret and Michael (2004).

3.2.3.4. Packed cell volume (PCV)

The procedure described by Hansen and Perry (1994) was used to estimate the packed cell volume. A jugular venous blood sample (about 3 to 5 ml) was taken from each animal, using a Vacutainer needle and tube (Vacutainer systems, Becton Dickinson, UK) containing EDTA as anticoagulant. After each blood sample had been taken, it was gently mixed. In the lab, the blood was again gently mixed and a 75 mm X 1.5 mm capillary tube was filled up to ¾ of its length and one end of it sealed with sealant. Then the blood filled tubes were centrifuged at 12,000 rpm for 5 min using a microhaematocrit centrifuge (Unico, C-MH30, NJ, USA). Finally, each tube was placed in a micro-haematocrit reader, to determine the packed red cell volume expressed as a percentage for each animal.

3.2.3.5. FAMACHA© score

To determine the FAMACHA© score, the colour of the mucous membrane of the lower eyelid of each animal was examined in good natural light and compared to the colours of the FAMACHA© chart (Van Wyk & Bath 2002). Each sheep was scored on a scale of 1–5 (1 = red, non-anaemic; 2 = red-pink, non-anaemic; 3 = pink, mildly anaemic; 4 = pink-white, anaemic; 5 = white, severely anaemic).

3.2.3.6. Body condition score

The body condition score is an estimation of fat and muscle condition over both the spinous processes and transverse processes of the spine of the loin which are felt and used to assess the body condition. It is a subjective score. The exact score is not as important as the relative scores and differences between scores. The system which was used in this work uses a scale of 1 to 5, with 1 being an emaciated sheep, 3 being a sheep in average condition, and 5 being an obese sheep. Scores of a 0.5 units were also used. On average, a condition score of 1 is equivalent to about 13 percent of the live weight of a sheep and a score of 3 to 5 equates to a moderate condition (Russel 1984).

3.3. Phytochemical analysis.

The primary objective of this study was to evaluate the potential use of *C. jamacaru* to control worm infection in livestock, but because of the increasing interest in the antioxidant, antibacterial and antifungal activities in plants, these activities were also investigated in the present plant..

The plant material extracted with acetone was separated by thin layer chromatography (TLC) on silica gel TLC plates (Merck® Germany). Extracts were spotted on to the TLC using 10 µl of a 100 mg extract/ml solution. After development of chromatograms with BEA (benzene : ethanol : ammonia) [18:2:0.2], CEF (chloroform : ethyl acetate : formic acid) [5:4:1] and EMW (ethyl acetate : methanol : water) [10:1.35:1], they were air-dried for 30 minutes and placed in tanks saturated with the eluent.

3.3.1. Antioxidant activity

Due to the presence of different antioxidant components in the crude extracts of biological tissue samples, it is relatively difficult to measure each antioxidant component separately. Therefore, several assay methods have been developed and applied to screen and evaluate the total antioxidant activity of extracts. In the present work, the qualitative diphenyl-picrylhydrazyl (DPPH) radical scavenging and vanillin-sulphuric acid assays and the quantitative Trolox equivalent anti-oxidant capacity (TEAC) assay were used to determine the antioxidant activity of *C. jamacaru* extracts.

3.3.1.1. DPPH and *vanillin-sulphuric acid assays*

The DPPH assay is a qualitative indicator of free radical scavenging activity based on the reduction of DPPH, a stable free radical. The DPPH is reduced from a stable free radical that is purple in colour to diphenylpicryl hydrazine that is yellow, in the presence of an anti-oxidant (Fig 3-3). The visual colour change is observed on the chromatograms. This technique shows the number of antioxidant compounds separated by TLC and also gives an indication of the polarity of the separated compounds.

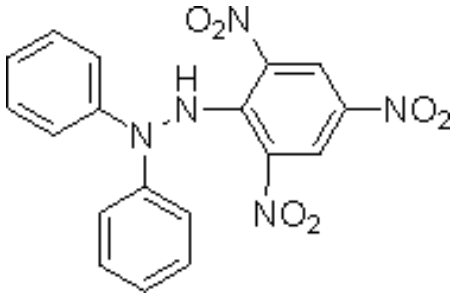


Fig. 3-3. Reaction of DPPH with hydroxyl groups of free radical (R-OH) to produce 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine and R-NO_2, 2-(4 nitrophenyl)-2 phenyl-1-picrylhydrazine.

In an attempt to characterize the antioxidant activity of *C. jamacaru* the TLC plates were eluted in EMW, BEA and CEF. After development the chromatograms were investigated under ultraviolet light (254 and 350 nm, Camag.Universal UV lamp TL-900/u) where fluorescent bands were marked with a solid pencil. Chromatograms developed with BEA and sprayed with 0.2% DPPH-radical had more bands with a visual colour change after 1 hour (Figure 5-1). For further compound identification, the TLC chromatograms were sprayed with vanillin-sulphuric acid (0.2 g of vanillin dissolved in 56 ml of methanol; 3 ml of sulphuric acid were carefully added). The plates were heated for *c*. 3 minutes at 110°C for optimal colour development (Figure 5-2).

3.3.1.2. Trolox equivalent anti-oxidant capacity (TEAC)

In this assay the ABTS [2,2–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] is oxidized to the coloured nitrogen centred radical cation $ABTS^+$ in a sulphate system as reported by Re, Pellegrini, Proteggente, Pannala, Yang & Rice-

Evans (1999). In the presence of an antioxidant the $ABTS^+$ radical changes from blue/green to colourless depending on the degree of the reaction. The extent of the discolouration as measured by spectrophotometry is expressed as the percentage of inhibition of the $ABTS^+$ radical. The result was compared to the percentage inhibition of the standard Trolox, a synthetic water soluble vitamin E analogue.

The ABTS stock solution was prepared by adding 50 ml of sterile water to 192 mg of ABTS to make up a 7 mM stock solution. The ABTS solution was mixed with 33 mg of potassium sulphate and was kept in the dark for 16 hours at room temperature prior to use. The procedure was adopted for a 96 well microtitre plate. The *C. jamacaru* extract and various fractions were made up in methanol, to a concentration of 0.5, 0.25, 0.125, 0.0625 mg/ml and the Trolox was made up to a concentration of 0.5, 0.25 and 0.125 mg/ml in methanol. The pre-formed $ABTS^+$ radical was diluted in methanol to an initial absorbance (A_i) of 0.70-0.9(±0.02), at a wavelength of 734nm (Versa Max microplate reader, Molecular devices-USA, blanked with methanol). Then the plant extract/Trolox (40µl) was mixed with the $ABTS^+$ radical (160µl) and the mixture was allowed to react for 6 minutes and absorbance (A) read at 734 nm.

3.3.2. Antibacterial assay

3.3.2.1. Experimental design for microbial assay

For the antibacterial and antifungal assays, extracts were tested using the following experimental design:

- Group 1 or test group: contained organisms plus different concentrations of extract (used to determine if the extract was effective).
- Group 2 or positive control: contained organisms plus a known antibiotic (was used to ensure that the organisms utilized were susceptible to common chemotherapeutics and were not resistant strains).

- Group 3 or negative control: contained organisms plus the pure extraction solvent (acetone and water). This was used to confirm that the extraction solvent had no inhibitory action of its own.

3.3.2.2. Evaluation of antibacterial activity

In the quantification of the antibacterial activity of the extracts, the microplate method was used (Eloff 1998). Four organisms were grown overnight in Muller-Hinton broth (Merck Chemicals, USA) at 37°C. *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29219) were used as representative Gram-positive organisms. *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as representative Gram-negative organisms. The test solution (25-100 µl) was serially diluted 50% with water and 25-100 µl of 3-hour old culture of one of the bacteria. After proper mixing 100 µl of the mixture was removed from well A and added to well B.

The process was repeated until well H was reached (Figure 3-4). This brought the final volume in each well to 100 µl (after discarding 100 µl from the last well). The microplates were incubated overnight at 37˚C prior to the addition of 40 µl of 0.2 mg/ml of p-iodonitrotetrazolium violet (INT Sigma®, Germany) solution in each well which aims to indicate bacterial growth.

Growth was detected by a red colour change and read visually. The MIC was determined as the lowest concentration at which growth was inhibited. The antibiotic gentamycin freshly made up to a stock solution of 500 µg/ml was used as the positive control (group 2); acetone and water were included as the negative control (group 3).

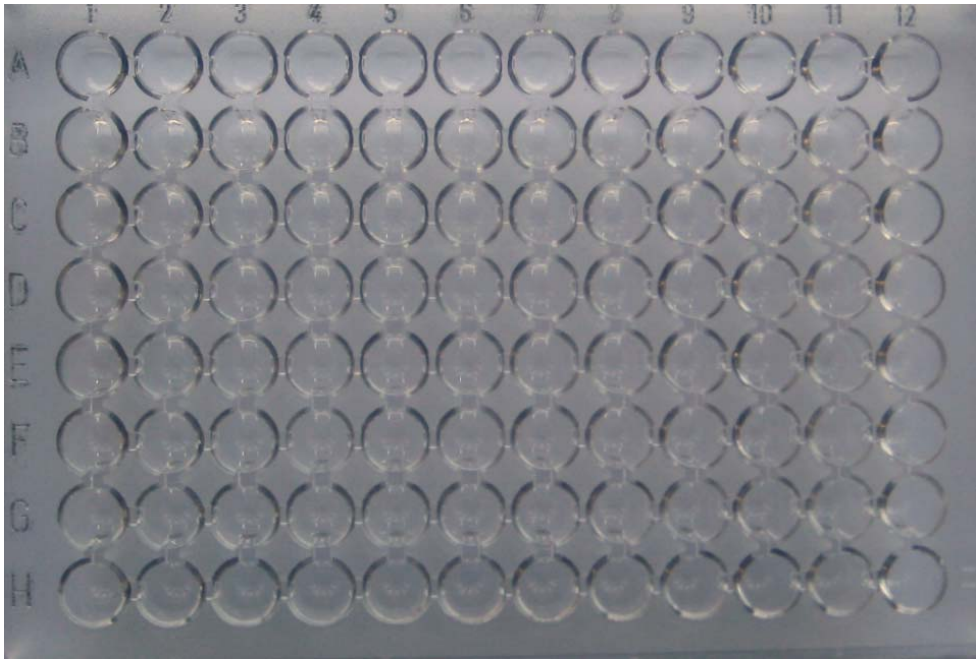


Fig. 3-4. Illustration of the microtitre plate made up of 12 columns (1-12) and 8 rows (A-H).

There are no validated criteria for the MIC end points for the *in vitro* assay of plant extracts but the following classification proposed by Aligiannis, Kalpotzakis, Mitaku & Chinou (2001) for essential oils is an efficient tool which was used in this study to classify the antibacterial activity.
In the Phytomedicine Programme we agree with the criteria of the highly regarded journal Phytomedicine's instructions to authors that only MICs of less than 0.1 mg/ml should be considered significant.

The following classification proposed by Aligiannis *et al*. (2001) was used in this study to classify the antibacterial activity:
≤ 0.5 mg/ml = strong inhibitors;
between 0.6 mg/ml and 1.5 mg/ml = moderate inhibitors and
≥ 1.6 mg/ml = weak inhibitors.

3.3.3. Antifungal assay

Measuring the antifungal activities was done by using the microplate method (Eloff 1998) as modified by Masoko, Picard & Eloff (2005). A serial microdilution assay with INT added as growth indicator (Eloff 1998) was used

to determine the minimum inhibitory concentration (MIC) values for the plant extracts. Residues of the plant extract were dissolved in acetone to a concentration of 10 mg/ml. The extract (100 µl) was serially diluted 50% with water in 96 well microtitre plates (Eloff 1998).

Fungal cultures (*Candida albicans*, *Aspergilus fumigatus* and *Cryptococcus neoformans*) were transferred from Sabouraud dextrose (SD) agar plates into fresh Sabouraud dextrose (SD) broth and 100 µl of this mixture were added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included.

As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in water were added to each of the micro plate wells. The plates were sealed in plastic to minimize infection of the environment with fungal spores and incubated for 2 days at 35ºC and 100% relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth after 24 and 48 hours.

3.4. Statistical analysis

The statistical analyses for the *in vivo* experiments were performed using GenStat® (Payne, Murray, Harding, Baird & Soutar 2007). Analyses of the three groups (double treated, single treated and control) for body condition score, PCV, FAMACHA© score, weight, weight gain and FEC were conducted using a one-way analysis of variance (ANOVA). All groups were compared per date starting from the date of treatment (day 0) until day 49 post-treatment. Arithmetic
means were used for all the parameters analyzed, except FEC that were $\log_{10}$ transformed to normalize the group variances.

Group comparison was performed using Fisher's t-test protected pair-wise least significant difference (LSD) per date when the overall F test of the ANOVA was significant at the 5% significance level. In the case of non-

significance of the F test for the ANOVA, pair-wise comparison did not proceed. All tests were performed two-sided.

The percentage of faecal egg count reduction (FECR) was calculated using the formula of Coles *et al.* (1992) and the arithmetic averages:

$$\text{FECR}\,(\%) = \left\{1 - \left|\frac{T_2}{C_2}\right|\right\} \text{x } 100$$

Where C_2 expresses the mean of the FEC for the control sheep and T_2 expresses the mean of the FEC for the sheep treated either with a double or a single dose of the plant material.

To calculate the percentage of egg hatch inhibition, the following formula was used:

$$\text{Egg hatch inhibition } \% = \frac{\text{Number of eggs}}{\text{Number of eggs} + \text{larvae}} \text{x } 100$$

To calculate the percentage of larval development inhibition, the following formula was used:

$$\text{Larval development inhibition } \% = \frac{\text{Number of dead larvae}}{\text{Number of (dead} + \text{living) larvae}} \text{x } 100$$

Chapter 4

Results and discussion of anthelmintic experiments

4.1. *In vitro* anthelmintic assays

4.1.1. Effect of crude extract and solvent-solvent derived fractions on egg hatching

The results of the larval cultures for third-stage nematode larvae from the donor animals are given in Table 4-1. These cultures indicate some contamination of both the *Haemonchus* and *Trichostrongylus* donor sheep with *Trichostrongylus* and *Haemonchus*, respectively. The reasons for this are unclear. For the actual *in vitro* assays, faeces from one of the two donor sheep were used and the percentage *Haemonchus* on larval culture was 77 or 100% and the percentage *Trichostrongylus* was 0 or 23%, based on two cultures made specifically from this animal.

The positive control, albendazole, inhibited 97.7% of eggs from hatching at 5.0 µg/ml, confirming the susceptibility to benzimidazoles of the strain of *H. contortus* used in the current study (Table 4-2; Annex: Table 1). The effect of the hexane and water fractions were comparable with 8.7 and 16.1 % inhibition, while chloroform, butanol and 35% water in methanol fractions were significantly ($p<0.001$) more active than the water fraction with values of 94, 100 and 91 % respectively (Table 4-3; Annex: Tables 2,3,4,5,6). The solvents used to dilute the plant extract and albendazole (0.1% DMSO and 35% acetone) inhibited egg hatchability to the same level as the control (Table 4-4; Annex Table 7). These results indicate that the compound(s) responsible for the anthelmintic activity must have an intermediate polarity because the higher non-polar hexane and highly polar water fractions were inactive.

Table 4-1. Larval culture percentages of *Haemonchus contortus* (*H. c.*) and *Trichostrongylus colubriformis* (*T. c.*) in the donor sheep.

Day	*H. contortus* donors		*T. colubriformis* donors	
	H. c.	***T. c.***	***H. c.***	***T. c.***
16/09	100	0	88	12
23/10	99	1	78	22
09/10	100	0	87	13
09/10	98	2	11	89
23/10	77	23	89	11
28/10	100	0	43	57
31/10	99	1	61	39
04/11	100	0	28	72
04/11	100	0	79	21
07/11	69	31	78	22
11/11	56	44	80	20
14/11	92	8	84	16
18/11	100	0	88	12
21/11	97	3	74	26
25/11	100	0	-	-
Average(%)	**92**	**7.5**	**69**	**31**

A dose dependent activity was observed with the acetone extract and with albendazole. The acetone extract of *C. jamacaru* significantly inhibited ($p < 0.01$) the hatching of a higher number of eggs compared to the controls (Table 4-4). The butanol, chloroform, 35% water in methanol, water and hexane fractions inhibited 100%, 93.96%, 90.57%, 16.14% and 8.76% of eggs from hatching at 1 mg/ml, respectively. This was the highest concentration tested (Table 4-3). The egg hatch assay indicated that the chloroform, butanol and 35% water in methanol fractions were significantly ($p<0.001$) more active than the water extract (Table 4-5). However, the water extract was more active than the hexane though the difference in activity was not significant ($p>0.05$) (Table 4-5).

4.1.2. Effects of C. jamacaru crude extract and solvent-solvent derived fractions on larval development and viability

At concentrations of 0.16–2.5 mg/ml the acetone extract inhibited development and killed between 83% and 94% of the larvae of *H. contortus* (Table 4-4). At all concentrations tested, the water and 35% water in methanol

fractions failed to inhibit 50% of *H. contortus* larvae from developing (Table 4-3). Despite the fact that the water fraction gave some inhibition of egg hatching and larval development in all concentrations tested, it was not as efficient as the other fractions. The effect of water and 35% water in methanol fractions are similar, while chloroform and hexane were significantly ($p<0.001$) more active than the water fraction (Table 4-5; Annex: Tables 8,9,10,11,12,13,14).

Table 4-2. Mean inhibition percentages for the egg hatch assay and larval development and viability assays for *H. contortus*, using different concentrations of albendazole

Concentration (µg/ml)	Egg hatch assay (% inhibition)	Larval development and viability
5.0	98±0.6	95±1.6
2.5	96±0.6	93±3.9
1.25	94±2.2	90±3
0.63	77±3.8	85±4
0.31	40±2.3	80±4.3
0.16	22±4.6	75±2
0.08	15±3.4	41.2±24
0.04	14±0.9	76.3±7.4
Control	11±5.3	9.5±3

Contrary to its weak activity on the egg hatch assay, the hexane extract had a strong anthelmintic effect even at a concentration of 0.16 mg/ml. Dose dependent activity was observed for the acetone extract (Table 4-4), but was less evident for the chloroform fraction and not evident for the hexane fraction (Table 4-3). Albendazole inhibited 95% of the larvae from developing at 5.0 µg/ml, providing further evidence of the susceptibility of the strain of *H. contortus* used in the current study (Table 4-2; Figs. 4-1; 4-2). The solvent

used to dilute the plant extract and albendazole (35% acetone and 0.1% DMSO) did not kill the larvae.

Table 4-3 Mean inhibition percentages for the egg hatch assay and the larval development and viabil different concentrations of fractions of an acetone extract of *C. jamacaru*

Conc. (mg/ml)	Egg hatch assay					Larval development a	
	Water	**Hexane**	**Chloroform**	**Butanol**	**35% H_2O in MeOH**	**Water**	**Hex**
1.0	16.14±13.42	8.76±8.18	93.96±5.75	100.00±0.0	90.57±11.4	30.90±2.74	84.92
0.5	12.07±4.26	4.18±1.78	13.45±2.20	6.23±2.73	29.42±6.22	27.53±5.79	83.40
0.25	21.18±0.22	3.66±1.84	10.16±2.50	4.36±3.42	5.38±3.05	24.92±4.58	85.52
0.125	11.62±3.46	4.81±3.55	5.32±1.64	13.34±2.45	2.37±1.45	24.14±6.65	88.57
0.063	5.68±3.20	5.58±1.54	12.43±1.40	8.76±2.53	8.99±4.14	23.33±3.66	89.10
0.031	3.68±2.91	2.25±0.96	5.73±2.56	6.26±0.92	5.69±1.59	19.54±0.81	86.51
0.016	4.40±0.88	6.31±2.34	3.44±2.59	3.80±2.18	0.70±0.61	19.54±1.10	86.81
0.008	1.41±1.63	1.03±1.02	1.44±0.56	0.78±0.68	1.40±0.60	20.39±4.98	83.67
Control	1.02±1.02	1.53±0,64	0.86±0.41	0.61±0.13	0.79±0.21	12.45±15.32	27.21

Table 4-4. Mean inhibition percentages and p values (compared with controls) for the egg hatch assays and larval development assay for *H. contortus*, using different concentrations (conc.) of a crude acetone extract of *C. jamacaru*

Conc. (mg/ml)	Egg hatch assay	p values	Larval development and viability assay	p values
2.5	100.00±0.00	p < 0.01	93.64±2.73	p < 0.01
1.25	79.67±2.31	p < 0.01	91.15±1.91	p < 0.01
0.63	79.01±5.82	p < 0.01	92.66±3.36	p < 0.01
0.31	69.25±2.58	p < 0.01	86.18±3.36	p < 0.01
0.16	24.46±9.58	p < 0.01	83.33±6.96	p < 0.01
0.08	14.75±3.70	p < 0.01	67.65±4.88	p < 0.01
0.04	1.40±1.63	p > 0.05	55.12±5.75	p < 0.01
0.02	0.40±0.70	p > 0.05	32.33±10.53	p < 0.01
Control	1.07±0.06		0.68±1.18	

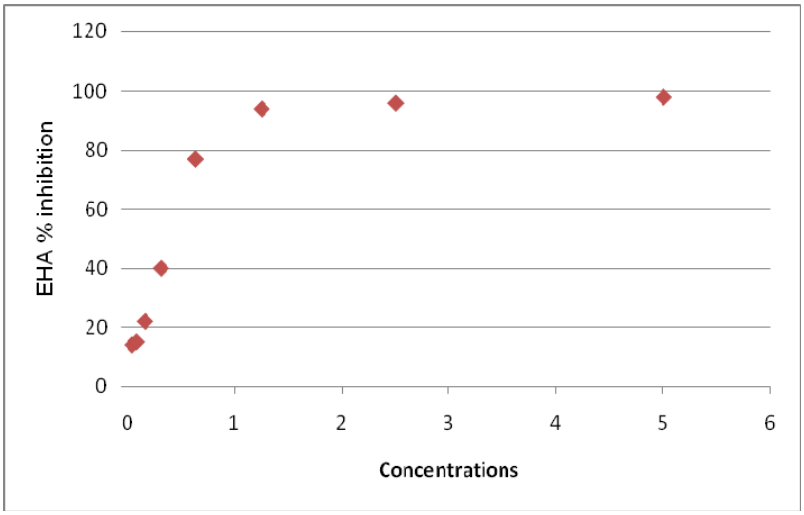


Fig. 4-1: Mean inhibition percentages for the egg hatch assay (EHA) for *H. contortus* different concentrations of albendazole

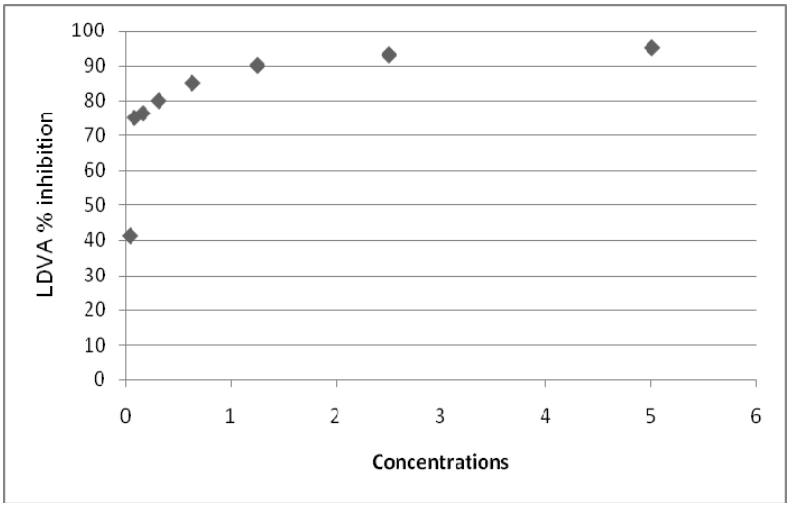


Fig. 4-2: Mean inhibition percentages for the larval development and viability assay (LDVA) for *H. contortus* at different concentrations of albendazole

Table 4-5. Tukey's multiple comparison tests comparing the percent activity values of fractions of *C. jamacaru*

Egg hatch assay		**Larval development and viability assay**	
Comparisons	**p value**	**Comparisons**	**p values**
Water vs hexane	p > 0.05	Water vs hexane	p < 0.001
Water vs chloroform	p < 0.001	Water vs chloroform	p < 0.001
Water vs butanol	p < 0.001	Water vs 35% water in methanol	p > 0.05
Water vs 35% water in methanol	p < 0.001	Hexane vs chloroform	p < 0.01
Hexane vs chloroform	p < 0.001	Hexane vs 35% water in methanol	p < 0.001
Hexane vs butanol	p < 0.001	Chloroform vs 35% water in methanol	p < 0.01
Hexane vs 35% water in methanol	p < 0.001		
Chloroform vs butanol	p > 0.05		
Chloroform vs 35% water in methanol	p > 0.05		
Butanol vs 35% water in methanol	p > 0.05		

4.2 *In vivo* anthelmintic assays

4.2.1. Live weight gain and body condition score of animals

The results of the live weight measurements are shown in Fig. 4-3 and Fig. 4-4. No statistically significant differences in live weight were observed among the groups ($p>0.05$). However significant difference in body weight gain were observed in the animals treated with the double dose ($p<0.05$) on days 14 and 28 days post-treatment compared to the controls. No statistical differences were observed between the group treated with a single dose and the control group for the whole period of observation.

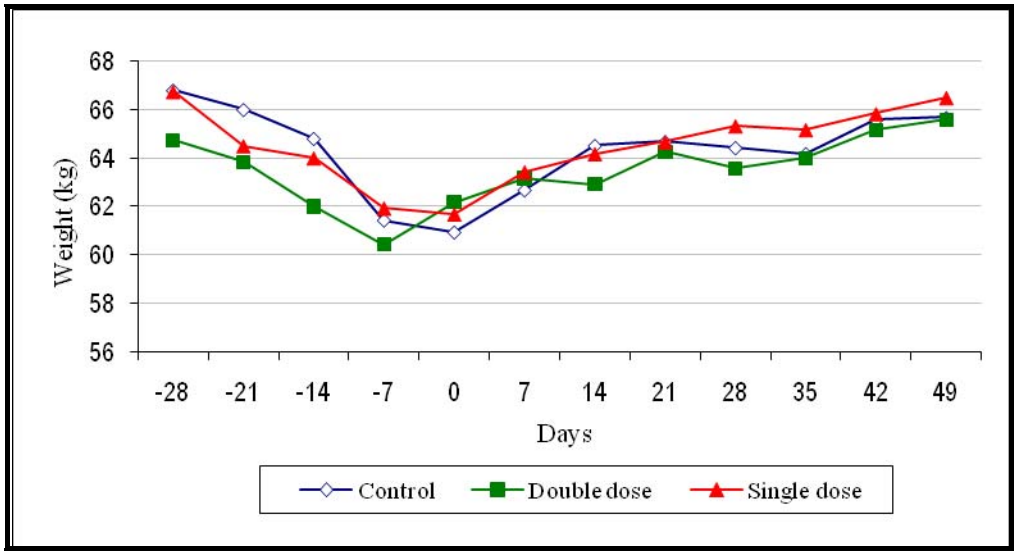


Fig. 4-3. Means of live weights for control, single and double dose groups of animals. Treatment started on day 0.

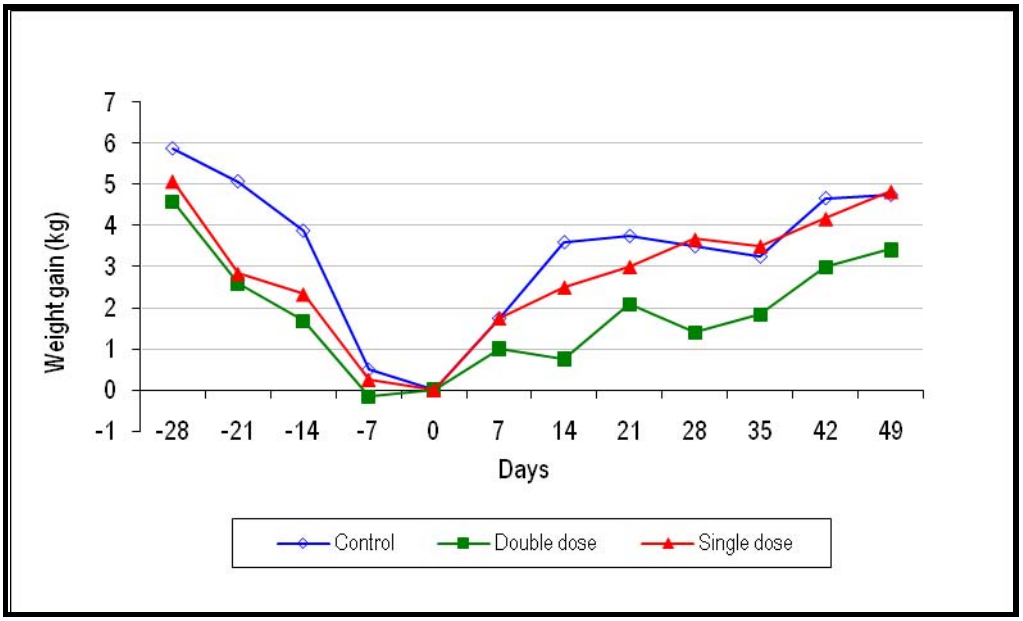


Fig. 4-4. Means of live weight gains for control, single and double dose groups of animals. Sheep were infected on week -6 and fed with product from day 0.

Figure 4-5 shows the means of the body condition scores. No significant differences in body condition scores were observed during the whole period of observation among the three groups ($p>0.05$).

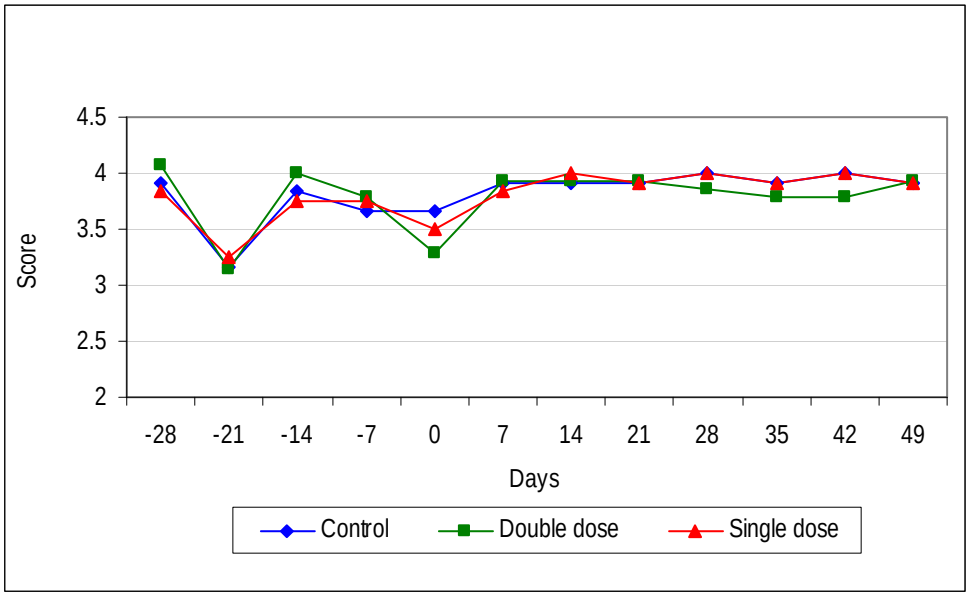


Fig. 4-5. Means of body condition score for control, single and double dose groups of animals. Treatment started on day 0.

4.2.2. Faecal egg counts and egg flotation

Figure 4-6 shows the arithmetic means of the FECs of the groups of sheep treated with single and double doses of plant material, and the control group. At treatment (day 0), there were no significant differences in FEC between the groups ($p>0.05$). Similarly no significant differences were observed in FECs between the groups on any of the other dates.

However, the FECs for the sheep given a double dose of *C. jamacaru* tended to be lower than those of the other two groups. The percentage reduction in FEC was 40% and 32% at three days post-treatment in the sheep treated with a single and a double dose, respectively, while the corresponding values at 49 days post-treatment were 3% and 65%, respectively (Annex: Table 15). The highest percentage reduction was seen on day 17 for the single dose treatment (41%) and on day 49 for the double dose treatment (65%).

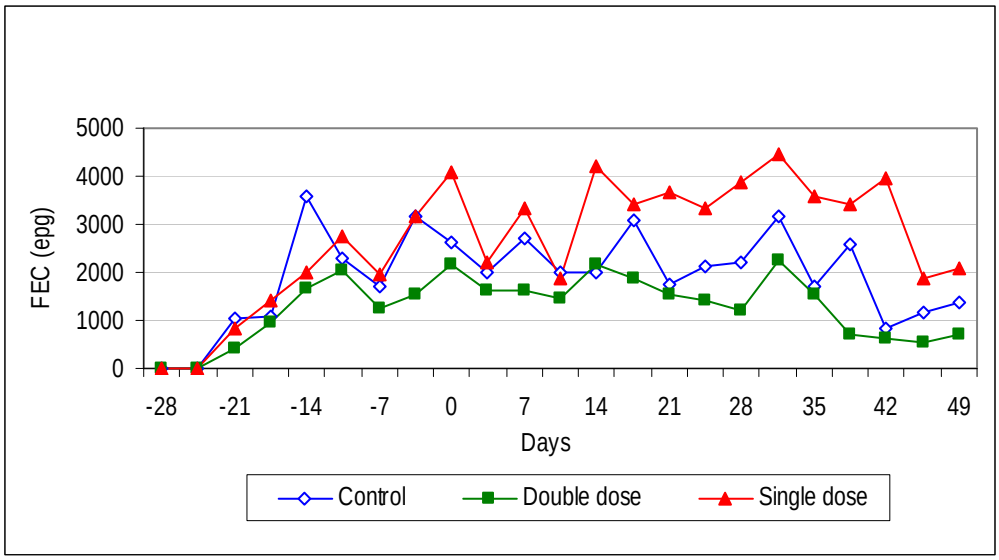


Fig. 4-6. Means of faecal egg counts (FEC) in eggs per gram of faeces for treated and control sheep. Treatment started on day 0.

4.2.3. PCV and FAMACHA© evaluation

The results of the PCV are presented in Fig. 4-7. Although there was a slight decline in PCV between days 21 and 28, in the animals treated with a single dose, no significant differences ($p>0.05$) in the mean PCVs were detected.

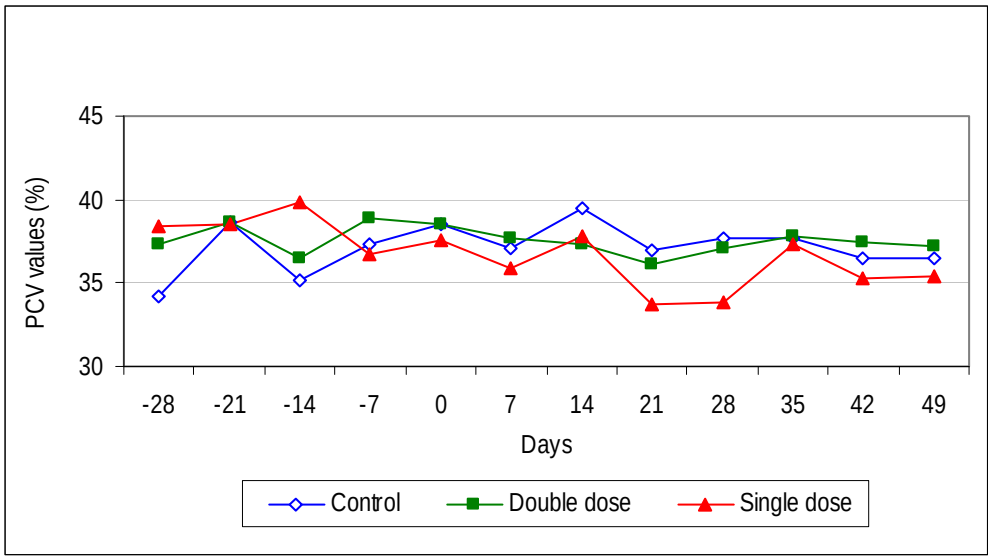


Fig. 4-7. Mean PCVs during the experiment in the control group and the groups treated with *C. jamacaru*.

A statistical difference was detected only on day 35 post-treatment between the single and double dosed groups and the control group in FAMACHA© scores (p>0.02) (Fig. 4-8). The sheep in this study were scored in categories 1 and 2. No animal was recorded in category 3, 4 or 5.

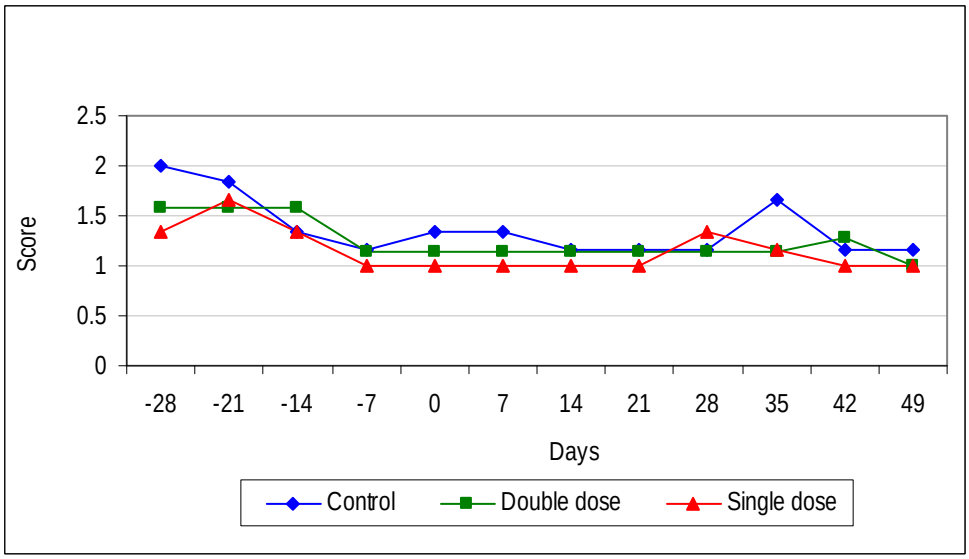


Fig. 4-8. Mean FAMACHA© scores of groups: control, single-dose and double-dose.

4.2.4. Larval culture

The results of the larval cultures (Table 4-6) revealed that *H. contortus* was the predominant helminth parasite in the experimental sheep.

Table 4-6. Faecal larval culture percentages of *H. contortus* (*H. c*) and *T. colubriformis* (*T. c*) of different experimental animal groups

	Double dose		Single dose		Control	
Day	***H. c***	***T. c***	***H. c***	***T. c***	***H. c***	***T. c***
15	57	43	68	32	67	33
22	55	45	85	15	64	36
25	53	47	35	65	56	44
29	21	79	76	24	34	66
32	61	39	55	45	46	54
36	45	55	84	16	53	47
39	81	19	90	10	53	47
43	72	28	90	10	71	29
49	60	40	70	30	46	54
Average (%)	**56**	**44**	**73**	**27**	**54**	**46**

4.3. Discussion

4.3.1. *In vitro* experiments

The *C. jamacaru* extracts were shown by means of egg hatch assay to exhibit anthelmintic activity. The *in vitro* assay showed clearly that the acetone extract inhibited egg hatching in a concentration-dependent manner. The acetone extract inhibited egg hatching at concentrations between 0.31 and 2.5 mg/ml significantly ($p<0.001$) compared to the controls, while larval development was inhibited significantly ($p<0.001$) compared to the negative control at all concentrations tested (Table 4-4). The chloroform, butanol and 35% water in methanol fractions inhibited egg hatching significantly ($p<0.001$), while the hexane and water fractions were not as effective (Table 4-3). Water and 35% water in methanol inhibited larval development comparably, while the chloroform and hexane fractions significantly inhibited the larval development (Table 4-3). The anthelmintic effects of the acetone extract and chloroform fraction of *C. jamacaru* seen in the egg hatch assay were confirmed by the larval development and viability assay.

The inhibition of the egg hatching occurred at lower concentrations of extract or fraction compared with some medicinal plants studied previously, for instance, an aqueous extract of *Annona senegalensis* inhibited only 11.5% of eggs at 7.1 mg/ml (Alawa *et al*. 2003) and a methanol extract of *Spigelia anthelmia* induced 97.4% egg hatch inhibition at a concentration of 50 mg/ml (Assis, Bevilaqua, Morais, Vieira, Costa & Souza 2003). Yousif *et al*. (2007) showed that *C. jamacaru* possesses a confirmed antischistosomal activity. The levels of activity observed in the acetone extract suggest that the anthelmintic component of *C. jamacaru* is a relatively polar compound. The chloroform and butanol fractions were only active against the nematode eggs at the higher concentrations. The hexane fraction demonstrated low egg hatch inhibition activity, but showed higher activity on *H. contortus* larvae.

The observed anthelmintic activity of the *C. jamacaru* extract *in vitro* could be attributable to its tannin or alkaloid content (Brunet, Montellano, Torres-Acosta, Sandoval-Castro, Aguilar-Caballero, Capetillo-Leal & Hoste 2008; Sousa *et al*. 2008). Tannins have the capacity to bind to proteins and may operate via several mechanisms to have an anthelmintic effect. Tannins may bind to the free protein available in the test plate wells for larval feeding, reducing the nutrient availability in the wells and this may result in larval starvation and death. Insects and insect larvae may ingest condensed tannins, which bind to the intestinal mucosa and cause autolysis (Schultz 1989); this could also apply to developing nematode larvae. In the LDVA, the *C. jamacaru* extract could bind to any macromolecule available in the growth medium, form stable complexes and via the mechanisms discussed above damage the parasite larvae.

We appreciate that *in vitro* conditions are *per se* different from conditions in the gastrointestinal tract of sheep. However, the larvae of *H. contortus* were susceptible to the presence of the *C. jamacaru* extract in the EHA and LDVA. The extraction of compounds from plant materials largely depends on the solvent or medium and the method of extraction. Extraction with water limits the amount and types of compounds extracted due to polarity (Eloff 1998). Farmers in many parts of Africa use hot water decoctions from plant materials

for treating animals (Abdu, Jagun, Gefu, Mohammed, Alawa & Omokanye 2000). Further work would therefore be required to find alternative ways in which farmers may extract a more active fraction from the plant.

4.3.2. *In vivo* experiments

This study was initiated after a report by a commercial farmer, Mr. Mike Bosch (Bosch, 2007) who uses *C. jamacaru* on his farm for gastrointestinal control in livestock. The experiment was carried out to assess the effectiveness of *C. jamacaru* as an anthelminthic in sheep experimentally infected with *H. contortus* and *T. colubriformis*. No reports on the anthelmintic effects of *C. jamacaru*, was found this work being the first investigation into the potential effects in livestock of *C. jamacaru*. Our results on FEC showed no significant differences ($p>0.050$) in faecal egg count reduction between groups on any of the days. However, during the course of the experiment, the FEC in the group receiving a double dose remained lower compared with the single dose and control groups.

The dosage rate (32.33g of the plant material per animal for a single dose and 64.66g per animal for a double dose) used in the sheep experiments may not have provided a sufficient knock-down effect. The dose is an extremely significant factor for the efficacy of the plant against the nematodes. Each *H. contortus* worm is responsible for a daily loss of about 0.05 ml of blood through ingestion of blood and seepage from lesions (Urquhart *et al*. 1996) which may lead to anaemia and emaciation. But in this study, for the groups of sheep infected and treated with both doses of *C. jamacaru* no significant differences were detected in the mean PCV, FAMACHA© scores during the whole period of observation among the three groups. Concerning the FAMACHA© scores, most of the sheep in this study were included in categories 1 and 2. No animal was recorded in categories 3, 4 or 5. This is probably related to a good feeding regimen and relatively low worm burdens.

No statistical differences in body weight gain were observed between the group treated with a single dose and the control group for the whole period of

observation while significant differences ($p<0.05$) were observed between animals treated with the double dose and the control group only on day 14 and 28 days post-treatment. However, when live weights were compared between the three groups, no statistically significant differences were observed ($p>0.05$). This lack of differences between the three groups for the live weights was also reflected in the body condition where no significant differences were detected during the whole period of observation among the three groups.

In conclusion, the plant used in this study did not exhibit a strong anthelmintic activity in sheep but better results may be obtained through using higher doses and by giving the plant for a longer period of time than was done in this experiment. This may explain the efficacy of the procedure used in the farmer. The *in vitro* work suggests that use of acetone extracts and chloroform fractions thereof may be a way of increasing efficacy.

CHAPTER 5

Result and Discussion of In vitro antioxidant, antibacterial and antifungal activity

5.1 Introduction

It was disappointing that the data of the *in vivo* animal experiment did not support the data found in the *in vitro* experiments and especially it did not support the observation of Mr Mike Bosch who has apparently been using this plant successfully for many years to control parasites in his stock. One explanation may be that the effect of the plant may not necessarily be directly on the parasites but could work indirectly. If the plant could stimulate the immune system of the animal or inhibit the growth of bacterial pathogens, the result would be healthier stock able to grow better and better able to resist the parasite attack.

Therefore it appears reasonable to investigate aspects such as the anti-oxidant activity that could lead to a stimulation of the immune system of the animal, or antimicrobial activity that could inhibit infections in the animal.

5.2. Percentage yields of *C. jamacaru*

The percentage yield of plant material following solvent-solvent fractionation is represented in Table 5-1. It was calculated by dividing the yield of the extract or fraction by the total quantity and multiplied by 100.

The yield can provide a tentative idea of the amount of active constituents that can be extracted from ground plant material. With the different solvent types used in extractions from the same plant material, the yield may give an indication of the polarity of compounds present in the crude extract and also the relationship with biological activity. It is remarkable that more than 60% of the compounds were very non-polar because they were extracted by hexane.

Table 5-1. The yields of different fractions separated by solvent-solvent fractionation of *C. jamacaru* tissue acetone extract (yield of acetone extract from plant material was 6%)

Extracts	Yield (g)	% Yield
Hexane	2.500	60.7
Chloroform	0.763	18.5
35% H_2O in Methanol	0.452	11.0
Water	0.321	7.8
Butanol	0.086	2.0
Total extract	**4.122**	**100.0**

5.3. Antioxidant activity of *C. jamacaru* extracts

Thin layer chromatography (TLC) was used to evaluate the chemical composition of the fractions on Merck TLC F 254 plates. Among the three mobile systems used, BEA was efficient in its ability to separate non-polar compounds and EMW was efficient in separating polar compounds. With the colour change after spraying with DPPH, the *C. jamacaru* extracts eluted in the CEF, BEA and EMW showed free scavenging activity (Fig. 5-1.). The active zones were better separated when developed in the BEA system. With vanillin-sulphuric acid, compounds were clearly identified as showed in Fig. 5-2.

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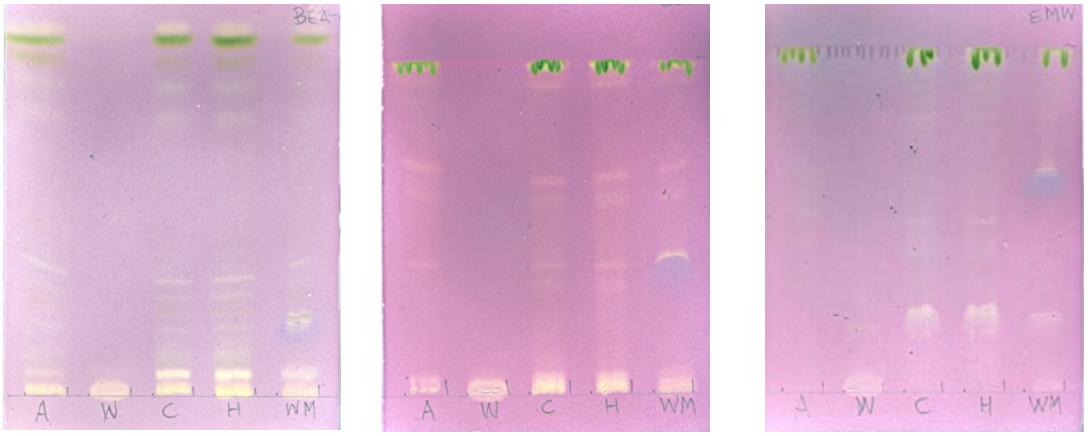

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Legend: **A**=acetone extract ; **W**=water extract; **C**=chloroform extract; **H**=hexane extract; **WM**= 35% water in methanol extract.

Fig. 5-1. Chromatogram developed in BEA (1), CEF (2) and EMW (3), and sprayed with DPPH, with the clear zones indicating the zones of anti-oxidant activity.

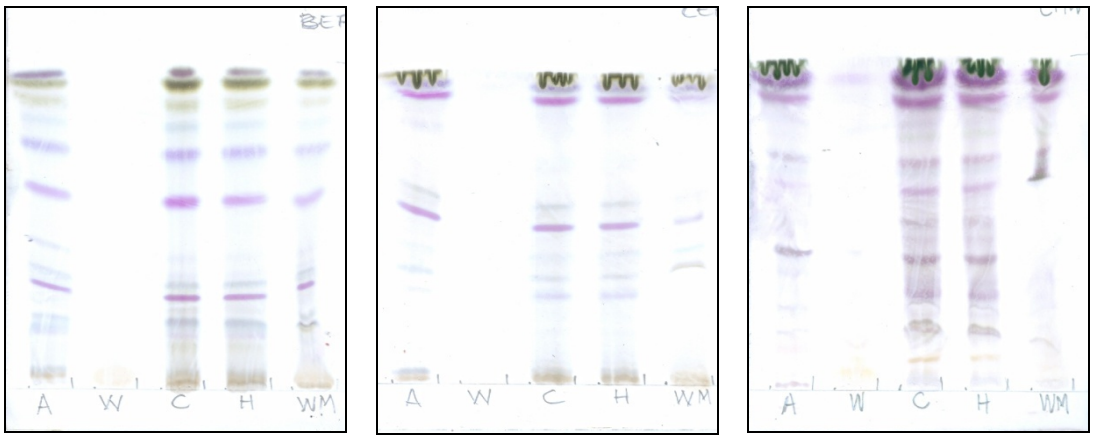

Fig. 5-2. Chromatogram developed in BEA (1'), CEF (2') and EMW (3'), and sprayed with vanillin-sulphuric acid, to identify several compounds present.

There were at least 8 antioxidant compounds present in the different fractions of the crude extract. It is surprising that several of these fractions were relatively non-polar with a high R_f value in the chromatogram separated by the non-polar BEA. Most compounds with antioxidant activity have a high polarity due to the presence of several phenolic moieties. The water fraction must

have contained highly polar antioxidant compounds because it did not move from the origin with the polar EMW solvent system.

The quantitative antioxidant activity was determined for the crude extract and fractions (hexane, chloroform, butanol, residual water) using the ABTS-radical scavenging capacity at concentrations in the ranged from 0.0039 to 0.5 mg/ml. Trolox and Vit C were used as positive controls in the antioxidant evaluation. The data obtained are shown in Fig. 5-3. The important factor is the gradient of inhibition of ABTS at different concentrations. These values were calculated by adding the trendline to the graph and calculating the formula of the line and the R^2 value with Microsoft Excel (Table 5-2).

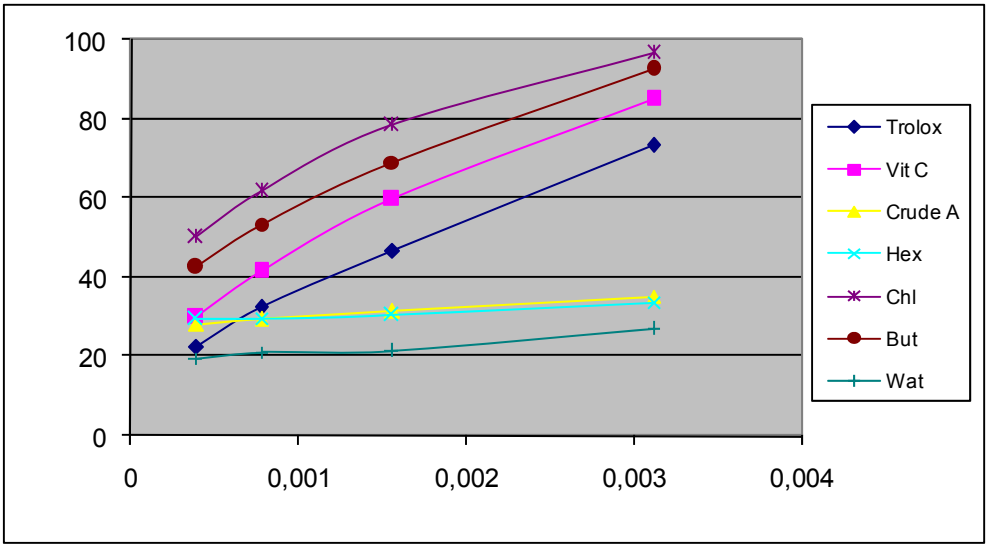

Fig. 5-3. Percentage ABTS-radical scavenging capacity of trolox, Vit C, crude extract and fractions of *C. jamacaru* at different concentrations in mg/ml after 6 minutes.

Table 5-2. Formulae calculated by Microsoft Excel describing the correlation between ABTS reduction and concentration and the correlation coefficient

Sample	Formula	R^2
Trolox	y = 18189x + 17.12	0.996
Ascorbic acid	y = 19720x + 25.17	0.984
Chloroform	y = 2386x + 41.81	0.991
Butanol	y = 1791x + 38.29	0.986
Acetone crude extract	y = 212x + 28.15	0.999
Water	y = 139x + 20.86	0.993
Hexane	y = 83x + 30.04	0.993

Table 5-3. Trolox equivalent antioxidant activity (TEAC) and ascorbic acid equivalent antioxidant capacity (AAEAC) of two positive controls, the crude extract and the solvent-solvent fractions of *C. jamacaru*.

	Trolox	Vit C	Chloroform	BuOH	Acet	Water	Hexane
Gradient	18189	19720	2386	1791	212	139	83
TEAC	100.0	108.4	13.1	9.8	1.2	0.8	0.5
AAEAC	92.2	100.0	12.1	9.1	1.1	0.7	0.4

The low level of activity seen in the DPPH qualitative antioxidant assay is reflected in the quantitative data (Table 5-3). Even the chloroform fraction with the highest antioxidant activity had 8 times lower activity than the trolox or ascorbic acid positive controls.

Based on the low antioxidant activity *C. jamacaru* extracts probably do not play an important role in stimulating the immune system of animals using this as part of their diet.

5.4. Antibacterial activity of *C. jamacaru* extracts

The crude acetone extract had excellent activity against *E. coli* with an MIC of 0.04 mg/ml (Table 5-4). This extract was not as active against *P. aeruginosa* (0.63 mg/ml), *S. aureus* (2.5 mg/ml) and *E. faecalis* (1.25 mg/ml). The higher activity against Gram negative organisms is a positive aspect because plant extracts are usually more active against Gram positive organisms (Vlietinck, Van Hoof, Totte, Lasure, Van den Berghe, Rwangabo & Mvukiyumwami 1995).

The activity in the solvent-solvent fractions varied but in all cases the crude extract had a higher or equal antibacterial activity pointing to a synergistic effect of compounds present in the crude extract, but separated by the fractionation process.

Table 5-4. Minimum inhibitory concentration (MIC) values of *C. jamacaru* crude acetone extract and 4 fractions on 4 microorganisms (*P. aeruginosa*, *E. faecalis*, *S. aureus* and *E. coli*)

Micro-organism		Fractions (mg/ml)					Control+
	Time (Hour)	Acetone	Butanol	Hexane	Chloroform	35% w MeOH	Gentam (µg/ml)
P. aeruginosa	1	0.63	0.63	0.63	0.63	1.25	0.19
	2	0.63	0.63	0.63	0.63	2.5	0.19
E. faecalis	1	1.25	0.63	2.5	0.63	2.5	0.19
	2	1.25	0.63	2.5	0.31	2.5	0.19
S. aureus	1	2.5	0.63	2.5	0.31	2.5	0.09
	2	2.5	0.63	2.5	0.31	2.5	0.09
E. coli	1	0.04	0.63	0.16	0.08	2.5	0.19
	2	0.04	0.31	0.16	0.08	2.5	0.19

% w MeOH= percentage water in methanol; Gentam=Gentamycin

5.5. Antifungal activity of *C. jamacaru* extracts

Three fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* were used in this assay. In general the crude extract and fractions had lower activity against the fungi than against the bacteria tested (Table 5-5). With the fungi, the chloroform fraction had a higher activity than the crude extract indicating that synergism may not play such a big role in this case. In contrast with the data for the bacteria there were not many differences in sensitivity between the different fungal pathogens.

Table 5-5. Minimum inhibitory concentration (MIC) values of *C. jamacaru* extracts on fungi (*C. albicans, A. fumigatus, C. neoformans*)

Micro-organisms		Extracts					Control+
	Time (Hour)	Acetone	Chloroform	Hexane	Water	35%w MeOH	AmB(µg/ml)
C. albicans	24	2.5	0.16	1.25	2.5	2.5	0.09
	48	2.5	0.16	0.63	2.5	2.5	0.09
A. fumigatus	24	1.25	0.16	2.5	2.5	2.5	0.19
	48	1.25	0.31	2.5	2.5	2.5	0.19
C. neoformans	24	0.16	0.16	0.63	2.5	2.5	0.09
	48	0.31	0.31	1.25	2.5	2.5	0.09

% w MeOH = percentage water in methanol; AmB = Amphotericin B

5.6. Discussion

5.6.1. Antioxidant activity

The antioxidants in plants may prevent damage caused by free radicals to DNA and other molecules, reduce inflammation, and promote good health and resistance to diseases. The antioxidant activity was evaluated because high activity may explain the positive results obtained by Mike Bosch. In the DPPH qualitative analysis of antioxidant activity there were a number of antioxidant compounds present in some of the extracts and fractions but the activity appeared to be low. This observation was confirmed in the TEAC quantitative analysis of antioxidant activity. Even the fraction with the highest activity was about 8 times less active than trolox or ascorbic acid.

The antioxidant activity of *C. jamacaru* may be due to polyphenols or tannins as it has been found to contain these compounds (Arizona 2006; Hollman 2001). It is however interesting that the activity in the most polar fraction was among the weakest. This is not what would have been expected if polyphenols or tannins were responsible for the antioxidant activity.

5.6.2. Antibacterial activity

The evolution of resistance to microbes is one of the most significant problems in modern medicine, posing serious threats to human and animal health. The early work on the use of antibiotics for bacterial infections gave much hope that infectious diseases were no longer a problem, especially in the human field. However, as their use, indeed mis-use, progressed, resistance which was often transferable between different strains and species of bacteria, emerged. Antimicrobial resistance is not confined to bacteria but occurs in viruses, protozoa, helminths and other parasites. In many of these, the mechanism of resistance is unknown, and hence their control is still in question. It is likely, however, that the mechanisms are no less complicated than those pertaining to bacteria.

The results recorded in this work showed that the plant had an antibacterial activity. This was seen especially with the chloroform fraction and acetone extract. Phenols have been held responsible for several types of activities: antibacterial, antiviral, antioxidant, diuretic, antirheumatic, and against gastric and hepatic problems (Carvalho & Amancio 2002). The presence of antibacterial compounds in *C. jamacaru* was established, confirming earlier work (Sousa *et al*. 2008). In the current work, the best MIC values of 0.08 mg/ml were obtained for the acetone extract against *S. aureus*. The best values for Gram-negative bacteria were 0.16 mg/ml for the acetone extracts of *C. jamacaru*. The data provides some evidence for the occurrence of synergism in the crude acetone extract.

5.6.3. Antifungal activity

In this study, a moderate antifungal activity was expressed by the chloroform fraction against all three fungal organisms tested, while the acetone extract showed a moderate activity only against *C. neoformans*. *Aspergillus fumigatus* was the most resistant organism even against AmpB. As reported by Masoko *et al*. (2005), *A. fumigatus* was the least sensitive of the test microorganisms while *C. neoformans* was the most sensitive, as indicated by the recorded MIC. Nevertheless, this moderate antifungal activity may confirm some of its ethnomedicinal uses. The antifungal activity of *C. jamacaru* could be related to the presence of tannins as stated by Monteiro, Almeida, Albuquerque, Lucena, Florentino & Oliveira (2006).

Chapter 6

Conclusions and recommendations

The aim of this study was to evaluate the use of *C. jamacaru* in protecting sheep against helminth infections in order to validate the success obtained by Mr Mike Bosch, a commercial farmer in Limpopo Province of South Africa, who apparently uses the plant as a dewormer. He reports this plant to be safe in the treated animals (Bosch 2007).

The following objectives were stated to attain the aim of the study:

- To evaluate any possible toxic effects of feeding animals with a higher dose of *C. jamacaru* than that used by Mr Mike Bosch.
- To evaluate the *in vitro* efficacy of *C. jamacaru* as an anthelmintic.
- To evaluate the *in vivo* efficacy against *H. contortus* and *T. colubriformis* at different dosage levels.
- To evaluate the antibacterial, antifungal and antioxidant properties of *C. jamacaru* extracts *in vitro* as an alternative explanation to the success of the commercial farmer.
- To make recommendations on using this procedure based on the results obtained.

Evaluating the safety of the procedure

Although plants have been fed to animals on the farm of Mr Mike Bosch without any negative effects for many years, we decided to test the activity at a double dose of the plant as well. It was important to ensure that this higher dose did not have a negative effect.

All the animals were monitored hourly for 4 hours after each administration. No abnormal behaviour or toxic effects were recorded during or after the experiment.

To evaluate the *in vitro* efficacy of *C. jamacaru* as an anthelmintic

Through egg hatching, larval development and viability assays, *C. jamacaru* was demonstrated to have anthelmintic activity (Githiori, Hoglund & Waller 2005). The percentage inhibition of egg hatching of the acetone extract and the butanol, chloroform, 35% water in methanol, hexane and water solvent-solvent fractions were 100%, 100%, 94%, 91%, 9% and 16%, respectively. The percentage inhibition of larval development of the acetone extract and chloroform, 35% water in methanol, hexane and water fractions were 93%, 84%, 49%, 85%, 31%, respectively. Nevertheless, the *in vitro* activity of *C. jamacaru* extracts may not necessarily be transferable wholesale to *in vivo* efficacy, as the latter is influenced by physiology and bioavailability factors in the body (Githiori *et al*. 2005).

To evaluate the *in vivo* efficacy against *H. contortus* and *T. colubriformis*

In the literature it is accepted that an FEC reduction of 70% constitutes an acceptable level of activity (Coles *et al*. 1992). Although a FEC reduction of only 65% was obtained in this study, a higher dose given for a longer period could be more efficacious. More research is required to investigate this hypothesis. Based on our *in vivo* experiments, *C. jamacaru* did not substantially reduce *H. contortus* and *T. colubriformis* infection in the sheep. Nevertheless, *in vivo* activity at double the dose, did result in lowered egg counts, although this was not statistically significant. Our data do not explain the positive effect Mr Mike Bosch observed in treating his animals. It may be that the answer lies in a long term effect via antioxidant activity or some other mechanism.

To evaluate the antibacterial, antifungal and antioxidant properties of *C. jamacaru* extracts *in vitro* as an alternative explanation

The crude extract had a relatively good activity against *E. coli*. In most cases the antibacterial and antifungal activity were relatively low and it does not seem likely that it could explain the success obtained by Mr Mike Bosch. The antioxidant activity was very low and this also does not provide a likely explanation for his success.

Possibly the combination of anthelmintic, antioxidant, antibacterial and antifungal activities as demonstrated in the *in vitro* experiments may have an effect *in vivo*.

To make recommendations on using this procedure

It is clear that the procedure used by Mr Bosch works in practice. It is disappointing that our results do not provide an explanation for the success he obtained. Even if we do not understand how the plant has an anthelmintic effect it may be useful to the use of a suitable dosage (using low level technology adaptable to rural conditions) for use by resource-poor rural communities where *C. jamacaru* occurs as an invasive weed.

It does not seem likely that further study of the plant will lead to the isolation of potent anthelmintic, antioxidant, antibacterial and antifungal compounds. From this investigation we have to bear in mind that *C. jamacaru* is a plant with moderate anthelmintic activity and it should still be considered possibly not as a sole alternative to anthelmintic drugs, but as part of an integrated approach specifically designed to achieve sustainable parasite control in ruminant production systems (Githiori *et al*. 2006).

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APPENDIX

Table 1. Inhibitory activity of egg hatch assay of *H. contortus* by albendazole (positive control)

Conc.(mg/ml)	Positive Control : Albendazole					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	98	3	99	2	98	2
1.25	95	4	97	4	96	5
0.63	89	7	96	4	91	8
0.31	77	22	83	21	79	30
0.16	46	71	43	68	46	61
0.31	18	85	27	74	23	83
0.16	19	81	12	83	14	89
0.08	14	89	16	89	13	82

Conc.: concentration

Table 2. Inhibitory activity of egg hatch assay of *H. contortus* by the water extract of *C. jamacaru*

Conc.(mg/ml)	Water					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	30	74	18	85	2	93
1.25	11	83	8	92	17	86
0.63	21	79	19	71	21	77
0.31	12	90	15	85	8	91
0.16	9	88	3	93	4	82
0.31	7	96	1	96	3	90
0.16	4	89	3	81	5	89
0.08	1	96	0	88	3	91

Conc.: concentration

Table 3. Inhibitory activity of egg hatch assay of *H. contortus* by the hexane extract of *C. jamacaru*

Conc.(mg/ml)	Hexane					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	4	82	18	81	3	84
1.25	2	79	3	71	5	78
0.63	4	66	2	91	3	93
0.31	1	95	5	89	8	91
0.16	4	101	7	98	6	90
0.31	1	86	3	101	3	107
0.16	4	79	5	93	9	91
0.08	0	91	2	96	1	94

Conc. : concentration

Table 4. Inhibitory activity of egg hatch assay of *H. contortus* by the chloroform extract of *C. jamacaru*

Conc.(mg/ml)	Chloroform					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	85	11	91	0	98	7
1.25	15	79	12	89	13	91
0.63	14	94	10	97	8	90
0.31	7	90	4	89	4	86
0.16	11	89	13	91	16	100
0.31	9	99	5	84	3	90
0.16	6	91	3	94	1	96
0.08	2	94	1	86	1	92

Conc.: concentration

Table 5. Inhibitory activity of egg hatch assay of *H. contortus* by the butanol extract of *C. jamacaru*

Conc.(mg/ml)	Butanol					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	88	0	91	0	98	0
1.25	3	79	6	95	9	90
0.63	1	89	4	95	8	93
0.31	14	91	18	96	11	90
0.16	7	94	12	91	8	96
0.31	5	88	7	90	6	91
0.16	3	94	2	95	6	90
0.08	1	89	1	80	0	79

Conc.: concentration

Table 6. Inhibitory activity of egg hatch assay of *H. contortus* by the 35% water in methanol extract of *C. jamacaru*

Conc.(mg/ml)	**35% H_2O in MEOH**					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	94	0	91	6	81	23
1.25	35	66	32	71	23	79
0.63	8	89	6	99	2	90
0.31	2	96	4	97	1	88
0.16	4	90	11	91	13	96
0.31	7	87	4	88	5	90
0.16	1	92	0	96	1	97
0.08	2	94	1	98	1	90

Conc.: concentration

Table 7. Inhibitory activity of egg hatch assay of *H. contortus* by the acetone crude extract of *C. jamacaru*

Conc.(mg/ml)	**Acetone**					
	Replicate 1		Replicate 2		Replicate 3	
	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
2.5	101	0	109	0	103	0
1.25	88	19	79	21	84	24
0.63	61	23	84	16	78	19
0.31	77	31	68	29	71	36
0.16	35	71	21	59	12	73
0.31	19	86	14	77	11	91
0.16	3	91	1	98	0	94
0.08	0	86	0	94	1	82

Conc.: concentration

Table 8. Mean inhibitory activity of different concentrations (conc.) of *C. jamacaru* acetone crude extract on larval development and viability of *H. contortus*

	Acetone			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	6	88	94	0.10
1.25	9	79	88	0.16
0.63	7	78	85	0.14
0.31	14	74	88	0.26
0.16	16	66	82	0.32
0.08	32	57	89	0.58
0.04	43	53	96	0.72
0.02	54	35	89	0.98

liv.=living; dv.=development

Table 9. Mean inhibitory activity of different concentrations (conc.) of *C. jamacaru* hexane extract on larval development and viability of *H. contortus*

	Hexane			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	13	81	94	0.23
1.25	16	76	92	0.29
0.63	14	78	92	0.25
0.31	11	72	83	0.22
0.16	10	78	88	0.19
0.08	13	71	84	0.25
0.04	14	71	85	0.27
0.02	16	76	92	0.28

liv.=living; dv.=development

Table 10. Mean inhibitory activity of different concentrations (conc.) of *C. jamacaru* chloroform extract on larval development and viability of *H. contortus*.

	Chloroform			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	16	74	90	0.29
1.25	23	73	96	0.39
0.63	21	72	93	0.37
0.31	22	66	88	0.40
0.16	28	61	89	0.50
0.08	67	54	121	0.89
0.04	71	61	132	0.87
0.02	51	45	96	0.86

liv.=living; dv.=development

Table 11. Mean activity inhibitory of different concentrations (conc.) of *C. jamacaru* butanol extract on larvae development and viability of *H. contortus*

	Butanol			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	14	80	94	0.24
1.25	15	75	90	0.27
0.63	14	74	88	0.26
0.31	12	72	84	0.23
0.16	14	69	83	0.27
0.08	15	76	91	0.27
0.04	14	71	85	0.27
0.02	16	76	92	0.28

liv=living; dv=development

Table 12. Mean inhibitory activity of different concentrations (conc.) of *C. jamacaru* 35% water in MeOH extract on larval development and viability of *H. contortus*

	35% water in MeOH			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	49	47	96	0.82
1.25	46	42	87	0.84
0.63	48	43	91	0.85
0.31	53	37	90	0.95
0.16	53	38	91	0.94
0.08	53	39	93	0.93
0.04	49	34	83	0.95
0.02	50	35	85	0.95

liv=living; dv=development

Table 13. Mean inhibitory activity of different concentrations (conc.) of *C. jamacaru* water extract on larval development and viability of *H. contortus*

	Water			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	52	36	88	0.95
1.25	53	41	94	0.91
0.63	55	44	99	0.89
0.31	61	41	102	0.96
0.16	59	39	98	0.97
0.08	57	45	102	0.90
0.04	59	40	99	0.96
0.02	58	43	101	0.92

liv.=living; dv.=development

Table 14. Mean inhibitory activity of different concentrations (conc.) of albendazole on larval development and viability of *H. contortus*

	Albendazole			
Conc.(mg/ml)	**Mean of liv. larvae**	**Mean of dead larvae**	**Mean of liv. + dead larvae**	**Larvae dv. rate**
2.5	5	85	90	0.09
1.25	7	84	91	0.12
0.63	9	86	95	0.16
0.31	15	86	101	0.24
0.16	21	81	102	0.33
0.08	26	76	102	0.41
0.04	51	37	88	0.93
0.02	23	72	95	0.39

liv.=living; dv.=development

Table 15. Arithmetic means of faecal egg counts (FEC) in eggs per gram of faeces for treated and control sheep and the percentage reduction in FEC (Coles *et al*. 1992)

Group	**Control (n=6)**	**Single dose (n=6)**		**Double dose (n=6)**	
Day	**Mean FEC (C)**	**Mean FEC (T)**	**% FEC Reduction**	**Mean FEC (T)**	**% FEC Reduction**
0	6028	5139	-	3855	-
3	3939	2367	40	2678	32
7	4853	3105	36	2833	42
10	3600	2255	37	2294	36
14	5194	5767	-11	3044	41
17	5955	3510	41	3272	45
21	3555	4517	-27	2472	30
24	4300	4094	5	2667	38
28	4367	5383	-23	3061	30
31	5611	5578	1	4616	18
35	3433	4228	-23	2639	23
38	3550	4489	-26	1622	54
42	2478	4606	-86	1378	44
45	3183	2700	15	1406	56
49	2889	2800	3	1000	65

C = Mean FEC for the corresponding day among the control sheep; T= Mean FEC for the corresponding day among the treated sheep.