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Research paper

Comparative efficacy and toxic effects of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes and mice

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ABSTRACT

Carvacrol is a compound isolated from some essential oils. It has been reported to possess anthelmintic activity. Acetylation of this monoterpene has been proposed as a potential way to reduce the toxicity and enhance the pharmacological effects of carvacrol. This study aimed to evaluate the effect of carvacryl acetate (CA) using *in vitro* and *in vivo* assays with gastrointestinal nematodes of small ruminants. The egg hatching test (EHT), larval development test (LDT) and adult worm motility (AWM) assessment were conducted to evaluate the effect of the acetylated product and pure carvacrol on *Haemonchus contortus* eggs, larvae and adults. The structural changes induced in adult *H. contortus* were assessed using scanning electron microscopy (SEM). CA and carvacrol acute toxicity was evaluated in mice. Finally, the efficacy of 250 mg/kg CA and 2.5 mg/kg monepantel (positive control) were evaluated in 30 sheep naturally infected with gastrointestinal nematodes by the fecal egg count reduction test (FECRT). *In vitro* tests were analyzed by analysis of variance (ANOVA) followed by comparison with Tukey's test. The efficacy was calculated by the Boot Street program using the arithmetic average. The number of eggs in feces (epg) of the groups were transformed to log $(x+1)$ and subjected to ANOVA to compare differences among the groups by Tukey's test. The level of significance was $P<0.05$. CA and carvacrol inhibited larval hatching by 89.3 and 97.7% at doses of 8.0 and 1.0 mg/ml, respectively. At the concentration of 2 mg/ml, CA and carvacrol inhibited 100% of larval development. At a concentration of 200 μg/ml, CA and carvacrol inhibited the motility of adult worms by 100% and 58.3% at 24 h post-exposure, respectively. CA caused cuticle and vulvar flap wrinkling and bubbles to emerge from the tegument. Carvacrol caused more discreet effects on the cuticle and vulvar flap. The LD_{10} and LD_{50} of CA were 566.7 mg/kg and 1544.5 mg/kg, respectively. The LD_{10} and LD_{50} of carvacrol were 546.8 mg/kg and 919 mg/kg, respectively. CA and monepantel reduced the epg of sheep by 65.9 and 96.4%, respectively, at 16 days post-treatment. CA showed *in vitro* and *in vivo* anthelmintic activity and was less toxic than carvacrol.

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1. Introduction

Gastrointestinal nematodes, especially *Haemonchus contortus*, endanger the health and well-being of sheep and goats worldwide and cause economic losses (Marie-Magdeleine et al., 2014; Zhong et al., 2014). These parasites are controlled with antiparasitic drugs. The broad-spectrum of action, good tolerability and low costs of anthelmintics were responsible for the prolonged use of these drugs over the last five decades (Lanusse et al., 2014). However, the overuse and misuse of these drugs favor the selection of resistant nematode populations (Dos Santos et al., 2014). Thus, it is necessary to develop complementary alternative methods to prevent infections with gastrointestinal nematodes, including pasture management, the selection of animals that are resistant to nematodes and the development of drugs based on plants with anthelmintic activity (Hoste and Torres-Acosta, 2011; Macedo et al., 2012).

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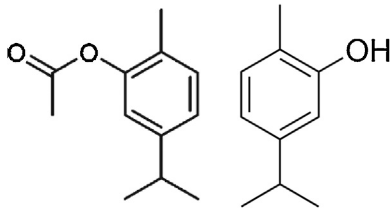

Fig. 1. Chemical structure of carvacryl acetate and carvacrol.

Essential oils are among the classes of vegetable substances reported to possess anthelmintic activity and can be used as an alternative to current therapies (Anthony et al., 2005; Ribeiro et al., 2013). These oils contain large amounts of terpenes, which are secondary metabolites that interfere with biochemical and physiological functions of parasites (Kaplan et al., 2014; Nordi et al., 2014).

Carvacrol is a phenolic monoterpene that is primarily found in essential oils of plants belonging to the genera *Origanum* and *Thymus* (Guimaraes et al., 2015). Because carvacrol is more toxic than many esters, carvacryl acetate (CA) was synthesized to obtain a semisynthetic derivative with an improved pharmacological profile and low toxicity (Damasceno et al., 2014). Thus, the presence of an ester group instead of the hydroxyl group found on carvacrol may provide different security characteristics and increase the efficacy of this compound.

Considering the potential use of natural products in new drug development, the aim of this study was to investigate the effect of carvacryl acetate (CA) and carvacrol against sheep gastrointestinal nematodes and to evaluate the toxicity of these compounds in mice.

2. Materials and methods

2.1. Ethics committee on animal welfare

This study was approved by the ethics committee for the use of animals of the Universidade Estadual do Ceará (Protocol number: 32228358/2014).

2.2. Carvacrol acetylation

CA (Fig. 1) was obtained *via* the acetylation of carvacrol (Sigma–Aldrich®, St. Louis, USA) (Fig. 1) using acetic anhydride as an acetylating agent and sodium acetate as a catalyst. Carvacrol was acetylated by the addition of acetic anhydride (15 ml) and sodium acetate (1.5 g) to carvacrol (1 g). The mixture was refluxed for 1 h, the solution was left at room temperature, and cold water was added (20 ml). The solution was neutralized to pH 7.0 with 5% sodium bicarbonate. The reaction mixture was transferred to a separating funnel and washed three times with chloroform (100 ml). The chloroform layer containing acetylated material was washed with water and then dried with sodium sulfate. The solvent was evaporated under reduced pressure (Matos, 1997). During the experiment, 50 g of carvacrol were used. The yield of carvacryl acetate was 83.1%.

2.3. Analysis of carvacryl acetate

The carvacryl acetate was subjected to thin layer chromatography and characterized by infrared spectroscopy (FTIR) using a model 8300 (Shimadzu Corporation, Japan).

2.4. *In vitro* assays

H. contortus population used in *in vitro* trials was resistant to benzimidazoles.

2.4.1. Egg hatch test (EHT)

The egg hatch test (EHT) test was performed according to Coles et al. (1992). Briefly feces were collected directly from the rectum of sheep harboring monospecific infection of *H. contortus*. *H. contortus* eggs were recovered according to Hubert and Kerboeuf (1992). Aliquots (250 μl) of a suspension containing approximately 100 fresh eggs were mixed with 250 μl of the following treatments: G1: 0.5 to 8 mg/ml CA; G2: 0.06 to 1.0 mg/ml carvacrol (Sigma–Aldrich®, St. Louis, USA); G3: 1% Tween 80 (negative control) and G4: 0.025 mg/ml thiabendazole (positive control). The eggs were incubated for 48 h at 25 °C, and drop of Lugol's iodine was added. The eggs and first-stage larvae (L1) were counted under a light microscope. We performed three repetitions with five replicates for each treatment and for each control.

2.4.2. Larval development test (LDT)

The larval development test (LDT) was performed using an aliquot of egg suspension obtained as described by Hubert and Kerboeuf (1992). The suspension was incubated for 24 h at 25 °C to obtain L1. The LDT was performed according to Camurça-Vasconcelos et al. (2007). A 500 μl aliquot of a suspension containing approximately 250 *H. contortus* L1 was mixed with the same volume of the following treatments: G1: 0.125–2 mg/ml CA; G2: 0.125 to 2 mg/ml carvacrol (Sigma–Aldrich®, St. Louis, USA); G3: 1% Tween 80 (negative control) and G4: 0.008 mg/ml ivermectin (Ivomec®, Merial Saúde Animal, São Paulo, Brazil). The L1 and treatments were added to 1 g of feces collected from a sheep free of gastrointestinal nematodes. After six days at room temperature (25 °C), third stage larvae (L3) were recovered according to the method of Roberts and O'Sullivan (1950), and drop of Lugol's iodine were added. The L3 were counted under a light microscope.

2.4.3. Adult worm motility (AWM)

The adult worm motility assay was performed based on the methodology described by Hounzangbe-Adote et al. (2005). Adult worms were collected from an experimentally infected lamb four weeks after infection. Immediately after slaughtering, the abomasum was removed, opened and placed in 37 °C saline solution. Mobile adult female worms were rapidly collected and put into 24-multiwell plates at a density of 3 worms per well in 1 ml of PBS at 37 °C in the presence of 4% penicillin/streptomycin (Sigma–Aldrich® St. Louis, USA). After 1 hour of incubation (37 °C, 5% carbon dioxide) 1 ml of 200, 100, 50, and 25 μg/ml of the following treatments were added to the worms: G1: CA; G2: carvacrol (Sigma–Aldrich®, St. Louis, USA); G3, PBS plus 4% penicillin/streptomycin (negative control) and G4: 100 μg/ml ivermectin (Ivomec®, Merial Saúde Animal, São Paulo, Brazil). The measurements were performed on eight replicates per dose for each treatment. The motility of adult worms was noted by careful observation under inverted microscope at a magnification of 40× after 6, 12 and 24 h.

2.5. Scanning electron microscopy (SEM)

H. contortus adult females were treated with 200 μg/ml CA or carvacrol for a period 24 hours. The worms were subsequently fixed in a 2.5% glutaraldehyde solution in a 0.1 M sodium cacodylate buffer (CACO) for 72 h. After three washes in the same buffer (0.1 M), the worms were placed in a 2% osmium in 0.1 M in CACO (pH 7.4) buffer fixative for 1 hour. Samples were washed two times with CACO and distilled water. The samples were dehydrated in a

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