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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 strongylid 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 (Bowker, 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

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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) crossbred 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 bodyweight (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 LD_{50} 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:

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