EVALUATION OF THREE IN VITRO BIOASSAYS FOR MEASURING THE ANTHELMINTIC ACTIVITY OF PLANT EXTRACTS CONTAINING CONDENSED TANNINS

by

JANET SUE HOWELL

(Under the Direction of Ray M. Kaplan)

ABSTRACT

Certain forages high in condensed tannins (CT) demonstrate anthelmintic activity, and appear to be a useful non-chemical adjunct to parasite control in small ruminants. *In vitro* bioassays have been used to evaluate this antiparasitic effect against several species of trichostrongyle nematodes. However, it remains unclear which *in vitro* bioassay is the most appropriate evaluation tool. The goal of this research is to evaluate different *in vitro* methods, and determine which are the most suitable for measuring the effective concentration (EC₅₀) of these extracts. In this research, 3 *in vitro* methods (egg hatch assay (EHA), larval development assay (LDA) and larval migration inhibition assay (LMIA) were performed using CT extracts from 4 plants with *Haemonchus contortus* eggs or larvae. Of the 3 bioassays, the LDA yielded the most consistent results, and therefore, appears to be the most appropriate bioassay for measuring the antiparasitic activity of CT plant extracts in small ruminants.

INDEX WORDS: anthelmintic resistance, condensed tannins, *Haemonchus contortus*, *in vitro* bioassays, plant extracts, Sericea lespedeza, small ruminants
EVALUATION OF THREE *IN VITRO* BIOASSAYS FOR MEASURING THE
ANTHELMINTIC ACTIVITY OF PLANT EXTRACTS
CONTAINING CONDENSED TANNINS

by

JANET SUE HOWELL

B.S., University of Alabama at Birmingham, 1992

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment
of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2009
EVALUATION OF THREE IN VITRO BIOASSAYS FOR MEASURING THE
ANTHELMINTIC ACTIVITY OF PLANT EXTRACTS
CONTAINING CONDENSED TANNINS

by

JANET SUE HOWELL

Major Professor: Ray M. Kaplan
Committee: James Noe
             Michael Yabsley

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009
DEDICATION

I would like to dedicate this work to all my family, past and present, who instilled an interest in education, and encouraged me along the way. I thank everyone who has supported me through this challenging process, particularly Bill and Jerry. I could never have accomplished this without you.
ACKNOWLEDGEMENTS

I would like to thank all of those who were instrumental in helping me obtain this challenging but rewarding degree. I would especially like to thank my mentor, Dr. Kaplan, whose patience and guidance these past years has been invaluable. I also want to thank my committee members, Dr. James Noe and Dr. Michael Yabsley for their guidance during this research. I would like to thank the “lab team”, particularly Bob Storey and Dr. Andy Moorhead, as well as, various undergraduate and graduate students, whose assistance has been greatly appreciated. I could never have completed this without you. I would like to recognize the USDA Sustainable Agriculture Research and Education (SARE) Grants Program for the funding of this project, and the SCSRPC members, in particular, Drs Tom Terrill, Joan Burke, and Jorge Mosjidis for their comments on the thesis. I would also like to recognize Dr. Jim Muir, Dr. B.R. Min and Richard Wolfe (Texas A & M) for the various plant extracts. I appreciated the comments and information regarding various aspects of the thesis work from Drs Jennifer Gill and Anand Vidyashankar. I would particularly like to thank my family, especially Bill and Jerry, who not only graciously prepared countless meals for me, but listened endlessly to my conversations about goats and parasites through many of those meals.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>Introduction to <em>Haemonchus contortus</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Haemonchus</em> life cycle and behavior</td>
<td>2</td>
</tr>
<tr>
<td><em>Haemonchus</em> morphology and structures</td>
<td>5</td>
</tr>
<tr>
<td>Clinical importance and pathogenicity of <em>H. contortus</em></td>
<td>10</td>
</tr>
<tr>
<td>History of anthelmintic use and the development and diagnosis of resistance</td>
<td>11</td>
</tr>
<tr>
<td>Alternatives to drug use in grazing ruminants</td>
<td>18</td>
</tr>
<tr>
<td>Introduction to plant tannins</td>
<td>19</td>
</tr>
<tr>
<td>Experimental plant extracts used in this study</td>
<td>26</td>
</tr>
<tr>
<td>Conclusions</td>
<td>29</td>
</tr>
<tr>
<td>2 INTRODUCTION</td>
<td>31</td>
</tr>
<tr>
<td>3 MATERIALS AND METHODS</td>
<td>34</td>
</tr>
<tr>
<td>Parasite isolate</td>
<td>34</td>
</tr>
<tr>
<td>Condensed tannin extracts</td>
<td>34</td>
</tr>
<tr>
<td>Egg Hatch/Larval Development Assays</td>
<td>35</td>
</tr>
</tbody>
</table>
Larval Migration Inhibition Assay ................................................................. 37
Data analysis ........................................................................................................... 40

4 RESULTS ............................................................................................................. 44
Egg Hatch Assay ................................................................................................. 44
Larval Development Assay .................................................................................. 44
Larval Migration Inhibition Assay ........................................................................ 45
Parasite isolate and plate replicates ..................................................................... 45

5 DISCUSSION ...................................................................................................... 51

REFERENCES ..................................................................................................... 58
LIST OF TABLES

Table 1.1: Percent condensed tannin present in various trees and plants………………………… 21
Table 3.1: Summary of assays performed................................................................................ 42
Table 4.1: 1-way ANOVA results for assays......................................................................... 47
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Light microscopy image of adult female <em>H. contortus</em></td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Scanning electron microscopy image of adult female <em>H. contortus</em></td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Light microscopy image of adult male <em>H. contortus</em> with bursa and spicules</td>
<td>7</td>
</tr>
<tr>
<td>1.4</td>
<td>Light microscopy image of adult female <em>H. contortus</em> with vulvar flap</td>
<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>Polarized microscopy image of <em>H. contortus</em> eggs</td>
<td>8</td>
</tr>
<tr>
<td>1.6</td>
<td>Light microscopy image of 1st stage free living larvae of <em>H. contortus</em></td>
<td>8</td>
</tr>
<tr>
<td>1.7</td>
<td>Light microscopy image of 3rd stage larvae of <em>H. contortus</em></td>
<td>9</td>
</tr>
<tr>
<td>1.8</td>
<td>Scanning electron microscopy image of 3rd stage larvae of <em>H. contortus</em></td>
<td>9</td>
</tr>
<tr>
<td>1.9</td>
<td>Scanning electron microscopy image of 3rd stage larvae of <em>H. contortus</em></td>
<td>9</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic for the Larval Migration Inhibition Assay (LMIA)</td>
<td>43</td>
</tr>
<tr>
<td>4.1</td>
<td>Graph of thiabendazole for the Egg Hatch Assay (EHA)</td>
<td>46</td>
</tr>
<tr>
<td>4.2</td>
<td>Graphs of condensed tannin extracts for the EHA</td>
<td>46</td>
</tr>
<tr>
<td>4.3</td>
<td>Graph of thiabendazole for the Larval Development Assay (LDA)</td>
<td>48</td>
</tr>
<tr>
<td>4.4</td>
<td>Graphs of condensed tannin extracts for the LDA</td>
<td>48</td>
</tr>
<tr>
<td>4.5</td>
<td>Graph of the EC$_{50}$ means of the dose responses for all 4 CT extracts for the LDA</td>
<td>49</td>
</tr>
<tr>
<td>4.6</td>
<td>Graph of levamisole for the LMIA</td>
<td>50</td>
</tr>
<tr>
<td>4.7</td>
<td>Graphs of condensed tannin extracts for the LMIA</td>
<td>50</td>
</tr>
</tbody>
</table>
CHAPTER I. LITERATURE REVIEW

Introduction to *Haemonchus contortus*

Virtually all grazing animals are infected with gastrointestinal nematode parasites (Zajac and Conboy 2006). Among ruminant hosts, nematodes of the family Trichostrongylidae are the most prevalent and important. Earliest details recorded about this parasite (Veglia 1915) credit Rudolphi in 1803 for the initial description. The most common and important species of trichostrongyles of small ruminants include *Haemonchus contortus, Trichostrongylus colubriformis, Teladorsagia circumcincta*. However, throughout the warm regions of the world, *H. contortus* is by far the most economically important and pathogenic parasite of domestic livestock, affecting sheep, goats, cattle, camelids and many wild ruminants (Craig 1986; Olsen 1986). Some small ruminants acquire little immunity to *Haemonchus* (as well as other GIN’s) and remain susceptible throughout their lives to diseases caused by these parasites (Kaplan 2003). Infections with *H. contortus* occur in many countries throughout the world; but, the highest prevalence is seen in warm, moist temperate and tropical regions (Olsen 1986).

The common name for *H. contortus* is the “barber pole worm” due to its appearance in the abomasum. The blood-filled intestine of female *Haemonchus* wraps around the white egg-filled uterus yielding an appearance similar to a barber pole. The classification of this parasite is as follows (Chitwood and Chitwood 1950):

Kingdom – Animalia

Phylum – Nematoda

Class – Secernentea

Order – Rhabditia
Suborder – Strongylina

Superfamily – Trichostrongyloidea

Family – Trichostrongylidae

Genus – Haemonchus (Cobb, 1898)

Species – *Haemonchus contortus* (Rudolphi, 1803)

*Haemonchus* life cycle and behavior

This parasite has a direct life cycle, meaning no intermediate host is required for its development. Adult male and female worms live and mate in the abomasum of ruminant animals. The female worms are extremely fecund and a single animal can shed millions of eggs per day onto a pasture. This can result in heavy pasture contamination because billions of eggs may be shed onto the pasture during the life cycle (Kaplan 2003), which is approximately 21 days. Also, infective larvae can survive months on pasture during favorable weather conditions. Eggs in the early morula stage pass out of the host with the feces. First stage larvae hatch from the eggs within 24 to 48 hours and feed on bacteria present in the feces. These free-living larvae molt and the 2\textsuperscript{nd} stage larvae continue to feed on bacteria as they develop into non-feeding infective 3\textsuperscript{rd} stage larvae. The 3\textsuperscript{rd} stage larvae retain the 2\textsuperscript{nd} stage cuticle as a protective sheath to yield a double cuticle. Third stage larvae are protected from adverse environmental conditions by this double cuticle, but can no longer feed and so must survive on stored energy reserves (Olsen 1986). The host becomes infected by ingesting the 3\textsuperscript{rd} stage larvae while grazing. Exsheathment of the 3\textsuperscript{rd} stage larvae occurs in the rumen after ingestion and is triggered by the high pH of the rumenal fluid. There, the infective larva rapidly shed the L\textsubscript{2} cuticle through a process in which a circular region of cuticle near the anterior end is digested releasing the
cuticular cap and allowing an opening for the L₃ to escape (Gamble, Lichtenfels et al. 1989). The young worms pass into the abomasum where they burrow into the mucosa, mostly at the level of the gastric pits. After a short period of feeding and growth, they undergo another molt, and the 4th stage larvae return to the surface of the mucosa. The final molt to the 5th stage occurs in the abomasal lumen and the worms mature into adults. Egg production commences when the sexually mature worms mate (Olsen 1986). This begins the cycle again with the eggs passing out of the host in the feces.

Parasitic nematodes have been very successful in adapting to different conditions to ensure their survival. An important adaptation of *H. contortus* is the ability to enter into a
dormant state known as hypobiosis, or arrested development. Hypobiosis takes place inside the abomasum of the host as the result of an environmental signal received by the immature stage of the parasite while on pasture. Because adult worms have a limited life span, this phenomenon helps to ensure continued existence of the parasite through harsh weather conditions. When conditions become favorable again, parasite development resumes. In regions with cool, temperate climates with pronounced seasonal changes, larvae will enter this state of arrested development when the cooler temperatures of autumn signal the approach of winter, a season threatening to free-living larvae on pastures. Development resumes in late winter or early spring when conditions become favorable again (Johnstone 2000). Although these larvae do little damage to their hosts during hypobiosis because they do not feed, there are serious consequences related to this phenomenon. When development of the hypobiotic parasites resume, large numbers of larvae will simultaneously mature into adults and start producing eggs. This leads to heavily contaminated pastures in the spring months which coincide with the host breeding season. Thus, when young susceptible animals are at greater risk for parasitic infections, they begin grazing the contaminated pastures. These infections not only affect the health of the young animals, but contribute even further to pasture contamination. This is often referred to as the “spring rise” (Michel 1974). Hypobiosis may also be seen in warm temperate, tropical or subtropical regions during summers and/or the dry season. Higher numbers of hypobiotic larvae have been found in animals during the dry months in these regions (Gatongi, Prichard et al. 1998), which ensures the parasite’s survival through these adverse dry conditions. The factors contributing to hypobiosis are complicated, to say the least, and are poorly understood, but it is clear that different seasonal and environmental patterns influence this event (Langrová, Makovcová et al. 2008).
Another successful adaptation of *H. contortus* according to Olsen (1986) is that during the infective stage some larvae may ascend the blades of grass during the twilight hours when dew is present or on dim rainy days, which makes host ingestion of the parasite more optimal. Other studies have demonstrated that the amount of rain and/or humidity, as well as, temperatures contribute greatly to the effect of larval migration on pastures (Skinner and Todd 1980; Dijk, David et al. 2008). In spite of different seasonal and environmental patterns, it is clear that considerable numbers of infective larvae are present on the upper regions of the grass, which are the areas most likely consumed by the grazing animals (Silva, Amarante et al. 2008).

*Haemonchus* morphology and structures

The adult males measure approximately 13 ± 2 mm in length while females are generally 19 ± 6 mm long (Lichtenfels, Pilitt et al. 1994). As mentioned previously, the uterus of the female worm spirals around the blood-filled intestine, giving rise to the so-called barber pole appearance (Figure 1.1). This parasite has several unique and interesting morphological features. Earliest descriptions by Veglia in 1915 include a buccal lancet structure on the anterior portion of the adult parasite. The presence of the buccal lancet was confirmed when electron microscopy studies revealed additional details about this structure (Weise 1977). A more recent electron micrograph confirmed an aperture in this lancet structure (Howell 2007) where blood is ingested by the parasite (Figure 1.2). Other morphological features present on the adults include lappets around the anterior opening and cervical papillae, which are located approximately 100 µm from the anterior of the worm (Howell 2007). The male worm has a copulatory organ called a bursa with asymmetrical dorsal rays and short, wedge-shaped spicules (Figure 1.3). The spicules are the main structure used to identify the various male species within the *Haemonchus* family.
There are spike-like structures called barbs present on the spicules that also aid in identification of the different species of *Haemonchus* (Lichtenfels, Pilitt et al. 1994). The female worm has a structure located about a quarter body length from the tail called the pars ejectrix by Veglia (1915). This structure has more recently been described as the vulva and may or may not be guarded by a vulvar flap (Figure 1.4). These flaps have variously shaped cuticular inflations (Bowman 2003) which are often used to identify different female species of *Haemonchus* (Lichtenfels, Pilitt et al. 1994).

The oval eggs of this parasite are approximately 70 µm long by 40 µm wide (Figure 1.5) with a thick protective shell. A first stage (L₁) larva (Figure 1.6) hatches from the eggs that have been deposited on pasture in the feces and continue the life cycle when conditions are favorable. When the larvae reach the infective stage (L₃) they are approximately 650 – 700 µm in length (Figure 1.7). Features that are present on the L₃ include a small, round sensory structure on the anterior portion of the worm called the amphid (Figure 1.8) and a long ribbon-like structure extending the length of the worm called the lateral alae (Lichtenfels, Gamble et al. 1990). Additional structures present on the anterior of the L₃ are the prominent lappets, which are arranged in a star-shaped pattern around the anterior opening (Figure 1.9).
Figure 1.1 - Light microscopy image of an adult female *H. contortus*. This demonstrates the explanation of the nickname “barber pole worm”.

Figure 1.2 - Scanning electron microscopy image of an adult female *H. contortus*. This image shows the aperture of the lancet structure.

Figure 1.3 - Light microscopy image of the adult male *H. contortus* showing bursa (A) and spicules (B).
Figure 1.4 - Light microscopy image of the vulvar flap (A) of an adult female *H. contortus*.

Figure 1.5 - Polarized microscopy image of *H. contortus* eggs. This image demonstrates the thick protective shell.

Figure 1.6 - Light microscopy image of the 1st stage free-living larvae of *H. contortus* shortly after hatching from the egg.
Figure 1.7 - Light microscopy image of the 3\textsuperscript{rd} stage larvae of \textit{H. contortus} still encased in the protective cuticle of the 2\textsuperscript{nd} stage larvae.

Figure 1.8 – Scanning electron microscopy image of the 3\textsuperscript{rd} stage larvae of \textit{H. contortus}. The small round amphid structure (A) is shown at the anterior of the worm.

Figure 1.9 - Scanning electron microscopy image of the 3\textsuperscript{rd} stage larvae of \textit{H. contortus} demonstrating the lappet structures (A) at the anterior of the worm.
Clinical importance and pathogenicity of *H. contortus*

Due to the increasing cultural diversity of the population in the United States and the demand for products derived from sheep and goats, small ruminant production has increased substantially in recent years (USDA 2009). Goat production is an attractive enterprise for farmers because of the low cost of breeding stock, high reproduction rates and their ability to thrive on native pastures and brush (Glimp 1995). In addition, sheep production has also risen in the southern United States in part because of increased availability of hair sheep, which are well adapted to southern climates (Burke 2009). Sheep and goat production is an important venture in many developing countries for the same reasons. However, infections with *H. contortus* are a major hindrance to small ruminant production. Large economic losses can result from reduced weight gains, reduced milk production in dairy animals, suppressed wool production, decreased quality of wool and reduced animal productivity in general (Mehlhorn 2008).

At peak infections, *H. contortus* acquired naturally by grazing animals may remove a fifth of the circulating erythrocyte volume per day from lambs and on average one tenth of the volume over the course of nonfatal infections lasting a few months (Georgi and Georgi 1990). The pathogenic effects result from the inability of the host to compensate for blood loss. If the blood loss is small and the host can adequately compensate for the loss, no measurable illness occurs. However, if the rate of blood loss exceeds the host’s hematopoietic capacity a progressive anemia leads to death. A hematocrit reading that is < 15% (normal mean = 28% for goats) is generally accompanied by extreme weakness, shortness of breath and listless behavior. Classical signs of haemonchosis are pale mucous membranes due to the anemia and submandibular edema (“bottle jaw”) due to severe protein loss. The animal’s appetite is normally good and weight loss is often minimal in acute outbreaks. Feces are frequently well
formed; however, diarrhea may occur with heavy infections or in combination with other parasitic infections. High fecal egg counts approaching 10,000 eggs per gram are typical of haemonchosis.

Haemonchosis may be classified as hyperacute, acute, or chronic. The hyperacute form occurs in animals exposed over a short period of time to thousands of parasites. Although it is rare, death may occur in this form of the disease from blood loss within 1 week of heavy infection without significant signs. The acute disease is characterized by severe anemia accompanied by generalized edema and dark feces. Animals lose weight, are weak and lethargic, lose wool, and have reduced milk production. Chronic haemonchosis may last for several months and is characterized by low worm burdens and progressive weight loss. Animals will be malnourished and iron and protein stores will become depleted (Mehlhorn 2008). This condition will become aggravated by poor quality grazing often seen in tropical regions. Young animals, older animals and females near parturition are most vulnerable to serious infections.

**History of anthelmintic use and the development and diagnosis of resistance**

Due to the severity of clinical disease for animals infected with *H. contortus*, anthelmintic treatments have routinely been used in an attempt to control these parasitic infections. Anthelmintics are drugs that kill parasitic helminths. However, overuse and misuse of anthelmintics, improper pasture management, and inadequate biosecurity when transferring infected animals from one farm to another has contributed to widespread anthelmintic resistance (Howell, Burke et al. 2008). Anthelmintic resistance is defined as a decline in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug, (Prichard, Hall et al. 1980; Wolstenholme, Fairweather et al. 2004). Many parasite species of multiple
animal hosts, as well as humans, have developed resistance to anthelmintic drugs (Sangster and Gill 1999). Resistance was first reported in the United States in the mid 1950’s in sheep, followed by reports of resistance in Australia a few years later (Prichard, Hall et al. 1980). Since that time resistance in parasitic nematodes of small ruminants has been documented worldwide (Waller 1994; Waller 1997). Now widespread reports of multiple-drug resistance has made veterinarians, producers and researchers aware of this global threat to small ruminant production (Waller 1999; Kaplan 2004). A high prevalence of multiple-drug resistant parasites were reported in a study involving goats in Georgia in 2003 (Mortensen, Williamson et al. 2003). Additionally, a more recent study including both sheep and goats farms throughout the southeastern region of the United States demonstrated total anthelmintic failure of H. contortus on 17% of the surveyed farms (Howell, Burke et al. 2008). These results demonstrate the severity of this problem for sheep/goat producers in the southern United States. As multiple-drug resistance is a worldwide trend, it is quite likely that similar problems exist throughout most regions of the United States. There are three broad spectrum classes of anthelmintics used for the control of GIN: benzimidazoles, imidazothiazoles/tetrahydropyrimidines and macrocyclic lactones. Benzimidazoles, the first of these drug classes that was developed, are commonly used because they are quite broad spectrum, safe and inexpensive (Brown, Matzuk et al. 1961; McKellar and Scott 1990). These drugs bind to the cytoskeletal protein tubulin, blocking the formation of the microtubule, which is an essential component of all eukaryotic cells (Lacey 1988; Sangster and Dobson 2002). Microtubules play an important role in cell division, but because benzimidazoles have a much higher affinity for nematode tubulin versus mammalian tubulin, there is selective activity against parasites (Bowman 2003). Thiabendazole was introduced in 1961 as the first benzimidazole anthelmintic licensed for use with sheep (Brown,
Matzuk et al. 1961). This drug was rapidly accepted because of the high efficacy against nematodes and low toxicity with animals (Kaplan 2004), but resistance to thiabendazole was reported in sheep to *H. contortus* within a few years (Conway 1964). Fenbendazole, oxfendazole, oxibendazole and albendazole are other benzimidazole drugs that have been developed since thiabendazole, however, albendazole is the newest and most potent. Albendazole has the potential to be teratogenic when used in higher dose concentrations than recommended during the early period of pregnancy, thus care should be taken when administering this drug during the first few weeks of pregnancy (Bowman 2003).

The discovery of the first drugs in the imidazothiazoles/tetrahydropyrimidines class was in the mid 1960’s. Of the imidazothiazoles, only levamisole is still readily available. There are several variations of pyrantel and morantel compounds used today in the tetrahydropyrimidine family. All of these drugs act as nicotinic agonists that disrupt the neuromuscular system causing spasmodic contraction and subsequent paralysis of the worm (Coles, East et al. 1975; Sangster and Dobson 2002), but these drugs should be used with caution. There have been occasional toxicity reports with levamisole given to sheep, even at the recommended dosages, and withholding times for slaughter must be adhered to. Also, ruminants are known to rapidly metabolize the compounds of the tetrahydropyrimidine family thus they require higher dosages that other monogastric animals (Campbell and Rew 1986).

The macrocyclic lactone drugs, first introduced in the 1980’s, are the newest class of anthelmintics and have revolutionized the treatment of parasitic disease. They are highly effective at low doses, are very safe, and provide broad-spectrum activity against nematodes as well as arthropods. This family of compounds are all similar in that they are produced by streptomycete microorganisms, and have large macrocyclic structures. These drugs bind with
high affinity to the glutamate-gated chloride channel and trigger a chloride influx which 
hyperpolarizes the neurons of the parasite and prevents initiation of normal action potentials 
(Arena, Liu et al. 1995; Shoop, Mrozik et al. 1995). The net effect is paralysis and death of the 
parasite (Bowman 2003). There are several drugs available in the avermectin-milbemycin class 
of anthelmintics including ivermectin, doramectin, eprinomectin, selamectin and moxidectin. 
Ivermectin was the first commercially available drug in this class but was shown to produce 
severe adverse reactions to some genetic lines of the canine collie breed if administered at 
typical therapeutic dosages (Pulliam, Seward et al. 1985). Moxidectin is the most potent 
nematocide in this class and has shown to produce high in vivo efficacy against ivermectin-
resistant H. contortus in sheep (Craig, Hatfield et al. 1992). However, a recent study done in 
Australia indicated that moxidectin use was associated with a higher prevalence of resistance to 
avermectin-milbemycin anthelmintics on sheep farms (Rendell, Rentsch et al. 2006).
Anthelmintic resistance is genetically based (Sangster and Dobson 2002); though, there are 
numerous factors that influence the rate with which resistance develops. Some of these factors 
are related to the pharmacokinetics of the anthelmintics, while others are related to the parasite 
biology, or environmental factors. Some drug related factors contributing to resistance include 
the mode of action, frequency, timing, dose rate, method of delivery, persistence of the drugs, 
and specific rates of metabolism for different hosts (Dobson, LeJambre et al. 1996). The genetic 
diversity in different parasite isolates and the frequency of resistant alleles also contribute to 
resistance (Prichard 2001). Other elements include biological factors such as, species, sex and 
stage of the parasite, generation time and fecundity, rate of establishment and life expectancy, 
and geographic variants of different species (Sangster and Gill 1999). Additionally, the 
untreated parasite population or “refugia” present on the pasture at the time of treatment, as well
as, environmental conditions all complicate the anthelmintic resistance dilemma (Sangster and Dobson 2002).

There have been a number of diagnostic methods developed to detect anthelmintic resistance. The traditional and definitive test for resistance is the controlled efficacy test (“drench and slaughter trial”), where worm-free hosts are artificially infected, treated with a particular drug then slaughtered to determine the number of worms which survived treatment in comparison to untreated controls. This procedure is the gold standard for detecting clinical resistance if it is properly conducted (Sangster and Dobson 2002), despite the fact that obvious drawbacks are associated with animal slaughter. Besides being very expensive and difficult to perform, this method is impractical for routine resistance testing. As a result, the fecal egg count reduction test (FECRT), which is suitable for all types of anthelmintics (Taylor, Hunt et al. 2002), has become the most commonly used in vivo method for determining anthelmintic resistance. The FECRT involves determining fecal egg counts in animals that have and have not been treated with anthelmintics and calculating the percentage reduction in fecal egg count among the treated animals (Coles, Bauer et al. 1992). This method is the procedure of choice for field studies (Waller 1986), but this method is also labor intensive and expensive if multiple anthelmintics are evaluated because it requires testing a large number of animals (Waller 1997). Regardless of the limitations of these experiments, they nevertheless have been the benchmark against which in vitro tests can be validated (Sangster and Gill 1999).

As a result of the shortcomings of in vivo testing, various in vitro bioassays have been developed as alternatives for diagnosing resistance. These tests measure the effects of anthelmintics on the inhibition of development in the egg and free-living larval stages (Lacey, Redwin et al. 1990; Gill, Redwin et al. 1995), or the impairment of worm motility (Wagland,
Jones et al. 1992). These bioassays provide several important advantages. They alleviate the need to sacrifice large numbers of animals, and they are cheaper and easier to perform than in vivo tests. Also, the efficacy of several anthelmintics can be analyzed at one time, and assays can be replicated to verify standardization (Dobson, LeJambre et al. 1996). Three in vitro bioassays are commonly used for resistance testing or for measuring the antiparasitic activity of novel compounds: the egg hatch assay (EHA), larval development assay (LDA) and larval migration inhibition assay (LMIA).

Research using the EHA in the mid-seventies demonstrated the ovicidal activity of benzimidazole anthelmintics (LeJambre 1976). Benzimidazole prevents egg embryonation and hatching, and this property can be used to detect benzimidazole resistance when nematode eggs are incubated in various concentrations of the drug. This test is fast, inexpensive to perform, and repeatable when a single species is involved (Prichard, Hall et al. 1980). One disadvantage with this assay, however, is the test must be performed on freshly collected eggs. Once aerobic metabolism occurs, the eggs are unaffected by benzimidazole anthelmintics. Since the initial development of this bioassay, a number of variations in the methodology have been described.

The LDA was first described in the late 1980’s (Coles, Tritschler et al. 1988), however, the development of an agar-based LDA resulted in the first in vitro assay capable of detecting resistance to all three classes of anthelmintics simultaneously (Lacey, Redwin et al. 1990). This method was initially validated for use with benzimidazole and imidazothiazoles anthelmintics, and then subsequently validated for use with avermectin/milbemycin anthelmintics. These preliminary validations used various susceptible and resistant isolates of the small ruminant nematodes H. contortus and Trichostrongylus colubriformis. This methodology is based on the fact that the activity of anthelmintics can be measured on the free-living stages of a particular
parasite isolate by determining the dose response against a drug. One critical component of any
LDA assay is the requirement of a nutritive media for feeding the larvae once they hatch so
development will continue for those larvae not inhibited by the compounds being tested. Again,
there have been several variations of this test described in the literature (Hubert and Kerboeuf

The LMIA measures changes in larval motility and was found to be useful for screening
anthelmintics which interfere with the neurophysiology or neuromuscular coordination of the
parasite (Wagland, Jones et al. 1992). The first description of an assay to measure motility was
in 1983 (Douch, Harrison et al. 1983). In this assay, mucus was mixed with exsheathed
*Trichostrongylus colubriformis* larvae, incorporated into agar blocks, and the proportion of
larvae that migrated out of the agar blocks was counted (Wagland, Jones et al. 1992). However,
several attempts to replicate this procedure were unsuccessful. A few modifications to this
procedure were described in the early 1990’s but it was the procedure described by Wagland,
Jones et al. (1992) that made the assay amenable to testing large numbers of samples. This
procedure utilized a 48 well plate with “homemade” 20 µm mesh sieves made from open ended
glass tubes. The mesh tubes were placed into the wells containing the testing fluid such that they
were suspended 2 mm above the bottom by a rubber “o” ring. This allowed the larvae to migrate
through the mesh into the fluid. Since that time there have been other modifications described
including the use of a commercially developed multiscreen mesh plate (Rabel, McGregor et al.
1994; Kotze, LeJambre et al. 2006). These assays have been used to measure the effects of
anthelmintics known to cause paralysis, and in recent years there has been an increase in
research utilizing these methods for investigating the effects of plant compounds on nematodes,
as well.
Alternatives to drug use in grazing ruminants

Given the current state of documented multi-drug resistance, and in some cases total anthelmintic failure, it is clear that small ruminant producers and veterinarians can no longer rely solely on anthelmintics for parasite control. There are a limited number of anthelmintics currently on the market today, and the few remaining effective drugs must be used carefully to extend their usefulness. With that goal in mind, it is imperative that non-anthelmintic approaches are found to help alleviate the burden of parasite control. An integral part of routine parasite control should include the use of sound principles in pasture management and maintaining strict quarantine procedures for new herd additions to prevent introduction of resistant parasites from newly acquired animals (Fleming, Craig et al. 2006). Routine testing of anthelmintic efficacy with the fecal egg count reduction test or the larval development assay should also be a part of herd management. In addition, the concepts of “smart drenching” should be employed. The term smart drenching refers to a number of strategies designed to maximize the effectiveness of anthelmintics while reducing the development of resistance (Kaplan 2003).

One component of smart drenching involves selectively treating only those animals that require anthelmintic treatment. It has been recognized for years that parasite populations are over-dispersed in naturally infected herds or flocks of grazing livestock (Waller 1999). This means that while the majority of the animals will have limited numbers of parasites, a few of the animals will have a disproportionately high parasite burden. Since the high egg shedders are responsible for most of the pasture contamination and subsequent reinfection of the herd or flock, identification and treatment of those specific animals could greatly reduce anthelmintic treatments. An additional advantage of this treatment scheme is limited anthelmintic exposure in the refugia. Since refugia are considered an important factor in limiting resistance (Wyk 2001),
allowing the refugia to escape selection for resistance is a significant benefit. The FAMACHA method aids in accomplishing this goal because it has proven to be effective in identifying animals most in need of treatment from heavy *H. contortus* burdens. The FAMACHA method is a novel system that was developed in South Africa for identifying sheep that are anemic based on the color of the lower conjunctiva (Malan, Wyk et al. 2001). A laminated color chart categorizes a range of scores based on different levels of anemia which were validated by hematocrit levels and fecal egg counts. The chart is compared to the color of the ocular membranes of the animals resulting in a FAMACHA score corresponding to the anemia state of the animal. The method, validated in South Africa, has subsequently been validated in the United States (Wyk and Bath 2002; Kaplan, Burke et al. 2004; Burke, Kaplan et al. 2007). Although FAMACHA sounds easy to use, it is critical to properly train individuals who intend to utilize this method in its limitations (Fleming, Craig et al. 2006).

Additional smart drenching techniques include monitoring changes in body weight, body condition and milk yields of dairy goats. These parameters can assist in making selective treatment decisions as well (Wyk, Hoste et al. 2006). Other novel alternatives to anthelmintic use that have shown promise in sheep and goats include the use of copper oxide wire particles (Bang, Familton et al. 1990; Burke, Miller et al. 2004), administering nematode-trapping fungi (Terrill, Larsen et al. 2004; Larsen 2006) and the feeding of plants or forages that contain condensed tannins (Min and Hart 2003; Shaik, Terrill et al. 2004).

**Introduction to plant tannins**

Tannins are a type of phenolic compounds of high molecular weight that are distributed throughout the plant kingdom. These plant polyphenols are usually water soluble and are
capable of precipitating proteins. The capacity of tannins to bind proteins has resulted in the use of these compounds in tanning animal hides into leather for centuries; hence the term “tannin” when referring to these compounds (Cannas 2008). The astringency from some tannins is a defense mechanism against pathogens and herbivores, and causes the dry, puckery feeling in the mouth associated with red wines or unripened fruit. Tannins may have a major impact on improved animal nutrition because of their ability to form these complexes with various types of molecules including carbohydrates and proteins. However, some tannins may also have a toxic or anti-nutritional effect when ingested depending on the animal’s tolerance level and the amount and type of tannin present in the plant. There are 2 types of tannins, hydrolyzable (HT) and condensed (CT). Hydrolyzable tannins contain a carbohydrate (usually D-glucose) as a center molecule and are hydrolyzed by weak acids or bases to produce carbohydrate and phenolic acids (Haslam 1989).

Condensed tannins (proanthocyanidins) are the most common type of tannin and are especially prominent in forage legumes, trees and shrubs (Min, Barry et al. 2003). Table 1.1 provides some examples of the CT concentrations present in various plants or trees. These tannins normally occur in cell vacuoles (Mosjidis, Peterson et al. 1990), and are complexes of oligomers and polymers of many flavonoid units joined by carbon-carbon bonds (Hagerman and Butler 1989). The term, proanthocyanidins, is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins upon heating proanthocyanidins in acidic alcohol solutions. The anthocyanidins pigments are responsible for the astringent taste in fruit juices and wines, as well as, the wide array of pink, scarlet, red, violet and blue colors in flowers, leaves and fruits. Depending on their chemical structure and the degree of polymerization, CT may or may not be soluble in aqueous organic solvents (Cannas 2008).
Table 1.1 – Percent condensed tannin present in various trees and plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>% CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sericea-AU Grazer</td>
<td>16.7</td>
</tr>
<tr>
<td>Sericea-AU Donnelly</td>
<td>14.9</td>
</tr>
<tr>
<td>Sericea-Wild Type</td>
<td>15.1</td>
</tr>
<tr>
<td>Almond Hulls</td>
<td>6</td>
</tr>
<tr>
<td>Black Locust</td>
<td>8.5</td>
</tr>
<tr>
<td>Black Wattle</td>
<td>10</td>
</tr>
<tr>
<td>Black Willow</td>
<td>3.3</td>
</tr>
<tr>
<td>Cranberry</td>
<td>8.7</td>
</tr>
<tr>
<td>Honeysuckle</td>
<td>0.4</td>
</tr>
<tr>
<td>Honey Locust</td>
<td>6.5</td>
</tr>
<tr>
<td>Mimosa</td>
<td>0.9</td>
</tr>
<tr>
<td>Shin Oak</td>
<td>3.9</td>
</tr>
<tr>
<td>Smilax</td>
<td>3</td>
</tr>
<tr>
<td>Sumac</td>
<td>0.3</td>
</tr>
<tr>
<td>Weeping Willow</td>
<td>0.6</td>
</tr>
<tr>
<td>Yucca cactus</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Considerable research relating to CT has been done for decades ranging from the antifungal, algicidal and antimicrobial properties present (Simeray, Chaumont et al. 1982; Ayoub and Yankov 1985; Hayashi, Funatogawa et al. 2008) to exploring the use of tannins as an additive in cooking oil to prolong the shelf life at frying temperatures (Sánchez-Muniz, Zulim Botega et al. 2007). However, the vast majority of research has been done on the nutritional value of CT and, more recently, as an anti-parasitic compound. Early research on animal nutrition has shown decreased bloat and improved animal production when ruminants were fed plant tannins (Reid, Ulyatt et al. 1974; Waghorn, Jones et al. 1990), though a number of investigations have reported detrimental effects of feeding tannins to ruminants, as well as,
beneficial effects (Terrill, Douglas et al. 1992; Schofield, Mbugua et al. 2001; Min, Barry et al.
2003; Min, Pomroy et al. 2004; Animut, Puchala et al. 2008; McSweeney, Collins et al. 2008;
Waghorn 2008).

Some issues regarding the negative effects of feeding some tannin forages include
toxicity to rumen microorganisms. However, this is balanced out by a beneficial change in the
microfloral and the lowering of fractional absorption of amino-acids from the intestine (Waghorn
2008). Also, a recent study investigating the effects of long-term feeding of low quality feeds
containing CT indicated reduced villus height and chronic inflammation in the intestine, as well
as, nephritis of the kidneys and hemorrhagic changes of the liver (Mahgoub, Kadim et al. 2008).
Another possible harmful effect due to the ingestion of CT is decreased feed intake and nutrition
leading to reduced weigh gains and animal productivity. These effects vary considerably
depending on the type of tannin ingested and the animal’s tolerance to the tannin. The
astringency, which is the sensation caused by the formation of complexes between salivary
glycoproteins and tannins, may also cause decreased palatability, as well as, feeding over-mature
forage. Additionally, the form in which the tannin forage is fed may influence the feed intake.
Dried forages rather than fresh or freshly frozen may be more palatable because the tannins are
more polymerized, resulting in fewer free hydroxyls available for binding proteins (Cannas
2008). Using a compound, such as polyethylene glycol (PEG), with a higher affinity to tannins
than proteins may also increase feed palatability and digestibility resulting in higher animal
productivity. Polyethylene glycol is relatively inexpensive, and selectively binds to CT. This
prevents the CT from binding to the plant proteins in the rumen (Min, Barry et al. 2003). It has
been suggested that PEG can be added to the diet or sprayed onto forages to improve feed
palatability. The utilization of this chemical to inactivate tannins has been very useful in
demonstrating the activity of tannins in many research studies as well (Min, Barry et al. 2003; Bahauaud, Martinez-Ortiz de Montellano et al. 2006; Brunet, Aufrere et al. 2007).

The beneficial effects of feeding CT are well recognized for their ability to complex with soluble rumen proteins thereby reducing degradation of protein to ammonia in the rumen. The host/CT interaction is very complex and begins in the oral cavity of the animal during mastication. The act of chewing the plant ruptures the cells and, when swallowed, exposes the proteins and carbohydrates to the tannins. As mentioned earlier, there is a strong interaction between tannins and these compounds based on hydrophobic and hydrogen bonding (Haslam 1989). The protein and the CT interactions bond in a pH-reversible manner which is influenced by the molecular weights and structures of these compounds. The reactions can be used to reduce the degradation of forage proteins in the rumen without reducing the microbial protein (Min, Barry et al. 2003). When ruminants are fed fresh forage diets high in nitrogen, carbohydrate digestion in the rumen is efficient, but most of the proteins are rapidly solubilized. Only about 60% of the nitrogen is released in the rumen, therefore, there are large nitrogen losses when ammonia absorption occurs from the rumen. This, in turn, results in only about 30% of the nitrogen escaping to the small intestine for absorption (MacRae and Ulyatt 1973; Min, McNabb et al. 2000). However, the binding from the CT protects the protein from rumen degradation and allows more dietary protein to flow into the small intestine increasing the supply of digestible protein to the host (Barry and McNabb 1999; Min, McNabb et al. 2000; Min, Attwood et al. 2002).

This improvement in nutrition has lead to a number of benefits including increased weight gain and immunity, enhanced wool and milk production, and heightened efficiency in reproduction (Waghorn 2008). Furthermore, the discovery of decreased gastrointestinal
parasitism, which has been demonstrated in ruminants fed CT in a number of studies worldwide, has been a tremendous benefit (Niezen, Waghorn et al. 1993; Molan, Waghorn et al. 2000; Athanasiadou, Kyriazakis et al. 2001; Paolini, Bergeaud et al. 2003; Min, Pomroy et al. 2004; Min, Hart et al. 2005; Shaik, Terrill et al. 2006). The mechanisms that relate to the reduction in parasites are not clearly understood, but is likely a combination of an indirect effect from improved animal nutrition and immunity from the ingestion of CT, as well as, a direct anthelmintic effect of the CT against the parasites themselves (Niezen, Waghorn et al. 1995; Barry and McNabb 1999; Molan, Waghorn et al. 2000; Athanasiadou, Kyriazakis et al. 2001).

The origins of anthelmintic medications are from plants or plant extracts such as garlic, aloes, cloves, cucurbit seeds and castor oil. Generally, these concoctions had low anthelmintic effect and were abandoned when safer, more effective synthetic anthelmintic compounds became available (Waller 1999). However, due to the severity of anthelmintic resistance, there has been resurgence in traditional health practices in the past decade. This renewed interest in traditional medicine has amplified research in the use of plants, grasses, forage crops, or herbal remedies that appear to act as vermifuges (Hammond, Fielding et al. 1997). In temperate regions of the world several leguminous plants have been reported to have anthelmintic properties (Anderson, Barger et al. 1987), and research reported in the mid 90’s has shown that forages containing CT provide sheep with the ability to withstand helminth infections (Niezen, Waghorn et al. 1995). Since that time there have been a number of studies worldwide that have shown a reduction in parasite burdens in vivo (when animals were fed forages containing CT) or using different in vitro methods (utilizing CT extracts or plants) (Niezen, Robertson et al. 1998; Athanasiadou, Kyriazakis et al. 2001; Butter, Dawson et al. 2001; Molan, Waghorn et al. 2002; Paolini, Bergeaud et al. 2003; Paolini, Frayssines et al. 2003; Barrau, Fabre et al. 2005; Bahuaud,
Martinez-Ortiz de Montellano et al. 2006; Ademola, Fagbemi et al. 2007; Brunet, Jackson et al. 2008; Moore, Terrill et al. 2008). The first report of anthelmintic activity in CT forages was an *in vivo* study published in a 1993 proceedings on animal health in New Zealand (Niezen, Waghorn et al. 1993) which demonstrated increased production in lambs infected with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* and less reliance on anthelmintics with the use of the CT forages *Lotus pedunculatus* and *Hedysarum coronarium* as compared to the non CT forages ryegrass and lucerne. Since then different *in vitro* studies have utilized extracts from trees or plants because of prior reports of medicinal effects or they were known to contain CT. The trees or plants include: *Lotus pedunculatus, Lotus corniculatus, Hedysarum coronarium, Onobrychis viciifolia, Dorycnium rectum, Dorycnium pentaphyllum, Rumex obtusifolius, Schinopsis balansae, Acacia mearnsii, Acacia gaumeri, Lespedeza cuneata, Castanea sativa, Pinus sylvestris, Erica erigena, Sarothamnus scoparius, Peltophotum africanum, Spigelia anthelmia, Prosopis cineraria,* and *Havardia albicans.* The majority of these *in vitro* studies utilized *Trichostrongylus colubriformis* or *Haemonchus contortus* parasites, however, other parasites studied have included *Teladorsagia circumcincta, Trichostrongylus vitrinus, Nippostrongylus brasiliensis, Trichinella spiralis, Heligmosomoides polygyrus,* or *Oesophagostomum columbianum.* Many of the studies utilized the LDA bioassay, or the LMIA procedure with L₃ larvae. A few studies evaluated the EHA, or simply observed adult worms in the plant extract solution to monitor toxicity effects. Although there have been many studies reporting the effects of plant extracts, no information was found in the literature of thorough evaluations validating any of these *in vitro* bioassays for use with plant extracts.
Experimental plant extracts used in this study

A. Sericea lespedeza (*Lespedeza cuneata*)

One forage with high levels of condensed tannins of particular interest in our laboratory is sericea lespedeza (*Lespedeza cuneata*), a perennial warm season legume. Specific anti-parasitic effects of sericea lespedeza in goats infected with *H. contortus* include reduced egg fecundity in female worms, decreased hatching of the eggs that are shed, and reduced numbers of adult worms in the abomasum and intestine (Min and Hart 2003; Min, Pomroy et al. 2004; Shaik, Terrill et al. 2004; Shaik, Terrill et al. 2006). Sericea lespedeza (Chinese bush clover) was first introduced into the United States in the late 19th century as a forage crop. The first sericea was coarse and stemmy, making it unpalatable to grazing animals. Most of the wild sericea around the pastures and grasslands of the southeast is of this type. However, long-term plant breeding for improved forage quality (mostly at Auburn University in Alabama), has lead to a more leafy and palatable type of sericea forage. The Auburn University variety of sericea (AU Grazer) has an average total condensed tannin content of 16.7 % as measured in several forage cuttings by the standard ether extraction method described by Terrill, (Terrill, Rowan et al. 1992) with some modifications (Wolfe, Terrill et al. 2008). This cultivar has numerous advantages as a forage crop. It is an economical, drought-resistant crop well adjusted to the hot climates of the southeast. It produces good forage yields, is resistant to diseases and insects, is tolerant to soil acidity compared to other legume plants, and is competitive with other grasses when mature. This plant does not require nitrogen fertilizer and very little phosphorus, potassium or lime. It has shown to be an excellent soil builder because it sheds leaves that add organic matter to the soil. One disadvantage of this plant is that it is sensitive to grazing, which limits its use as a pasture crop. However, AU Grazer tolerates close and frequent grazing more
readily than other sericea cultivars and can be used as a hay crop, as well as, a forage crop (Mosjidis 2001). This plant is widely adapted throughout the southeastern United States and could be grown in other regions throughout the world. For these reasons, the use of this plant to aid in parasite control could be extremely beneficial to producers worldwide.

B. Cranberry (*Vaccinium oxycoccus*)

The cranberry plant (*Vaccinium oxycoccus*) is from a group of evergreen dwarf shrubs or trailing vines found in acidic bogs in the cooler regions of the Northern Hemisphere. This berry, which is dark red when fully ripe, has an acidic, tart taste. The cranberry plant is a major commercial crop in some northern states and in Canada. Like CT from other plants, cranberries are abundant in phenolic acids, flavonoids, and antioxidant and nutrient properties (Dew, Day et al. 2005). Research has indicated that cranberries promote many health benefits ranging from anti-bacterial effects against oral disease and other bacterial infections, reduced risk of cardiovascular disease, and apoptosis of cancer cells (Puupponen-Pimiä, Nohynek et al. 2001; Vattem, Ghaedian et al. 2005; Seeram, Adams et al. 2006; Bodet, Grenier et al. 2008; Kresty, Howell et al. 2008; Watson 2008). These berries have moderate levels of vitamin C, manganese and dietary fiber, and ranks high among foods measured by Oxygen Radical Absorbance Capacity (ORAC), and the tannin content of cranberries is approximately 8.7% (Vinson, Su et al. 2001). Besides the known health advantages attributed to these berries, suggestions that this fruit may also have anti-parasitic properties make it a viable candidate for parasite control research.

C. Black Locust (*Robinia pseudoacacia*)

The Black Locust tree (*Robinia pseudoacacia*) is native to the southeastern United States, but has been cultivated in Europe, Asia and other temperate regions of the U.S. This tree is in
the subfamily Faboideae of the pea family. One of the benefits of this tree is an expansive root system, which takes up nitrogen from the atmosphere in the root nodules, and grows on poor soils. This tree is a major honey plant in the eastern U.S., and the leaves can be used as a maintenance fodder for livestock (Barman and Rai 2008). The average total condensed tannin content from several forage cutting was 8.51% as measured by the standard ether extraction method described by Terrill (1992) with some modifications described by Wolfe (2008). Since this tree is readily available in the southern U.S., and is known to contain CT, there is interest in determining if any anthelmintic activity is present.

D. Black Wattle (*Acacia mearnsii*)

Black Wattle (*Acacia mearnsii*), also called mimosa, is a fast growing leguminous tree native to Australia. These trees are considered a vast economic resource. The timber is used in housing construction, furniture production, paper pulp industry, fiber board manufacturing, and cellulose for rayon. The foliage and green pods are eaten by stock animals around the world, especially in draught years. The seeds have a high nutritional value and are good sources of protein, fat and carbohydrates. For these reasons, they have been introduced in many other countries for commercial purposes. There are a number of different species of this tree, each with certain beneficial uses. As with the *Robinia* species, Black Wattle has a nitrogen-fixing root system, which allows it to grow on poor soils. Large plantations of *Acacia mearnsii* are grown for the commercial tannin business because the bark is high in tannin content. The tannins are extracted by steaming or boiling the bark. The resulting liquid is evaporated off, leaving an extracted powder high in tannic acid. Besides tanning leather, this powder is also used in adhesives, dyes and pharmaceutical products among other things. Research has shown that CT from Black Wattle has strong anti-oxidation activity, antimicrobial effects, and is toxic
to *E. coli* (Yao, Lu et al. 2002; Smith, Imlay et al. 2003; Liu and Wang 2007). This extract was commercially prepared and stated to contain 67% tannic acid (lot # ME-HOU-734; North American Minerals Corporation, West Des Moines, IA). The fact that this is a readily available commercial tannin product, and that the leaves contains approximately 10% CT, has stimulated interest in exploring the possible anthelmintic properties of this tree.

**Conclusions**

*Haemonchus contortus* is the most clinically important parasite of small ruminants worldwide causing serious pathogenicity in heavily infected animals. Frequent treatments with anthelmintics in an attempt to control this parasite have lead to severe anthelmintic resistance. This is now a critical problem for the small ruminant industry in numerous countries. An added constraint for many farmers in developing nations is the cost of anthelmintics, which also can be an impediment to parasite control. Thus, cheaper, locally available alternatives to anthelmintics are also needed in developing countries. The awareness of these important issues has lead to increased research investigating non-chemical alternatives to parasite control. Several strategies have shown potential in helping alleviate some of the parasite burdens of these animals; however, one of the most promising has been the demonstration of anthelmintic properties of some forage plants containing high levels of CT. A number of *in vivo* studies have confirmed anthelmintic properties of these forages when fed to sheep and goats. Thus, the goal of this research project is to validate a bioassay method for identifying CT-containing plants with anthelmintic properties. Such an assay would serve as an excellent screening test to identify plants with antiparasitic properties that may be candidates for more detailed evaluation using *in vivo* trials. If plants indigenous to specific areas can be identified as having anthelmintic
properties, those plants might be useful adjuncts to parasite control in the context of an integrated parasite management strategy. Such an alternative to anthelmintic treatment should provide vast and long term benefits to the small ruminant industry.
CHAPTER 2. INTRODUCTION

Small ruminant production has increased substantially in recent years in the United States (USDA 2009) due to the demand for products derived from sheep and goats. Goat production is an attractive enterprise for farmers because of the low cost of breeding stock, high reproduction rates and their ability to thrive on native pastures and brush (Glimp 1995). In addition, sheep production has also risen in the southern United States (USDA 2009) in part because of increased availability of hair sheep, which are well adapted to southern climates (Burke 2009). Small ruminant production is an important venture in many developing countries, as well, for the same reasons. However, gastrointestinal nematode (GINs) infections, particularly *Haemonchus contortus*, are a major constraint to small ruminant production worldwide. Large economic losses can result from reduced weight gains, reduced milk production in dairy animals, suppressed wool production, decreased quality of wool and reduced animal productivity in general (Mehlhorn 2008).

Due to the severity of clinical disease for animals infected with *H. contortus*, anthelmintic treatments have routinely been used in an attempt to control these parasitic infections. However, overuse and misuse of anthelmintics, improper pasture management, and reduced biosecurity when transferring infected animals from one farm to another has contributed to widespread anthelmintic resistance (Prichard 1994; Howell, Burke et al. 2008). In the U.S., reports from Georgia have shown the prevalence of resistance reaching alarming levels in goats (Terrill, Kaplan et al. 2001; Mortensen, Williamson et al. 2003). In addition, multiple drug resistant worms have become an issue in many areas of the world which has elevated the problem of resistance (Miller and Craig 1996; Waller 1999; Kaplan 2004). A study including
both sheep and goat farms throughout the southeastern region of the United States has demonstrated total anthelmintic failure of *H. contortus* on 17% of the surveyed farms (Howell, Burke et al. 2008). These results demonstrate the severity of this problem for producers in the US, and are likely a worldwide trend.

There are several methods for detecting anthelmintic resistance. The principle *in vivo* method is the fecal egg count reduction test (FECRT). This test involves determining fecal egg counts in animals that have and have not been treated with anthelmintics and calculating the percentage reduction in fecal egg count among the treated animals (Coles, Bauer et al. 1992). This method is considered the “gold standard” for field studies (Waller 1986), but has several disadvantages. It is expensive and labor intensive due to handling animals multiple times for repeated sampling (Waller 1997; Craven, Bjorn et al. 1999; Ancheta, Dumilon et al. 2004), and is characterized by low precision and reproducibility due to inter-animal variation and the pharmacology of the drugs used (Sangster 1996; Sangster and Gill 1999). These limitations often make this a poor choice for resistance testing among producers.

As a result of these disadvantages, various *in vitro* bioassays have been developed that have made the diagnosis of resistance easier. These tests measure the effectiveness of anthelmintics on the inhibition of egg development, free-living larval stages (Lacey, Redwin et al. 1990; Gill, Redwin et al. 1995), or impairment of the motility of the worms (Wagland, Jones et al. 1992), and offer several important advantages. They alleviate the need to sacrifice large numbers of animals, are cheaper and easier to perform than *in vivo* tests, and the efficacy of several compounds can be analyzed at one time (Dobson, LeJambre et al. 1996). Three commonly used bioassays include the egg hatch assay (EHA), larval development assay (LDA) and larval migration inhibition assay (LMIA).
Because of the seriousness of anthelmintic resistance, alternatives to drug control are of vital importance. Research has shown that certain forages high in condensed tannins (CT) demonstrate anthelmintic activity, and appear to be a useful non-chemical adjunct to parasite control in small ruminants (Niezen, Waghorn et al. 1993; Molan, Waghorn et al. 2000; Athanasiadou, Kyriazakis et al. 2001; Paolini, Bergeaud et al. 2003; Min, Pomroy et al. 2004; Min, Hart et al. 2005; Shaik, Terrill et al. 2006). Therefore, the ultimate goal of this research project is to develop a method for identifying plants with anthelmintic properties that may be suitable for in vivo trials. The first step is to assess which plants may be potential candidates for future studies. This can be accomplished by estimating the antiparasitic effect using in vitro bioassays. These bioassays have previously been used to evaluate the antiparasitic effect against several species of trichostrongyle nematodes using CT plant extracts. However, no studies had validated these bioassays for use with CT plant compounds, and it is uncertain which ones are the most appropriate evaluation tools for this purpose. Consequently, the dose response characteristics and repeatability of 3 different in vitro bioassays will be assessed to determine which methods are the most suitable for measuring the effective concentration (EC$_{50}$) of these plant extracts. An isolate of H. contortus (UGA), which is known to be resistant to benzimidazoles, imidazothiazoles/tetrahydropyrimidines and macrocyclic lactones plus low resistant to moxidectin according to the DrenchRite® assay (Microbial Screening Technologies, Armidale, New South Wales, Australia), will be used in these studies. Sericea lespedeza (SL) is known to reduce parasite burdens in small ruminants, thus this extract will be used to initially validate the bioassays. Once confidence is gained in the bioassays, further testing with SL in conjunction with additional CT extracts will be performed to assess the effectiveness of CT extracts, and determine if any of these extracts may be suitable for future in vivo trials.
CHAPTER 3. MATERIALS AND METHODS

Parasite isolate

An isolate of *H. contortus* originating from sheep at the University of Georgia research farm was maintained by periodic passage through worm-free goats. The parasite isolate was confirmed to be resistant to benzimidazoles, imidazothiazoles/tetrahydropyrimidines, and macrocyclic lactones and low resistant to moxidectin using the DrenchRite® larval development assay on 2 separate occasions. The goats, identified as Red, Black, Blue, and Green, were infected at varying intervals, resulting in each animal having an assortment of worms of different ages. Feces containing *H. contortus* eggs were collected from these goats over the course of several months in canvas bags harnessed to the animals. The fecal pellets were used for egg isolations and coprocultures to obtain both eggs and L₃ larvae for the bioassays being performed. Table 3.1 lists the number of times each goat was used for the different assays.

Condensed tannin extracts

Four condensed tannin (CT) plant extracts were evaluated. Pure CT extracts from fresh leaves of sericea lespedeza (*Lespedeza cuneata*) and black locust (*Robinia pseudoacacia*) (provided by Texas AgriLife Research and Extension Center, Stephenville, TX) were purified by a standard ether extraction protocol (Terrill, Rowan et al. 1992) with some modifications (Wolfe, Terrill et al. 2008). The black wattle (*Acacia mearnsii*) extract (provided by Texas AgriLife Research and Extension Center, Stephenville, TX) was prepared from a commercially available tannin source (North American Minerals Corporation, West Des Moines, IA) by the same
protocols mentioned above. Cranberry (*Vaccinium oxycoccos*) extract (provided by Texas Agricultural Experiment Station, Vernon, TX) was extracted using a standard ether extraction protocol (Terrill, Rowan et al. 1992; Min, Hart et al. 2005) from whole, frozen cranberries (Dole-Fresh Fruit, Westlack Village, CA). The extracts were kept at -20° C until use. A 50 mg/ml stock solution was prepared from each extract using 10% DMSO and thoroughly mixed to dissolve the powdered extract. Following dissolution in DMSO the sample was centrifuged at 12,000 x g for 90 seconds, and the supernatant was drawn off and transferred to a fresh tube. Stock solution was diluted with deionized water to reduce the DMSO concentration to 0.6% in all working dilutions.

**Egg Hatch/Larval Development Assays**

Both the EHA and LDA were performed on the same test plate following the protocol described by Lacey et al. (1990) with modifications. Nematode eggs were isolated from the feces following the protocol in the DrenchRite® LDA Users Guide (HorizonTechnology 1996) with modification. In brief, water was added to the feces from one of the infected goats to obtain a fecal slurry and the eggs were isolated by filtering the slurry through a series of sieves (425 µm, 180 µm, 85 µm, 30 µm). Material retained on the 30 µm sieve was then added to a sucrose gradient (Marquardt 1961) and centrifuged at 2000 x g for 7 minutes at 4 °C using slow acceleration and deceleration speed. The egg layer was retrieved from the sucrose gradient and rinsed with deionized water to remove the sucrose residue. The volume of water was then adjusted to yield a final concentration of approximately 70 eggs/20 µl. Ninety µl of amphotericin B (Sigma, St. Louis, MO) per ml of egg solution was added just before pipetting the eggs into the assay plates.
These assays were carried out using 96 well plates with wells in each row containing doubling concentrations of drug or CT extract. Deionized water containing 0.6% DMSO served as a negative control in the 8 wells in column 1. Wells 2-12 in rows A and B served as positive control for the assays. These wells contained thiabendazole (Sigma, St. Louis, MO) which for many years has been used in EHA and LDA due to its ovicidal and larvicidal properties. TBZ was dissolved in DMSO and then diluted with deionized water down to 0.6% DMSO for all working concentrations used in the assays. TBZ concentrations used for the assay wells were 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25(µg/ml), with each concentration tested in triplicate. Rows C-E and F-H contained 2 different CT extracts, each of which was tested in triplicate. Concentrations of CT extracts tested in the assays were 1.5, 3.05, 6.1, 12.2, 24.4, 48.8, 97.5, 195, 390, 780, and 1560 (µg/ml). The sericea lespedeza (SL) extract was assayed on 14 separate occasions, and the cranberry (Cr), black locust (BL) and black wattle (BW) extracts were assayed 6 times each.

Assay plates were prepared by adding 75 µl of the appropriate concentration of CT extract or TBZ (or water for negative control wells) and 75 µl of 2% Bacto™ agar (Difco Laboratories, Becton-Dickinson and Company, Sparks, MD) to each well. A final DMSO concentration of 0.3% was present in all wells after the addition of the agar. The plates were immediately placed in a heated incubator shaker at 60° C and 160 RPM for 30 minutes to ensure the contents of the wells were thoroughly mixed. The plates were sealed with parafilm and refrigerated overnight at 4° C to allow the agar to solidify. All plates were used within 3 weeks of preparation.

Just prior to performing the assay, the assay plates were warmed to room temperature and 20 µl of deionized water was added to the wells to ensure adequate moisture content on the agar
surface. Freshly isolated eggs (as described above) were then added to all wells and the plates were sealed with parafilm. The plates were incubated at 25°C in a humidifying incubator for approximately 24 – 40 hours. At this point, the hatched and unhatched eggs were counted in all wells using a 100 x magnification on an inverted compound microscope. This point marked the end of the EHA. The LDA was then performed using the same assay plates and eggs. To start the LDA process, 20 µl nutritive media was added to all the wells as a nutritive source for hatched larvae. The nutritive media consisted of equal parts Yeast Extract (Difco, Sparks, MD) and Earle’s Balanced Salt Solution (Sigma, St. Louis, MO) adjusted to pH 7.0, and then diluted 50% with deionized water prior to adding to the LDA plate. The plates were then resealed and returned to the incubator for an additional 6 days. In the LDA it is important that a thin layer of moisture be maintained on the agar surface throughout the entire assay period, thus the plates were examined every 2 to 3 days and 20 µl of deionized water was added to all wells if the wells appeared to be drying. The assays were terminated 6 days after the addition of the nutritive media by adding 20 µl of 10% Lugols iodine to all wells. The larvae were counted and identified as L₃ stage or as eggs/immature (L₁/L₂) larvae using an inverted compound microscope at 100 x magnification.

**Larval Migration Inhibition Assay**

The LMIA required third-stage (L₃) larvae, which were obtained from coprocultures. Feces from each goat was mixed with vermiculite (Sargent-Welch, Buffalo, NY) and incubated for 10-14 days at room temperature. The samples were examined and mixed every few days and proper moisture content was maintained by addition of water as needed. L₃ were recovered
using the Baermann technique (Dinaburg 1942) and stored at 10° C in deionized water until use, usually within 2 weeks of recovery.

The LMIA procedure was performed using standard 96 well plates, as well as, a 2-stage 20 µm multi-screen mesh filter plate consisting of a receiver plate and a corresponding insert plate containing the mesh screen wells (Millipore, Billerica, MA) as previously described (Kotze, LeJambre et al. 2006), with modifications (Figure 3.1). In brief, the assay was performed such that the receiver plate contained concentrations of drug or extracts and the 20 µm mesh contained diluted agar. The purpose of the agar was to simulate the mucous that would be present in the abomasum of the animal. The mesh screen insert containing the agar was lowered into the receiver plate and the drug or extract concentrations were allowed to equilibrate through the agar before the exposed L3 were added to the mesh wells. The assay began when a 96 well “pre-incubation” plate was prepared using 25 µl of larvae solution (containing approximately 50 L3 in deionized water) and 25 µl of the appropriate control or extract concentrations (with a final DMSO concentration of 0.3%) in all wells. The plate consisted of triplicate concentrations of levamisole (Sigma, St. Louis, MO) as a positive control (µg/ml): 0.05, 0.5, 5, 50, and 500 in row A and part of row B. Levamisole was used as the positive control because it is known to kill nematodes by paralyzing them, thus inhibiting their motility and ability to migrate (Rabel, McGregor et al. 1994). Deionized water with 0.6% DMSO was the negative control in wells 1-9 of row B. The first well in rows C-H contained only water and wells 2-12 in rows C-H contained 2 different extracts (µg/ml): 1.5, 3.05, 6.1, 12.2, 24.4, 48.8, 97.5, 195, 390, 780, and 1560, each done in triplicate. The assays were repeated 11 times for the SL extract, 5 times for the Cr extract and 6 times each for the BL and BW extracts.
The larvae in all wells of the pre-incubation plate were counted using a 100 x magnification on an inverted compound microscope and recorded. This plate was then sealed with parafilm and allowed to incubate at 27° C in a humidifying incubator for 24 hr. A duplicate “rinse plate” was prepared in a second 96 well plate with each well containing 225 µl of the same concentrations and in the same format as the pre-incubation plate, and stored at 4° C overnight. The following morning the rinse plate was allowed to equilibrate to room temperature, and 165 µl of this solution was transferred into the corresponding well of the multiscreen receiver plate. The remaining solution was saved for later use in a rinsing step. Seventy-five µl of 0.125% Bacto™ agar (Difco, Sparks, MD) solution, which was made fresh and maintained at 45° C, was pipetted onto the 20 µm mesh screen of each well of the multiscreen plate. This plate was supported on a plastic box containing about 1 inch of cool water such that the wells were suspended over water. This served as a humidifying chamber to keep the agar from drying. The agar was allowed to equilibrate in this fashion for approximately 1 hr, and then the mesh plate containing the agar was lowered into the receiver plate containing the concentrations duplicated from the pre-incubation plate. This plate was allowed to equilibrate for several hours at ambient temperature. At the end of the first 24 hr incubation period, the L3 that were exposed to the drug or extracts were transferred into the corresponding wells of the agar/mesh plates using a multi-channel pipette. Following the transfer of the L3, plates were examined under microscopy to confirm that all L3 had been transferred and any remaining worms were collected by the addition of 50 µl of the appropriate rinse solution into each well, mixed with the pipette, and then transferred into the corresponding well of the agar/mesh plate. The final contents of the wells in the agar/mesh plate consisted of: 165 µl of rinse solution, 75 µl of agar solution, 50 µl of pre-incubated worm solution and 50 µl of final
rinse solution. The plate was sealed with parafilm and placed under a light at 27° C in a humidifying incubator for 24 hr to allow the worms to migrate through the agar and 20 µm mesh. After the second 24 hr incubation period, the screen mesh plate was removed, and all the worms present in the receiver plate were killed and stained with 5 µl of 50% Lugols solution. The migrated larvae were counted using a 100 x magnification on an inverted compound microscope and recorded.

An important issue with the LMIA is the expense of the multiscreen plates; therefore, a secondary objective was to test the impact of reusing plates in multiple assays. One plate was used for 6 assays, a second plate was used for 5 assays, and a third plate was used 3 times. To clean and maintain the integrity of the mesh plates, they were gently soaked in a 10% bleach/detergent solution for a minimum of 30 minutes in an ultrasonic cleaner and thoroughly rinsed with deionized water. The plates were examined under a dissection microscope after every use to ensure the mesh was intact, properly attached and not blocked by debris.

**Data analysis**

For the EHA, all hatched and unhatched eggs were counted and the percent inhibition of egg hatching was calculated based on the average hatching rate of the negative control wells. For the LDA, the percentage of development to the L3 stage was calculated based on the development of the negative control wells. For the LMIA, the numbers of worms that were inhibited from migrating through the agar/mesh filter in each drug or extract well were expressed as a percentage and compared to the numbers observed in the negative control wells. The effective concentration 50, (EC$_{50}$), where 50% of the eggs or larval are inhibited from development or migration, were analyzed using the “One population” logistic regression model.
of the Fit Logit Program (Waller, Dobson et al. 1985; Dobson, Griffiths et al. 1987) to determine
the dose response characteristics. Graph Pad Prism® 5.02 (San Diego, CA) was used for 1-way
ANOVA statistics. A mixed model approach was used to look at the variability between assays,
variability between the response of different extracts, variability between the response of
parasites obtained from the different goats, and variability in the numbers of time the multiscreen
plates were used.
Table 3.1 - Summary of assays performed.

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Plant Extracts</th>
<th>Goat</th>
<th>Number of Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>EH/LDA</td>
<td>SL/Cr</td>
<td>Black</td>
<td>3</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>SL/Cr</td>
<td>Red</td>
<td>3</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>SL/BL</td>
<td>Black</td>
<td>3</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>SL/BL</td>
<td>Blue</td>
<td>1</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>SL/BW</td>
<td>Black</td>
<td>2</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>SL/BW</td>
<td>Red</td>
<td>2</td>
</tr>
<tr>
<td>EH/LDA</td>
<td>BL/BW</td>
<td>Black</td>
<td>2</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/Cr</td>
<td>Black</td>
<td>5</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/BL</td>
<td>Black</td>
<td>1</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/BL</td>
<td>Red</td>
<td>1</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/BL</td>
<td>Green</td>
<td>1</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/BW</td>
<td>Black</td>
<td>2</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL/BW</td>
<td>Green</td>
<td>1</td>
</tr>
<tr>
<td>LMIA</td>
<td>BL/BW</td>
<td>Black</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 3.1 - Schematic for the Larval Migration Inhibition Assay and diagram of the multiscreen mesh filter plate used for the assay (reprinted from Kotze, LeJambre et al. 2006).
CHAPTER 4. RESULTS

**Egg Hatch Assay**

The TBZ positive control for the EHA demonstrated repeatable results with a mean EC$_{50}$ of 5.4 µg/ml and a range from 3.5 – 7.1 µg/ml (Fig. 4.1). In contrast, the EC$_{50}$ for the condensed tannin extracts for the EHA could not be calculated because even at the highest CT concentration tested there was minimal inhibition of hatching for all extracts (Figure 4.2). ANOVA analysis demonstrated there were no significant differences (p = 0.05) between the replicates of the assays for TBZ; however, all 4 CT extracts demonstrated significant differences among the assay replicates (Table 4.1).

**Larval Development Assay**

The TBZ positive control for the LDA also demonstrated repeatable results among assay replicates with a mean EC$_{50}$ of 3.85 µg/ml and a range from 3.3 – 4.1 µg/ml (Fig. 4.3). Results of the LDA for the four CT extracts also demonstrated consistency between replicates. The mean and range EC$_{50}$ were 42.3 µg/ml (26.5 – 66.2 µg/ml) for SL, 41.5 µg/ml (23.2 – 58.6 µg/ml) for Cr, 31.0 µg/ml (19.6 – 52.1 µg/ml) for BL, and 54.0 µg/ml (39.0 – 69.1 µg/ml) for BW (Fig. 4.4). ANOVA analysis demonstrated there were no significant differences (p = 0.9940) for TBZ or any of the 4 CT extracts between the assay replicates (Table 4.1). In addition, there were no significant differences between the mean values for each of the four extracts compared to each other (Fig. 4.5). The mixed model analysis also indicated there were no significant differences in the variability of the 4 extracts with this assay.
Larval Migration Inhibition Assay

The LEV positive control for the LMIA yielded EC$_{50}$ values that ranged from 1.2 – 13.6 µg/ml (Fig. 4.6). All 4 CT extracts yielded inconsistent EC$_{50}$ values ranging from 9.3 – 470 µg/ml for SL, 56.6 – 283.4 µg/ml for Cr, 50.7 – 576.4 µg/ml for BL and 109.0 – 996.1 µg/ml for BW (Fig. 4.7). ANOVA analysis demonstrated there was a significant difference (p = 0.0445) for the LEV positive control, and there were significant differences (p <0.0001) for SL, Cr, BL and BW (Table 4.1). The mixed model approach indicated there were no significant differences in the variability of the 4 different extracts.

Parasite isolate and plate replicates

Eggs and L$_3$ larvae from each of the 4 goats infected with *H. contortus* were used in the 3 bioassays. The mixed model analysis indicated there were no significant differences (p = 0.101) between the results of various assays due to the animal host from which the parasites were collected. The mixed model analysis was also used to determine if there was a difference in the performance of the multiscreen plate with multiple uses. This analysis indicated there were no significant differences (p = 0.102) in plate performance when the plates were used multiple times.
Figure 4.1 – Dose response curves for TBZ (positive control) for the Egg Hatch Assay. Figure shows the results of 10 assays.

Figure 4.2 – Dose response results from 4 CT extracts for the Egg Hatch Assay. Figure shows the results of 14 assays using Sericea lespedeza and 6 assays each using Cranberry, Black Locust and Black Wattle extracts. The EC$_{50}$ results could not be detected under the assay conditions.
Table 4.1 - 1 way ANOVA analysis of bioassays with CT plant extracts and drugs

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Plant Extract/ Control</th>
<th>p value</th>
<th>EC₅₀ Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHA</td>
<td>TBZ</td>
<td>0.05</td>
<td>No</td>
</tr>
<tr>
<td>EHA</td>
<td>SL</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>EHA</td>
<td>Cr</td>
<td>&lt; 0.0569</td>
<td>No</td>
</tr>
<tr>
<td>EHA</td>
<td>BL</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>EHA</td>
<td>BW</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>LDA</td>
<td>TBZ</td>
<td>0.9940</td>
<td>No</td>
</tr>
<tr>
<td>LDA</td>
<td>SL</td>
<td>0.9654</td>
<td>No</td>
</tr>
<tr>
<td>LDA</td>
<td>Cr</td>
<td>0.7389</td>
<td>No</td>
</tr>
<tr>
<td>LDA</td>
<td>BL</td>
<td>0.7096</td>
<td>No</td>
</tr>
<tr>
<td>LDA</td>
<td>BW</td>
<td>0.9513</td>
<td>No</td>
</tr>
<tr>
<td>LMIA</td>
<td>LEV</td>
<td>0.0445</td>
<td>Yes</td>
</tr>
<tr>
<td>LMIA</td>
<td>SL</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>LMIA</td>
<td>Cr</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>LMIA</td>
<td>BL</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>LMIA</td>
<td>BW</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 4.3 – Dose response curves for TBZ (positive control) for the Larval Development Assay. Figure shows the results of 10 assays.

Figure 4.4 – Dose response results from 4 CT extracts for the Larval Development Assay. Figure shows the results of 14 assays using Sericea lespedeza and 6 assays each using Cranberry, Black Locust and Black Wattle extracts.
Figure 4.5 – EC$_{50}$ means of the dose response curves for the four CT extracts for the Larval Development Assay.
Figure 4.6 - Dose response curves for LEV (positive control) for the Larval Migration Inhibition Assay. Figure shows the results of 8 assays.

Figure 4.7 – Dose response results from 4 CT extracts for the Larval Migration Inhibition Assay. Figure shows the results of 11 assays using Sericea lespedeza, 5 assays each using Cranberry and 6 assays using Black Locust and Black Wattle extracts.
CHAPTER 5. DISCUSSION

The primary objective of this study was to evaluate the dose response characteristics and repeatability of 3 in vitro bioassays with CT plant extracts, and to determine which methods were the most suitable for measuring anthelmintic activity. This was accomplished using an isolate of Haemonchus contortus originating from the UGA Sheep Unit. The 3 bioassays evaluated were the Egg Hatch Assay (EHA), Larval Development Assay (LDA), and Larval Migration Inhibition Assay (LMIA). Each of these assays was repeated multiple times in triplicate using 4 different CT plant extracts along with an appropriate drug control. Of the 3 bioassays, the LDA yielded the most consistent results for all 4 extracts. The LDA is routinely used in our laboratory to detect anthelmintic resistance in small ruminants, and the use of doubling concentrations of anthelmintics in this assay is standard procedure. However, using this procedure with the CT extracts, there was a very steep slope in the dose response curve. This meant that over just a few concentration increases we saw a change from 0% activity to 100% activity. From a clinical standpoint, this yielded acceptable values for comparing the EC$_{50}$ concentrations between the various assay repeats and extract repeats (Figure 4.4). But, for statistical analysis, we had limited numbers of data points for accurately measuring the EC$_{50}$. Thus, for future studies, we would use smaller dilutions in that range to obtain more precise data for analysis.

Sericea lespedeza was chosen as the primary extract for these experiments, because previous in vivo studies have demonstrated reduced H. contortus burdens in small ruminants fed this forage (Athanasiadou, Kyriazakis et al. 2001; Min and Hart 2003; Shaik, Terrill et al. 2004; Moore, Terrill et al. 2008). Since SL was known to decrease H. contortus burdens, our initial approach was to evaluate SL, and then use the values obtained from SL to compare the effectiveness of other CT extracts. The discovery that there was very little variability between
SL and the other 3 CT plant extracts was unexpected. In retrospect, each plant was prepared such that the final product was a pure CT extract, thus, it is logical that the bioassays utilizing these CT extracts could yield similar results. Additional factors should be regarded when evaluating possible forages for in vivo trials. The actual amount of CT present in the leaves of the plant, along with other nutritional benefits that plant may offer should be considered. A plant such as SL with 16.7% CT present in the forage may likely be a better candidate for limiting parasite infections compared to Honeysuckle with < 1% CT present in the leaves (Figure 1.1), although the bioassay evaluation of the extracts may yield similar results. Certainly it is premature to judge whether all CT extracts will behave in the same manner in the bioassays, or whether all CT plants will cause a decrease H. contortus burdens, therefore more research evaluating CT plant extracts is warranted.

There have been previous reports which have shown decreased hatching rates from shed H. contortus eggs in animals that have been fed SL for a period of time (Molan, Waghorn et al. 1999; Min, Pomroy et al. 2004). Although these results for the EHA did not indicate any decrease in hatching rate within the parameters tested, the drug controls indicated the assays was functioning properly. It is probable that the eggs were not in contact with the CT solution long enough to measure an affect, but due to the rapid hatching rate of the eggs, it is not possible to prolong the contact time of the CT. Thus, this particular bioassay may not provide the proper conditions to detect this effect. It may be possible to detect a change in the EHA for eggs shed from animals fed CT for several weeks. A study addressing this particular question is ongoing in our laboratory in conjunction with a feeding trial presently being performed at Fort Valley State University. Perhaps this current work will indicate whether a decrease in hatching rate can be observed under these conditions. Also, it may be possible to detect some decrease in hatching
rates if higher concentrations of the extract were tested; however, concentrations above 1560 µg/ml for these particular extracts yielded wells that were too opaque to observe the eggs. Therefore, the extract pigments were a limiting factor in the concentrations used for these assays.

A number of studies using the LMIA to evaluate larval motility in parasites exposed to plant extracts have been reported (Molan, Hoskin et al. 2000; Barrau, Fabre et al. 2005), but, at best, most assays are only performed a few times in triplicate. The evaluation of the LMIA demonstrated inconsistent results when tested repeatedly. Several factors can contribute to inconsistent results. First, the initial age, health, and motility of the larvae being tested is always a varying factor. The negative control values are supposed to minimize this effect, but accurate pre-counts of larvae present in each well before and after migration are critical to properly evaluate the true percentage of larvae that are inhibited from migrating. In most of the reported studies, larvae in a few wells are killed and counted, and the average number of larvae obtained in those wells was used as the pre-count value. This would seem to be a reasonable approach because of the extreme difficulty in counting the rapidly moving larvae. However, in my experience, there were always at least 10% of the wells that had differences as much as a 50% from the average estimate, even when extreme care was taken to provide a uniform mixture of larvae while pipetting into the wells. Therefore, this source of error could certainly alter the accuracy of the calculations.

Due to these difficulties, alternative approaches to obtain the most accurate pre-count data were explored. It was quickly discovered that there were a number of complex factors associated with counting the moving larvae. First, the living larvae were able to move into different planes of focus, which was a large source of error. Also, when using a flat-bottom plate, the moving larvae would quickly migrate toward the periphery of the wells, where they
could not be clearly examined. The use of a round-bottom plate allowed the larvae to aggregate into large clumps in the bottom of the wells, again making an accurate count impossible. The use of a phase contrast inverted microscope was a significant advantage contributing to our ability to maximize the accuracy of the counts. The limitations associated with this bioassay are inherent for this particular biological system, and extreme care should be taken to verify the accuracy of the pre-counts when utilizing this bioassay. Although the LMIA technique is cumbersome with this biological system, it could still be a valuable bioassay for other biological systems, such as cyathostomes, filarial worms, adult parasites or plant nematodes.

For this study, 4 goats (Red, Black, Blue and Green) were infected with the same isolate of *H. contortus*. This consistently provided a steady supply of eggs or larvae for use in the research. An additional question we were interested in addressing was “could differences be detected in the bioassays due to host variability”? The mixed model analysis indicated there were no differences due to host variability; however, Black was the only goat that consistently maintained a high fecal egg count, and this could have biased the statistical analysis. The other 3 goats always cleared their infections rapidly, so consequently, the numbers of eggs and larvae from the other 3 animals were limited. This verified the resilience of these particular animals and further corroborated the concept that parasites tend to be over-dispersed in a group of animals.

Another factor worth investigating regarding the LMIA was the ability to reuse the multiscreen plates. The latest information regarding the cost of these plates indicated that each multiscreen plate cost approximately $50 each (Millipore MANMN2050). Since the statistical analysis indicated there were no significant differences in plate performance when the plates were reused up to 6 times, reusing these plates would be a tremendous advantage if this method
is utilized for future research or diagnostic testing.

Both *in vivo* and *in vitro* research has shown that CT forages reduce the parasite burdens in small ruminants (Molan, Waghorn et al. 2000; Paolini, Dorchies et al. 2002; Min, Pomroy et al. 2004; Shaik, Terrill et al. 2006). Various bioassays have been used in an attempt to assess this effect; however, no assays have been validated for this purpose until now. The research accomplished for this project has demonstrated a more accurate and consistent method for detecting anthelmintic activity in various CT plant extracts. There has been much speculation that other plants without CT, such as pineapple or pumpkin, may also serve as anthelmintic agents (Diaz, Lloja et al. 2004; Stepek, Behnke et al. 2004; Fu, Shi et al. 2006). This research indicates that the LDA could be used to evaluate these plants, as well. Even though research has shown that feeding CT forages to small ruminants contributes to reducing parasite infections, the mechanisms of action that lead to these reductions are unclear. It is probable that there are a number of different factors contributing to this phenomenon, but it is evident that more research is needed to explore the reasons for the decrease in parasite burdens as it relates to ingestion of CT forages. Although it is unlikely that CT forages can totally eliminate the need for anthelmintic treatments, even moderate reductions of parasite burdens due to feeding CT forages can result in fewer anthelmintic treatments, thus improving the ability to sustain small ruminant production. This could potentially benefit small ruminant farmers in many countries, including the United States.

Results of our studies highlight the need for additional research into the use of CT forages and/or extracts as parasiticides. Three important avenues for future research are: (1) is it possible to predict the *in vivo* anthelmintic activity of CT containing forages based on the *in vitro* activity of extracts from these plants, (2) whether parasite resistance is likely to develop to CT,
and (3) what are the primary anthelmintic mechanisms of action of CT.

Because CT-containing forages have a wide range of CT content, in vitro anti-parasitic activity of a purified extract might not translate to in vivo activity of the actual forage. Thus an important direction for future CT research is to address the question of whether it is possible to predict the in vivo anti-parasitic activity of CT containing forages based on the in vitro activity of extracts from these plants. The most direct approach would be by performing dose-titration feeding trials with various CT plants, while also characterizing the type, content and in vitro activity of the purified CT. Since previous trials with SL demonstrate that a high percentage of the diet as SL is required to achieve high levels of anti-parasitic activity, it is possible that plants containing lesser amounts of CT than SL may not be effective when fed as a forage. Such plants may need to have the CT extracted and added to another feed source such as in a pellet to achieve sufficient levels in the diet.

Given the rapid development of anthelmintic resistance by parasites of small ruminants, a logical question regarding CT is: “will parasites develop resistance to CT over time?” This is an important question that could be addressed in future research studies. One approach would be to collect worm eggs from adult parasites that survived exposure to high levels of CT. These eggs could be cultured to the L3 stage and used to infect a new group of animals. Those animals could in turn be fed CT forages and the cycle repeated a number of times through passage in new animals. By examining the EC$_{50}$ using the LDA and the percent reduction in FEC after each feeding cycle, changes could be detected over time that might indicate the development of resistance. This would require a lengthy study, but may indicate whether resistance to CT is likely to develop or not.

The anthelmintic mechanisms of CTs are poorly understood, but it is speculated that CTs
may work through multiple mechanisms. Understanding these mechanisms is fundamental to gaining a deeper appreciation of how best to manage and use CT containing forages for parasite control. The first question to ask might be to determine whether CT is being taken up by the worm and causing internal damage or whether it is only causing external damage. This could be addressed by examination of the cuticle and other external structures of the adult parasites to look for changes associated with CT exposure, which could be achieved by visualization using electron microscopy. Likewise, mechanisms of internal damage could be addressed by examining differential gene expression in worms exposed or not exposed to CT.
REFERENCES


Kaplan, R. M., J. M. Burke, et al. (2004). "Validation of the FAMACHA(C) eye color chart for detecting clinical anemia in sheep and goats on farms in the southern United States." Veterinary Parasitology 123(1/2): 105-120.


