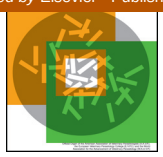
Veterinary Parasitology 218 (2016) 52–58

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar



Research paper

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

Weibson P.P. Andre[a], Wesley L.C. Ribeiro[a], Géssica S. Cavalcante[a], Jessica M.L.dos Santos[a], Iara T.F. Macedo[a], Haroldo C.B.de Paula[b], Rivelilson M. de Freitas[c], Selene M. de Morais[a], Janaina V.de Melo[d], Claudia M.L. Bevilaqua[a,*]

[a] Programa de Pós-Graduação em Ciências Veterinárias, Universidade Estadual do Ceará, Brazil
[b] Departamento de Química Analítica e Físico-Química, Universidade Federal do Ceará, Brazil
[c] Programa de Pós-Graduação em Ciência Farmacêutica, Universidade Federal do Piauí, Brazil
[d] Centro Estratégico de Tecnologia do Nordeste, Brazil

ARTICLE INFO

Article history:
Received 14 August 2015
Received in revised form
17 December 2015
Accepted 4 January 2016

Keywords:
Acetylation
Carvacrol
Sheep
Haemonchus contortus

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.

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).

* Corresponding author at: Claudia Maria Leal Bevilaqua Programa de Pós-graduação em Ciências Veterinárias/FAVET/UECE Av. Dr. Silas Munguba, 1700, Campus do Itaperi CEP 60714-903 Fortaleza, Ceará Brazil. Fax: + 55 85 31019840.
E-mail address: bevilaqua.uece@gmail.com (C.M.L. Bevilaqua).

http://dx.doi.org/10.1016/j.vetpar.2016.01.001
0304-4017/

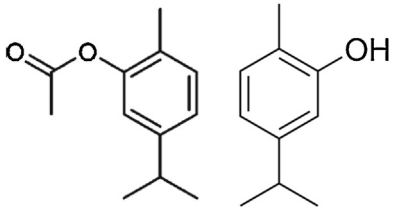


Fig. 1. Chemical structure of carvacryl acetate and carvacrol.

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

graded acetone series. Critical point drying was completed using a CPD 030 (Bal-Tec, Liechtenstein), and samples on metal stubs were coated with a 10 nm layer of gold in a sputter coating machine (Leica SCD 500, Leica Microsystems, Wetzlar, Germany). Parasites were then observed with a scanning electron microscope (FEI QUANTA 200 FEG ESEM, FEI Company, USA) at an accelerating voltage of 20 kV.

2.6. Acute toxicity for mice

In the acute toxicity test, 90 female Swiss albino mice (*Mus musculus*) with average weight of 25 g were kept in polypropylene boxes and given Labina Purina® (Paulinia, Brazil) feed and water *ad libitum*. The mice were randomly divided into treatment groups ($n = 10$). G1 to G4 received 500, 1000, 2000 and 4000 mg/ml of CA; G5–G8 received 250, 500, 1000 and 2000 mg/ml carvacrol; and G9 received 1% Tween 80. The treatments were administered by esophageal gavage. The animals were observed for 15 days, and changes in behavior and mortality were recorded.

2.7. Fecal egg count reduction test (FECRT)

We used thirty sheep of both sexes ranging in age from 6 to 16 months, weighing on average 18 kg. The sheep were reared in a semi-arid region of northeastern Brazil and were fed on native pasture and with water *ad libitum*. The animals were selected according to the number of eggs in feces (epg) over 500 using the McMaster technique (Ueno and Gonçalves, 1998). A lower LD50 value established in the acute toxicity test was used to ensure that the carvacryl acetate would not cause any toxic effects on sheep. Sheep were divided into 3 homogeneous groups ($n = 10$) according to the epg and randomly assigned to the following treatments: G1: 250 mg/kg CA; G2: 2.5 mg/kg of monepantel (Zolvix®, Novartis, New Zealand) as positive control and G3, water as negative control. Sheep received a single treatment. Fecal samples from each animal were collected on days 0, 8 and 16 post-treatment to determine the epg. Coprocultures were performed using the method described by Roberts and O'Sullivan (1950).

3. Statistical analysis

The larvae hatching percentage was determined according to the following equation: (number of hatched larvae/number of hatched larvae + number of eggs) × 100. The inhibition of larval development was calculated using the following formula: [(L1 control group – L1 treated group)/L1 control group] × 100. Adult worm motility was evaluated as the number of motile worms/total number of worms per well.

The results of *in vitro* tests were analyzed by analysis of variance (ANOVA) followed by comparison with Tukey's test using Graph Pad Prism® 5.0 software. The effective concentrations to inhibit 50% (EC_{50}) of the egg hatching and larval development were determined by linear regression using SPSS 17.0 program for Windows. The results of the worm motility inhibition were expressed as the mean ± standard error (SE).

In the acute toxicity test for mice, the total number of dead animals was verified, and the lethal doses were calculated (LD50 and LD10) by probit analysis with SPSS 17.0 software.

The total number of dead animals was verified, and the lethal doses were calculated (LD50 and LD10) in the acute toxicity test for mice were calculated by probit analysis with SPSS 17.0 software.

The FECRT efficacy was calculated by the Boot Street program through the arithmetic average using the formula100 (1 − XT/XC), where XT and XC are the average epg in the treatment and control groups, respectively (Coles et al., 1992). The data were transformed to log ($x + 1$) and subjected to ANOVA and Tukey's test using GraphPad Prism® 5.0 software. The significance level was $P < 0.05$.

Table 1
Mean efficacy (±standard error) of carvacryl acetate and carvacrol on *Haemonchus contortus* egg hatching.

Concentration (mg/ml)	Carvacryl acetate	Concentration (mg/ml)	Carvacrol
8	89.3 ± 1.4^{Aa}	1	99.7 ± 0.1^{Ab}
4	$82.2 \pm 1,1^{Ba}$	0.5	94.8 ± 0.7^{Ac}
2	64.3 ± 1.3^{Ca}	0.25	73.4 ± 2.3^{Bc}
1	26.2 ± 1.2^{Da}	0.12	25.0 ± 2.1^{Ca}
0.5	8.7 ± 1.0^{Eab}	0.06	6.1 ± 1.5^{Da}
Tween 80 (1%)	4.6 ± 0.3^{Ea}	Tween 80 (1%)	3.5 ± 0.3^{Da}
TBZ (0.025 mg/ml)	94.5 ± 0.5^{Aa}	TBZ (0.025 mg/ml)	94.5 ± 0.5^{Aa}

The capital letters indicate comparisons of the means in the columns, and the lowercase letters denote comparisons of the means in the rows. Different letters indicate significantly different values ($P < 0.05$). Fifteen replicates for each treatment and for each control were performed.

Table 2
Mean efficacy (±standard error) of of carvacryl acetate and carvacrol on *Haemonchus contortus* larval development.

Concentration (mg/ml)	Carvacryl acetate	Carvacrol
2	100 ± 0.0^{Aa}	100 ± 0.0^{Aa}
1	97.7 ± 0.3^{Aa}	99.5 ± 0.1^{Aa}
0.5	52.0 ± 1.2^{Ba}	63.9 ± 2.1^{Bb}
0.25	31.7 ± 1.1^{Ca}	33.7 ± 1.2^{Ca}
0.12	8.6 ± 0.8^{Da}	21.1 ± 0.8^{Da}
Tween 80 (1%)	2.1 ± 0.6^{Da}	5.1 ± 1.7^{Da}
Ivermectin (0.008 mg/ml)	99.7 ± 0.08^{Aa}	99.8 ± 0.09^{Aa}

The capital letters indicate comparisons of the means in the columns, and the lowercase letters denote comparisons of the means in the rows. Different letters indicate significantly different values ($P < 0.05$). Fifteen replicates for each treatment and for each control were performed.

4. Results

After acetylation, the band at 3214 cm^{-1}, which is characteristic of hydroxyl groups disappeared, whereas those at 2963 cm^{-1} assigned to CH remain, as expected. Moreover, a new band due to an inserted acetyl group appears at 1760 cm^{-1} for carvacryl acetate. The remaining main 1″ bands (at 1206 cm^{-1} and 1370 cm^{-1}) were also detected, as previously reported in the literature (Pires et al., 2015).

The effects of CA and carvacrol on EHT were dose-dependent, and the results are shown in Table 1. The CA dose of 8 mg/ml inhibited 89.3% of larval hatching, while carvacrol inhibited 99.7% at a dose of 0.5 mg/ml. These results did not differ statistically from the positive control ($P > 0.05$). The EC_{50} for EHT was 1.7 (1.9–2.4) mg/ml and 0.17 (0.15–0.19) mg/ml for CA and carvacrol, respectively.

The LDT results are shown in Table 2. The most effective dose was 2 mg/ml for CA and carvacrol. These results did not differ statistically from the positive control ($P > 0.05$). The EC_{50} values for LDT were 0.3 (0.1–0.7) mg/ml and 0.2 (0.1–0.6) mg/ml for CA and carvacrol, respectively.

The concentration of 200 μg/ml CA and carvacrol inhibited worm motility by 100% and 41.8%, respectively, at 24 h post-exposure (Fig. 2). The adult motilities in PBS and ivermectin were 87.5 and 0%, respectively, at 24 h post-exposure. The worm motility was dose-dependent.

The SEM micrographs show the changes in adult *H. contortus* after *in vitro* exposure to CA and carvacrol (Fig. 3). The main change that can be observed is the wrinkling of the cuticle. The vulvar flap has lost its normal aspect and shows wrinkling and bubbles emerging from the tegument. Carvacrol caused more discrete effect on the cuticle and on the vulvar flap.

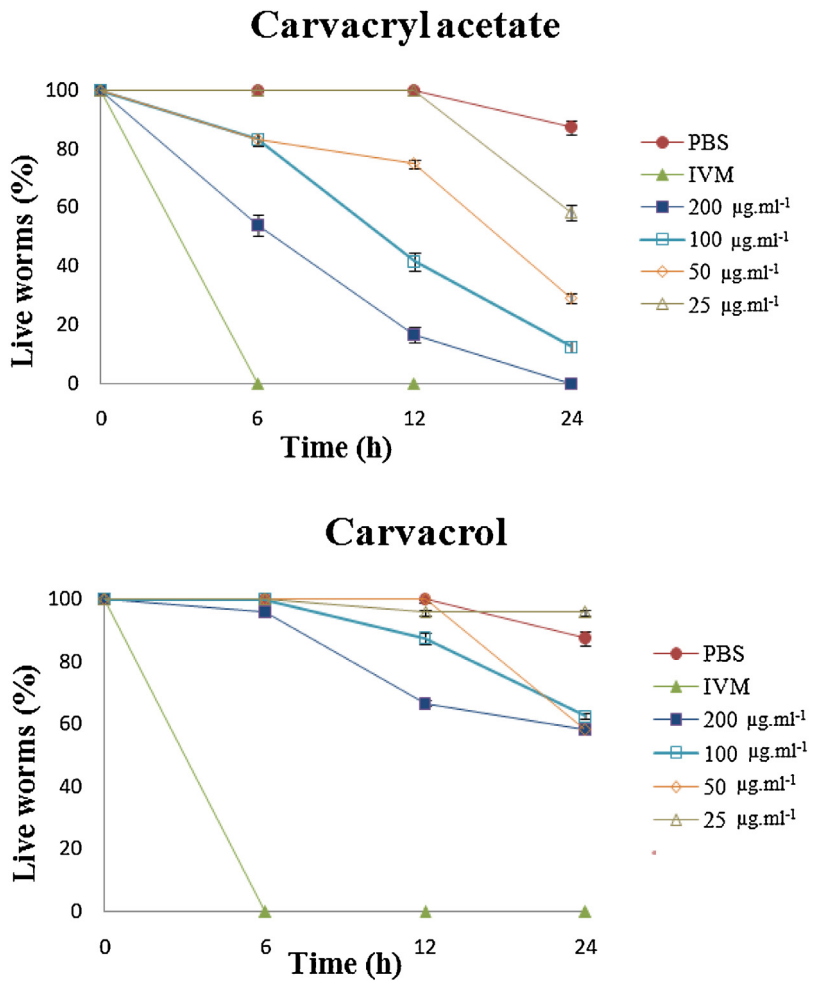


Fig. 2. Percent (±standard error) inhibition of carvacryl acetate, carvacrol, PBS (negative control) and 100 µg/ml ivermectin (positive control) of *H. contortus* adult motility.

Table 3
Mean efficacy and egg counts per gram of feces (epg ± standard error) of carvacryl acetate and monepantel.

Treatments	Day 0	Day 8	Day 16
Carvacryl acetate			
Mean epg	2065 ± 1526^{Aa}	1405 ± 1080^{Aa}	975 ± 605.2^{Aa}
Efficacy (%)	–	35.4	65.9
Monepantel			
Mean epg	2670 ± 2387^{Aa}	165.3 ± 141,5^{Bb}	100.4 ± 122,5^{Bb}
Efficacy (%)	–	92.4	96.4
Water			
Mean epg	2260 ± 1586^{Aa}	2175 ± 1254^{Aa}	2865 ± 1977^{Ac}

Capital letters compare mean in the lines, and the lowercase letters denote comparisons of the means in the rows. Different letters indicate significantly different values ($P < 0.05$)

In the acute toxicity test the LD_{10} and LD_{50} CA were 566.7 (176.3–930.9) mg/kg and 1,544.5 (943.6–2,475.8) mg/kg, respectively. Already the LD_{10} and LD_{50} of carvacrol were 546.8 (266.3–718) mg/kg and 919 (693.3–1,251.9) mg/kg, respectively. No changes were observed in the behavior of mice during the acute toxicity test. Substances with a LD_{50} value above 1000 mg/kg *via* oral route are safe or of low toxicity (Clarke and Clarke, 1977) justifying the use of the CA in the FECRT.

The FECRT results of CA and monepantel are expressed as the mean epg on days 0, 8 and 16 post-treatment (Table 3). CA and monepantel reduced epg by 65.9 and 96.4%, respectively, by 16 days post-treatment. The CA results are significantly different from the negative control ($P < 0.05$).

The larvae recovered by fecal cultures of sheep before treatment were identified as *Haemonchus* spp. (90%), *Trichostrongylus* spp. (7%) and *Oesophagostumum* spp. (3%), while those recovered 16 days after treatment with carvacryl acetate were *Haemonchus* spp. (43%), *Trichostrongylus* spp. (51%) and *Oesophagostumum* spp. (6%), while the larvae recovered after monepantel treatment were *Haemonchus* spp. (53%), *Trichostrongylus* spp. (48%) and *Oesophagostumum* spp. (9%).

5. Discussion

The anthelmintic effect of essential oils depends on the chemical components of the essential oils, and the isolation of these compounds is important for the development of new anthelmintic drugs (Ribeiro et al., 2013; Qi et al., 2015). Carvacrol is a monoterpene that has several pharmacological actions, including anthelmintic (Zhu et al., 2013), acaricide (Cruz et al., 2013), anti-*Leishmania infantum* and anti-*Trypanosoma cruzi* activity (Escobar et al., 2010).

In vitro tests are used to screen substances with antiparasitic properties (Marie-Magdeleine et al., 2014). In preliminary EHT, 2 mg/ml of carvacrol achieved 100% efficacy (data not shown). Then the doses used for carvacrol were lower than those of the CA. This difference is possibly related to the reduced ability of acetylated products to penetrate the three basic layers (outer vitelline, intermediate chitin and inner layer of lipids)

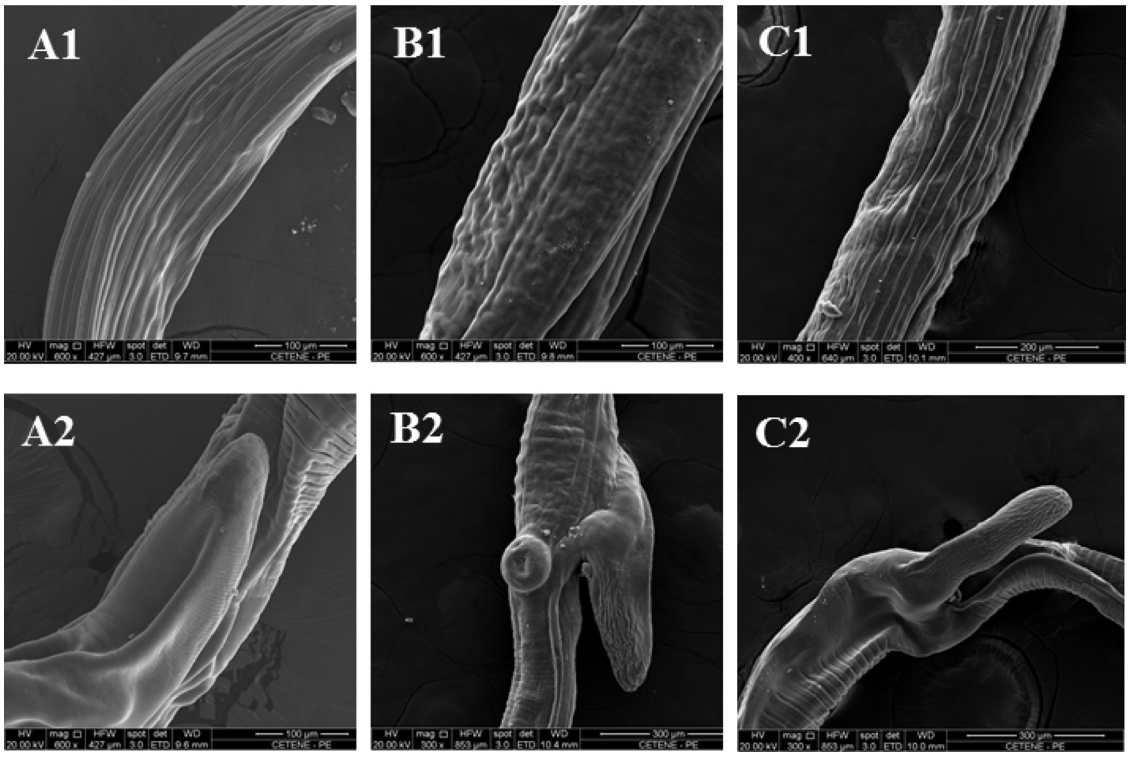


Fig. 3. Scanning electron microscopic image of cuticle and vulvar flap of *Haemonchus contortus* adult female after incubation with PBS (A1 and A2), carvacryl acetate (B1 and B2) or carvacrol (C1 and C2).

that form the *H. contortus* eggshell (Mansfield et al., 1992). The intermediate layer of chitin is an association of chitinous fibrils surrounded by a protein layer that can provide the eggshell resistance to chemical substances (Wharton, 1980). This intermediate layer can be a barrier to the penetration of acetylated compounds, since the higher liposolubility conferred by the acetyl radical only allows greater penetration in lipid membranes, the inner layer. Carvacrol ovicidal effect (EC_{50} = 0.1 mg/ml) was superior to other compounds isolated from essential oils that exhibit anthelmintic effect, such as thymol (EC_{50} = 0.5 mg/ml), anethol (EC_{50} = 0.6 mg/ml), camphor (EC_{50} = 1.8 mg/ml) and borneol (EC_{50} = 1.5 mg/ml) (Camurça-Vasconcelos et., 2007; Zhu et al., 2013; Qi et al., 2015). The carvacrol ovicidal effect could be attributed to the phenolic group in its chemical structure. This radical possibly inhibit enzymes (proteases, lipases, chitinases, beta glucosidase and leucine aminopeptidase) that are responsible for inhibiting hatching of eggs (Molan and Faraj, 2010). This mechanism is similar to the polyphenols or tannins that have the hydroxyl radical in their chemical structure (Vargas-Magaña et al., 2014).

In the LDT, the results of carvacryl acetate and carvacrol revealed no significant difference ($P > 0.05$). The CA (EC_{50} = 0.3 mg/ml) and carvacrol (EC_{50} = 0.2 mg/ml) larvicide activity was superior to thymol (EC_{50} = 2.4 mg/ml), anethol (EC_{50} = 2.1 mg/ml), camphor (EC_{50} = 7.8 mg/ml) and borneol (EC_{50} = 1.9 mg/ml) (Camurça-Vasconcelos et., 2007; Zhu et al., 2013; Qi et al., 2015). Carvacrol caused changes in the cuticle and intestine of L3 larvae of *Anisakis simplex* and the efficacy could be attributed to the phenolic group in its structure (Hierro et al., 2004). The larvicidal effect of carvacrol could be associated with the effect that this compound has on bacterial membrane, consisting of the phenolic group connection with the amine and hydroxylamine groups of bacterial membrane proteins. The interaction of hydroxyl radical with the proteins affects the stability of the lipid layer increases the passive flow of protons through the membrane, and consequently, alter the permeability resulting in the cell death. In contrast, although carvacryl acetate do not exhibit the hydroxyl group, it inhibited the exchange of protons by changing the membrane permeability, and yet prevent bacterial growth (Lambert et al., 2001; Arfa et al., 2006). The substitution of the hydroxyl radical by the radical acetyl did not change the CA efficiency.

AWM is an *in vitro* test that allows for a more realistic assessment of the nematicidal activity of these products (Elandalousi et al., 2013). The adult motility test is used to assess the interaction of bioactive compounds with the cuticle of helminths in a short time by SEM (Hoste et al., 2006; Behnke et al., 2008; Martínez-Ortíz-de-Montellano et al., 2013). The action of CA on adult worms was nearly two times better than carvacrol, and this may be due to greater liposolubility of CA that is conferred by the acetyl radical added during the process of acetylation. CA and carvacrol both have low molecular weights of 192.2 g/mol and 150.2 g/mol, respectively, and the octanol/water partition coefficient (logP) of carvacryl acetate (3.59) is higher than carvacrol (3.52) which gives it greater liposolubility (Arfa et al., 2006). Substances which have logP greater than 3 are considered lipophilic compounds having high affinity for cell membranes and their inserts can induce physicochemical changes in the cell membrane (Arfa et al., 2006). This property may lead to increased penetration of the compounds *via* transcuticular diffusion, which a common way for non-nutrient and non-electrolyte substances to gain entry to helminths (Eguale et al., 2007). CA is responsible for causing cuticular lesions (swelling) on the integument of *Schistosoma mansoni* (Moraes et al., 2013). Essential oils of *Thymus vulgaris*, which are rich in thymol and carvacrol, cause damage to the cuticle and digestive apparatus of *Anisakis* larvae (Giarratana et al., 2014). In addition to the cuticular changes, carvacrol is neurotoxic to the free-living nematode *Caenorhabditis elegans* because it interact with SER-2 tyramine (Lei et al., 2010). The cuticular changes and possible neurotoxicity caused by CA and carvacrol may interfere with the permeability of the cuticle and motility, hindering the maintenance of homeostasis within these parasites.

Acetylated substances have low toxicity due to the replacement of the hydroxyl radical by the acetyl radical (Morais et al., 2014). This property has been demonstrated by the reduced toxicity of CA compared to carvacrol in rodents.

The effect of CA on sheep gastrointestinal nematodes maybe be related to changes in the cuticle and reproductive tract of parasites. The cuticle is a barrier that protects the worm, and it is involved in motility and metabolic exchanges that take place in the digestive tract of sheep (Martínez-Ortíz-de-Montellano et al., 2013). The structural changes in the external reproductive organs of female worms could also affect nematode reproduction and reduce the production of eggs (Hoste et al., 2006). CA action has also been show to interfere with the production of *S. mansoni* eggs (Moraes et al., 2013). It is possible that the activity of CA against *H. contortus* reproductive system was responsible for the decrease in the egg counts of the feces of treated animals. CA and monepantel were more effective against *H. contortus*, as there was a reduction in the number of *H. contortus* L3 obtained in coprocultures after treatment. Carvacrol has antifungal activity at acidic pH values (Chavan and Tupe, 2014). It is possible that the activity of CA is related to its effect on pH. The accumulation of CA in the abomasum favors transticular absorption by parasites in addition to reaching the target parasite through the plasma after oral ingestion (Lifschitz et al., 2014).

Carvacrol and CA were effective against different stages of *H. contortus*, in addition acetylation increased CA effect on the motility of adult worms and it was less toxic in mice. Nonetheless, it is necessary to increase the effectiveness of CA against sheep gastrointestinal nematodes and the use of encapsulation techniques may be one potential solution.

Conflict of interests

None of the authors have any conflict of interest.

Acknowledgements

The authors are grateful to CNPq (503908/2012-6) and CAPES (23038.003104/2014-58, AUXPE - PROEX - 533/2014) for financial support. Dr. Bevilaqua, Dr. Freitas and Dr. Morais received a fellowship from CNPq.

References

Anthony, J.P., Fyfe, L., Smith, H., 2005. Plant active components—a resource for antiparasitic agents? Trends Parasitol. 21, 462–468.
Arfa, A.B., Combes, S., Preziosi-Belloy, L., Gontard, N., Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical Structure. Lett. Appl. Microbiol. 43, 149–154.
Behnke, J.M., Buttle, D.J., Stepek, G., Lowe, A., Duce, I.R., 2008. Developing novel anthelmintics from plant cysteine proteinases. Parasites Vectors 1, 29.
Camurça-Vasconcelos, A.L.F., Bevilaqua, C.M.L., Morais, S.M., Maciel, M.V., Costa, C.T.C., Macedo, I.T.F., Oliveira, M.L.B., 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.
Chavan, S.P., Tupe, S.G., 2014. Antifungal activity and mechanism of action of carvacrol and thymol against vineyard and wine spoilage yeasts. Food Control 46, 115–120.
Clarke, E.G.C., Clarke, M.L., 1977. Veterinary Toxicology. Cassel and Collier Macmillan, Publishers London, pp. 268–277.
Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World association for the advancement of veterinary parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.
Cruz, E.M.O., Costa-Junior, L.M., Pinto, J.A.O., Santos, D.A., Araujo, S.A., Arrigoni-Blank, M.F., Bacci, L., Alves, P.B., Cavalcanti, S.C.H., Blank, A.F., 2013. Acaricidal activity of *Lippia gracilis* essential oil and its major constituents on the tick *Rhipicephalus* (*Boophilus*) *microplus*. Vet. Parasitol. 195, 198–202.
Damasceno, S.R.B., Oliveira, F.R.A.M., Carvalho, N.S., Brito, C.F.C., Silva, I.S., Sousa, F.B.M., Silva, R.O., Sousa, D.P., Barbosa, A.L.R., Freitas, R.M., Medeiros, J.V.R., 2014. Carvacryl acetate a derivative of carvacrol, reduces nociceptive and inflammatory response in mice. Life Sci. 94, 58–66.
Dos Santos, J.M.L., Monteiro, J.M.L., Ribeiro, W.L.C., Macedo, I.T.F., Camurça-Vasconcelos, A.L.C., Vieira, L.S., Bevilaqua, C.M.L., 2014. Identification and quantification of benzimidazole resistance polymorphisms in *Haemonchus contortus* isolated in Northeastern Brazil. Vet. Parasitol 199, 160–164.
Eguale, T., Tilahun, G., Debella, A., Feleke, A., Makonnen, E., 2007. In vitro and in vivo anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus*. J. Ethnopharmacol. 110, 428–433.
Elandalousi, R.B., Akkari, H., B'chir, F., Gharbi, M., Mhadhbi, M., Awadi, S., Darghouth, M.A., 2013. *Thymus capitatus* from Tunisian arid zone: Chemical composition and *in vitro* anthelmintic effects on *Haemonchus contortus*. Vet. Parasitol. 197, 374–378.
Escobar, P., Leal, S.M., Herrera, L.V., Martinez, J.R., Stashenko, E., 2010. Chemical composition and antiprotozoal activities of Colombian *Lippia* spp. essential oils and their major components. Mem. Inst. Oswaldo Cruz. 105, 184–190.
Giarratana, F., Muscolino, D., Beninati, C., Giuffrida, A., Panebianco, A., 2014. Activity of *Thymus vulgaris* essential oil against *Anisakis* larvae. Exp. Parasitol. 142, 7–10.
Guimaraes, A.G., Oliveira, M.A., Alves, R.S., Menezes, P.P., Serafini, M.R., Araujo, A.A.S., Bezerra, D.P., Junior, L.J.Q., 2015. Encapsulation of carvacrol a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. Chem. Biol. Interact. 227, 69–76.
Hierro, I., Valero, A., Pérez, P., González, P., Cabo, M.M., Montilla, M.P., Navarro, M.C., 2004. Action of different monoterpenic compounds against *Anisakis simplex* s.l. L3 larvae. Phytomedicine 11, 77–82.
Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253–261.
Hoste, H., Torres-Acosta, J.F.J., 2011. Non chemical control of helminths in ruminants: adapting solutions for changing worms in a changing world. Vet. Parasitol. 180, 144–154.
Hounzangbe-Adote, M.S., Paolini, V., Fouraste, I., Moutairou, K., Hoste, H., 2005. In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode *Haemonchus contortus*. Res. Vet. Sci. 78, 155–160.
Hubert, J., Kerboeuf, D., 1992. A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. Vet. Rec. 130, 442–446.
Kaplan, R.M., Storey, B.E., Vidyashankar, A.N., Bissinger, B.W., Mitchell, S.M., Howell, S.B., Mason, M.E., Lee, M.D., Pedroso, A.A., Akashe, A., Skrypec, D.J., 2014. Antiparasitic efficacy of a novel plant-based functional food using an *Ascaris suum* model in pigs. Acta Tropica 139, 15–22.
Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nycs, G.J.E., 2001. A study of minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462.
Lanusse, C., Alvarez, L., Lifschitz, A., 2014. Pharmacological knowledge and sustainable anthelmintic therapy in ruminants. Vet. Parasitol. 204, 18–33.
Lei, J., Leser, M., Enan, E., 2010. Nematicidal activity of two monoterpenoids and SER-2 tyramine receptor of *Caenorhabditis elegans*. Biochem. Pharmacol. 79, 1062–1071.
Lifschitz, A., Ballent, M., Virkel, G., Sallovitz, J., Lanusse, C., 2014. Accumulation of monepantel and its sulphone derivative in tissues of nematode location in sheep: pharmacokinetic support to its excellent nematodicidal activity. Vet. Parasitol. 2013, 120–126.
Macedo, I.T.F., Bevilaqua, C.M.L., Oliveira, L.M.B., Camurça-Vasconcelos, A.L.F., Morais, S.M., Machado, L.K.A., Ribeiro, W.L.C., 2012. In vitro activity of *Lantana camara*, *Alpinia zerumbet*, *Mentha villosa* and *Tagetes minuta* decoctions on *Haemonchus contortus* eggs and larvae. Vet. Parasitol. 190, 504–509.
Mansfield, L.S., Gamble, H.R., Fetterer, R.H., 1992. Characterization of the eggshell of *Haemonchus contortus*: I structural components. Comp. Biochem. Physiol. 103, 681–686.
Marie-Magdeleine, C., Udino, L., Philibert, L., Bocage, B., Archimede, H., 2014. In vitro effects of *Musa paradisiacal* extracts on four developmental stages of *Haemonchus contortus*. Res. Vet. Sci. 96, 127–132.
Matos, F.J.A. (Ed.), 1997. Edições UFC, Fortaleza, p. 141.
Martínez-Ortíz-de-Montellano, C., Arroyo-López, C., Fourquaux, I., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Hoste, H., 2013. Scanning electron microscopy of *Haemonchus contortus* exposed to tannin-rich plants under *in vivo* and *in vitro* conditions. Exp. Parasitol. 133, 281–286.
Molan, A.L., Faraj, A.M., 2010. The effects of condensed tannins extracted from different plant species on egg hatching and larval development of *Teladorsagia circumcincta* (*Nematoda*: *Trichostrongylidae*). Folia Parasitol. 57, 62–68.
Moraes, J., Carvalho, A.A.L., Nakano, E., Almeida, A.A.C., Marques, T.H.C., Andrade, L.N., Freitas, M.R., Sousa, D.P., 2013. Anthelmintic activity of carvacryl acetate against *Schistosoma mansoni*. Parasitol. Res. 112, 603–610.
Morais, S.M., Vila-Nova, N.S., Bevilaqua, C.M.L., Rondon, F.C.M., Lobo, C.H., Moura, A.A.A.N., Sales, A.D., Rodrigues, A.P.R., Figuereido, J.R., Campello, C.C., Wilson, M.E., Junior, H.F.A., 2014. Thymol and eugenol derivatives as potential antileishmanial agents. Bioorg. Med. Chem. 22, 6250–6255.
Nordi, E.C.P., Costa, R.L.D., David, C.M.G., Parren, G.A.E., Freitas, A.C.B., Lameirinha, L.P., Katiki, L.M., Bueno, M.S., Quirino, C.R., Gama, P.E., Bizzo, H.R., Chagas, A.C.S., 2014. Supplementation of moist and dehydrated citrus pulp in the diets of sheep artificially and naturally infected with gastrointestinal nematodes on the parasitological parameters and performance. Vet. Parasitol. 205, 532–539.
Pires, F.L., Costa, L.M., Silva, O.A., Almeida, A.A.C., Cerqueira, G.S., Sousa, D.P., Pires, R.M.C., Satyal, P., Freitas, R.M., 2015. Neuropharmacological effects of carvacryl acetate on δ-aminolevulinic dehydratase Na^+, K^+-ATPase activities and amino

acids levels in mice hippocampus after seizures. Chem. Biol. Interact. 226, 49–57.
Qi, H., Wang, W.X., Dai, J.L., Zhu, L., 2015. In vitro *Zanthoxylum simulans* essential oil against *Haemonchus contortus*. Vet. Parasitol. 211, 223–227.
Ribeiro, W.L.C., Macedo, I.T.F., Dos Santos, J.M.L., Oliveira, E.F., Camurça-Vasconcelos, A.L.C., De Paula, H.C.B., Bevilaqua, C.M.L., 2013. Activity of chitosan-encapsulated *Eucalyptus staigeriana* essential oil on *Haemonchus contortus*. Exp. Parasitol. 135, 24–29.
Roberts, F.H.S., O'Sullivan, P.J., 1950. Methods for egg counts and larval cultures for strongyles infecting the gastrointestinal tract of cattle. Aust. J. Agric. Res. 1, 99–102.
Ueno, H., Gonçalves, P.C., 1998. Manual para Diagnóstico das Helmintoses de Ruminantes. JIICA, Tokyo, Japan, p. 143.
Vargas-Magaña, J.J., Torres-Acosta, J.F.J., Aguilar-Caballeroa, A.J., Sandoval-Castroa, C.A., Hosteb, H., Chan-Péreza, J.I., 2014. Anthelmintic activity of acetone–water extracts against *Haemonchus contortus* eggs: interactions between tannins and other plant secondary compounds. Vet. Parasitol. 206, 322–327.
Wharton, D.A., 1980. Nematode egg-shells. Parasitology 81, 447–463.
Zhong, R.Z., Li, H.Y., Sun, H.X., Zhou, D.W., 2014. Effects of supplementation with dietary green tea polyphenols on parasite resistance and acute phase protein response to *Haemonchus contortus* infection in lambs. Vet. Parasitol. 205, 199–207.
Zhu, L., Dai, J., Yang, L., Qiu, J., 2013. Anthelmintic activity of *Arisaema franchetianum* and *Arisaema lobatum* essential oils against *Haemonchus contortus*. J. Ethnopharmacol. 148, 311–316.