Efficacy of an orange oil emulsion as an anthelmintic against *Haemonchus contortus* in gerbils (*Meriones unguiculatus*) and in sheep

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**Abstract**

*Haemonchus contortus* is a blood-sucking abomasal parasite responsible for major losses to small ruminant producers worldwide. The recent increase in populations of anthelmintic resistant parasites has produced a demand for alternative control methods. An orange oil emulsion that has shown activity against plant parasitic nematodes and *H. contortus* in vitro was assessed for activity against *H. contortus* in a gerbil model and in the natural ovine host. In gerbil experiments, animals were infected with 600 infective third stage (L3) *H. contortus* larvae. In one experiment, gerbils were treated with 600 milligrams per kilogram bodyweight (mg/kg BW) orange oil once or daily for 5 days. In a second experiment, gerbils were treated with 1200 mg/kg BW orange oil once or daily for 5 days. On Day 9 post-infection, gerbils were killed, their stomachs removed, and the worms counted. The 600 mg/kg BW dosage caused 7% and 62.6% parasite reduction compared to a control group when given once or daily for 5 days, respectively. The 1200 mg/kg BW dosage of orange oil caused 25% and 87.8% parasite reduction compared to a control group when given once or daily for 5 days, respectively. The difference between the multiple treatment and control group were significant at both dosages (\(P<0.005\)). In the sheep trial, 18 lambs were orally inoculated with 10,000 L3 *H. contortus*. One month later, two groups of six lambs each were dosed with 600 mg/kg BW orange oil either once or daily for 3 days. Fecal egg counts were monitored daily starting on the first day of treatment (Day 0) and continuing for 14 days. Results showed that a single dose of the product caused high fecal egg count reduction (97.4%) compared to control sheep. Egg counts were significantly reduced by Day 2 (\(P<0.0001\)). Thus, the orange oil emulsion may potentially be useful in the control of ovine haemonchosis.

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1. Introduction

Increasing levels of anthelmintic resistance in strongyloid parasites of small ruminants has stimulated interest in naturally occurring plant nematocides. Citrus peels and their extracted oils have shown activity against several infective agents including fungi (Vargas et al., 1999) and the root-knot nematode *Meloidogyne incognita* (Tsai, 2008). The disinfectant use of emulsions containing orange terpene oil, orange Valencia oil, polysorbate 80, hydrogen peroxide, and water has recently been patented (Bower, 2008). Evaluations of various formulations of these orange oil emulsions also showed them to be effective in reducing damage to tomato plant roots caused by *M. incognita* and in reducing the number of nematode eggs produced per gram of root tissue (Rosskopf et al., 2008).

Another series of experiments evaluated varying concentrations of an orange oil emulsion against egg and larval
stages of *H. contortus* (Rosskopf et al., 2008). Concentrations ranging from 2 to 100% inhibited egg hatching by at least 90%. Exposure of third stage larvae (L3) to a concentration of 3% or more of the emulsion resulted in greater than 50% inhibition of larval motility or death. These findings led to another patent application addressing the use of orange oil emulsions in treatment of gastrointestinal nematode infections in ruminants (Rosskopf et al., 2008). The purpose of the current study was to evaluate the activity of the orange oil emulsion against *H. contortus* in vivo. Investigations were initially conducted using a gerbil (*Meriones unguiculatus*) model of *H. contortus* infection (Conder et al., 1990, 1991). Infections in gerbils progress to the fourth larval stage, allowing this model to be used effectively in preliminary drug testing. Following the gerbil component of the study, efficacy of treatment was evaluated against *H. contortus* in the natural ovine host.

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. Sheep

Recently weaned (approximately 90 days of age) cross-bred ram lambs from the Virginia Tech teaching and research flock were housed indoors in pens containing six sheep per pen. They were fed hay and whole shelled corn and provided with water *ad libitum*. All sheep were dewormed twice with 8 milligrams per kilogram body-weight (mg/kg BW) levamisole orally at the time of housing to remove naturally acquired strongylid infection. Daily health observations were performed throughout the experiment.

2.3. Animal welfare

All experimental protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

2.4. *H. contortus*

For use in the gerbil studies, infective *H. contortus* L3 were cultured from the feces of a monospecifically infected lamb according to standard parasitological techniques. Larvae for the sheep study were provided by Dr. Ray Kaplan, University of Georgia, Athens, GA, USA.

2.5. Orange oil emulsions

Experimental composition 1 (EC1) orange oil emulsion used in the gerbil studies contained 10% (v/v) orange terpene oil, 5% (v/v) orange Valencia oil, 10% (v/v) polysorbate 80, 5.25% (v/v) hydrogen peroxide and 69.75% (v/v) water. Due to concern over dose volume and emetic effects of hydrogen peroxide in sheep, the formulation of the orange oil emulsion was altered for the sheep study to increase the proportion of orange oils and decrease the amount of hydrogen peroxide. This formulation (EC2) contained 40% (v/v) orange terpene oil, 20% (v/v) orange Valencia oil, 4% (v/v) polysorbate 80, 1.5% (v/v) hydrogen peroxide and 34.5% (v/v) water. Both EC1 and EC2 were provided by Robert Bowker (Knock-Out Technologies, Dover Plains, NY, USA). Orange oils were products of Florida Chemical Co. Inc. (Winter Haven, FL, USA).

2.6. Gerbil studies

2.6.1. *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.

2.6.2. 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.

2.6.3. Experiment 1

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: 600 mg/kg BW orange oil (4 ml/kg EC1) Day 6 after infection.

Group 2: 600 mg/kg BW orange oil (4 ml/kg EC1) daily for 5 days (Days 4–8 after infection).

Group 3: Control, water daily for 5 days (Days 4–8 after infection).

Because no information on efficacy of the orange oil emulsion against internal parasites was available, an initial dosage of EC1 was established at approximately 10% the oral LD50 of orange oil in lab animals (Anonymous, 2006). The total orange oil concentration was the only constituent of EC1 used to calculate the dose given to the gerbils. All treatments were administered via oral gavage in a total volume of 0.3 ml. Deionized water was used to equalize individual treatment volumes.

2.6.4. Experiment 2

Based on the results of the first experiment, a second study was conducted to assess the effect of a higher dose of EC1. The procedure was identical to Experiment 1 with the exception of the dosage. The following treatments were administered to 3 groups of 10 gerbils:
Group 1: 1200 mg/kg orange oil BW (8 ml/kg BW EC1) once (Day 6).
Group 2: 1200 mg/kg orange oil BW (8 ml/kg BW EC1) daily for 5 days (Days 4–8) after infection.
Group 3: Control, water daily for 5 days (Days 4–8) after infection.
Doses were administered via oral gavage in a total volume of 0.38 ml.

2.6.5. Statistics

Normal probability plots were generated based on larval counts to verify that data followed an approximately normal distribution. In the second gerbil study, the parasite distribution was not normal. A logarithmic (base e) transformation was applied to counts to normalize worm burdens and geometric means and 95% confidence intervals were determined. Groups were compared using ANOVA followed by Tukey’s procedure for multiple comparisons. The analyses were performed using SAS version 9.2 (Cary, NC, USA). Efficacy of the orange oil emulsion against H. contortus was calculated as percent larval reduction:

\[
\% \text{ efficacy} = 100 \times \frac{C - T}{C}
\]

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.

2.7. Sheep study

Eighteen lambs were inoculated via oral drench with 10,000 H. contortus L3 approximately one month after housing and 28 days before treatment with the orange oil emulsion. Shortly before treatment lambs were individually weighed and allocated into three groups of six animals each blocked by fecal egg count:

Group 1: 600 mg/kg BW orange oil (1 ml/kg 2 EC2 BW) once (Day 0).
Group 2: 600 mg/kg BW orange oil (1 ml/kg EC2 BW) daily for 3 days (Days 0–2).
Group 3: Control, water (1 ml/kg BW) daily for 3 days (Days 0–2).

2.7.1. Fecal collection and egg counts

Rectal fecal samples were collected in the preinfection period to confirm efficacy of deworming at the time of housing and shortly before the start of the study for allocation to groups. Fecal samples were also collected daily from all lambs from Day 0 (for pre-treatment egg counts) through Day 14 post-initial treatment. Fecal egg counts (FEC) were determined using the Modified McMaster Test (Zajac and Conboy, 2006).

2.7.2. Statistics

Arithmetic mean group fecal egg counts were compared each day using the exact Kruskal–Wallis test followed by Dunn’s procedure for multiple comparisons. Efficacy against H. contortus was calculated as a percent FEC reduction:

\[
\% \text{ FEC reduction} = 100 \times \frac{C - T}{C}
\]

where C is the arithmetic mean FEC of the untreated control group and T is the arithmetic mean FEC of a group that received EC2. The analyses were performed using SAS version 9.2 (Cary, NC, USA). Confidence intervals for percent FEC reduction in sheep were calculated based on the World Association for the Advancement of Veterinary Parasitology methods for the detection of anthelmintic resistance (Coles et al., 1992).

3. Results

3.1. Gerbil experiment 1

Parasite burdens from gerbils were normally distributed and thus not transformed for analysis. The groups of gerbils receiving single or multiple treatments of 600 mg/kg BW orange oil (EC1) averaged 74.8 and 30.1 H. contortus larvae, respectively, compared to a mean of 80.4 larvae in the control group (Table 1). The multiple dose treatment mean was significantly lower (P < 0.005) than the means of the control group and single dose group. The efficacy of the multiple dose treatment was 62.6%.

3.2. Gerbil experiment 2

Parasite burdens were not normally distributed, thus a logarithmic (base e) transformation was applied to the larval counts before group differences were analyzed and geometric means with 95% confidence intervals were determined. Compared to a mean of 76.1 H. contortus larvae in the control group, treatment for 1 or 5 days with 1200 mg/kg BW orange oil emulsion (EC1) produced means of 56.1 and 4.8 larvae, respectively (Table 2). As in Experiment 1, only the multiple dose group mean was significantly different from that of the control (P < 0.005). Treatment for 5 days reduced parasite numbers with an efficacy of 87.8%.

3.3. Sheep study

On Day 0 of the study there were no significant differences (P < 0.05) in treatment group mean FEC. On Day 14 after the initial treatment of 600 mg/kg BW orange oil, mean FEC were 25 (±42), 50 (±78), and 975 (±715) for the 1- and 3-day treatment and control groups respectively (Fig. 1). A significant effect of EC2 on mean FEC was seen on Day 2 post-dosing (P < 0.0001) and continued to Day 14 post-dosing (P < 0.002). There were no significant differences between the FEC of the 2 groups treated with EC2 from Days 0 to 14. On Day 14, FEC were reduced by 97.4% in the single treatment group and 94.9% in the 3-day treatment group.

4. Discussion

Our studies showed that the orange oil emulsion EC1 significantly reduced numbers of H. contortus by Day 9 after
Table 1
Arithmetic mean Haemonchus contortus burdens and treatment efficacy in experimentally infected gerbils dosed with water (control) or 600 mg/kg orange oil (4 ml/kg Experimental Composition 1) once or on 5 successive days post-infection.

<table>
<thead>
<tr>
<th>Treatment (n = 10)</th>
<th>Mean worm burden (± standard deviation)</th>
<th>%Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Days 4–8 post-infection (control)</td>
<td>80.4 (26.4)a</td>
<td>70</td>
</tr>
<tr>
<td>Orange oil Day 6 post-infection</td>
<td>74.8 (45.7)a</td>
<td>7</td>
</tr>
<tr>
<td>Orange oil Days 4–8 post-infection</td>
<td>30.1 (26.2)b</td>
<td>62</td>
</tr>
</tbody>
</table>

+ Experimental Composition 1 contained 10% (v/v) orange terpene oil, 5% (v/v) orange Valencia oil, 10% (v/v) polysorbate 89, 5.5% (v/v) hydrogen peroxide and 69.5% (v/v) water.

+ Values with different letters are significantly different at P < 0.005.

Table 2
Geometric mean H. contortus burdens and treatment efficacy (based on arithmetic means) in experimentally infected gerbils dosed with water (control) or 1200 mg/kg orange oil (4 ml/kg BW Experimental Composition 1) once or on 5 successive days post-infection.

<table>
<thead>
<tr>
<th>Treatment (n = 10)</th>
<th>Mean worm burden (95% confidence intervals)</th>
<th>%Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Days 4–8 post-infection (control)</td>
<td>76.1 (40.1–144.3)a</td>
<td>25</td>
</tr>
<tr>
<td>Orange oil Day 6 post-infection</td>
<td>56.1 (29.6, 106.4)a</td>
<td>87</td>
</tr>
<tr>
<td>Orange oil Days 4–8 post-infection</td>
<td>4.8 (2.5, 9.0)b</td>
<td></td>
</tr>
</tbody>
</table>

+ Experimental Composition 1 contained 10% (v/v) orange terpene oil, 5% (v/v) orange Valencia oil, 10% (v/v) polysorbate 89, 5.5% (v/v) hydrogen peroxide and 69.5% (v/v) water.

+ Values with different letters are significantly different at P < 0.005.

infection in gerbils that received at least 600 mg/kg on Days 4–8 after infection. Although both levels of EC1 tested produced significant parasite reduction, the higher orange oil dose (1200 mg/kg BW) was approximately 20% more effective than the 600 mg/kg dose.

For the sheep study, the orange oil emulsion was formulated (EC2) before it was tested to reduce the dose volume and decrease the amount of hydrogen peroxide administered and the lower dosage level from the gerbil trials was used. Even with the lower level of orange oil and hydrogen peroxide, a single treatment of the emulsion (EC2) was highly effective in reducing FEC in sheep experimentally infected with H. contortus. This effect was seen within a day of treatment and the two additional treatments given to sheep in Group 2 did not provide any additional benefit. Because fecal egg counts of both sheep treatment groups remained low for the entire 14 days of the study, it seems likely that parasite numbers were reduced, as was seen in the gerbil trials. However, this could not be confirmed by assessing total worm burdens.

While a single dose of 600 mg/kg BW of orange oil in sheep rapidly caused a 98% FEC reduction, the same dose in gerbils reduced parasite burdens by only 7%. The observed host variation may be due to metabolic differences that make the active compounds in the orange oil emulsion less available in gerbils. In sheep, the rumen may serve as a reservoir, slowing the passage of the emulsion, thus prolonging the exposure of H. contortus to the orange oil emulsion.

Alternatively, adult H. contortus may be more susceptible to the effects of the orange oil than the larval stages, although in vitro work suggests that H. contortus L3 and eggs are quite susceptible to the effects of the orange oil emulsion (Rosskopf et al., 2008).

Fifteen percent of the formulation of EC1 and 60% of EC2 is orange terpene and orange Valencia oil. These oils are approximately 95% d-limonene (Shaw and Coleman, 1974; Vora et al., 1983), a terpene that is lipophilic with strong solvent capability. We believe that the terpenes alone are responsible for the anthelmintic activity of the formulations tested because the small quantity of hydrogen peroxide used in our studies is probably quickly deactivated in animals, especially in the rumen. In combination with hydrogen peroxide, the orange oils are capable of degrading the cell walls of microbes (R. Bowker, personal communication). However, the mode of action of orange oils against eukaryotic parasites is unknown. 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.

The major component of orange oil, d-limonene, is generally recognized as safe and has low oral and dermal toxicity (LD50 > 5 g/kg; Anonymous, 2006) in rabbits.
We saw no clinical evidence of toxicity in gerbils following administration of EC1. Furthermore, histopathological examination of the gerbil stomachs did not identify any lesions in EC1 treated animals. Data not shown. A few lambs demonstrated head shaking and lack of interest in food after dosing with EC2, but these behaviors did not persist for more than 15–20 min after treatment. In general, EC2 was well tolerated by the sheep. Some treated sheep passed soft but formed stool for a few days following treatment, although none of the sheep developed diarrhea. Additionally, EC2 was well tolerated by the sheep. Some treated sheep passed soft but formed stool for a few days following treatment, although none of the sheep developed diarrhea. In general, EC2 was well tolerated by the sheep. Some treated sheep passed soft but formed stool for a few days following treatment, although none of the sheep developed diarrhea.

In the 14 days following the first treatment. An in vitro rumen fermentation assay of EC1 showed a reduction of dry matter disappearance with an orange oil concentration of 2% (Rosskopf et al., 2008), but the effect was not greater than that observed with the same amount of soybean oil, a dietary supplement often provided at this level in mixed rations for ruminants (Bateman and Jenkins, 1998). While detrimental interactive effects of hydrogen peroxide and orange oil on rumen microorganisms are possible, the emulsion did not appear to adversely affect rumen function or digestion in our experiment. Further studies are needed to assess the emulsion for parasiticidal activity and negative health effects in sheep.

5. Conclusion

Studies of two formulation of orange oil emulsion have demonstrated efficacy against Haemonchus contortus infection in gerbils and sheep. Terpenes are likely to be the active compounds, however to clearly determine the mechanism of action of the orange oil emulsion, further studies should be conducted. Additional studies are also needed to evaluate the feasibility and safety of orange oil emulsions for use in livestock. However, based on our initial studies, the emulsion shows promise as an alternative to commercial anthelmintics.

Conflict of interest statement

The authors declare no conflicts of interest.

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