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# Effects of artemisinin and *Artemisia* extracts on *Haemonchus contortus* in gerbils (*Meriones unguiculatus*)

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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. Plants in the genus *Artemisia* have traditionally been used as anthelmintics and whole plants and plant extracts have demonstrated activity against gastrointestinal nematodes in several studies. In addition, *Artemisia annua* is the sole commercial source of artemisinin, the raw material used to produce drugs effective against the hemoprotozoan malaria parasites (*Plasmodium* species). Artemisinin derivatives have also shown efficacy against some trematodes, including *Fasciola hepatica* and *Schistosoma* species. In this study, artemisinin was tested for efficacy against *H. contortus* in a gerbil model of infection. Also tested in the gerbil model were an aqueous extract, an ethanolic extract and the essential oil of *A. annua*, and an ethanolic extract of *Artemisia absinthium*. In all experiments, gerbils were infected with 600 third-stage *H. contortus* larvae. In experiment 1, gerbils were treated orally with 400 milligrams per kilogram body weight (mg/kg BW) artemisinin once or 200 mg/kg BW artemisinin daily for 5 days (Days 4–8 post-infection). In experiment 2, gerbils were treated daily for 5 days with 600 mg/kg BW of *A. annua* ethanolic or aqueous extract. In Experiment 3, gerbils were treated with 1000 mg/kg BW of *A. annua* or *A. absinthium* ethanolic extract or with 300 mg/kg BW of *A. annua* essential oil daily for five consecutive days (Days 4–8 post-infection). No significant effects of treatment were seen with artemisinin or any of the *Artemisia* species extracts at the dosages studied. The non-ionic surfactant Labrosol® was an effective nontoxic solvent for delivery of hydrophilic plant extracts and the lipophilic essential oil used in the study.



## 1. Introduction

*Haemonchus contortus* is an important abomasal helminth of small ruminants responsible for disease and major production losses worldwide. Heavy burdens of this blood-feeding parasite can cause severe anemia and rapid death in affected livestock. Modern control programs have relied heavily on the use of commercial anthelmintics. However, extensive resistance of *H. contortus* to these drugs has developed. As an alternative, research is being conducted to identify plant products with anthelmintic properties. Numerous studies have been published investigating the potential nematocidal activity of whole plants and plant extracts against *H. contortus* and related trichostrongylid parasites. Both in vitro assays and efficacy testing in infected small ruminants have been employed, but results are not always consistent between methods or even within methods used (Hoste et al., 2006). The most common in vitro assays are conducted with non-parasitic stages and are not always predictive of efficacy in the host.

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Trials conducted in infected sheep and goats are subject to wide variation in experimental conditions, are often restricted to small animal numbers, and may be useful for evaluation of specific plants in a particular region, but cannot be easily used to identify active components of plants or their mechanism of action. In addition, extracts of plants or putative active compounds may be difficult to produce in quantities needed for trials in the natural host. Conder et al. (1990, 1991) described a gerbil (*Meriones unguiculatus*) model of *H. contortus* infection for anthelmintic testing, which offers the advantages of requiring smaller product quantities and greater standardization in testing conditions.

*Artemisia annua* (Asteraceae), commonly known as sweet Annie, is a weed that grows throughout temperate areas worldwide and is currently grown as a pharmaceutical crop in China, Vietnam, and north and east Africa (Ferreira et al., 2005). For centuries, *A. annua* has been used for the treatment of malaria caused by *Plasmodium* species. The compound present in *A. annua* responsible for its antimalarial properties is artemisinin, a sesquiterpene lactone that contains an endoperoxide bridge, which is rarely found in natural compounds. Heme released by *Plasmodium* species digestion of hemoglobin is postulated to react with the endoperoxide bridge, breaking the oxygen bonds and causing the production of free radicals that damage the parasite (Pandey et al., 1999; Robert et al., 2005). Artemisinin has also been shown to alkylate parasite proteins, although a specific molecular target is yet to be found (O'Neill et al., 2010). Artemether, a derivative of artemisinin, similarly affects *Schistosoma* species, especially immature parasites (Utzinger et al., 2001; Keiser and Utzinger, 2007) and has also shown activity against *Fasciola hepatica* infection in sheep (Keiser et al., 2008).

Artemisinin is not the only medicinal compound in *Artemisia* species. The genus is a rich source of other sesquiterpene lactones and flavonoids that also might have anthelmintic activity with low risk of mammalian toxicity (Kerboeuf et al., 2008). Whole plants of *Artemesia herba-alba* showed activity against *H. contortus* in goats (Idris et al., 1982). Whole plant aqueous and methanolic extracts of *Artemisia brevifolia* were tested against *H. contortus* in vitro and in sheep with natural trichostrongylid infections (Iqbal et al., 2004). The methanolic extract killed *H. contortus* in vitro and use of the aqueous extract was associated with a reduction (67.2%) in fecal egg counts. Aqueous and ethanolic extracts of the stems and leaves of *A. absinthium* have also been evaluated for anthelmintic activity in vitro and in vivo (Tariq et al., 2008). Both extracts significantly affected motility and viability of *H. contortus* in vitro and significantly reduced fecal egg counts in naturally infected sheep. However, in another study (Worku et al., 2009) *A. absinthium* aqueous extract did not show activity against gastrointestinal nematodes in goats. Extracts of *A. absinthium* and *A. vulgaris* also reduced numbers of *Trichinella spiralis* larvae in the muscle of rats (Caner et al., 2008).

Our goal was to evaluate the anthelmintic effects of ethanolic extracts of *A. annua* and *A. absinthium*, artemisinin, and of an aqueous extract and essential oil of *A. annua* in the gerbil model.

## 2. Materials and methods

### 2.1. Gerbils

Visually healthy, non-pregnant, non-lactating female Mongolian gerbils approximately 5 weeks of age and weighing about 50 g were caged in pairs and provided commercial rodent chow and water *ad libitum*. Daily health observations were performed throughout the experiments.

### 2.2. Animal welfare

The Virginia Tech Institutional Animal Care and Use Committee approved all experimental protocols.

### 2.3. H. contortus

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 donor lamb according to standard parasitological techniques.

### 2.4. Artemisia products

#### 2.4.1. Artemisinin

Artemisinin was provided by Allergy Research Group (Alameda, CA, USA) and was analyzed by high performance liquid chromatography with ultraviolet detectors to ensure purity (approximately 98% pure). Artemisisin was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution containing 160 mg per milliliter (mg/ml) of artemisisin. The appropriate volume of stock solution was mixed with light olive oil to produce the desired dose in a constant volume for administration to gerbils.

#### 2.4.2. A. annua and A. absinthium ethanolic extracts

All plants used in this work were cultivated at the Appalachian Farming Systems Research Center experimental farm in Beaver, WV, USA. Plants were started from seeds in a greenhouse and transplanted to the field in June for harvest in late August or early September of 2008. Oven dried (45 °C) leaves of *A. annua* (seeds from University of Campinas, Brazil, cultivar 3 M) and *Artemisia absinthium* (seeds from Horizon Herbs, Williams OR, USA) were ground in a cyclone grinder to 2 millimeter size particles. Twenty five grams of ground leaves were stirred with 150 ml of 70% aqueous ethanol (70:30 ethanol:water) for 2 h at 60 °C. The extract was then sonicated for 30 min and filtered through #2 Whatman filter paper (Whatman, Inc. Piscataway, NJ, USA). The ground leaves were re-extracted, the extracts were combined, rotoevaporated to dryness at 40 °C, redissolved in pure ethanol, and sonicated to remove all extract from the rotoevaporator flask. The extract was concentrated by evaporation under nitrogen using an N-Evap 111 (Organomation Associates, Inc., Berlin, MA, USA) and freeze dried. For use in Experiment 2, the extract was mixed with 3% Tween 80 to produce a final concentration of 75 mg/ml.

Solubility of the ethanolic extracts was improved by dissolving them in Labrosol® (Gatefossé USA, Paramus, NJ, USA). Labrasol® is a non-ionic amphiphilic excipient

used as a surfactant and bioenhancer for pharmaceutical drugs of poor bioavailability or of high molecular weight. Labrasol® is a mixture of caprylocaproyl polyoxyglycerides and polyethylene glycol esters. It is very soluble in ethanol and water, but insoluble in mineral oils. To prepare the extracts for administration in Experiment 3, lyophilized ethanolic extract was dissolved in 50% Labrasol® in water with sonication. Following sonication, additional water was added to produce a final extract concentration of 143 mg/ml in 25% Labrasol®.

#### 2.4.3. *A. annua* aqueous extract

Leaves of the Brazilian *A. annua* cultivar were oven dried and ground as described above. Ground leaves (25 g) were placed in 200 ml water and boiled on a hotplate with stirring for 1.5 h. The samples were then concentrated in a rotoevaporator at 60 °C, frozen at −4 °C, then freeze dried to produce 8.0 g (32% yield) of a light brown extract. The extract was dissolved in 3% Tween 80 to provide a final concentration of 75 mg/ml.

#### 2.4.4. *A. annua* essential oil

*A. annua* (sweet Annie) essential oil was provided by the Lebermuth Company (Bremen, IN, USA). Essential oils are obtained by distillation in comparison to the extraction methods previously described. For use in Experiment 3, a solution of 43 mg/ml was prepared by dissolving the essential oil in olive oil containing 16.6% Labrasol as a bioenhancer.

### 2.5. Parasite infection and recovery

#### 2.5.1. *H. contortus* exsheathment and gerbil infection

*H. contortus* L3 used in gerbil studies were exsheathed using carbon dioxide as described by Conder and Johnson (1996). Gerbils were inoculated via oral gavage with 600 exsheathed *H. contortus* L3 in Earle's Balanced Salt Solution in a total volume of 0.5 ml.

#### 2.5.2. Parasite recovery

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 h following the method of Conder et al. (1991). The incubation fluid and stomach were preserved with formaldehyde for later enumeration of *H. contortus*. Parasites were counted using a dissecting microscope by personnel blind to the treatment groups.

### 2.6. Experimental protocol

Published reports or preliminary testing in gerbils were used as guides for selecting experimental dosages. No signs of toxicity were observed at any of the dosages tested in the experiments.

#### 2.6.1. Experiment 1

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

Group 1: Control, water daily for 5 days (Days 4–8 after infection).
Group 2: Control, DMSO and olive oil (1:4) daily for 5 days (Days 4–8 after infection).
Group 3: 400 mg per kilogram body weight (mg/kg BW) artemisinin in DMSO and olive oil (day 6 after infection).
Group 4: 200 mg/kg BW artemisinin in DMSO and olive oil daily for 5 days (Days 4–8 after infection).

All treatments were given by oral gavage in a volume of 0.3 ml/treatment. Dosages were based on a study describing the efficacy of artemether (an artemisinin derivative) against schistosomes (Shuhua and Catto, 1989).

#### 2.6.2. Experiment 2

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

Group 1: Control, 3% Tween 80 daily for 5 days (Days 4–8 after infection).
Group 2: 600 mg/kg BW *A. annua* ethanolic extract in 3% Tween 80 daily for 5 days (Days 4–8 after infection).
Group 3: 600 mg/kg BW *A. annua* aqueous extract in 3% Tween 80 daily for 5 days (Days 4–8 after infection).

Treatments were administered by oral gavage in a total volume of 0.36 ml. Dosages were based on results of a study describing effects of *Artemisia* species extracts on *T. spiralis* infection in rats (Caner et al., 2008).

#### 2.6.3. Experiment 3

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

Group 1: Control, water daily for 5 days (Days 4–8).
Group 2: 1000 mg/kg BW *A. annua* ethanolic extract in 25% Labrasol® daily for 5 days (Days 4–8 after infection).
Group 3: 300 mg/kg BW *A. annua* essential oil in olive oil with 16.6% Labrasol® daily for 5 days (Days 4–8 after infection).
Group 4: 1000 mg/kg BW *A. absinthium* ethanolic extract in 25% Labrasol® daily for 5 days (Days 4–8 after infection).

Treatments were delivered in a volume of 0.35 ml. Because there was no available data to guide dosage selection of the essential oil, a pilot study was conducted to establish safety of the mulltiple day 300 mg/kg dosage.

### 2.7. 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. Groups were compared using ANOVA followed by Tukey's procedure for multiple comparisons. Analyses were performed using SAS

**Table 1**
Arithmetic mean *Haemonchus contortus* burdens and treatment efficacy in experimentally infected gerbils dosed with either 400 mg/kg artemisinin in DMSO/olive oil once, or water, DMSO/olive oil (1:4) or 200 mg/kg artemisinin in DMSO/olive oil daily for 5 days (Days 4–8 after infection). No significant differences were observed.

| Treatment ($n = 10$) | Mean worm burden (±standard deviation)[2] | % Reduction |
|---|---|---|
| Water Days 4–8 post-infection (control) | 58.9 (±32.1) | – |
| DMSO/olive oil (1:4) Days 4–8 after infection | 57.0 (±19.2) | – |
| 400 mg/kg artemisinin day 6 after infection | 72.6 (±18.5) | −25.2 |
| 200 mg/kg artemisinin Days 4–8 after infection | 78.2 (±22.1) | −34.8 |

version 9.2 (Cary, NC, USA). Efficacy against *H. contortus* was calculated as percent parasite reduction:

$$\%\,\text{reduction} = 100 \times \frac{C - T}{C}$$

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.

## 3. Results

### 3.1. Experiment 1

Data from the artemisinin trial were normally distributed and arithmetic data was used for analysis. There were no significant differences in mean parasite burden among the groups ($P = 0.14$). The groups treated with artemisinin once or daily for 5 days had a mean *H. contortus* burden of 72.6 and 78.2 larvae, respectively, while the DMSO/olive oil and water control groups averaged 57 and 58.9 larvae, respectively (Table 1).

### 3.2. Experiment 2

Parasite counts in groups from Experiment 2 were not normally distributed. A logarithmic (base e) transformation was applied to the larval counts and data were summarized as geometric means with geometric 95% confidence intervals (Table 2). Groups treated with *A. annua* ethanolic and aqueous extracts averaged 54.8 and 68.5 *H. contortus* larvae, respectively, compared to a mean of 74.5 larvae in the control group. Although the group treated with the ethanolic extract showed a parasite reduction of 24.7% compared to the control group this difference was not significant ($P = 0.37$).

### 3.3. Experiment 3

Parasite numbers in Experiment 3 were normally distributed. No difference was seen between mean parasite numbers in the control group (mean 97.1) and the *A. annua* ethanolic extract group (mean 96) in this study (Table 3). Similarly, neither the *A. absinthium* ethanolic extract (mean 111.6) nor the *A. annua* essential oil (mean 94.7) produced a significant reduction in parasite numbers compared to the control group following daily treatment for 5 days.

## 4. Discussion

Published reports of effects of *Artemisia* species on trichostrongylid nematodes of small ruminants stimulated our experiments to specifically identify species and/or components of *Artemisia* with the greatest antiparasitic activity using the gerbil model of *H. contortus* infection. We first hypothesized that the compound artemisinin might contribute to the efficacy of *A. annua* against *H. contortus* through the same mode of action reported for artemisinin's antimalarial activity, by which reactive oxygen species are produced following the interaction of artemisinin with heme released by the action of the parasite on hemoglobin (Pandey et al., 1999; Robert et al., 2005). We reasoned that digestion of host blood containing artemisinin might result in internal damage to *H. contortus*. However, treatment with artemisinin did not reduce *H. contortus* burdens in infected gerbils. These results may merely be the result of selecting ineffective dosages of artemisinin, either because the dose level or length of contact with the parasite was insufficient. The dosages used in our experiment were based on studies of schistosome infected mice treated with semi-synthetic artemisinin derivatives with higher stability and bioavailability than artemisinin (Klayman, 1993; Utzinger et al., 2001). We used a combination of DMSO and olive oil to improve artemisinin delivery and uptake, but unfortunately, blood levels of the compound and its active metabolite, dihydroartemisinin (Hien et al., 2004), could not measured. As a result, we do not know if systemic levels were adequate for antiparasitic activity. Even higher doses of artemisinin may be ineffective, however, if *H. contortus* is able to protect itself from oxidative stress caused by artemisinin. Fourth stage *H. contortus* larvae in vitro exhibited a 4.6-fold induction of catalase in the presence of hydrogen peroxide (Kotze, 2003).

We also investigated whether other components of *Artemisia* species contain activity against *H. contortus* by testing plant extracts and an essential oil. Again, our results

**Table 2**
Geometric mean *Haemonchus contortus* burdens and treatment efficacy in experimentally infected gerbils dosed with 3% Tween 80, 600 mg/kg *Artemisia annua* ethanolic extract in 3% Tween 80 or 600 mg/kg *A. annua* aqueous extract in 3% Tween 80 daily for 5 days. No significant differences were observed.

| Treatment ($n = 10$) | Mean worm burden (95% confidence intervals) | % Reduction |
|---|---|---|
| 3% Tween 80 Days 4–8 after infection | 74.5 (45.7, 121.4) | – |
| 600 mg/kg *A. annua* ethanolic extract Days 4–8 infection | 54.8 (33.6, 89.3) | 24.7 |
| 600 mg/kg *A. annua* aqueous extract Days 4–8 after infection | 68.5 (42.0, 111.6) | 2.1 |

**Table 3**
Arithmetic mean *Haemonchus contortus* burdens and treatment efficacy in experimentally infected gerbils dosed with water (control), 1000 mg/kg *Artemisia annua* ethanolic extract in 25% Labrasol®, 300 mg/kg *A. annua* essential oil in 16.6% Labrasol® or 1000 mg/kg *A. absinthium* ethanolic extract in 25% Labrasol® daily for 5 days. No significant differences were observed.

| Treatment ($n = 10$) | Mean worm burden (±standard deviation)[2] | % Reduction |
|---|---|---|
| Water Days 4–8 post-infection (control) | 97.1 (±40.1) | – |
| 1000 mg/kg *A. annua* ethanolic extract Days 4–8 after infection | 96 (±61.7) | 1.1 |
| 300 mg/kg *A. annua* essential oil Days 4–8 after infection | 94.7 (±39.0) | 1.3 |
| 1000 mg/kg *A. absinthium* ethanolic extract Days 4–8 after infection | 111.6 (±61.41) | −14.9 |

were disappointing. No antiparasitic effects were seen with the ethanolic extract of *A. absinthium*, or with the aqueous extract or essential oil of *A. annua*. The ethanolic extract of *A. annua* resulted in a small, but non-significant reduction in parasite burden (24%) in Experiment 2, but no effect of the ethanolic extract was seen in Experiment 3 when the dose was increased.

One of the difficulties associated with investigating anthelmintic effects of some non-aqueous plant extracts in laboratory animals is their low solubility in commonly used carriers like DMSO or Tween 80 (Abad et al., 1996). The highly lipophilic nature of essential oils also makes mixtures with these products difficult. In order to dissolve high doses of the *Artemisia* ethanolic extracts and deliver the *A. annua* essential oil in a constant volume, a commercial product, Labrasol® was used. Labrasol® is a bioenhancer for the oral delivery of pharmaceutical compounds of poor solubility, or of good solubility but high molecular weight. It is a non-ionic surfactant based on vegetable oils esterified with polyethylene glycol. Labrasol® also appears to be relatively nontoxic. The oral LD50 in rats is 22 g/kg BW (Delongeas et al., 2010). Although we did not have the resources to include a gerbil group that received only Labrasol®, our results indicate that the solvent alone probably had no effect on *H. contortus*. Labrasol® has recently been reported to improve the absorption and bioavailability of anti-diabetic compounds from *Artemisia dracunculus* ethanolic extracts (Ribnicky et al., 2009) and may be a useful alternative solvent in animal studies investigating anthelmintic activity of plant extracts.

Our consistently negative results with the *Artemisia* species extracts products are perplexing in view of reports in the literature. *A. absinthium* extracts in vitro were highly effective and reduced fecal egg counts in naturally infected sheep when used at the same 1000 mg/kg BW dose that was ineffective in the gerbils (Tariq et al., 2008). Single treatments with much lower levels of *A. absinthium* extracts also reduced levels of *T. spiralis* larvae in the tissues of rats (Caner et al., 2008). This discrepancy emphasizes the difficulties associated with efforts to evaluate and standardize antiparasitic effects of plants and plant-derived products. Even though the use of extracts allows delivery of a consistent amount of plant product based on host body weight, results cannot be easily compared across studies because of variation that may occur in active components due to variation in extract preparation and phytochemical differences in plant species, cultivars or maturity when harvested. The gerbil model of *H. contortus* infection provides a method of testing plant extracts that is easier to standardize and requires smaller amounts of plant extracts than livestock studies. However, if the mode of action of plant compounds requires treatment for an extended period, the short lived infection model in gerbils may not allow sufficient contact time for the full effects of a compound to be evident. For example, when sericea lespedeza (a forage high in condensed tannins) was fed to naturally infected goats in pelleted form, an effect on fecal egg counts was not seen for several weeks (Terrill et al., 2009). Also, products with adulticidal, but little larvicidal activity cannot be adequately tested in the gerbil model, where parasites do not progress beyond the fourth larval stage. Finally, there are physiological differences between gerbils and ruminants that may affect the bioavailability and activity of plant compounds.

## 5. Conclusion

At the dosages given, artemisinin, crude ethanolic extracts of *A. annua* and *A. absinthium*, and the essential oil of *A. annua* did not have anthelmintic activity against *H. contortus* in the gerbil model of *H. contortus*. However, reports of antiparasitic effects from field studies justify further investigation of these plants and possible modes of anthelmintic activity in the gerbil model and natural hosts.

## Conflict of interest

The authors declare no conflicts of interest and the mention to proprietary names is solely for the convenience of the reader and does not mean any endorsement by the USDA of the products cited here over other similar products.

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