The Effects of Naturally Occurring Plant Products on Experimental *Haemonchus contortus* Infection in Gerbils and Sheep

Jill M. Squires

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Dr. Anne Zajac, Chair
Dr. David Lindsay
Dr. Terry Swecker

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ABSTRACT

Haemonchus contortus is a blood-sucking abomasal helminth of small ruminants responsible for major economic losses to producers worldwide. Widespread resistance to commercial anthelmintics has created a need for alternative methods of parasite control. One method involves using plant products with natural anthelmintic properties. This thesis assessed the efficacy of several plant products against experimental *Haemonchus contortus* infection in gerbils and sheep.

In gerbil assays, animals were orally infected with 600 third-stage *Haemonchus* larvae and treated once or daily for 5 days with artemisinin, *Artemisia annua* aqueous or ethanolic extract, an orange oil emulsion, or *Asimina triloba* ethanolic extract. Nine days post-infection, gerbils were euthanized, their stomachs removed, and the worms counted. Significant anthelmintic activity was not found for artemisinin, *A. annua* extracts, or *A. triloba* extract. The orange oil product caused significant parasite reductions up to 87.8% when administered for 5 days.

The orange oil emulsion was tested in sheep to evaluate the product against *Haemonchus* in its natural host. Sheep were orally inoculated with 10,000 *Haemonchus* larvae and, one month later, dosed with the emulsion once or daily for 3 days. Fecal egg counts were monitored daily starting on the first day of dosing and continuing to 14 days post-dosing. Results showed that a single dose of the product caused highly significant fecal egg count reduction (97.4%) compared to control sheep and that there is no advantage to treating for 3 days. Thus, the orange oil emulsion shows promise as an alternative to commercial dewormers.

TABLE OF CONTENTS

Page

CHAPTER 1. LITERATURE REVIEW 1
Life Cycle of *Haemonchus contortus* 1
Hypobiosis 1
Pathogenesis 2
Importance and Distribution 2
Commercial Anthelmintics for the Control of *Haemonchus* 3
Benzimidazoles 3
Imidazothiazoles 4
Macrocyclic lactones 4
Anthelmintic Resistance 5
Alternative Control Strategies 7
Pasture Management 7
Selective Deworming 7
Breeding/Selection for Resistant Animals 8
Dietary Supplementation 9
Alternatives to Commercial Anthelmintics 9
Previous Research in Forages and Plant Products 10
Tanniferous plants 10
Plants with various other constituents 10
Challenges in research 12
Plants and Plant Products Addressed in This Work 13
Artemisia annua 13
Orange oils 14
Asimina triloba 16
The Gerbil Model for Anthelmintic Studies 17
References 17

CHAPTER 2. Effects of *Artemisia annua* products on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*) 29

Abstract 29
Introduction 29
Materials and Methods 30
Results 33
Discussion 34
References 37

CHAPTER 3. The effect of an orange oil emulsion on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*) 42
Abstract 42
Introduction 42
Materials and Methods 43
Results 45
Discussion 46
References 48

CHAPTER 4. The effect of an orange oil emulsion on experimental *Haemonchus contortus* infection in sheep (*Ovis aries*) 51
Abstract 51
Introduction 51
Materials and Methods 52
Results 53
Discussion 54
References 56

APPENDIX A. The Effect of *Asimina triloba* on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*) 58
Abstract 58
Introduction 58
Materials and Methods 59
Results 61

Discussion 61
References 63

LIST OF TABLES

Page

Table 1.1 Recent publications related to the effects of condensed tannins on *Haemonchus contortus*……26

Table 1.2 Recent publications related to activity of non-tannin-rich plants against *Haemonchus contortus*……....28

Table 2.1 Effect of artemisinin on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean *H. contortus* (±S.D.) burdens and parasite reduction (with 95% C.I.) according to group ……..……40

Table 2.2 Effect of *Artemisia annua* extracts on *Haemonchus contortus* infection in gerbils; Individual and geometric mean (with 95% C.I.) *H. contortus* burdens and parasite reduction (with 95% C.I.) according to group…….…..……...41

Table 3.1 Effect of an orange oil emulsion on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean (±S.D.) *H. contortus* burdens and parasite reduction (with 95% C.I.) according to group .……...……49

Table 3.2 Effect of an orange oil emulsion on *Haemonchus contortus* infection in gerbils; Individual and geometric mean (with 95% C.I.) *H. contortus* burdens and parasite reduction (with 95% C.I.) according to group. ……..….50

Table A.1 Effect of *Asimina triloba* ethanolic extract on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean *H. contortus* burdens and parasite reduction (with 95% C.I.) according to group ……..…….…….65

LIST OF FIGURES

Page

Fig. 4.1 Effect of an orange oil emulsion on *Haemonchus contortus* infection in sheep; mean fecal egg count (±S.E.) by day post-first dose according to group ..……..…..…57

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CHAPTER 1

LITERATURE REVIEW

Life Cycle of *Haemonchus contortus*

Haemonchus contortus is a gastrointestinal (GI) parasite capable of causing severe disease in small ruminants and substantial economic loss to producers. Although *H. contortus* is equally important in sheep and goats, this thesis will focus on sheep. The blood-sucking strongylid nematode belongs to the family Trichostrongylidae, which includes other important GI nematodes such as *Trichostrongylus* spp. and *Ostertagia* spp. The life cycle is direct. Adult male and female worms reside in the abomasum and reproduce sexually. Female *H. contortus* are prolific, producing 5,000 – 10,000 eggs per worm per day (Levine, 1980) that are passed in the feces of the host. If environmental conditions are ideal (warm and humid), a first stage larva will hatch from an egg within a day. This larva is small and slender and feeds on fecal bacteria. It molts to a larger second stage larva which continues to feed on bacteria before molting to the third stage. The span from hatching to the third stage larva (L3) under ideal conditions is approximately 7 days. The L3 retains the cuticle from the second stage over its own cuticle as a protective sheath. This sheath increases the resistance of the larva to adverse weather conditions. However, at this stage the L3 is prevented by its sheath from eating and must survive on energy reserves. The L3 is infective to hosts. As the larvae are dispersed from fecal pellets by rain and mechanical disruption, they are able to move onto vegetation via surface films of moisture. Once ingested by a grazing host, the larva emerges from its sheath in the rumen and then moves into the gastric glands of the abomasal mucosa to feed. Another series of molts occur as the parasite moves through a fourth and fifth larval stage to become an adult in the abomasal lumen 14 - 21 days after ingestion. The cuticle of *H. contortus* forms a small lancet in its oral opening that is used to pierce the mucosa, causing capillary bleeding on which the worm feeds. Blood feeding begins at the fourth larval stage.

Hypobiosis. *H. contortus* is capable of undergoing a period of developmental arrest known as hypobiosis (O'Connor et al., 2006). When this takes place, larvae in the host do not develop directly into adults but remain as L4 in the gastric glands of the abomasum for weeks or months. The portion of hypobiotic worms is usually greatest when conditions outside the host

are unfavorable for parasite development so that any eggs shed into the environment would be unlikely to develop and survive (Levine, 1980, Gatongi et al., 1998, O'Connor, 2006). The mechanism of hypobiosis has yet to be fully elucidated, although responses to environmental conditions (Langrova et al., 2008), host immunity (Adams, 1986), and genetic programming (Capitini et al., 1990) have all been implicated.

Pathogenesis

Due to the worm's blood-consuming lifestyle, the major effect of haemonchosis is anemia. Logically, higher worm burdens result in greater blood loss. Baker et al. (1959) reported that an adult worm is capable of consuming 0.08 ml of blood per day. With low level infections, blood loss would not be clinically significant. However, with favorable conditions infections can easily increase to tens of thousands of worms, producing a rate of blood loss that exceeds the host's ability to regenerate red blood cells. Stress and poor nutrition worsen the effects of infection because they weaken the host's immune response to the parasite. Characteristic signs of severe *H. contortus* infection are pale mucous membranes and "bottle jaw", submandibular edema due to hypoproteinemia. Haemonchosis can result in reduced weight gain, weight loss, general unthriftiness, and death. Susceptibility varies from animal to animal due to individual genetic and environmental factors.

Importance and Distribution

Sheep are a worldwide commodity. They provide a number of products: meat, milk, skins, and wool or hair (Zygoyiannas, 2006). While in most cases they cannot compete with cattle in meat and milk production, sheep have characteristics that give them value over cattle. One main advantage is wool production. A second value is their smaller size, which is beneficial on small farms that do not possess the land or resources required for cattle (Zygoyiannas, 2006). Furthermore, sheep have a unique ability to perform well on forages and terrain on which cattle would not (Zygoyiannas, 2006). In 2006, Zygoyiannas estimated the world population of sheep to be 1024 million, with the major sheep producing regions located in Europe, the Near East, South America, Australia, and New Zealand. Sheep are raised in all regions of the United States, and in 2005, the Food and Agricultural Organization of the United Nations ranked the U.S. #18 in sheep meat producing countries. For countries where sheep are economically critical, such

as Syria, where indigenous sheep meat is the second ranked commodity (FAO, 2005), a decline in production could seriously impact the livelihood of millions.

Where there are sheep, there are parasites; so based on its pathogenicity, it is not surprising that *H. contortus* is the most important parasite of sheep in numerous regions throughout the world. In the past 10 years, estimates for the cost of treatment due to *H. contortus* alone have approached $30 million, $50 million, and $105 million in Kenya, South Africa, and India, respectively (Waller and Chandrawathani, 2005). *H. contortus* is especially a problem in tropical and subtropical areas with high temperatures and abundant rainfall. *H. contortus* is distributed throughout the United States; it is the predominant ovine helminth in the eastern U.S. whereas in the more arid western U.S., it is more likely to be a problem in irrigated areas. Temperatures suitable for the development of *H. contortus* range from 11°C - 40°C, the optimum temperatures being between 31°C - 34°C (O'Connor et al., 2006). Eggs will not hatch below 9°C, and high humidity is important especially at high temperatures where desiccation becomes a serious threat to both eggs and larvae (O'Connor et al., 2006).

Commercial Anthelmintics for the Control of *H. contortus*

For more than half a century, the pharmaceutical industry has been distributing what are considered "modern" anthelmintic drugs aimed at a variety of animal parasites (Getachew et al., 2007). Some have been more successful than others; ideal modern anthelmintics have a wide margin of safety and spectrum of action, are easy to administer, have short residual periods, and are cost effective (Aiello and Mays, 1998). Prophylaxis for or treatment of nematode diseases can be achieved via the use of anthelmintics. Anthelmintics interfere with vital functions of the parasite, leading to starvation and/or paralysis followed by expulsion of the parasite. There are various products in multiple drug classes available to combat parasitic nematodes; however, most of the commonly used ovine anthelmintics in the United States belong to one of three drug classes: the benzimidazoles, the imidazothiazoles, and the macrocyclic lactones.

Benzimidazoles. The benzimidazoles are a large class of anthelmintics characterized by a broad spectrum of activity and low toxicity (Bowman, 2008). They are poorly water soluble and thus given orally. They are effective in removing the major adult GI nematodes and many of

their larval stages (Aiello and Mays, 1998). Currently, albendazole and fenbendazole are approved in the U.S. for use in sheep and goats, respectively (Zajac, 2006). The primary mode of action of these drugs is to bind to β-tubulin, a structural protein necessary for the formation of microtubules (Aiello and Mays, 1998), and affect cellular transport within the parasite, leading to its death. Because benzimidazoles have a higher affinity for nematode tubulin over mammalian tubulin, the drugs are selective for parasites. Albendazole should be administered to sheep at a dose rate of 7.5 mg/kg; however, the drug has teratogenic effects and should not be used in the first 30 days of pregnancy (Zajac, 2006). Repeating doses or withholding feed to slow GI passage can be used to enhance absorption of benzimidazoles (Aiello and Mays, 1998, Zajac, 2006). Residues from these drugs may persist, requiring withholding periods up to two weeks before slaughter (Aiello and Mays, 1998).

Imidazothiazoles. The imidazothiazoles act as nicotinic acetylcholine receptor agonists that disrupt the parasite neuromuscular system, causing paralysis (Bowman, 2008, Zajac, 2006). Paralysis could result in starvation of the parasite through inhibition of feeding, or the worm could simply be paralyzed and be swept away with ingesta. Selectivity for parasites may be due to differing receptor physiology and distribution between invertebrate parasites and mammals (Bowman, 2008), although toxicosis in the host animal is due to the same mechanism of action (Aiello and Mays, 1998). Levamisole is a widely used imidazothiazole marketed as a soluble powder for oral administration to sheep (Zajac, 2006). The drug is easily water soluble with broad spectrum activity and withdrawal periods much shorter than that of the benzimidazoles (Aiello and Mays, 1998). Given at 8 mg/kg to sheep, levamisole is efficacious against adult and larval stages of *H. contortus* and other GI nematodes (Bowman, 2008). Despite a reasonable safety margin, occasional side effects occur even at the recommended dose; debilitated sheep apparently are at greater risk for toxicosis (Bowman, 2008). The withdrawal period for levamisole is short; only 72 hours are required before slaughter of sheep (Bowman, 2008).

Macrocyclic lactones. Also known as macrolides, this class of anthelmintics has become a major contender in the battle against parasitic disease. Macrolides are highly effective at low doses and thus very safe. The drugs also provide very broad spectrum activity against adult and larval (even hypobiotic) nematodes as well as arthropods (Aiello and Mays, 1998). While

synthetic macrolides have been created (White et al., 1995), the original products in this class are antibiotics produced by streptomycete microorganisms that act by binding with high affinity to glutamate-gated chloride channels (Bowman, 2008). Binding of macrolides to the channels results in hyperpolarization of parasite neurons which causes muscular block and results in paralysis and death (Bowman, 2008). The two products approved in the U.S. for oral use in sheep are ivermectin and moxidectin (Zajac, 2006). Both are recommended at a dose of 0.2 mg/kg in sheep but are not approved for use in goats (Zajac, 2006). The drawbacks to macrolide use are that the drugs are ineffective against cestodes and trematodes due to their lack of glutamate-gated chloride channels (Aiello and Mays, 1998), and some preparations can be more expensive than other anthelmintics (Bowman, 2008). The withdrawal period before slaughter of sheep is 11 days and 7 days after treatment with ivermectin and moxidectin, respectively.

Anthelmintic Resistance

The introduction of modern anthelmintics was a double-edged sword, particularly for the small ruminant production industry. On one hand, anthelmintics provided broad spectrum, safe, highly effective elimination of helminths that resulted in more productive and more profitable animals. On the other hand, the great dependability of these drugs to kill parasites within the host caused a shift in production practices such that producers relied on application of anthelmintics alone and began to abandon other control methods to minimize parasite populations in the environment. Several years following the advent of modern dewormers, resistant strains of *H. contortus* were reported. The first broad-spectrum anthelmintic to become associated with resistant parasite strains in North America was thiabendazole, a benzimidazole, followed in the 1980's by levamisole and ivermectin (Craig, 2006). As early as 1997 (Waller), reports of resistance to all three major drug classes were confirmed in multiple countries in Africa and 80% of sheep farms in high rainfall areas of Australia reported resistance to benzimidazoles and levamisole/morantel. In southern states in the U.S. such as Louisiana and Florida, no major drug class could effectively control *H. contortus*, and South America, where even combination products were failing, was reputed to have the worst resistance problem in the world (Waller, 1997). Since then, due in part to the fact that few new drugs have emerged to provide relief, resistance is on the rise (Kaplan, 2004). Modern commercial anthelmintics were initially so effective that the efficacy standard for these drugs is a parasite reduction of 90% or

more; however in light of the current resistance problem, many producers are forced to settle for lesser reductions with these products.

Genetic variation is responsible for resistance; spontaneous mutations or pre-existing alleles render a worm invulnerable to the mechanism of action of a drug (Silvestre and Humbert, 2002, Craig, 2006). When all susceptible worms are killed by treatment, resistant worms survive and thus pass resistant alleles to further generations. When a worm is resistant to one member of a drug class, it is also resistant to the other members of that drug class because of the similar mode of action of the drugs, a phenomenon known as side resistance (Sangster, 1999). A mutation can also result in cross resistance where drugs from different classes with different mechanisms are ineffective (Sangster, 1999).

Inappropriate use of anthelmintics by producers has contributed significantly to the development of resistance in *H. contortus* and other GI helminths. To save money, some producers underdose animals, potentially conferring a selective advantage to heterozygote worms with a higher level of tolerance. Similarly, use of an average weight to determine a dose for an entire flock results in underdosing and selection for resistance in the largest animals (Craig, 2006). Furthermore, goats metabolize anthelmintics faster than sheep (Sundlof, 1992), so the amount of bioavailable drug may be lower in goats given a dosage intended for sheep. Finally, drugs with long residuals eventually reach a sub-therapeutic level in the body that kills only incoming worms without resistant alleles and favors heterozygotes, allowing only resistant worms to establish in the animal (Craig, 2006). Some producers treat their entire flock rather than only the animals that appear to need treatment. Under this practice, only resistant worms are left after treatment; thus, more resistant than susceptible alleles will be shed onto pasture in the parasite eggs. With each round of treatment, resistant worms increase in the population and dilute out any genetically susceptible worms acquired from the environment. If stocking rates are high, the level of these resistant worms on pasture may rise quickly. The result is rapid reinfection and possible development of clinical disease, more frequent need for treatment, and greater opportunities for selection of resistant worms.

Alternative Control Strategies

Anthelmintics alone cannot combat ever-changing strains of *H. contortus*. The current state of crisis regarding widespread anthelmintic resistance calls for alternative control strategies. A multifaceted approach that includes pasture management, careful planning and observation, and individualized treatment is required to control gastrointestinal helminths. The goal of alternative control is to develop methods or products that can be used in place of commercial dewormers to reduce dependency on them. The options available to individual producers will depend on availability of pasture and labor as well as cost.

Pasture Management. Effective pasture management will reduce animal exposure to infective larvae. One option is to reduce stocking density. This practice decreases the amount of egg-laden feces shed over a certain area, increasing the likelihood that an animal can avoid grazing near concentrations of larvae (Stear et al., 2007). This option is not necessarily feasible, however, for those with limited pasture or if production of a greater number of animals is needed to make a profit. For farms raising more than one type of livestock, alternating or mixing species, for example, sheep and cattle or sheep and horses, provides safer pasture due to the fact that few nematode species infective to horses or cattle are able to infect sheep, and vice versa (Stear et al., 2007). Alternate or mixed grazing results in a lower frequency of exposure of either host to infective larvae than would be observed on an equally stocked pasture with one livestock species. This practice does not apply to the mixture of sheep and goats, however, as they are parasitized by the same helminths. Rotational grazing is a practice in which animals are allowed to graze a pasture and are then removed from it for a period of time before returning. This practice may allow the level of pasture contamination to fall drastically so that animals can be periodically introduced to cleaner pastures, reducing the number of larvae they are exposed to year-round (Getachew et al., 2007). However, this strategy is more likely to be successful in extreme climates where *H. contortus* larvae would die quickly from intense heat or cold or desiccation (Craig, 2006).

Selective Deworming. The goal of selective deworming is to increase the refugia, which is the parasite population not exposed to a drug treatment (Kaplan, 2004), so that drug susceptible alleles do not get diluted out of the population. Maintaining as high a refugia as

possible slows the development of anthelmintic resistance (Kaplan et al., 2004). In view of the exorbitant cost of bringing a new drug to market and the lack of such products on the horizon, the efficacy of currently available anthelmintics must be preserved for as long as possible. Rather than an entire flock, only animals at risk or with signs of clinical disease should be treated. Restricting anthelmintic use helps to maintain the refugia by targeting the major sources of pasture contamination; only a small proportion of highly susceptible animals in a population harbor the majority of worms (Kaplan et al., 2004). The FAMACHA system is an extremely useful tool to determine which animals carry the greatest number of adult *H. contortus*. Developed in South Africa, the system allows producers to rank animals based on their level of anemia (Burke et al., 2007a). Animals are scored on a scale of 1 to 5 following examination of the color of their conjunctival membrane: 1 and 2 indicating normal, 5 signifying severe anemia. By using these scores to assign individual treatments, anthelmintic use is minimized. A small hurdle in this otherwise successful program is that optimal performance of the system requires training and is subject to human error (Burke et al., 2007a). The savings on the cost of anthelmintics may be lost to additional labor, but the benefit of maintaining the refugia is certain.

Breeding/Selection for Resistant Animals. Just as genetic variation produces nematodes resistant to anthelmintics, genetic variation has rendered certain breeds of sheep more resistant to *H. contortus* infection than others. For example, hair sheep breeds such as the St. Croix have been shown to be more resistant to GI helminths than wool breeds such as the Dorset (Gamble and Zajac, 1992). The benefits of using resistant breeds include fewer and less fertile worms, which results in greater production, less need for anthelmintic treatment, and reduced pasture contamination (Getachew, 2007). Resistance can be identified by criteria such as consistent low fecal egg count and high packed cell volume, which can be used as a selection tool in production schemes (Craig, 2006). Producers with heavily infected sheep could benefit from culling susceptible animals and introducing replacements from a more resistant breed. Breeders may choose to cross-breed animals to bring in resistance from one breed while preserving another desirable production trait from the other. For those unwilling to substitute new breeds, there is also variation within the members of a single breed, and the same criteria can be used in breeding or selection of replacements. There are other markers of resistance such as anti-helminth antibody levels and genetic polymorphisms that could provide additional information to aid in

selection, but they are not consistent enough to be commercially useful.

Dietary Supplementation. Appropriate nutrition is important for a host to be able to mount an effective immune response to *H. contortus* infection. The protein loss caused by this hematophagous nematode can negatively affect growth and reproduction (Coop and Kyriazakis, 2001). Supplementing adequate amounts of protein to the diet of sheep has been shown to boost immunity and improve the expulsion of worms and reduction of fecal egg counts in both young and mature animals (Coop and Kyriazakis, 2001).

Alternatives to Commercial Anthelmintics. Several alternative treatments for *H. contortus* infection have been evaluated in the place of commercial anthelmintics. Copper oxide wire particles reduced *H. contortus* fecal egg counts when given in a single capsule containing 2.5 to 5 g (Knox, 2002), and capsules with 2 g or less reduced fecal egg counts in lactating ewes and lambs (Burke et al., 2007b). However, caution should be used with regular dosing as sheep are subject to chronic copper poisoning (Craig, 2006, Burke et al., 2007b). Several species of fungi have potential for controlling nematode larvae on pasture. For example, the spores of *Duddingtonia flagrans*, a nematophagous fungus, can survive passage through the gastrointestinal tract of sheep and are then shed with nematode eggs in feces (Larsen, 2000). Hyphae grow from these spores that trap and use hatched larvae as a nutrient source. The major drawbacks to this approach, however, are the influence of temperature on the spores (Larsen, 2000) and the requirement for daily feeding of the spores to effectively contain larvae within feces (Getachew, 2007). Vaccination for *H. contortus* has been considered; various nematode proteins have been tested as potential targets of the host immune response. Surface antigen or excretory/secretory product vaccines have created some level of protection, although probably not enough to protect lambs from high level infection (Getachew, 2007). Other vaccines made from nematode intestinal cells have generated host antibodies that successfully reduced worm burdens (Craig, 2006, Getachew, 2007). However, a cost-effective method of producing *H. contortus* antigen on a commercial level has yet to be found. Finally, ongoing research is aimed at confirming the efficacy of the age-old use of plants for their anti-parasitic properties. Not only is treatment with natural plant products a way to decrease dependency on and reduce genetic selection by commercial anthelmintics, it may be a money-saver, an option especially valuable to

producers in poor countries who cannot afford commercial dewormers.

Previous Research in Forages and Plant Products

Long before the dawn of modern medicine, ancient healers used plants to combat parasitic disease. Ethnobotanical medicine, which is based on traditional remedies passed down from generation to generation to treat both human and animal ailments, is still practices in many countries. Entire books are dedicated to listing the apparent medicinal properties of plants and there are seemingly endless choices in the treatment of gastrointestinal parasites. However, much of the evidence supporting the efficacy of ancient remedies is based on subjective observation and word of mouth reports. Researchers have focused on scientifically validating (or invalidating) those claims via *in vitro* and *in vivo* assays of plants and plant products. The sources reviewed here relate to plants tested for activity against *H. contortus*.

Tanniferous plants. The most extensive research on plants as alternatives to commercial anthelmintics has focused on those plants that are rich in condensed tannins. Condensed tannins (CTs) are secondary plant metabolites that have been reported to have direct and indirect effects on gastrointestinal helminths. An indirect effect may be due to the capacity of CTs to complex with proteins in the rumen so that they pass to the abomasum and small intestine for digestion (Nguyen et al., 2005). This increase in digestible protein supports improved growth and resistance against GI nematodes (Iqbal et al., 2007). However, too high a concentration of CTs in the diet has adverse effects on intake, rumen function, and digestion due to unpalatability of high-CT plants, destruction of rumen microbes, and binding of digestive enzymes by CTs, respectively (Nguyen et al., 2005). Reports of direct anthelmintic effects of CTs have included claims such as inhibited egg hatching and larval development, decreased fecal egg counts, hindrance of exsheathment, and reduced worm burdens. The mechanisms of anthelmintic activity are likely to vary between different CTs from different plant species (Nguyen et al., 2005), and the differences in anti-parasitic effects could be related to the various chemical structures of CTs (Brunet and Hoste, 2006). Table 1.1 summarizes recent publications related to the effects of CT on *Haemonchus contortus*.

Plants with other constituents. In addition to forages known to contain high levels of

condensed tannins, numerous other plants have been chosen for anthelmintic study simply based on their mention in folk medicine or previous reports of antiparasitic, antibacterial, antifungal, or antiviral activity. Table 1.2 summarizes recent studies describing activity against *H. contortus*. These plants appear promising due to the fact that the reported antiparasitic activities were significant (at least 70% or greater efficacy against one or more life stages). Some of these studies mention the presence of specific constituents that could be responsible for the observed anthelmintic effects. Pessoa et al. (2002) noted that eugenol, the main component of *Ocimum gratissimum*, was as efficient in inhibiting egg hatching as the essential oil of the plant, indicating that eugenol may be the active constituent. *Spigelia anthelmia* was reported to contain spiganthine, a compound capable of causing paralysis in worms (Assis et al., 2003), and spigelline, a toxic alkaloid (Ademola et al., 2007). *Nicotiana tabacum*, a species of tobacco plant, contains nicotine, an alkaloid that binds nicotinic receptors on nematode muscles causing paralysis (Iqbal et al., 2006b).

Other plants that have shown significant but less efficacious activity include, but are not limited to, *Zingiber officinale*, commonly known as ginger (66.6% fecal egg count reduction, Iqbal et al., 2006a), *Azadirachta indica* (69% worm burden reduction in sheep, Chandrawathani et al., 2006), and *Hedera helix* (66.6% kill of adults *in vitro*, 44% worm burden reduction in sheep, Eguale et al., 2007). Iqbal et al. (2006a) suggested that because ginger activates cholinergic receptors of the gut, it may activate the same receptors in nematodes, causing paralysis.

Terpenes are the largest group of plant secondary metabolites, and they are the primary constituents of many essential oils. Numerous references list terpenes or terpenoids as compounds identified in the plant of interest (Hördegen et al., 2003, Gathuma et al., 2004, Iqbal et al., 2005, Eguale et al., 2007a, Jabbar et al., 2007). While the main component of a plant is not necessarily responsible for an observed biological activity, terpenes have exhibited a wide range of medicinal activities that include the inhibition of parasites. For example, Kaur et al. (2009) reviewed various terpenes isolated from a range of plants and their efficacies as antimalarials. Suggested modes of action include inhibitory effects on growth, parasite enzymes or plasma membrane pumps, and interference with metabolic pathways or host cell entry. Kaur et al. (2009) also noted the wide variety of chemical structures of these compounds and how small changes in the structure increased or decreased the antimalarial activity of a terpene. This

observation of structure-activity relationships may be linked to the varying levels of anthelmintic activity observed between different plant species.

Challenges in research. One must not take the results reported in medicinal plant studies at face value. There are strengths and weaknesses associated with the methods used to assess the antiparasitic activity of plants. *In vitro* tests such as larval or adult motility and migration assays are cheaper and more convenient than *in vivo* assays. However, they do not recreate actual physiological conditions within the host and possible confounding interactions between plant compounds and host molecules cannot be observed. *In vivo* studies are more appropriate measures of anthelmintic activity but also have disadvantages. *In vivo* studies are costly and more time consuming. Also in these trials, a fecal egg count reduction test may sometimes be the only measure of efficacy when killing animals to determine worm burdens is not an option. A reduction in egg counts may be due to an impact on fecundity rather than actual death of the parasite, so interpretation of the result can be difficult. Also, the time allotted for an *in vivo* study may not be long enough to observe an effect that might occur with consumption of a plant over a long period of time.

Another factor to consider when interpreting any study results is the preparation of a test substance. Athanasiadou et al. (2007) highlighted common concerns. Many studies today opt to test extracts or essential oils rather than the whole plant. These preparations deliver higher concentrations of plant constituents than an animal would be exposed to while grazing, so what may work in the form of an extract may not be efficacious as a forage. Furthermore, the concentration of a compound in the plant or extract does not directly relate to its bioavailability in an animal. The solvent used in preparing an extract is important as well because, due to polarity, an active compound may be present in an alcoholic but not an aqueous extract or vice versa. Also, differing methods relating to storage of plant material prior to a trial may result in changes in the presence of various constituents at the time of use. Similarly, seasonal and environmental factors alter how one plant to the next within the same species will perform. Changes in temperature, daylight, rainfall, and soil nutrients can affect the level of a compound within a plant such that it may be higher during one season than another, or lower in a plant from one geographic location as compared to the same species elsewhere. Active constituents may also vary in presence or concentration between different parts of the same plant. Finally, an

effort must be made to determine which compounds in a plant are actually responsible for its anthelmintic activity because the active compound can also be responsible for toxic effects in the host. A medicinal plant assigned to treat helminths is of little use if it suppresses appetite and weight gain, for example.

Plants and Plant Products Addressed in This Work

Artemisia annua. This weed is a member of the Asteraceae family; there are over 300 *Artemisia* spp., which in the past have been used commonly as spices, essential oils, and insect repellants (Klayman, 1993). *Artemisia annua* is not native to the United States but can be found along rivers in the U.S. *A. annua* also grows in China and many temperate areas worldwide. *A. annua*'s most important role is in the treatment of malaria, a disease caused by invasion of the blood by the protozoan parasite *Plasmodium* spp. Klayman (1993) described the plant's history. Centuries ago, Chinese healers prescribed this plant, often prepared as a tea, for fever and chills associated with the disease. The first effort to validate the prescription was made in the 1960's. Chinese researchers first experimented unsuccessfully with the tea; but by 1971, a diethyl ether extract showed encouraging results in *Plasmodium* spp.-infected mice and monkeys. Further investigation yielded the responsible constituent, a compound eventually named artemisinin. The highest concentration of artemisinin is found in the leaves and flowers of *A. annua* during its vegetative state, which is just before flowering or during flowering (Lommen et al., 2005). By 1973, artemisinin and its derivatives were already being prescribed to thousands of malaria patients all over China. Years later, artemisinin's structure was determined to be a sesquiterpene lactone that contains a rare endoperoxide bridge. This bridge is believed to be responsible for the activity of artemisinin and its derivatives against *Plasmodium* spp. These parasites ingest host blood and breakdown hemoglobin to produce free heme. The heme then reacts with the endoperoxide bridge, breaking the oxygen bonds and causing the production of free radicals that specifically alkylate parasite proteins and heme and damage food vacuole membranes, leading to the death of the parasite (Pandey et al., 1999, Meshnick, 2002, Robert et al., 2005, Efferth, 2007). Artemether, a derivative of artemisinin, kills another blood-feeding parasite, the blood fluke *Schistosoma* spp. Schistosomes died when incubated with artemether and haemin *in vitro* (Xiao et al., 2001, 2003). Also, juvenile schistosomes were killed and marked morphological changes were inducd in adult schistosomes in artemether-treated mice (Xiao et al., 2004).

Researchers have suggested that the mechanism of artemether is similar to the mechanism of artemisinin, involving cleavage of the endoperoxide bridge and generation of free radicals (Utzinger et al, 2001, Xiao et al., 2003, 2004).

Artemisinin is not the only medicinal compound in *Artemisia annua*. Artemisinin is only one of 29 sesquiterpenes in the plant, and *A. annua* also contains monoterpenes, triterpenoids, flavanoids, coumarins, and aromatic and aliphatic compounds (Willcox et al., 2004). It would be surprising if none of these other compounds exhibited activity against parasites. In light of this, other *Artemisia* species have been tested for activity against *H. contortus*. Idris et al. (1982) completely cleared adult *H. contortus* from the abomasums of 4 of 6 treated goats using powdered shoots of *Artemisia herba-alba*. Whole plant aqueous and methanolic extracts of *Artemisia brevifolia* were tested against *H. contortus in vitro* and in sheep with natural helminth infections (Iqbal et al., 2004). The methanolic extract killed *H. contortus in vitro* while the aqueous extract only temporarily inhibited motility, indicating a greater presence of anthelmintic compounds in the methanolic extract. However, a maximum reduction in eggs per gram of feces (67.2%) was observed with the aqueous extract *in vivo* whereas the methanolic extract caused only a slight reduction, indicating water soluble compounds are responsible for an anthelmintic effect *in vivo*. Finally, aqueous and ethanolic extracts of the aerial parts of *A. absinthium* were evaluated *in vitro* and *in vivo* (Tariq et al., 2008). Both extracts significantly decreased motility and viability of *H. contortus in vitro*; however, the ethanolic extract was more efficacious than the aqueous extract. In sheep with natural helminth infections, both extracts fed orally significantly reduced fecal egg counts; again, the ethanolic extract performed better. *Artemisia* spp. have also shown activity against other nematodes. *Artemisia vulgaris* inhibited egg hatching and caused juvenile stage mortality of a plant parasitic nematode, *Meloidogyne megadora* (Costa et al., 2003). Methanolic extracts of both *A. vulgaris* and *Artemisia absinthium* reduced the number of *Trichinella spiralis* larvae in the muscles of treated rats, with *A. vulgaris* causing greater reductions (Caner et al., 2008).

The various *Artemisia* species all seem to show promise as commercial anthelmintic alternatives; however, the aformentioned factors affecting anthelmintic studies should be considered when interpreting these results.

Orange oils. There are 17 *Citrus* spp. which belong to the Rutaceae family and produce

fruits such as lemon, lime, orange, and grapefruit. These plants, native to India, China, and Australia, are cultivated worldwide in tropical and subtropical areas. Oranges are well known for their supply of the essential nutrient and antioxidant Vitamin C. Historically, sailors stricken with scurvy had a deficiency of this vitamin that was remedied by the supply of citrus fruits. Orange oils have long been used as fragrances and flavorings and for medicinal purposes. Bitter orange, for example, is purported to soothe headaches, stimulate digestion, and boost the immune system (Chevallier, 1996). Orange oil is obtained by pressing or steam distilling the oil from the peel. The main component of orange oil (>90%) is a terpene, limonene ($C_{10}H_{16}$), which is responsible for the characteristic aroma of the fruit and has many uses. It is generally regarded as safe by the U.S. Food and Drug Administration, can be used as flavoring or a cleaning product, and has been well-documented as an insecticide (Raina et al., 2007). Orange oil can also serve as an antimicrobial or antifungal agent; Sharma and Tripathi (2006) showed complete inhibition of *Aspergillus niger* by *Citrus sinensis* (Valencia orange) oil even at high heat. Finally, Tsai (2008) showed utility of orange oils against nematodes by showing that pulpified citrus peel killed and inhibited root infection by second-stage juvenile larvae and inhibited hatching of eggs of the plant parasitic nematode *Meloidogyne incognita*.

Based on the many uses of orange oil and the safety of its main constituent, it is not surprising that when the ozone-depleting fumigant methyl bromide was to be phased out of use, orange oil seemed a likely replacement. An entirely food-grade orange oil emulsion which contains orange terpene oil, orange valencia oil, polysorbate 80, hydrogen peroxide, and water, has been patented for purposes of reducing plant pests such as nematodes and fungi; it also has antimicrobial activity (R. Bowker, creator of the product, president of Knock-Out Technologies, personal communication). In initial experiments, some formulations of the product were found to be effective in reducing damage to tomato plant roots caused by the root-knot nematode *Meloidogyne incognita* and all formulations reduced the number of eggs produced per gram of root tissue (Rosskopf et al., 2008). Researchers hypothesized that because plant and animal parasitic nematodes are closely related, the formulation may also work against gastrointestinal helminths. A series of experiments followed that evaluated varying concentrations of the emulsion against egg and larval stages of *H. contortus* (Rosskopf et al., 2008). Eggs were exposed to concentrations of 0.1% to 100% of the emulsion, and a positive and negative control substance (Prohibit ® or Ivomec® and water, respectively), then incubated at 23° C. for 48

hours. The experiment was replicated 8 times. Non-embryonated and embryonated eggs were counted and the number of hatched eggs was estimated as the difference from the negative control. All concentrations significantly inhibited egg hatch, with concentrations of 2% to 100% resulting in at least 90% inhibition. Next, L3 were cultured from egg-laden feces following standard parasitological procedures, exsheathed, and assayed for motility following incubation with the aformentioned controls or the orange oil emulsion at concentrations from 1% to 10%. Incubation lasted 2 hours at which point larvae and media were transferred to sieves. Eighteen hours later the number of larvae that had moved through the sieves was counted and a percent inhibition of migration was calculated after six replicates of the assay. More than 50% of larvae were inhibited from movement or killed by concentrations of 3% or higher. These encouraging *in vitro* results prompted the *in vivo* trials of the orange oil product that will be discussed in later chapters.

Asimina triloba. A member of the Annonaceae family, the paw paw tree is native to the eastern part of North America. The "paw paw" is the edible fruit produced by the tree, the flavor of which is likened to a combination of banana and mango. *A. triloba* is known to be resistant to pests and disease, probably due to the presence of acetogenins (Johnson et al., 1996), which have been shown to have activity against various insects (Mikolajczak et al., 1988). The majority of acetogenins can be found in the bark, roots, twigs, and seeds (Johnson et al., 1996). The mode of action of the compounds involves inhibition of Complex I in mitochondria involved in cellular respiration, resulting in a decreased production of ATP (Johnson et al., 1996). A purified acetogenin, asimicin, and a crude extract of the bark *Asimina triloba* were both shown to cause 100% mortality of the free-living nematode, *Caenorhabditis elegans*, after 72 hours of exposure to an aqueous test solution, although asimicin caused mortality at 0.1 ppm whereas the crude extract caused mortality at 10 ppm (Mikolajczak et al., 1988).

Souza et al. (2008) showed that acetogenins from another member of the Annonaceae family, *Annona squamosa*, had activity against *H. contortus* eggs *in vitro*. Ethyl acetate, methanol-water, and aqueous extracts of the seeds of the plant and an isolated acetogenin were incubated at various concentrations with *H. contortus* eggs for 48 hours. Both the isolated acetogenin and the ethyl acetate extract completely inhibited egg hatching; the methanol-water extract caused 81% inhibition, and the aqueous extract caused only 52% inhibition. These

results show that acetogenins do have anthelmintic activity against at least one stage of *H. contortus*, and that they and possibly other anthelmintic compounds are less soluble in water than in alcohol.

The Gerbil Model for Anthelmintic Studies

Prior to 1990, no suitable *in vivo* laboratory animal model was available to evaluate possible anthelmintics against *H. contortus*. Conder et al. (1990) described a novel assay using Mongolian gerbils (*Meriones unguiculatus*) in which several modern broad-spectrum anthelmintics were evaluated for activity against *H. contortus*. The gerbils were orally inoculated with exsheathed third stage *H. contortus* larvae, treated 10 days post-infection, and killed on day 13 post-infection at which point their stomachs were removed and examined for worms. Conder et al. concluded that many of the drugs exhibited parasite clearance similar to that in ruminants (≥95%) at comparable doses. Conder et al. (1992) went on to confirm the utility of the gerbil model by showing that *H. contortus* is able to establish and grow to immature adult stages in the glandular portion of the gerbil stomach, the same anatomical location that is parasitized in sheep. They reported that worm numbers did not begin to decline until 10-14 days after infection (Conder et al., 1992). These studies support the use of the gerbil assay to screen plant products for anthelmintic activity prior to more costly and time-consuming studies in sheep. This assay is currently in wide use by pharmaceutical companies for testing candidate anthelmintics.

References

Adams, D.B., 1986. Developmental arrest of *Haemonchus contortus* in sheep treated with a corticosteroid. Int J Parasitol 16, 659-664.

Ademola, I.O., Fagbemi, B.O., Idowu, S.O., 2007. Anthelmintic activity of *Spigelia anthelmia* extract against gastrointestinal nematodes of sheep. Parasitol Res 101, 63-69.

Ademola, I.O., Idowu, S.O., 2006. Anthelmintic activity of *Leucaena leucocephala* seed extract on *Haemonchus contortus* infective larvae. Vet Rec 158, 485-486.

Aiello, S.E., Mays, A. (Ed.), 1998. The Merck Veterinary Manual 8th edition. Merck & Co, Inc., Whitehouse Station, NJ.

Alonso-Diaz, M.A., Torres-Acosta, J.F., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Hoste, H., 2008. *In vitro* larval migration and kinetics of exsheathment of *Haemonchus contortus* larvae exposed to four tropical tanniniferous plant extracts. Vet Parasitol 153, 313-319.

Assis, L.M., Bevilaqua, C.M., Morais, S.M., Vieira, L.S., Costa, C.T., Souza, J.A., 2003. Ovicidal and larvicidal activity *in vitro* of *Spigelia anthelmia* Linn. extracts on *Haemonchus contortus*. Vet Parasitol 117, 43-49.

Athanasiadou, S., Githiori, J., Kyriazakis, I., 2007. Medicinal plants for helminth parasite control: facts and fiction. Animal 1, 1392-1400.

Bahuaud, D., Martinez-Ortiz de Montellano, C., Chauveau, S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the *in vitro* exsheathment of third-stage larvae of parasitic nematodes. Parasitology 132, 545-554.

Baker, N.F., Cook, E.F., Douglas, J.R., Cornelius, C.E., 1959 Dec. The pathogenesis of trichostrongyloid parasites. III. Some physiological observations in lambs suffering from acute parasitic gastroenteritis. J Parasitol 45, 643-651.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology 131, 531-538.

Bowman, D.D., 2008. Georgis' Parasitology for Veterinarians. Saunders, Philadelphia.

Brunet, S., Aufrere, J., El Babili, F., Fouraste, I., Hoste, H., 2007. The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both *in vitro* and *in vivo*. Parasitology 134, 1253-1262.

Brunet, S., Jackson, F., Hoste, H., 2008a. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int J Parasitol 38, 783-790.

Brunet, S., Montellano, C.M., Torres-Acosta, J.F., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Capetillo-Leal, C., Hoste, H., 2008b. Effect of the consumption of *Lysiloma latisiliquum* on the larval establishment of gastrointestinal nematodes in goats. Vet Parasitol 157, 81-88.

Burke, J.M., Kaplan, R.M., Miller, J.E., Terrill, T.H., Getz, W.R., Mobini, S., Valencia, E., Williams, M.J., Williamson, L.H., Vatta, A.F., 2007a. Accuracy of the FAMACHA

system for on-farm use by sheep and goat producers in the southeastern United States. Vet Parasitol 147, 89-95.

Burke, J.M., Morrical, D., Miller, J.E., 2007b. Control of gastrointestinal nematodes with copper oxide wire particles in a flock of lactating Polypay ewes and offspring in Iowa, USA. Vet Parasitol 146, 372-375.

Camurca-Vasconcelos, A.L., Bevilaqua, C.M., Morais, S.M., Maciel, M.V., Costa, C.T., Macedo, I.T., Oliveira, L.M., Braga, R.R., Silva, R.A., Vieira, L.S., 2007. Anthelmintic activity of *Croton zehntneri* and *Lippia sidoides* essential oils. Vet Parasitol 148, 288-294.

Caner, A., Doskaya, M., Degirmenci, A., Can, H., Baykan, S., Uner, A., Basdemir, G., Zeybek, U., Guruz, Y., 2008. Comparison of the effects of *Artemisia vulgaris* and *Artemisia absinthium* growing in western Anatolia against trichinellosis (*Trichinella spiralis*) in rats. Exp Parasitol 119, 173-179.

Capitini, L.A., McClure, K.E., Herd, R.P., 1990. Effect of environmental stimuli on pre-infective and infective stages of *Haemonchus contortus* in the northern United States for the induction of hypobiosis. Vet Parasitol 35, 281-293.

Chandrawathani, P., Chang, K.W., Nurulaini, R., Waller, P.J., Adnan, M., Zaini, C.M., Jamnah, O., Khadijah, S., Vincent, N., 2006. Daily feeding of fresh Neem leaves (*Azadirachta indica*) for worm control in sheep. Trop Biomed 23, 23-30.

Chevallier, A., 1996, *Citrus aurantium* (Rutaceae) Bitter Orange, In: The Encyclopedia of Medicincal Plants. Dorling Kindersley Limited, London.

Conder, G.A., Jen, L.W., Marbury, K.S., Johnson, S.S., Guimond, P.M., Thomas, E.M., Lee, B.L., 1990. A novel anthelmintic model utilizing jirds, *Meriones unguiculatus*, infected with *Haemonchus contortus*. J Parasitol 76, 168-170.

Conder, G.A., Johnson, S.S., Hall, A.D., Fleming, M.W., Mills, M.D., Guimond, P.M., 1992. Growth and development of *Haemonchus contortus* in jirds, *Meriones unguiculatus*. J Parasitol 78, 492-497.

Coop, R.L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends Parasitol 17, 325-330.

Costa, S., Santos, M., Ryan, M., 2003. Effect of *Artemisia vulgaris* Rhizome Extracts on Hatching, Mortality, and Plant Infectivity of *Meloidogyne megadora*. J Nematology 35, 437-442.

Craig, T.M., 2006. Anthelmintic resistance and alternative control methods. Vet Clin North Am Food Anim Pract 22, 567-581.

Efferth, T., 2007. Willmar Schwabe Award 2006: antiplasmodial and antitumor activity of artemisinin--from bench to bedside. Planta Med 73, 299-309.

Eguale, T., Tilahun, G., Debella, A., Feleke, A., Makonnen, E., 2007a. *Haemonchus contortus*: *in vitro* and *in vivo* anthelmintic activity of aqueous and hydro-alcoholic extracts of *Hedera helix*. Exp Parasitol 116, 340-345.

Eguale, T., Tilahun, G., Debella, A., Feleke, A., Makonnen, E., 2007b. *In vitro* and *in vivo* anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus*. J Ethnopharmacol 110, 428-433.

FAO, 2005. Major Food and Agricultural Commodities and Producers; Commodities by Country. Food and Agriculture Organization of the United Nations Statistics Division. http://www.fao.org/es/ess/top/country.html;jsessionid=6861F237E560BC39F631510F663641C6?lang=en&country=212&year=2005 (accessed January 10, 2009).

Gamble, H.R., Zajac, A.M., 1992. Resistance of St. Croix lambs to *Haemonchus contortus* in experimentally and naturally acquired infections. Vet Parasitol 41, 211-225.

Gathuma, J.M., Mbaria, J.M., Wanyama, J., Kaburia, H.F., Mpoke, L., Mwangi, J.N., 2004. Efficacy of *Myrsine africana*, *Albizia anthelmintica* and *Hilderbrantia sepalosa* herbal remedies against mixed natural sheep helminthosis in Samburu district, Kenya. J Ethnopharmacol 91, 7-12.

Gatongi, P.M., Prichard, R.K., Ranjan, S., Gathuma, J.M., Munyua, W.K., Cheruiyot, H., Scott, M.E., 1998. Hypobiosis of *Haemonchus contortus* in natural infections of sheep and goats in a semi-arid area of Kenya. Vet Parasitol 77, 49-61.

Getachew, T., Dorchies, P., Jacquiet, P., 2007. Trends and challenges in the effective and sustainable control of *Haemonchus contortus* infection in sheep. Review. Parasite 14, 3-14.

Heckendorn, F., Haring, D.A., Maurer, V., Senn, M., Hertzberg, H., 2007. Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemonchus contortus* and *Cooperia curticei*. Vet Parasitol 146, 123-134.

Heckendorn, F., Haring, D.A., Maurer, V., Zinsstag, J., Langhans, W., Hertzberg, H., 2006. Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs. Vet Parasitol 142, 293-300.

Hordegen, P., Hertzberg, H., Heilmann, J., Langhans, W., Maurer, V., 2003. The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. Vet Parasitol 117, 51-60.

Idris, U.E., Adam, S.E., Tartour, G., 1982. The anthelmintic efficacy of *Artemisia herba-alba* against *Haemonchus contortus* infection in goats. Natl Inst Anim Health Q (Tokyo) 22, 138-143.

Iqbal, Z., Lateef, M., Akhtar, M.S., Ghayur, M.N., Gilani, A.H., 2006a. *In vivo* anthelmintic activity of ginger against gastrointestinal nematodes of sheep. J Ethnopharmacol 106, 285-287.

Iqbal, Z., Lateef, M., Ashraf, M., Jabbar, A., 2004. Anthelmintic activity of *Artemisia brevifolia* in sheep. J Ethnopharmacol 93, 265-268.

Iqbal, Z., Lateef, M., Jabbar, A., Ghayur, M.N., Gilani, A.H., 2006b. *In vitro* and *In vivo* anthelmintic activity of *Nicotiana tabacum* L. leaves against gastrointestinal nematodes of sheep. Phytother Res 20, 46-48.

Iqbal, Z., Lateef, M., Jabbar, A., Muhammad, G., Khan, M.N., 2005. Anthelmintic activity of *Calotropis procera* (Ait.) Ait. F. flowers in sheep. J Ethnopharmacol 102, 256-261.

Iqbal, Z., Sarwar, M., Jabbar, A., Ahmed, S., Nisa, M., Sajid, M.S., Khan, M.N., Mufti, K.A., Yaseen, M., 2007. Direct and indirect anthelmintic effects of condensed tannins in sheep. Vet Parasitol 144, 125-131.

Jabbar, A., Zaman, M.A., Iqbal, Z., Yaseen, M., Shamim, A., 2007. Anthelmintic activity of *Chenopodium album* (L) and *Caesalpinia crista* (L) against trichostrongylid nematodes of sheep. J Ethnopharmacol 114, 86-91.

Johnson, H.A., Gordon, J., McLaughlin, J.L., 1996, Monthly variations in biological activity of *Asimina triloba*, In: Janick, J. (Ed.) Progress in new crops. ASHS Press, Arlington, VA, pp. 609-614.

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

Kaplan, R.M., Burke, J.M., Terrill, T.H., Miller, J.E., Getz, W.R., Mobini, S., Valencia, E., Williams, M.J., Williamson, L.H., Larsen, M., Vatta, A.F., 2004. Validation of the FAMACHA eye color chart for detecting clinical anemia in sheep and goats on farms in the southern United States. Vet Parasitol 123, 105-120.

Kaur, K., Jain, M., Kaur, T., Jain, R., 2009. Antimalarials from nature. Bioorg Med Chem. In press.

Klayman, D.L. 1993. *Artemisia annua*: From weed to respectable antimalarial plant. In Amercian Chemical Society Symposium Series, Human Medicinal Agents from Plants, Kinghorn, A.D., Balandin, M.F., eds. (Washington DC).

Knox, M.R., 2002. Effectiveness of copper oxide wire particles for *Haemonchus contortus* control in sheep. Aust Vet J 80, 224-227.

Lange, K.C., Olcott, D.D., Miller, J.E., Mosjidis, J.A., Terrill, T.H., Burke, J.M., Kearney, M.T., 2006. Effect of sericea lespedeza (*Lespedeza cuneata*) fed as hay, on natural and experimental *Haemonchus contortus* infections in lambs. Vet Parasitol 141, 273-278.

Langrova, I., Makovcova, K., Vadlejch, J., Jankovska, I., Petrtyl, M., Fechtner, J., Keil, P., Lytvynets, A., Borkovcova, M., 2008. Arrested development of sheep strongyles: onset and resumption under field conditions of Central Europe. Parasitol Res 103, 387-392.

Larsen, M., 2000. Prospects for controlling animal parasitic nematodes by predacious micro fungi. Parasitology 120 Suppl, S121-131.

Levine, N.D., 1980. Nematode parasites of domestic animals and of man, 2d Edition. Burgess Publishing Company, Minneapolis.

Lommen, W.J., Schenk, E., Bouwmeester, H.J., Verstappen, F.W., 2006. Trichome dynamics and artemisinin accumulation during development and senescence of *Artemisia annua* leaves. Planta Med 72, 336-345.

López, J., Ibarra, O.F., Cantó, G.J., Vásquez, C.G., Tejada, Z.I., Shimada, A., 2005. *In vitro* effect of condensed tannins from tropical fodder crops against eggs and larvae of the nematode *Haemonchus contortus*. J Food Ag Environ 3, 191-194.

Meshnick, S.R., 2002. Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 32, 1655-1660.

Mikolajczak, K.L., McLaughlin, J.L., Rupprecht, J.K. 1998. Control of Pests with Annonaceous acetogenins (United States Patent Number 4721727).

Nguyen, T.M., Binh, D.V., Orskov, E.R., 2005. Effect of foliages containing condensed tannins and on gastrointestinal parasites. An Feed Sci Tech 121, 77-87.

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

Pandey, A.V., Tekwani, B.L., Singh, R.L., Chauhan, V.S., 1999. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. J Biol Chem 274, 19383-19388.

Paolini, V., Bergeaud, J.P., Grisez, C., Prevot, F., Dorchies, P., Hoste, H., 2003. Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. Vet Parasitol 113, 253-261.

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

Raina, A., Bland, J., Doolittle, M., Lax, A., Boopathy, R., Folkins, M., 2007. Effect of orange oil extract on the formosan subterranean termite (Isoptera: Rhinotermitidae). J Econ Entomol 100, 880-885.

Robert, A., Benoit-Vical, F., Claparols, C., Meunier, B., 2005. The antimalarial drug artemisinin alkylates heme in infected mice. Proc Natl Acad Sci U S A 102, 13676-13680.

Rosskopf, E.N., Therrien, L.L., Adkins, S.T., Iriarte, F., Foster, J.G., Kokalis-burelle, N. 2008. Methods of reducing pests and treating gastrointestinal nematode infections (Patent Application 20080166437, filed 1/3/2008), http://www.freshpatents.com/Methods-of-reducing-pests-and-treating-gastrointestinal-nematode-infections-dt20080710ptan20080166437.php?type=description 7/22/08 - 4/23/09.

Sangster, N.C., 1999. Anthelmintic resistance: past, present and future. Int J Parasitol 29, 115-124; discussion 137-118.

Shaik, S.A., Terrill, T.H., Miller, J.E., Kouakou, B., Kannan, G., Kaplan, R.M., Burke, J.M., Mosjidis, J.A., 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infection in goats. Vet Parasitol 139, 150-157.

Sharma, N., Tripathi, A., 2008. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. Microbiol Res 163, 337-344.

Silvestre, A., Humbert, J.F., 2002. Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. Int J Parasitol 32, 921-928.

Souza, M.M., Bevilaqua, C.M., Morais, S.M., Costa, C.T., Silva, A.R., Braz-Filho, R., 2008. Anthelmintic acetogenin from *Annona squamosa* L. Seeds. Ann Brazilian Acad Sci 80, 271-277.

Stear, M.J., Doligalska, M., Donskow-Schmelter, K., 2007. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 139-151.

Sundlof, S.F., Whitlock, T.W., 1992. Clorsulon pharmacokinetics in sheep and goats following oral and intravenous administration. J Vet Pharm Ther 15, 282-291.

Tariq, K.A., Chishti, M.Z., Ahmad, F., Shawl, A.S., 2008. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes Vet Parasitol 160, 83-88.

Tsai, B.Y., 2008. Effect of peels of lemon, orange, and grapefruit against *Meloidogyne incognita*. Plant Pathol Bull 17, 195-201.

Utzinger, J., Xiao, S., N'Goran, E.K., Bergquist, R., Tanner, M., 2001. The potential of artemether for the control of schistosomiasis. Int J Parasitol 31, 1549-1562.

Waller, P.J., 1997. Anthelmintic resistance. Vet Parasitol 72, 391-405; discussion 405-312.

Waller, P.J., Chandrawathani, P., 2005. *Haemonchus contortus*: Parasite problem No. 1 from Tropics - Polar Circle. Problems and prospects for control based on epidemiology. Tropical Biomedicine 22, 131-137.

White, J.D., Bolton, G.L., Dantanarayana, A.P., Fox, C.M.J., Hiner, R.N., Jackson, R.W., Sakuma, K., Warrier, U.S., 1995. Total Synthesis of the Antiparasitic Agent Avermectin B1a. J Amer Chem Soc 117, 1908-1939.

Willcox, M., Bodeker, G., Bourdy, G., Dhingra, V., Falquet, J., Ferreira, J., Graz, B., Hirt, H., Hsu, E., de Magalhães, P., Provendier, D., Wright, C., 2004, *Artemisia annua* as a Traditional Herbal Antimalarial, In: Willcox, M., Bodeker, G., Rasoanaivo, P., Addae-Kyereme, J. (Eds.) Traditional Medicinal Plants and Malaria. CRC Press, Boca Raton, FL, pp. 43-59.

Xiao, S., Chollet, J., Utzinger, J., Matile, H., Mei, J., Tanner, M., 2001. Artemether administered together with haemin damages schistosomes in vitro. Trans R Soc Trop Med Hyg 95, 67-71.

Xiao, S.H., Wu, Y.L., Tanner, M., Wu, W.M., Utzinger, J., Mei, J.Y., Scorneaux, B., Chollet, J., Zhai, Z., 2003. *Schistosoma japonicum*: In vitro effects of artemether combined with haemin depend on cultivation media and appraisal of artemether products appearing in the media. Parasitol Res 89, 459-466.

Xiao, S.H., Guo, J., Chollet, J., Wu, J.T., Tanner, M., Utzinger, J., 2004. Effect of artemether on *Schistosoma mansoni*: dose-efficacy relationship, and changes in worm morphology and histopathology. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 22, 148-153.

Zajac, A.M., 2006. Gastrointestinal nematodes of small ruminants: life cycle, anthelmintics, and diagnosis. Vet Clin North Am Food Anim Pract 22, 529-541.

Zygoyiannas, D., 2006. Sheep production in the world and in Greece. Small Rum Res 62, 143-147.

Table 1.1

Recent publications related to the effects of condensed tannins on *Haemonchus contortus*

Reference	Forage Species	Preparation	Results
Iqbal et al., 2007	Quebracho (*Schinopsis* spp)	Extract of commercial tannin	Egg hatch inhibition but no effect on adults *in vitro*, fecal egg count reduction in sheep
Brunet et al., 2008a	Sainfoin (*Onobrychis viciifolia*)	Whole plant extract	Dose-dependent, reduced association of larvae with fundic explants *in vitro*
Heckendorn et al., 2007	Chicory (*Chicorium intybus*), Birdsfoot trefoil (*Lotus corniculatus*), *Onobrychis viciifolia*	Fresh cut plant	All reduced fecal egg counts in lambs, none significantly reduced worm burdens
Brunet et al., 2007	*Onobrychis viciifolia*	Whole plant extract	Dose-dependent inhibition of larval exsheathment *in vitro* and *in vivo*
Barrau et al., 2005	*Onobrychis viciifolia*	Whole plant extracts and fractions of an acetone/water extract	Some but not all inhibited larval migration *in vitro*
Heckendorn et al., 2006	*Onobrychis viciifolia*	Hay and Silage	Both reduced adult burden and fecal egg counts in lambs, but only hay results were significant
Paolini et al., 2003	Quebracho	Extract	Reduced fecal egg counts in goats but no reduction of adult worm burden
Shaik et al., 2006	*Lespedeza cuneata*	Hay	Reduced fecal egg counts in goats and decreased larval development from eggs in culture
Lange et al., 2006	*Lespedeza cuneata*	Hay	Reduced fecal egg counts in naturally and experimentally infected lambs, reduced adult burden in naturally infected lambs
Ademola and Idowu, 2006	*Leucaena leucocephala*	Aqueous seed extract	Concentration-dependent kill of L3 *in vitro*
Alonso-Díaz et al., 2008	*Acacia pennatula, Lysiloma latisiliquum, Piscidia piscipula, Leucaena leucocephala*	Leaf extracts	All inhibited L3 exsheathment and all but *P. piscipula* dose-dependently inhibited migration *in vitro*

López et al., 2005	*Arachis pintoi, Guazuma ulmyfolia, Leucaena leucocephala, Manihot esculenta*	CT extracts	All were larvicidal but not ovicidal *in vitro*
Brunet et al., 2008b	*Lysiloma latisiliquum*	Fresh leaves	Reduced larval establishment in goats
Bahuaud et al., 2006	*Sarothamnus scoparius, Erica erigena, Pinus sylvestris, Castanea sativa*	Whole plant extracts Leaf extract External tegument of fruit extract	All but *S. scoparius* delayed or fully inhibited exsheathment *in vitro*

Table 1.2

Recent publications related to activity of non-tannin-rich plants against *Haemonchus contortus*

Reference	Forage Species	Preparation	Results
Camurça-Vasconcelos et al., 2007	*Croton zehntneri, Lippia sidoides*	Essential oil	Both inhibited egg hatch and larval development *in vitro*
Jabbar et al., 2007	*Chenopodium album*	Whole plant aqueous methanolic extract	Killed adults and inhibited egg hatch *in vitro*
Pessoa et al., 2002	*Ocimum gratissimum*	Essential oil	Inhibited egg hatching *in vitro*
Assis et al., 2003	*Spigelia anthelmia*	Extracts of dried aerial parts	Some but not all extracts inhibited egg hatching and/or larval development *in vitro*
Ademola et al., 2007	*Spigelia anthelmia*	Whole plant ethanolic extract	Killed L3 *in vitro* and reduced fecal egg counts in sheep
Iqbal et al., 2005	*Calotropis procera*	Flower crude powder, aqueous and methanolic extract	Aq. and methanolic extracts inhibited motility *in vitro*, powder and aq. but not methanolic extract reduced fecal egg counts in sheep
Eguale et al., 2007b	*Coriandrum sativum*	Aqueous and hydro-alcoholic extract	Both inhibited egg hatching *in vitro*, aqueous extract reduced male worm burden in sheep
Iqbal et al., 2006b	*Nicotiana tabacum*	Leaf aqueous and methanolic exctract	Both inhibited adult worm motility *in vitro*
Gathuma et al., 2004	*Albizia anthelmintica*	Aqueous extract	Egg count reduction in sheep
Hördegen et al., 2003	*Fumaria parviflora*	Ethanolic extract	Fecal egg count and adult worm burden reductions in lambs

CHAPTER 2

Effects of *Artemisia annua* products on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*)

Abstract

Haemonchus contortus is a blood-sucking abomasal parasite of small ruminants that is responsible for major losses to producers worldwide. Resistance of this nematode to commercial anthelmintics has produced a demand for alternative control methods. *Artemisia annua* is a herb used as an ancient Chinese remedy for various ailments including malaria and helminth infections. Artemisinin, a compound isolated from *A. annua*, has been shown experimentally to have activity against schistosomes. In this study, artemisinin and *A. annua* aqueous extract (AE) and ethanolic extract (EE) were evaluated against *H. contortus* in a gerbil model. In all experiments, gerbils were infected with 600 third-stage larvae. In one experiment, gerbils were treated orally with 400 mg/kg artemisinin once or 200 mg/kg artemisinin daily for 5 days. In a second experiment, gerbils were treated daily for 5 days with 600 mg/kg AE or EE. On day 9 post-infection, gerbils were killed, their stomachs removed, and the worms counted. Artemisinin once or daily for 5 days did not show anthelmintic activity (-25% and -35% parasite reduction, respectively) compared to untreated control groups. *Artemisia annua* AE and EE caused 2% and 25% parasite reduction, respectively, compared to an untreated control group. Differences were not statistically significant in either experiment. Artemisinin and *A. annua* extracts did not significantly affect *H. contortus* in gerbils at the given dosages.

Introduction

Haemonchus contortus is an important abomasal helminth of small ruminants responsible for disease and major production losses worldwide. This blood-feeding parasite can cause severe anemia and rapid death in afflicted livestock. Modern control programs for infection with the parasite have relied heavily on the use of commercial anthelmintics. However, extensive resistance of *H. contortus* to these drugs has developed and there is a need for alternative control strategies. One such strategy is to employ plant products that have natural anthelmintic properties.

Artemisia annua (Asteraceae), commonly known as sweet wormwood, is a weed that

grows throughout temperate areas worldwide. For centuries, practitioners of folk medicine have prescribed this plant for the treatment of malaria, a disease caused by invasion of the blood by the protozoan parasite *Plasmodium* spp. The compound present in *A. annua* purported to be responsible for its medicinal properties is artemisinin, a sesquiterpene lactone that contains a rare endoperoxide bridge. Studies indicate that as *Plasmodium* spp. digests host hemoglobin, free heme is produced that reacts with the endoperoxide bridge of artemisinin, breaking the oxygen bonds and causing the production of free radicals which specifically alkylate parasite proteins and heme and damage food vacuole membranes, leading to the death of the parasite (Pandey et al., 1999, Meshnick, 2002, Robert et al., 2005, Efferth, 2007). Artemether, a derivative of artemisinin, kills blood flukes (*Schistosoma* spp.) via a similar mechanism (Utzinger et al, 2001, Xiao et al., 2001, 2003, 2004). We hypothesize that *Haemonchus contortus* will also be negatively affected by artemisinin due to its blood-consuming lifestyle.

Artemisinin may not be the only medicinal compound in *A. annua*. Extracts from *Artemisia* spp. contain many compounds and have exhibited activity against *H. contortus*. Idris et al. (1982) successfully cleared *H. contortus* infection in 4 of 6 treated goats using powdered shoots of *Artemisia herba-alba*. Whole plant aqueous and methanolic extracts of *Artemisia brevifolia* were tested against *H. contortus in vitro* and in sheep with natural helminth infections (Iqbal et al., 2004). The methanolic extract killed *H. contortus in vitro* whereas a reduction in eggs per gram of feces (67.2%) was observed with the aqueous extract *in vivo*. Finally, aqueous and ethanolic extracts of the stems and leaves of *A. absinthium* were evaluated *in vitro* and *in vivo* (Tariq et al., 2008). Both extracts significantly affected motility and viability of *H. contortus in vitro*. In sheep with natural helminth infections, both extracts fed orally significantly reduced fecal egg counts. Based on these findings, we hypothesized that extracts of *A. annua* would also clear *H. contortus* burdens *in vivo*.

The purpose of our experiments was to evaluate artemisinin as well as aqueous and ethanolic extracts of *A. annua* for anthelmintic activity against larval stages of *H. contortus* in a gerbil model of infection.

Materials and Methods

Animals

Visually healthy, non-pregnant, non-lactating female gerbils (*Meriones unguiculatus*) at

approximately 5 weeks of age and weighing about 50 g were purchased from Charles River Laboratories International, Inc. They were housed 2 per cage and provided commercial rodent chow and water *ad libitum*. The gerbils were allowed to acclimate for a period of two weeks before infection. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Parasites

Third stage infective *H. contortus* larvae (L3) were provided in water by Dr. James Miller, Louisiana State University, or were cultured from the feces of a monospecifically infected lamb according to standard parasitological techniques.

Infection

Third stage larvae for infection were exsheathed according to Conder and Johnson, 1996. Briefly, carbon dioxide was bubbled into a flask containing Earle's Balanced Salt Solution (EBSS) and *H. contortus* larvae that were incubated overnight at 37°C. Gerbils were inoculated via oral gavage with 600 exsheathed *H. contortus* L3 in EBSS in a total volume of 0.3 - 0.5 ml. Daily health observations were performed throughout the experiment.

H. contortus Counts

In all experiments, gerbils were euthanized by carbon dioxide asphyxiation followed by thoracotomy 9 days after infection. Their stomachs were removed, opened longitudinally, placed in deionized water, and incubated at 37°C for 2-3 hours following the method of Conder et al., 1991. Formaldehyde was added to create a final concentration of 10% formalin to preserve samples for subsequent counting. The collected fluid and stomachs were examined for parasites using a dissecting microscope by investigators blind to the treatment groups. Lugol's iodine solution was used to enhance the appearance of *H. contortus* for easier counting.

Statistical Analysis

Normal Probability plots were generated based on larval counts to verify that data followed an approximate normal distribution. When distributions were not normal, a logarithmic (base e) transformation was applied to the larval counts to normalize worm burdens. Base e

transformation was selected to maintain resolution that may be lost following base 10 transformation. Groups were compared using ANOVA followed by Tukey's procedure for multiple comparisons. Statistical significance was set to $\alpha=0.05$. These analyses were performed using SAS version 9.2 (Carry, NC, USA). For experiments two and three, efficacy against *H. contortus* was calculated as a percent parasite reduction:

$$\% \text{ reduction} = 100 \times (C-T)/C$$

where C is the arithmetic mean number of worms in an untreated control group and T is the arithmetic mean number of worms in a treatment group.

Experimental Protocol

Experiment One

An initial infection of gerbils was carried out in an attempt to verify the utility of the *H. contortus* gerbil model and determine the range of worm burdens for use in designing further experiments. Ten gerbils were infected with 600 *H. contortus* L3 (day 0). No treatments were administered and parasite counts were determined as previously described.

Experiment Two

Forty gerbils were infected with 600 *H. contortus* L3 (day 0) and randomly allocated into 4 groups of 10 gerbils that were given the following treatments:

Group 1: 400 mg/kg artemisinin in DMSO and olive oil once (day 6)

Group 2: 200 mg/kg artemisinin in DMSO and olive oil daily for 5 days (days 4-8)

Group 3: Control, DMSO and olive oil daily for 5 days (days 4-8)

Group 4: Control, water daily for 5 days (days 4-8)

Treatments

Dosages were based on available information on efficacy of artemether against another blood feeding helminth (Shuhua and Catto, 1989). Artemisinin was provided by Allergy Research Group (Alameda, CA) and was analyzed by high performance liquid chromatography with ultraviolet detectors to ensure purity (app. 98% pure). All gerbils were dosed via oral gavage. A mixture of dimethyl sulfoxide (DMSO) and olive oil was used to dissolve the artemisinin to achieve a constant volume (0.3 ml) and avoid potential DMSO toxicosis.

Experiment Three

Thirty gerbils were infected (day 0) with 600 *H. contortus* L3 cultured from the feces of a monospecifically infected donor lamb and randomly allocated into 3 treatment groups:
Group 1: 600 mg/kg *A. annua* ethanolic extract (E.E.) in 3% Tween 80 daily for 5 days (days 4-8)
Group 2: 600 mg/kg *A. annua* aqueous extract (A.E.) in 3% Tween 80 daily for 5 days (days 4-8)
Group 3: Control, 3% Tween 80 daily for 5 days (days 4-8)

Treatments

Artemisia annua extracts were prepared by Dr. Jorge Ferreira, USDA, ARS as follows: oven dried (45°C) leaves of a Brazilian cultivar of *A. annua* (University of Campinas, Brazil) were ground in a cyclone grinder to 2 mm size particles. 25 g of the ground leaves were extracted using 150 ml of 70% ethanol (70:30 ethanol:water) at 60°C with stirring on a hot plate for 2 hours. The ethanolic extract was then sonicated for 30 minutes and filtered through #2 Whatman filter paper. The extraction was repeated and the extracts were combined, rotoevaporated to dryness, redissolved in pure ethanol, and sonicated to remove all the extract from the rotoevaporator flask. The extract was then concentrated under air flow and freeze dried. The resulting crystals were crushed and suspended in 3% Tween 80 before use. For the aqueous extract, 25 g of the ground leaves was placed in 200 ml water and brought to a boil on a hot plate with stirring for 1.5 hours. The sample was then concentrated in a rotoevaporator at 60°C, frozen at -4°C, and then freeze dried. The extract was dissolved in 3% Tween 80 before use. Tween 80 was used rather than DMSO for solubility reasons.

Dosages were based on the efficacy and lack of toxicity of other *Artemisia* spp. against *Trichinella spiralis* in rats (Caner et al., 2008). All gerbils were dosed via oral gavage with a total volume of 0.36 ml.

Results

Experiment One

The results of the initial inoculation of gerbils with *H. contortus* infective larvae showed a mean parasite burden of 111 ± 51 larvae. This confirmed establishment of the parasite and the

observed variability of infection was expected due to individual animal susceptibility.

Experiment Two

Data from the artemisinin trial were normally distributed and arithmetic data was used for analysis. Percent larval reduction caused by artemisinin equaled -25.2% and -34.8% for the groups treated once or daily for 5 days, respectively (Table 2.1). Although there were no significant differences in mean parasite burden between all four groups (P = 0.14), artemisinin treated groups exhibited higher mean parasite burdens than control groups. The groups treated with artemisinin once or daily for 5 days had a mean *H. contortus* burden of 72.6 (±18.5) and 78.2 (±22.1) larvae, respectively, while the DMSO/olive oil and water control groups averaged 57 (±19.2) and 58.9 (±32.1) larvae, respectively.

Experiment Three

Parasite counts from the *A. annua* trial were not normally distributed most likely due to natural variability in parasitic infection, thus a logarithmic (base e) transformation was applied to the larval counts to normalize burdens. Therefore, means data were summarized as geometric means with geometric 95% confidence intervals. Results are shown in Table 2.2. Groups treated with *A. annua* E.E. and *A. annua* A.E. averaged 54.8 and 68.5 *H. contortus* larvae, respectively, compared to a mean of 74.5 larvae in the control group. Gerbils treated with *A. annua* E.E. had 24.7% fewer *H. contortus* larvae as compared to the control group; however, this was not significant (P = 0.37). *A. annua* A.E. produced a parasite reduction of only 2.14% compared to the control group; this also was not significant (P = 0.81).

Discussion

Artemisinin

Treatment with artemisinin did not reduce *Haemonchus contortus* larvae in infected gerbils. The bioavailability of artemisinin is known to be low due to its poor oil- and water-solubility (Klayman, 1993, Utzinger et al., 2001), so a combination of DMSO and olive oil was selected to aid in the delivery and uptake of artemisinin. DMSO is a relatively non-toxic solvent capable of dissolving both polar and nonpolar molecules and easily crossing membranes without causing damage. These properties make DMSO an ideal carrier for a wide variety of

compounds. However, to avoid any potential DMSO toxicity, we limited the amount given to the gerbils and used olive oil, which is rich in lipids that also aid in cellular uptake, to complete the volume. The exact bioavailability of artemisinin in this trial is unknown, but it may not have reached its target, heme. According to the mode of action of artemisinin reported against *Plasmodium* spp. and schistosomes, heme is produced from the digestion of hemoglobin by the parasite (Pandey et al., 1999, Utzinger et al, 2001, Meshnick, 2002, Xiao et al., 2003 & 2004, Robert et al., 2005, Efferth, 2007). Heme then reacts with the endoperoxide bridge of artemisinin, causing the production of reactive oxygen species that go on to damage host proteins. Ingestion of both blood and artemisinin by *H. contortus* may be necessary to produce the reaction. We began dosing gerbils with artemisinin 4 days post-infection. According to Conder et al. (1992), all *H. contortus* larvae in infected gerbils have reached the fourth stage by 4 days post-infection. Although blood feeding does occur during the fourth stage in sheep (Gamble and Mansfield, 1996), it is unknown how long after entrance into the fourth stage feeding begins. Perhaps the larvae in our experiment had not yet begun feeding, in which case heme would not be available, nor would artemisinin be present in the worm to react. Further studies are needed to determine the feeding status of *H. contortus* larvae at the time of treatment.

If, however, *H. contortus* L4 were feeding during treatment, another explanation for the lack of efficacy of artemisinin is needed. The dosages of artemisinin in this experiment were based on Shuhua and Catto (1989) who reported that mice infected with adult *Schistosoma japonicum* and treated with a total dose of 400 to 800 mg/kg over a 1 to 4 day period exhibited worm reduction rates of up to 79.9%. Mice infected with *S. mansoni* and treated with up to 1200 mg/kg over a 3 to 6 day period exhibited worm reductions up to only 39.1%. Artemisinin derivatives are more soluble and active than artemisinin itself (Utzinger et al., 2001), thus, it may be possible that the doses administered in our study were not sufficient to provide anthelmintic activity. Quite the contrary, artemisinin treated groups exhibited higher worm burdens than controls. Wang et al. (2007) studied the effects of artemether on immunity and found that the artemisinin derivative inhibited T-cell activation and cell cycle progression as well as cytokine production. If artemisinin is also immunosuppressive, an inhibited T-cell response could have aided parasite establishment in the gerbil stomachs. Further studies would be necessary to determine the existence and degree of immunosuppression and to assess higher doses of artemisinin needed for efficacy despite reduced immunity.

Another explanation for a lack of efficacy of artemisinin is that *H. contortus* is able to cope with the oxidative stress caused by artemisinin. Kotze and McClure (2001) provided evidence that *H. contortus* utilizes the enzyme catalase to protect itself from the effects of hydrogen peroxide *in vitro*. Kotze (2003) also showed that fourth-stage larvae exhibited up to 4.6-fold induction of catalase in the presence of hydrogen peroxide generated *in vitro*. This ability to respond to the production of reactive oxygen species may explain the survival of *H. contortus* in spite of exposure to artemisinin in our experiment. Worms were eventually overwhelmed, however, by higher concentrations of hydrogen peroxide (Kotze, 2003); perhaps higher doses of artemisinin could have a similar effect.

Finally, there are physiological differences between gerbils and sheep that should be considered, such as metabolic rates and differences in the digestive tract, that may affect the bioavailability and activity of artemisinin in either species. Thus, artemisinin should be tested in the natural host for *H. contortus* before it is ultimately accepted or discarded as a possible ruminant anthelmintic.

Artemisia annua extracts

Following the determination of a lack of efficacy of artemisinin against *H. contortus* in gerbils, *A. annua* extracts were tested in a second gerbil assay in an attempt to identify if any anthelmintic activity was present in other compounds in the plant. There was a slight decrease in parasite burdens in extract treated gerbils compared to control gerbils; however, the reductions were not significant. The 24.7% reduction seen in *A. annua* ethanolic extract-treated gerbils indicates that there may be some alcohol soluble active compounds, but perhaps the dosage was not high enough to result in a significant reduction. Perazzo et al. (2003) reported the LD_{50} of intraperitoneally-administered *A. annua* ethanolic extract to be more than 2.0 g/kg in mice. Our dosage of 600 mg/kg is less than half of that dosage and was delivered orally, which is considered to result in lower concentrations of extract in the body than i.p. delivery due to slower absorption (M. Ehrich, verbal communication). There were no signs in the gerbils of toxicosis reported in mice (stereotypy, convulsions, ataxia) even after 5 days of dosing. A future study is planned to evaluate a higher dosage of *A. annua* ethanolic extract for greater efficacy against *H. contortus* larvae in gerbils.

The smaller reduction in parasite burdens in aqueous extract-treated gerbils suggests that

potential components of *A. annua* responsible for anthelmintic activity are not water soluble. This point brings to light the issue of the carrier selected in this experiment: 3% Tween80 in water. The ethanolic extract contains non-water-soluble compounds, so delivery in water is less than ideal. However, the extracts could not be delivered in DMSO and olive oil due to toxicity and solubility issues. Labrasol, a drug carrier composed of glycerides and esters of polyethylene glycol, poses a less toxic threat than DMSO and enhances the bioavailability of poorly soluble drugs. Further investigation of the extract will include delivery with Labrasol rather than 3% Tween 80.

Perazzo et al. (2003) analyzed the ethanolic extract of *A. annua* with gas chromatography-mass spectrometry and found that the main component was phytol, an acyclic diterpene alcohol. They also indentified camphor, a terpenoid, β-cubeben, a tri-cyclic sesquiterpene, and *trans*-caryophyllene, a bicyclic sesquiterpene. It is unknown whether or not these main constituents are responsible for the possible small larval reduction observed in this experiment; further investigation would be required to determine the exact compound and mode of action. However, terpenes have been described as having activity against parasites. Camphor was identified in the essential oil (29.2%) of flowerheads of *Chrysanthemum coronarium* (Asteraceae), which showed significant nematocidal activity against the root-knot nematode *Meloidogyne artiellia in vitro* by reducing egg hatching and larval survival (Perez et al., 2003). Kaur et al. (2009) reviewed various terpenes isolated from a range of plants and their efficacies as antimalarials. Suggested modes of actions include inhibitory effects on growth, parasite enzymes or plasma membrane pumps, and interference with metabolic pathways. *In vitro* tests of a similar concentration of the ethanolic extract used in this experiment resulted in a significant reduction of larval motility (J. Ferreira, verbal communication), suggesting that interference with neuromuscular activity may be the mode of action of the extract but, as mentioned before, problems with solubility may explain the lack of a significant affect *in vivo*.

References

Caner, A., Doskaya, M., Degirmenci, A., Can, H., Baykan, S., Uner, A., Basdemir, G., Zeybek, U., Guruz, Y., 2008. Comparison of the effects of *Artemisia vulgaris* and *Artemisia absinthium* growing in western Anatolia against trichinellosis (*Trichinella spiralis*) in rats. Exp Parasitol 119, 173-179.

Conder, G.A., Johnson, S.S., 1996. Viability of infective larvae of *Haemonchus contortus*, *Ostertagia ostertagi*, and *Trichostrongylus colubriformis* following exsheathment by various techniques. J Parasitol 82, 100-102.

Conder, G.A., Johnson, S.S., Guimond, P.M., Cox, D.L., Lee, B.L., 1991. Concurrent infections with the ruminant nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* in jirds, *Meriones unguiculatus*, and use of this model for anthelmintic studies. J Parasitol 77, 621-623.

Conder, G.A., Johnson, S.S., Hall, A.D., Fleming, M.W., Mills, M.D., Guimond, P.M., 1992. Growth and development of *Haemonchus contortus* in jirds, *Meriones unguiculatus*. J Parasitol 78, 492-497.

Efferth, T., 2007. Willmar Schwabe Award 2006: antiplasmodial and antitumor activity of artemisinin--from bench to bedside. Planta Med 73, 299-309.

Gamble, H.R., Mansfield, L.S., 1996. Characterization of excretory-secretory products from larval stages of *Haemonchus contortus* cultured in vitro. Vet Parasitol 62, 291-305.

Idris, U.E., Adam, S.E., Tartour, G., 1982. The anthelmintic efficacy of *Artemisia herba-alba* against *Haemonchus contortus* infection in goats. Natl Inst Anim Health Q (Tokyo) 22, 138-143.

Iqbal, Z., Lateef, M., Ashraf, M., Jabbar, A., 2004. Anthelmintic activity of *Artemisia brevifolia* in sheep. J Ethnopharmacol 93, 265-268.

Kaur, K., Jain, M., Kaur, T., Jain, R., 2009. Antimalarials from nature. Bioorg Med Chem.

Klayman, D.L. 1993. *Artemisia annua*: From weed to respectable antimalarial plant. In Amercian Chemical Society Symposium Series, Human Medicinal Agents from Plants, Kinghorn, A.D., Balandin, M.F., eds. (Washington DC).

Kotze, A.C., 2003. Catalase induction protects *Haemonchus contortus* against hydrogen peroxide in vitro. Int J Parasitol 33, 393-400.

Kotze, A.C., McClure, S.J., 2001. *Haemonchus contortus* utilises catalase in defence against exogenous hydrogen peroxide in vitro. Int J Parasitol 31, 1563-1571.

Meshnick, S.R., 2002. Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 32, 1655-1660.

Pandey, A.V., Tekwani, B.L., Singh, R.L., Chauhan, V.S., 1999. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. J Biol Chem 274, 19383-19388.

Perazzo, F.F., Carvalho, J.C., Carvalho, J.E., Rehder, V.L., 2003. Central properties of the essential oil and the crude ethanol extract from aerial parts of *Artemisia annua* L. Pharmacol Res 48, 497-502.

Perez, M.P., Navas-Cortés, J.A., Pascual-Villalobos, M.J., Castillo, P., 2003. Nematicidal activity of essential oils and organic amendments from Asteraceae against root-knot nematodes. Plant Pathology 52, 395-401.

Robert, A., Benoit-Vical, F., Claparols, C., Meunier, B., 2005. The antimalarial drug artemisinin alkylates heme in infected mice. Proc Natl Acad Sci U S A 102, 13676-13680.

Shuhua, X., Catto, B.A., Sept. 1989. *In vitro* and *in vivo* studies of the effect of artemether on *Schistosoma mansoni*. Antimicrob Agents Chemo 33, 1557-1562.

Tariq, K.A., Chishti, M.Z., Ahmad, F., Shawl, A.S., 2008. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes Vet Parasitol 160, 83-88.

Utzinger, J., Xiao, S., N'Goran, E.K., Bergquist, R., Tanner, M., 2001. The potential of artemether for the control of schistosomiasis. Int J Parasitol 31, 1549-1562.

Wang, J.X., Tang, W., Shi, L.P., Wan, J., Zhou, R., Ni, J., Fu, Y.F., Yang, Y.F., Li, Y., Zuo, J.P., 2007. Investigation of the immunosuppressive activity of artemether on T-cell activation and proliferation. Br J Pharmacol 150, 652-661.

Xiao, S., Chollet, J., Utzinger, J., Matile, H., Mei, J., Tanner, M., 2001. Artemether administered together with haemin damages schistosomes in vitro. Trans R Soc Trop Med Hyg 95, 67-71.

Xiao, S.H., Guo, J., Chollet, J., Wu, J.T., Tanner, M., Utzinger, J., 2004. Effect of artemether on *Schistosoma mansoni*: dose-efficacy relationship, and changes in worm morphology and histopathology. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 22, 148-153.

Xiao, S.H., Wu, Y.L., Tanner, M., Wu, W.M., Utzinger, J., Mei, J.Y., Scorneaux, B., Chollet, J., Zhai, Z., 2003. *Schistosoma japonicum*: *In vitro* effects of artemether combined with haemin depend on cultivation media and appraisal of artemether products appearing in the media. Parasitol Res 89, 459-466.

Table 2.1

Effect of artemisinin on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean *H. contortus* (±S.D.) burdens and parasite reduction (with 95% C.I.) according to group: single dose was 400 mg/kg artemisinin in DMSO/Olive Oil once; multiple dosage was 200 mg/kg artemisinin in DMSO/Olive Oil daily for 5 days; carrier control was DMSO/Olive Oil daily for 5 days; water control was water daily for 5 days. No significant differences were observed.

Group	Single	Multiple	Carrier control	Water control
Individual # *H. contortus* larvae	76	93	27	40
	71	54	75	28
	65	72	69	40
	51	59	73	10
	74	77	56	51
	41	114	40	96
	109	91	73	66
	76	41	55	87
	87	96	74	58
	76	85	28	113
Mean # larvae (±S.D.)	72.6 (±18.5)	78.2 (±22.1)	57.0 (±19.2)	58.9 (±32.1)
% larval reduction	-25.2 (-64.0, 4.46)	-34.8 (-78.6, -0.72)	-	-

Table 2.2

Effect of *Artemisia annua* extracts on *Haemonchus contortus* infection in gerbils; Individual and geometric mean (95% C.I.) *H. contortus* burdens and parasite reduction (95% C.I.) according to group: (E.E.) 600 mg/kg *A. annua* ethanolic extract in 3% Tween 80 daily for 5 days; (A.E.) 600 mg/kg *A. annua* aqueous extract in 3% Tween 80 daily for 5 days; (Control) 3% Tween 80 daily for 5 days. No significant differences were observed.

Group	E.E.	A.E.	Control
Individual # *H. contortus* larvae	110	75	206
	17	25	36
	55	134	55
	63	57	23
	56	75	111
	15	64	65
	37	22	73
	76	81	64
	58	46	58
	216	334	242
Geometric mean # larvae	54.8 (33.6, 89.3)	68.5 (42.0, 111.6)	74.5 (45.7, 121.4)
% larval reduction	24.7 (-57.7, 64.0)	2.14 (-122.5, 57.0)	-

CHAPTER 3

The effect of an orange oil emulsion on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*)

Abstract

Haemonchus contortus is a blood-sucking abomasal parasite responsible for major losses to small ruminant producers worldwide. Resistance of this nematode to commercial anthelmintics has produced a demand for alternative control methods. An orange oil emulsion (experimental composition 1, EC1) with known activity against plant parasitic nematodes, was assessed for activity against *H. contortus* in a gerbil model. In all experiments, gerbils were infected with 600 infective third-stage *H. contortus* larvae. In one experiment, gerbils were treated with 600 mg/kg orange oil (4 ml/kg EC1) once or daily for five days. In a second experiment, gerbils were treated with 1200 mg/kg orange oil (8 ml/kg EC1) once or daily for five days. On day 9 post-infection, gerbils were killed, their stomachs removed, and the worms counted. The 600 mg/kg dosage of EC1 caused 7% and 62% parasite reduction compared to an untreated control group when given once or daily for five days, respectively. The 1200 mg/kg dosage of EC1 caused 25% and 88% parasite reduction compared to an untreated control group when given once or daily for five days, respectively. Analysis of variance and Tukey's test revealed a significant difference between the multiple dose group and the other groups in both experiments ($P < 0.005$). The orange oil emulsion may be an effective alternative to commercial dewormers.

Introduction

A food grade emulsion containing orange terpene oil, orange valencia oil, polysorbate 80, hydrogen peroxide, and water, has been patented for reducing plant pests such as nematodes and fungi; it also has antimicrobial activity (R. Bowker, personal communication). In initial experiments, various formulations of the product were found to be effective in reducing damage to tomato plant roots caused by the root-knot nematode *Meloidogyne incognita* and all tested formulations reduced the number of eggs produced per gram of root tissue (Rosskopf et al., 2008). It has been hypothesized that because plant and animal parasitic nematodes are closely related, the formulation may also work against gastrointestinal helminths (Rosskopf et al., 2008).

A series of experiments followed that evaluated varying concentrations of the emulsion against egg and larval stages of *Haemonchus contortus* (Rosskopf et al., 2008). Results showed that all concentrations significantly inhibited egg hatching, with concentrations of 2% to 100% resulting in at least 90% inhibition. Also, more than 50% of larvae were inhibited from movement or killed by concentrations of 3% or higher. Based on this, we hypothesized that the orange oil emulsion would show anthelmintic activity against *Haemonchus contortus in vivo*. The orange oils, which have shown previous nematocidal activity (Tsai, 2008), may be responsible for the anthelmintic properties of the product. We evaluated the emulsion against larval stages of *Haemonchus contortus* in a gerbil model of infection.

Materials and Methods

Animals

Visually healthy, non-pregnant, non-lactating female Mongolian gerbils (*Meriones unguiculatus*) at approximately 5 weeks of age and weighing about 50 g were purchased from Charles River Laboratories International, Inc. They were housed 2 per cage and provided commercial rodent chow and water *ad libitum*. The gerbils were allowed to acclimate for a period of two weeks before infection. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Parasites

Third stage infective *H. contortus* larvae (L3) were cultured from the feces of a monospecifically infected lamb according to standard parasitological techniques.

Infection

Third stage larvae for infection were exsheathed according to Conder and Johnson, 1996. Briefly, carbon dioxide was bubbled into a flask containing Earle's Balances Salt Solution (EBSS) and *H. contortus* larvae that was incubated overnight at 37°C. Gerbils were inoculated via oral gavage with 600 exsheathed *H. contortus* L3 in EBSS in a total volume of 0.5 ml. Daily health observations were performed throughout the experiment.

H. contortus Counts

In all experiments, gerbils were euthanized by carbon dioxide asphyxiation followed by thoracotomy 9 days after infection. Their stomachs were removed, opened longitudinally, placed in deionized water, and incubated at 37°C for 2-3 hours following the method of Conder et al., 1991. Formaldehyde was added to create a final concentration of 10% formalin to preserve samples for subsequent counting. The collected fluid and stomachs were examined for parasites using a dissecting microscope by investigators blind to the treatment groups. Lugol's iodine solution was used to enhance the appearance of *H. contortus* for easier counting.

Statistical Analysis

Normal Probability plots were generated based on larval counts to verify that data followed an approximate normal distribution. When distributions were not normal, a logarithmic (base e) transformation was applied to the larval counts to normalize worm burdens. Base e transformation was selected to maintain resolution that may be lost following base 10 transformation. Groups were compared using ANOVA followed by Tukey's procedure for multiple comparisons. Statistical significance was set to $\alpha=0.05$. These analyses were performed using SAS version 9.2 (Carry, NC, USA). Efficacy against *H. contortus* was calculated as a percent larval reduction:

$$\% \text{ efficacy} = 100 \times (C-T)/C$$

where C is the arithmetic mean number of worms in an untreated control group and T is the arithmetic mean number of worms in a treatment group. Confidence limits for percent larval reduction were calculated using arithmetic means and based on the World Association for the Advancement of Veterinary Parasitology methods for the detection of anthelmintic resistance (Coles et al., 1992).

Experimental Protocol

Experiment One

Thirty gerbils were infected with 600 *H. contortus* L3 (day 0) and randomly allocated to 3 groups of 10 gerbils that received the following treatments:

Group 1: 600 mg/kg orange oil (4 ml/kg experimental composition 1, EC1) once (day 6)

Group 2: 600 mg/kg orange oil (4 ml/kg EC1) daily for 5 days (day 4-8)

Group 3: Control, water daily for 5 days (day 4-8)

Treatment

The orange oil emulsion , EC1, was provided by Robert Bowker (Knock-Out Technologies, Dover Plains, NY). EC1 contains 10% orange terpene oil, 5% orange valencia oil, 10% Polysorbate 80, 5.5% hydrogen peroxide and 69.5% water. Because no information on efficacy against internal parasites was available, an initial dosage of EC1 was established at 1/10 the oral LD_{50} of orange oil in mice. The total orange oil concentration alone was used to calculate the dose. Doses were administered via oral gavage in a total volume of 0.3 ml, the volume being completed with deionized water.

Experiment Two

Based on the results of the first experiment, a second study was performed to assess the effect of higher doses of EC1. The procedure was identical to experiment one with the exception of the dosage. Three groups of 10 gerbils received the following treatments:
Group 1: 1200 mg/kg orange oil (8 ml/kg EC1) once (day 6)
Group 2: 1200 mg/kg orange oil (8 ml/kg EC1) daily for 5 days (day 4-8)
Group 3: Control, water daily for 5 days (day 4-8)

Treatment

Doses were administered via oral gavage in a total volume of 0.38 ml.

Results

Experiment One

Parasite counts from experiment one were normally distributed and thus analyzed as actual larval counts; arithmetic means with standard deviations were generated. Results of experiment one are shown in Table 3.1. Groups 1 and 2 averaged 74.8 (±45.7) and 30.1 (±26.2) *H. contortus* larvae, respectively, compared to a mean of 80.4 (±26.4) larvae in the control group. A highly significant difference was observed in the 5-day treatment group (2) compared to the control group ($P<0.005$); the treated gerbils had 62.6% [95% C.I. (31.6, 79.5)] fewer parasites than controls. However, the single dose group (1) was not significantly different from the control ($P>0.05$); only a 6.97% parasite reduction was observed.

Experiment Two

Due to a high proportion of gerbils without parasites, the data for this experiment were not normally distributed, thus a logarithmic (base e) transformation was applied to the larval counts from experiment two to normalize burdens. Therefore, means were summarized as geometric means with geometric 95% confidence intervals. The results of experiment two are shown in Table 3.2. Compared to a geometric mean of 76.1 *H. contortus* larvae in the control group, groups 1 and 2 averaged geometric means of 56.1 and 4.76 larvae, respectively. Groups 1 and 2 exhibited greater parasite reductions than in the prior experiment. Group 1 was not significantly different from the control ($P>0.05$), exhibiting a parasite reduction of 25.0%. However, group 2 was highly significantly different from the control ($P<0.005$) with a resultant 87.8% [95% C.I. (61.2, 96.2)] reduction.

Discussion

We conclude that, upon repeated dosing, EC1 is effective against *Haemonchus contortus* larvae in infected gerbils with greater efficacy at 1200 mg/kg than 600 mg/kg of orange oil. In the first experiment, a single dose of 600 mg/kg of orange oil (4 ml/kg of EC1) caused no significant reduction in parasite burdens, but the dose over 5 days resulted in a 62.2% reduction. With an increased dosage (1200 mg/kg of orange oil or 8 ml/kg of EC1) in the second experiment, a 25% reduction, although not significant, was seen on the first day with a significant, greater reduction (87.8%) exhibited following 5 days of treatment. It appears that prolonged exposure at this dosage is necessary for a significant effect. There is also a dose-dependent affect of EC1 evident by the greater larval reductions seen after 1 or 5 days of treatment with the larger dosage.

Initial tests of EC1 were performed to evaluate the product for use against plant parasitic nematodes, specifically *Meliodogyne* spp. (Rosskopf et al., 2008). Within 4 days of treatment *in vitro*, concentrations of 1% or higher dose-dependently inhibited egg hatching, and concentrations of 2% or higher killed all juvenile nematodes. In trials with infected tomato plants, application of the emulsion reduced root damage by *Meliodogyne* spp. and reduced the number of eggs produced by the nematodes. These results are consistent with Tsai (2008), who reported that that pulpified citrus peel killed and inhibited mung bean root infection by second-

stage juvenile larvae and inhibited hatching of eggs of the plant parasitic nematode *Meloidogyne incognita*.

The orange oil emulsion provided similar results *in vitro* against *H. contortus* eggs and larvae where concentrations of 2% to 100% resulting in at least 90% inhibition of egg hatching and concentrations of 3% or higher inhibited the migration of or killed more than 50% of third-stage larvae (Rosskopf et al., 2008). Third-stage larvae are prevented from feeding by their larval sheath and are affected *in vitro* by EC1, suggesting that the mechanism of this product does not require ingestion by the parasite. Our *in vivo* trials also show that EC1 is effective against larvae, in this case the fourth stage.

Fifteen percent of the formulation of EC1 used in these trials is orange terpenes and orange valencia oil. Orange valencia oil contains aldehydes, ketones, and esters (Moshonas and Lund, 1969) but is at least 90% limonene, a terpene that is lipophilic with strong solvent capability. It is believed that the terpenes are responsible for the anthelmintic activity of EC1; they along with the action of hydrogen peroxide are capable of degrading the cell walls of microbes (R. Bowker, personal communication). The cuticle of *H. contortus* is composed primarily of protein with some lipids and carbohydrates (Fetterer and Rhoads, 1993). Perhaps limonene or other terpenes are capable of softening the casing of eggs or the cuticle of *H. contortus* larvae, making them vulnerable to the affects of hydrogen peroxide or other compounds present in the valencia oil *in vitro*, or even to digestion *in vivo*. Kaur et al. (2009) reviewed a wide variety of terpene structures isolated from a range of plants and their efficacies as antimalarials. Suggested modes of actions include inhibitory effects on growth, parasite enzymes or plasma membrane pumps, and interference with metabolic pathways. It may be possible that any of these modes of actions are also associated with the terpenes in EC1; however, this is only speculation as very little is known about modes of action of terpenes against nematodes in general.

Limonene is generally recognized as safe by the FDA and has low acute oral and dermal toxicity in laboratory animals. The amount of hydrogen peroxide administered to the gerbils in EC1 was also well below LD_{50} values for laboratory animals. We can therefore assume and confirm through observation that EC1 reduced larval burdens in gerbils without significant toxicity. However, whether the solvent capability of EC1 affects the gerbil stomach mucosa is a concern and is under investigation.

Previous studies as well as this study have shown that the orange oil emulsion is effective against multiple stages of *H. contortus in vitro* and *in vivo* through inhibition of egg hatching, inhibition of movement, and death of larvae. Terpenes are likely to be the active compounds, however to further determine the mechanism of action of EC1, further studies should test hydrogen peroxide, the oils and specific terpenes individually for activity against the various stages of *H. contortus*.

References

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

Conder, G.A., Johnson, S.S., Guimond, P.M., Cox, D.L., Lee, B.L., 1991. Concurrent infections with the ruminant nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* in jirds, *Meriones unguiculatus*, and use of this model for anthelmintic studies. J Parasitol 77, 621-623.

Fetterer, R.H., Rhoads, M.L., 1993. Biochemistry of the nematode cuticle: relevance to parasitic nematodes of livestock. Vet Parasitol 46, 103-111.

Kaur, K., Jain, M., Kaur, T., Jain, R., 2009. Antimalarials from nature. Bioorg Med Chem.

Moshonas, M.G., Lund, E.D., 1969. Aldehydes, Ketones, and Esters in Valencia Orange Peel Oil. J Food Science 34, 502-503.

Rosskopf, E.N., Therrien, L.L., Adkins, S.T., Iriarte, F., Foster, J.G., Kokalis-burelle, N. 2008. Methods of reducing pests and treating gastrointestinal nematode infections (Patent Application 20080166437, filed 1/3/2008), http://www.freshpatents.com/Methods-of-reducing-pests-and-treating-gastrointestinal-nematode-infections-dt20080710ptan20080166437.php?type=description 7/22/08 - 4/23/09.

Tsai, B.Y., 2008. Effect of peels of lemon, orange, and grapefruit against *Meloidogyne incognita*. Plant Pathol Bull 17, 195-201.

Table 3.1

Effect of an orange oil emulsion on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean (±S.D.) *H. contortus* burdens and parasite reduction (95% C.I.) according to group: single dose was 600 mg/kg orange oil (4 ml/kg EC1) once; multiple dosage was 600 mg/kg orange oil (4 ml/kg EC1) daily for 5 days; control dosage was water daily for 5 days

Group	Single	Multiple	Control
Individual # *H. contortus* larvae	20	0	29
	140	53	64
	88	10	114
	39	37	90
	90	23	72
	31	87	86
	70	22	67
	94	39	103
	28	1	65
	148	29	114
Mean # larvae (±S.D.)	74.8^{a} (±45.7)	30.1^{b} (±26.2)	80.4^{a} (±26.4)
% larval reduction	6.97 (-45.8, 40.6)	62.6 (31.6, 79.5)	-

[a,b]In the same rows, unlike superscripts indicate significant difference ($P < 0.005$).

Table 3.2

Effect of an orange oil emulsion on *Haemonchus contortus* infection in gerbils; Individual and geometric mean (95% C.I.) *H. contortus* burdens and parasite reduction (95% C.I.) according to group: single dose was 1200 mg/kg orange oil (8 ml/kg EC1) once; multiple dosage was 1200 mg/kg orange oil (8 ml/kg EC1) daily for 5 days; control dosage was water daily for 5 days

Group	Single	Multiple	Control
Individual # *H. contortus* larvae	27	0	70
	11	56	45
	103	15	98
	53	1	52
	70	2	88
	55	1	38
	53	10	56
	96	0	52
	47	24	271
	163	1	132
Geometric mean # larvae	56.1 (29.6, 106.4)[a]	4.76 (2.51, 9.04)[b]	76.1 (40.1, 144.3)[a]
% larval reduction	25.0 (-44.1, 60.8)	87.8 (61.2, 96.2)	-

[a,b]In the same rows, unlike superscripts indicate significant difference ($P < 0.005$)

CHAPTER 4

The effect of an orange oil emulsion on experimental *Haemonchus contortus* infection in sheep (*Ovis aries*)

Abstract

An orange oil emulsion with previously confirmed activity against *Haemonchus contortus* larvae in a gerbil model was evaluated against adult stages of the parasite in sheep. Ram lambs were orally infected with 10,000 third-stage larvae and, one month later, dosed daily for 3 days with water or dosed once or daily for three days with 600 mg/kg orange oil (1 ml/kg experimental composition 2, EC2). Rectal fecal samples were collected daily from all sheep starting on the first day of dosing and continuing to 14 days post-dosing. Mean fecal egg counts were compared between each group for each day of the dosing period and percent fecal egg count reduction compared to the control group was calculated for day 14 post-dosing. Both treated groups' fecal egg counts were significantly lower than the control from days 2-14 post-dosing ($P < 0.05$). The mean fecal egg counts of the treated groups were not significantly different during the dosing period ($P > 0.05$) and the day 14 percent egg count reductions for the single and multiple-dose group compared to the control were 97.4% and 94.9%, respectively. These results indicate that a single dose of the orange oil emulsion is highly effective in reducing fecal egg counts in sheep and there was no advantage to treating animals for 3 days. Thus, the product shows potential as an alternative to commercial anthelmintics for the control of *H. contortus* in sheep.

Introduction

An orange oil emulsion tested in a gerbil model of *Haemonchus contortus* infection effectively reduced parasite burdens *in vivo*. However, gerbils are not a natural host for *H. contortus* and the parasite does not reach the adult stage in these animals. Therefore, we evaluated a slightly different formulation of the emulsion (experimental composition 2, EC2) against adult stages of *H. contortus* in its natural host, sheep (*Ovis aries*), using the fecal egg count reduction test as a measure of efficacy and hypothesizing that the product would be efficacious in reducing fecal egg counts.

Materials and Methods

Animals

Eighteen recently weaned hair-wool cross ram lambs were randomly selected from the Virginia Tech teaching and research herd. They were housed in 12' x 9' pens with 6 sheep per enclosure. They were provided hay, whole shelled corn and water *ad libitum*. All sheep were dewormed twice with levamisole (8 mg/kg orally) and allowed to acclimate for a period of one month before infection. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Parasites

Third stage infective *H. contortus* larvae were provided by Dr. Ray Kaplan, University of Georgia.

Experimental Protocol

Infection. All lambs were inoculated via oral drench with 10,000 *H. contortus* L3 in 36 ml water (day -28) and allocated into 3 groups that were blocked by fecal egg count. They received the following treatments:

Group 1: 600 mg/kg orange oil (1 ml/kg EC2) once (day 0)

Group 2: 600 mg/kg orange oil (1 ml/kg EC2) daily for 3 days (day 0-2)

Group 3: Control, water daily for 3 days (day 0-2)

Treatments

Daily health observations were performed throughout the experiment. Experimental *H. contortus* infections were given 28 days to reach patency. Packed cell volume was monitored weekly beginning on day -28. EC2 was provided by Robert Bowker (Knock-Out Technologies, Dover Plains, NY). Due to concerns over volume and effects of hydrogen peroxide in sheep, a more concentrated formulation of the orange oil emulsion (40% orange terpene oil, 20% orange valencia oil, 4% polysorbate 80, 1.5% hydrogen peroxide, 34.5% water) was provided and administered at the lower orange oil dosage used in the gerbil trials. Lambs were weighed on day -1 (mean weight = 51 kg) and doses were administered orally to each lamb in a total volume of 52-72 ml.

Fecal Collection

Rectal fecal samples were collected daily from all lambs from day 0 (for pre-treatment egg counts) through day 14 post-dosing and fecal egg counts were determined using the Modified McMaster Test (Zajac and Conboy, 2006).

Statistical Analysis

On each day, the groups were compared using the exact Kruskall-Wallis Test followed by Dunn's procedure for multiple comparisons. Statistical significance was set to $\alpha=0.05$. These analyses were performed using SAS version 9.2 (Carry, NC, USA). Efficacy against *H. contortus* was calculated as a percent fecal egg count reduction:

$$\% \text{ fecal egg count reduction} = 100 \times (C-T)/C$$

where C is the arithmetic mean fecal egg count of an untreated control group and T is the arithmetic mean fecal egg count of a treatment group. Confidence limits for percent fecal egg count reduction were calculated based on the World Association for the Advancement of Veterinary Parasitology methods for the detection of anthelmintic resistance (Coles et al., 1992).

Results

Mean fecal egg counts for each day post-dosing according to group are displayed in Figure 4.1. Day 0 mean fecal egg counts for groups 1, 2 and 3 were 620 (±653), 1050 (±1325), and 708 (±571), respectively. Day 14 mean fecal egg counts for groups 1, 2 and 3 were 25 (±42), 50 (±78), and 975 (±715), respectively. A highly significant effect of EC2 on mean fecal egg counts was seen on day 2 post-dosing ($P < 0.0001$) that continued to day 14 post-dosing ($P < 0.002$). Groups 1 and 2 were both significantly different from group 3 from days 2-14 post-dosing ($P < 0.05$). There was no significant difference between the fecal egg counts of the two treated groups from days 0-14 post-dosing ($P > 0.05$).

Percent fecal egg count reductions compared to the control group on day 14 post-dosing were: Group 1: 97.4% [95% C.I. (88.0, 99.4)] and Group 2: 94.9% [95% C.I. (78.6, 98.8)]. Packed cell volumes remained within the normal range throughout the experiment (data not shown).

Discussion

The orange oil emulsion has anthelmintic activity against eggs and L3 *in vitro* (Rosskopf et al., 2008). When dosed for 5 days, the emulsion reduced fourth stage larvae *in vivo* in gerbils (Chapter 3). This study was designed to determine the activity of EC2 against adult *H. contortus* in its natural host, sheep. Originally, the higher dosage given to gerbils was to be tested in sheep. However, it became apparent that the volume needed to administer the same amount of orange oil would be cumbersome and impractical. There were also concerns that the amount of hydrogen peroxide administered could induce vomiting. Therefore, the formulation of the orange oil emulsion generated for this experiment contained less hydrogen peroxide (1.5% compared to 5.5% previously) and a higher concentration of orange oils (60% compared to 15% previously).

After administering EC2 to the experimental groups and monitoring their fecal egg counts for 3 days, fecal egg counts were highly reduced (98% in both groups) and we were still concerned about potential toxicity in ruminants, thus the fourth and fifth days of dosing originally planned were cancelled. Statistical analysis revealed a highly significant effect of EC2 on mean fecal egg counts in both treated groups by day 2 post-dosing. Since fecal egg counts of both groups remained very low until the end of the experiment, it is more likely that EC2 caused fecal egg count reduction in both groups by killing the parasites rather than by reducing their fecundity. However, this could not be confirmed because we were not able to euthanize the sheep for examination of the abomasa. There was no significant difference between the fecal egg counts of the two treated groups from days 0-14 post-dosing and the percent fecal egg count reductions compared to the control group on day 14 post-dosing were not significantly different (97% and 94% for the single and multiple dose group, respectively). Thus, a single dose of EC2 is highly effective in reducing fecal egg counts in sheep and there was no advantage to treating animals for 3 days.

A single dose of 600 mg/kg of orange oil in sheep caused a 98% fecal egg count reduction in 2 days, which could be interpreted as an almost complete kill of parasites in these animals. This same dose in gerbils reduced parasite burdens by only 7%. This may be due to metabolic differences that make the active compounds in the orange oil emulsion less available in gerbils. Similarly, the rumen serves as a reservoir, slowing the passage of the emulsion in

sheep, thus prolonging the exposure of *H. contortus* to the product. This would explain why the most marked reduction in fecal egg counts was not seen in the sheep until 2 days after treatment.

Another explanation could be that adults may be more susceptible to the effects of the orange oil emulsion than larval stages. Varying susceptibility of different life stages has been described in *H. contortus*; more 4-day old larvae were removed by levamisole from sheep than 12- or 26-day old worms of the same strain (Sangster and Bjorn, 1994). Although these results are opposite ours, they may indicate a difference in receptor expression between life stages that alters susceptibility.

As previously discussed in Chapter 3, terpenes may be responsible for the efficacy of the emulsion, perhaps by weakening the protective covering of eggs or larvae, making them vulnerable to the affects of hydrogen peroxide or other compounds present in the valencia oil *in vitro*, or even to digestion *in vivo*. The lower amount of hydrogen peroxide in EC2 administered in this experiment (only 1.5% compared to 5.5% previously) confirms the likelihood that orange oils are more important to the activity. Terpenes have shown inhibitory effects on growth, parasite enzymes or plasma membrane pumps, and interference with metabolic pathways in *Plasmodium* spp. (Kaur, 2009), but we can only speculate that these effects occur in nematodes as very little is known about the mechanism of action of orange oils or terpenes against nematodes.

A few lambs demonstrated head shaking and disinterest in food after dosing, but this passed within 15-20 minutes and in general, EC2 was well tolerated by the sheep and no signs of toxicosis were observed. An *in vitro* rumen fermentation assay (unpublished) of the orange oil emulsion showed a reduction of dry matter disappearance at a 2% concentration. Some treated sheep in our study passed soft but formed stool during the days following treatment, but it is unknown if this was due to stress or a disruption by EC2 of rumen function and no sheep developed diarrhea. Further studies are needed to assess the emulsion for negative health effects in sheep.

Collectively, studies of the orange oil emulsion have demonstrated efficacy of the product against all the life stages of *H. contortus*; eggs, larvae, and adults. More studies are needed to evaluate the plausibility and safety of the product for use in livestock. However, based on our initial studies, the emulsion shows promise as an alternative to commercial anthelmintics.

References

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

Kaur, K., Jain, M., Kaur, T., Jain, R., 2009. Antimalarials from nature. Bioorg Med Chem.

Rosskopf, E.N., Therrien, L.L., Adkins, S.T., Iriarte, F., Foster, J.G., Kokalis-burelle, N. 2008. Methods of reducing pests and treating gastrointestinal nematode infections (Patent Application 20080166437, filed 1/3/2008), http://www.freshpatents.com/Methods-of-reducing-pests-and-treating-gastrointestinal-nematode-infections-dt20080710ptan20080166437.php?type=description 7/22/08 - 4/23/09.

Sangster, N.C., Bjorn, H., 1995. Levamisole resistance in *Haemonchus contortus* selected at different stages of infection. Int J Parasitol 25, 343-348.

Zajac, A.M., Conboy, G.A., 2006. Veterinary Clinical Parasitology, 7th Edition. Blackwell Publishing, Ames.

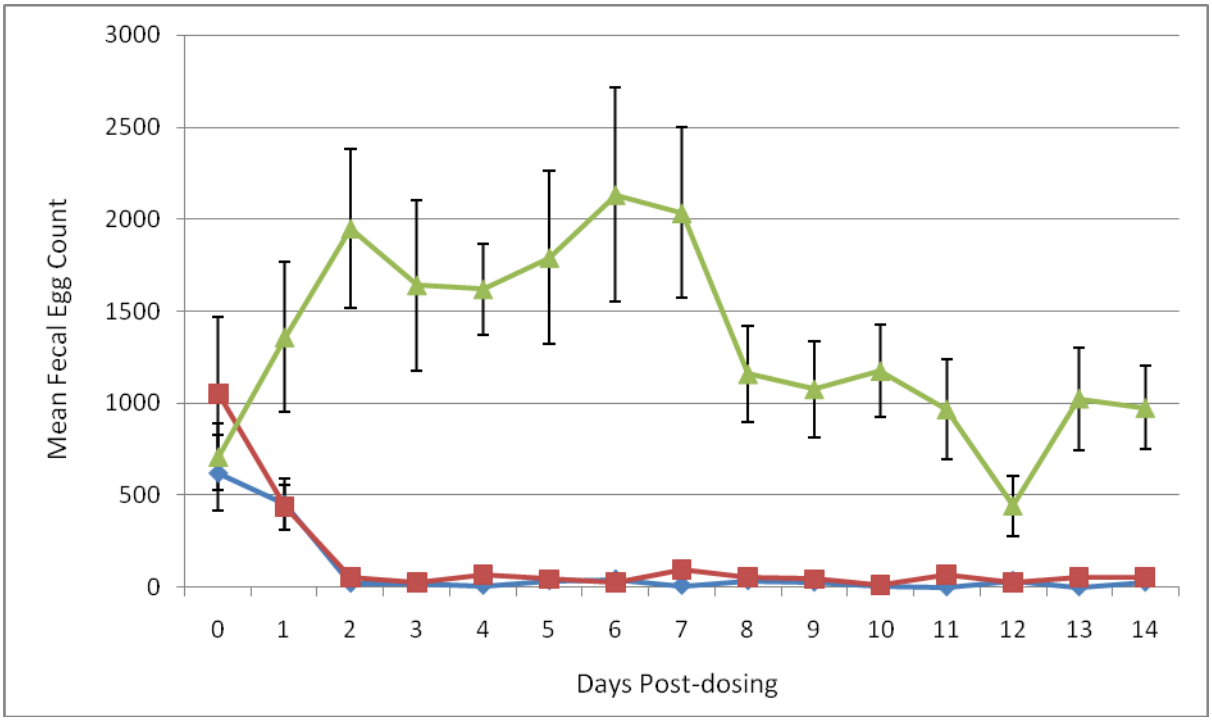

Fig. 4.1. Effect of an orange oil emulsion on *Haemonchus contortus* infection in sheep; mean fecal egg count (±S.E.) by day post-first dose according to group: (diamonds) 600 mg/kg orange oil (1 ml/kg EC2) once; (squares) 600 mg/kg orange oil (1 ml/kg EC2) daily for 3 days; (triangles) control, water daily for 3 day

APPENDIX A

The Effect of *Asimina triloba* on experimental *Haemonchus contortus* infection in gerbils (*Meriones unguiculatus*)

Abstract

Haemonchus contortus is a blood-sucking abomasal parasite responsible for major losses to small ruminant producers worldwide. Resistance of this nematode to commercial anthelmintics has produced a demand for alternative control methods. *Asimina triloba*, commonly known as paw paw, contains acetogenins that have been shown to be lethal to the free-living nematode, *Caenorhabditis elegans*, *in vitro*. Acetogenins have also inhibited *H. contortus* eggs from hatching *in vitro*. *Asimina triloba* ethanolic extract (EE) was evaluated against *H. contortus* in a gerbil model. Twenty gerbils were orally infected with 600 third-stage larvae and dosed daily for 5 days with water or *A. triloba* EE. On day 9 post-infection, gerbils were killed, their stomachs removed, and the worms counted. There was no statistically significant difference between the treated group and the control ($P > 0.05$); the extract caused only a 10.3% parasite reduction. It was concluded that, at the given dosage, paw paw ethanolic extract is not effective in reducing *H. contortus* larval burdens in gerbils.

Introduction

Asimina triloba (Annonaceae), commonly known as paw paw, is a fruit-producing tree native to the eastern part of North America. The tree is known to be resistant to pests and disease, probably due to the presence of acetogenins (Johnson et al., 1996), which have been shown to have activity against various insects (Mikolajczak et al., 1988). The majority of acetogenins can be found in the bark, roots, twigs, and seeds (Johnson et al., 1996). The mode of action of the compounds involves inhibition of Complex I in mitochondria involved in cellular respiration, resulting in a decreased production of ATP (Johnson et al., 1996). A purified acetogenin, asimicin, and a crude extract of the bark of *Asimina triloba* were both shown to cause 100% mortality of the free-living nematode, *Caenorhabditis elegans*, after 72 hours of exposure to an aqueous test solution (Mikolajczak et al., 1988). Souza et al. (2008) concluded that acetogenins from another member of the Annonaceae family, *Annona squamosa*, had activity against *Haemonchus contortus* eggs *in vitro*. Ethyl acetate, methanol-water, and

aqueous extracts of the seeds of the plant and an isolated acetogenin were incubated at various concentrations with *H. contortus* eggs for 48 hours. Both the isolated acetogenin and the ethyl acetate extract completely inhibited egg hatching; the methanol-water extract and aqueous extracts caused lesser inhibition. Thus, acetogenins do have anthelmintic activity against at least one stage of *H. contortus*, and they and possibly other anthelmintic compounds are less soluble in water. Therefore, we evaluated an ethanolic extract of *Asimina triloba* against the larval stages of *Haemonchus contortus* in a gerbil model of infection, hypothesizing that the extract would reduce larval burdens.

Materials and Methods

Animals

Twenty visually healthy, non-pregnant, non-lactating female Mongolian gerbils (*Meriones unguiculatus*) at approximately 5 weeks of age and weighing about 50 g were purchased from Charles River Laboratories International, Inc. They were housed 2 per cage and provided commercial rodent chow and water *ad libitum*. The gerbils were allowed to acclimate for a period of two weeks before infection. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Parasites

Third stage infective *H. contortus* larvae (L3) were cultured from the feces of a monospecifically infected lamb according to standard parasitological techniques.

Experimental Protocol

Infection

Third stage larvae for infection were exsheathed according to Conder and Johnson, 1996. Briefly, carbon dioxide was bubbled into a flask containing Earle's Balanced Salt Solution (EBSS) and *H. contortus* larvae that was incubated overnight at 37°C. Gerbils were inoculated via oral gavage with 600 exsheathed *H. contortus* L3 in EBSS in a total volume of 0.3 ml (day 0) and randomly allocated into 2 groups of 10 animals that received the following treatments:
Group 1: 40 mg/kg *A. triloba* ethanolic extract in DMSO and olive oil daily for 5 days (day 4-8)
Group 2: Control, DMSO and olive oil daily for 5 days (day 4-8)

Treatments

Daily health observations were performed throughout the experiment. *A. triloba* extract was prepared by Dr. Jorge Ferreira, USDA, ARS as follows: The contents of capsules of Paw Paw Cell Reg® (Nature's Sunshine, Spanish Fork, Utah), standardized to contain 12.5 mg *A. triloba* twig powder each, were pooled and 30 g were extracted using 300 ml 100% ethanol. The solution was stirred for 30 minutes at room temperature, sonicated at 30°C for 15 minutes, then filtered though #1 Whatman filter paper. A second extraction was performed following the same procedure, and the extractions were combined. The extract was then concentrated in a rotoevaporator at 40°C. Before freeze drying, 5 ml of water was added to the sample to facilitate freeze drying before the sample was placed into an ultra-low freezer for 8 days. A dark, thick concentrate was obtained into which air was blown for approximately 5 hours to eliminate water that had condensed in the sample following removal from the freezer. The sample was then refrozen in an ultra low freezer, then freeze dried for 5 more days. The extract was emulsified in DMSO and olive oil (1:7) before use.

The dosage was based on Cuendet et al. (2008). All gerbils were dosed via oral gavage with a total volume of 0.36 ml. DMSO was given with olive oil to complete the volume and avoid potential DMSO toxicity.

H. contortus Counts

All gerbils were euthanized by carbon dioxide asphyxiation followed by thoracotomy 9 days after infection. Their stomachs were removed, opened longitudinally, placed in deionized water, and incubated at 37°C for 2-3 hours following the method of Conder et al., 1991. Formaldehyde was added to create a final concentration of 10% formalin to preserve samples for subsequent counting. The collected fluid and stomachs were examined for parasites using a dissecting microscope by investigators blind to the treatment groups. Lugol's iodine solution was used to enhance the appearance of *H. contortus* for easier counting.

Statistical Analysis

Normal Probability plots were generated based on larval counts to verify that data followed an approximate normal distribution. The two groups were compared using a Student's

t-test. Statistical significance was set to $\alpha=0.05$. These analyses were performed using SAS version 9.2 (Carry, NC, USA). Efficacy against *H. contortus* was calculated as a percent larval reduction:

$$\% \text{ efficacy} = 100 \times (C-T)/C$$

where C is the arithmetic mean number of worms in an untreated control group and T is the arithmetic mean number of worms in a treatment group.

Results

The extract treated group had a mean parasite burden of 85.7 (±64.6) *H. contortus* larvae that was not significantly different from the control group mean of 95.5 (±50.8) larvae ($P = 0.49$). The parasite reduction in the treated group equaled 10.3% (Table A.1).

Discussion

The mode of action of acetogenins, the compounds responsible for the supposed anthelmintic activity of *Asimina triloba*, involves inhibition of mitochondrial Complex I, an electron carrier that is involved in cellular respiration and the production of ATP (Johnson et al., 1996). Without ATP, many cellular functions would cease. Nafuredin, an inhibitor of complex I in helminth mitochondria, has shown activity against *H. contortus* in sheep by causing greater than 90% fecal egg count reduction, confirming that *H. contortus* is susceptible to mitochondrial Complex I inhibition (Omura et al., 2001).

However, that at the dosage given, paw paw ethanolic extract was not effective in reducing *H. contortus* larval burdens in gerbils. The dosage in this trial was based on Cuendet et al. (2008), who found that the maximum tolerated dose in rats of the Nature's Sunshine paw paw extract (the same commercial paw paw extract used in this experiment) was 2500 mg/kg of diet. Based on feed the intake of both rats and gerbils, the dosage converted to an oral dosage of 125 mg/kg of body weight. The Paw Paw Cell Reg® capsules also contain fillers such as cellulose and magnesium stearate, thus the paw paw was extracted from the contents of the capsules with ethanol. The resultant extract was 3 times more concentrated than the capsules, so the dosage was reduced to 40 mg/kg. This is a low dosage compared to other extracts we have tested; each gerbil ultimately received less than 2 mg of the extract in a single dose, equaling an extract concentration of 5.6 mg/ml. This is a lower concentration than that of the extract of *Annona*

muricata that killed 50% of filarid nematode larvae (*Molinema dessetae*) after 7 days *in vitro* (Bories et al., 1991). Our results are inconsistent with Souza et al. (2008), who showed that 5 mg/ml of an ethyl acetate extract of *Annona squamosa* seeds inhibited 99% of *H. contortus* eggs from hatching *in vitro*. They are also inconsistent with Alawa et al. (2003), who reported that 7.1 mg/ml of an extract of the bark of *Annona senegalensis* inhibited 88% of *H. contortus* eggs from hatching *in vitro*. A very low concentration of an extract of the bark of *Asimina triloba* was shown to cause 100% mortality of the free-living nematode, *Caenorhabditis elegans*, after 72 hours of exposure *in vitro* (Mikolajczak et al., 1988). One difference between these studies and ours is the sources of extract are different; they are either from a different species within the Annonaceae family or from a different part of the plant (i.e. not from twigs as in our study). Different sources could results in varying levels of acetogenins in the extract; perhaps there are fewer acetogenins in twigs than in the bark. Another factor may be the varying susceptibility of different nematode species to acetogenins; perhaps *C. elegans* is somehow more susceptible than *H. contortus* larvae. Finally, it is important to note that these studies were performed *in vitro*. The concentration of our extract was similar to the other studies; however, *in vitro* studies cannot recreate actual physiological conditions within the host. There may have been confounding interactions between the acetogenins and host molecules that limited the activity of the paw paw extract *in vivo*.

In this study, the carrier for the paw paw extract was DMSO with olive oil. We did not also include a water control because this was done in a prior experiment (see Chapter 2, artemisinin trial) and results indicated that DMSO and olive oil did not produce a difference in larval burdens compared to water-dosed animals. Therefore, the lack of efficacy of paw paw in this experiment cannot be attributed to interference by the carrier.

The paw paw extract dosage in this experiment was chosen to avoid potential toxicity. Generally, paw paw is considered safe because, in cases of overdose, emesis is triggered (McLaughlin, 2008). However, because gerbils cannot vomit, they cannot avoid toxicosis this way. In beagles, dosages similar to that in our study caused vomiting and loose stools and no dogs died even at much higher doses (D'Ver, 2001). Signs of toxicosis were not observed in the gerbils, suggesting that the dosage of paw paw extract did not approach toxic levels in these animals. Further studies could test higher dosages of the extract for activity against *H. contortus*.

References

Alawa, C.B., Adamu, A.M., Gefu, J.O., Ajanusi, O.J., Abdu, P.A., Chiezey, N.P., Alawa, J.N., Bowman, D.D., 2003. *In vitro* screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. Vet Parasitol 113, 73-81.

Bories, C., Loiseau, P., Cortes, D., Myint, S.H., Hocquemiller, R., Gayral, P., Cave, A., Laurens, A., 1991. Antiparasitic activity of *Annona muricata* and *Annona cherimolia* seeds. Planta Med 57, 434-436.

Conder, G.A., Johnson, S.S., 1996. Viability of infective larvae of *Haemonchus contortus*, *Ostertagia ostertagi*, and *Trichostrongylus colubriformis* following exsheathment by various techniques. J Parasitol 82, 100-102.

Conder, G.A., Johnson, S.S., Guimond, P.M., Cox, D.L., Lee, B.L., 1991. Concurrent infections with the ruminant nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* in jirds, *Meriones unguiculatus*, and use of this model for anthelmintic studies. J Parasitol 77, 621-623.

Cuendet, M., Oteham, C.P., Moon, R.C., Keller, W.J., Peaden, P.A., Pezzuto, J.M., 2008. Dietary administration of *Asimina triloba* (Paw Paw) extract increases tumor latency in N-methyl-N-nitrosourea-treated rats. Pharm Biol 46, 3-7.

D'Ver, A.S. 2001. Ascending Oral Dosing of PAWPAW Extract in Male Beagle Dogs (Doylestown, PA, White Eagle Toxicology Laboratories), p. 6.

Johnson, H.A., Gordon, J., McLaughlin, J.L., 1996, Monthly variations in biological activity of *Asimina triloba*, In: Janick, J. (Ed.) Progress in new crops. ASHS Press, Arlington, VA, pp. 609-614.

McLaughlin, J.L., 2008. Paw paw and cancer: annonaceous acetogenins from discovery to commercial products. J Nat Prod 71, 1311-1321.

Mikolajczak, K.L., McLaughlin, J.L., Rupprecht, J.K. 1998. Control of Pests with Annonaceous acetogenins (United States Patent Number 4721727).

Omura, S., Miyadera, H., Ui, H., Shiomi, K., Yamaguchi, Y., Masuma, R., Nagamitsu, T., Takano, D., Sunazuka, T., Harder, A., Kolbl, H., Namikoshi, M., Miyoshi, H., Sakamoto, K., Kita, K., 2001. An anthelmintic compound, nafuredin, shows selective inhibition of complex I in helminth mitochondria. Proc Natl Acad Sci U S A 98, 60-62.

Souza, M.M., Bevilaqua, C.M., Morais, S.M., Costa, C.T., Silva, A.R., Braz-Filho, R., 2008. Anthelmintic acetogenin from *Annona squamosa* L. Seeds. Ann Brazilian Acad Sci 80, 271-277.

Table A.1

Effect of *Asimina triloba* ethanolic extract on *Haemonchus contortus* infection in gerbils; Individual and arithmetic mean *H. contortus* burdens and parasite reduction (with 95% C.I.) according to group: treated group received 40 mg/kg *A. triloba* ethanolic extract in DMSO and olive oil daily for 5 days; control group received DMSO and olive oil daily for 5 days. No significant difference was observed.

Group	Treated	Control
Individual # *H. contortus* larvae	17	33
	17	35
	26	63
	31	68
	59	88
	97	93
	109	109
	156	113
	165	167
	180	186
Mean # larvae (±S.D.)	85.7 (±64.6)	95.5 (±50.8)
% larval reduction	10.3 (-63.1, 50.6)	-