Experimental Parasitology 197 (2019) 36–42

Contents lists available at ScienceDirect

Experimental Parasitology

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

Evaluation of encapsulated anethole and carvone in lambs artificially- and naturally-infected with *Haemonchus contortus*

L.M. Katiki[a,*], R.C. Araujo[b], L. Ziegelmeyer[a], A.C.P. Gomes[a], G. Gutmanis[a], L. Rodrigues[a], M.S. Bueno[a], C.J. Veríssimo[a], H. Louvandini[c], J.F.S. Ferreira[d], A.F.T. Amarante[e]

[a] Instituto de Zootecnia (IZ/SAA), Rua Heitor Penteado 56, CEP 13460-000, Nova Odessa, SP, Brazil
[b] GRASP Ind. e Com. LTDA, Av. Juscelino Kubitschek de Oliveira, CEP 81.260-000, Curitiba, PR, Brazil
[c] Centro de Energia Nuclear na Agricultura (CENA/USP), Rua do Centenário 303, CEP 13400-970, Piracicaba, SP, Brazil
[d] US Salinity Lab (USDA-ARS), 450 W. Big Springs Rd., Riverside, CA, 92507, USA
[e] Instituto de Biociências (UNESP), CEP 18618-689, Botucatu, SP, Brazil

ARTICLE INFO

Keywords:
Haemonchus
Anethole
Carvone
Encapsulation
Essential oil

ABSTRACT

Molecules from natural sources, such as essential oils, have shown activity against parasites *in vitro*, but have not yet been explored extensively *in vivo*. Anethole and carvone (10% each), encapsulated with 80% of a solid matrix, referred to as EO (encapsulated oils), were tested *in vivo* in 2 experiments. In Experiment 1: Lambs were artificially infected with multidrug resistant *Haemonchus contortus*, or left uninfected, and treated (or not) with 50 mg/kg bw (body weight) of EO in a controlled environment. Thirty-two male lambs were kept in individual cages for a period of 45 days, after which animals were evaluated for parasitological, hematological, toxicological, and nutritional parameters. After 45 days of treatment, EO at 50 mg/kg bw provided a significant ($P \leq 0.05$) reduction in fecal egg count (FEC). Although FEC was reduced, animals from both treatments had similar counts of total adult worms. The low FEC was caused probably by a significant reduction ($P \leq 0.05$) in both male worm size and female fecundity. Dry matter intake of uninfected controls was significantly ($P \leq 0.05$) reduced, although no toxicity was observed in treated animals. Thus, in Experiment 2, conducted for five months we used an EO dose of 20 mg/kg bw. Thirty-four weaned lambs, free of parasites, were divided in two groups and kept in collective pens. One group received EO at 20 mg/kg bw mixed with concentrate for 5 months and the other was kept as a control group (CTL). Parasitological and hematological parameters as well as body weight were evaluated. In the first 2.5 months, CTL and EO groups were confined, and both presented similar clinical parameters. Then, animals were allotted to graze on contaminated pastures to acquire natural infection for the next 2.5 months. The infection was patent after 25 days and both groups had similar decreases in weight gain, increases in FEC, and decreases in blood parameters. Coprocultures from CTL and EO groups established that parasite population was 90% *Haemonchus* sp. We concluded that the technology of encapsulation is safe and practical to deliver to lambs at the farm level and anethole and carvone at 50 mg/kg bw caused a significant decrease in FEC and, consequently, in pasture contamination by free living stages of *H. contortus*. However, EO at 20 mg/kg bw was not effective to prevent or treat sheep naturally-infected with gastrointestinal nematodes.

1. Introduction

Gastrointestinal nematodes (GIN) are a major cause of economic losses in sheep production systems, leading to low productivity and, in severe cases, to animal death. The treatment for GIN is based on commercial anthelmintics. However, *Haemonchus contortus*, a hematophagous nematode with high prevalence and pathogenicity in the tropics, has developed multidrug-resistant strains worldwide (Almeida et al., 2010). This widespread resistance of *Haemonchus*, the lack of new, safe, and effective anthelmintics for livestock, and the growing concerns about chemical residues, passed on to consumers through meat and milk of treated animals, have sparked a growing interest in the search for novel anthelmintics from natural sources. Based on past promising reports with the use of essential oils and their isolated compounds *in vivo* (Squires et al., 2010; Carvalho et al., 2012; Ribeiro et al., 2013), plant products can be viable alternatives to develop natural anthelmintics for livestock.

Some essential oil components have synergistic activity when used

* Corresponding author.
E-mail address: lmkatiki@iz.sp.gov.br (L.M. Katiki).

https://doi.org/10.1016/j.exppara.2019.01.002
Received 26 September 2018; Received in revised form 30 November 2018; Accepted 7 January 2019
Available online 08 January 2019
0014-4894/

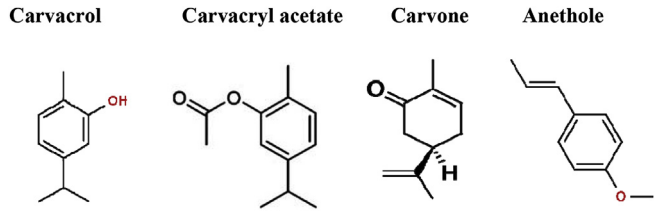

Fig. 1. Structures of carvacrol ($C_{10}H_{14}O$), carvacryl acetate ($C_{12}H_{16}O_2$, derived from carvacrol), and L-carvone ($C_{10}H_{14}O$), and anethole ($C_{10}H_{12}O$), the latter two were encapsulated and tested in sheep infected with *Haemonchus contortus* in this work.

in binary systems (paired). Katiki et al. (2017) evaluated the anthelmintic activity of 10 essential oil components (carvacrol, carvone, cineole, linalool, limonene, and thymol, and the phenylpropanoids cinnamaldehyde, anethole, vanillin, and eugenol). These compounds were evaluated individually and in binary, ternary, and quaternary combinations. The authors found that some components had synergistic interactions. Anethole and carvone mixed in equal parts had the best synergistic effect against *H. contortus in vitro* and were chosen for *in vivo* evaluation in this work. Carvacrol and carvacryl acetate (Fig. 1) have been reported to reduce adult motility of *Haemonchus contortus*, but carvacryl acetate (the acetylated form of carvacrol) was more potent than carvacrol and reduced fecal egg count (FEC) in sheep in 66%, while monepantel reduced FEC in 96% (Andre et al., 2016). As carvacrol is structurally very similar to carvone, both $C_{10}H_{14}O$, and carvone to anethole (Fig. 1), it is possible that carvone and anethole may also have anthelmintic activities against *H.* contortus *in vivo*.

Although essential oils have potential anthelmintic activity, some essential oils (depending on the dose and length of use) may be toxic, causing adverse reactions or even death on treated animals (Camurça-Vasconcelos et al., 2005). However, anethole and carvone have no known toxicity to the environment and their toxicity to mammals, established though their LD_{50} in rats, are 2090 mg/kg for anethole and 5400 mg/kg for L-carvone, according to the National Library of Medicine HSDB Database (2018). The low solubility in water (anethole) and easy oxidation by air (carvone) suggest that encapsulation would be a good strategy to stabilize both before administration to animals.

Essential oils are highly volatile compounds and disseminate through its gaseous form if the material is not properly contained through a physical barrier. Encapsulation of essential oils can reduce losses to the environment by volatilization, while increasing their stability. Another advantage of encapsulation is the reduction of the oxidation in contact with air (Edris, 2007). The encapsulation of liquid compounds in solid compounds adds the convenience of allowing a homogenous mixture with the feed concentrate and easy handling, even in small doses. Encapsulated essential oils had over 90% stability after 6 months of storage (Li et al., 2012).

Considering the information obtained from past literature and from our previous work on anthelmintic activity of essential oils and isolated compounds *in vitro* and *in vivo* (Katiki et al., 2011, 2012, 2017), both safety and anthelmintic efficacy of essential oils and its main components would be better determined *in vivo*, and evaluated through the FEC reduction test, parasitological necropsy, and hepatic function. These tests offer an estimated anthelmintic efficacy and could be associated to clinical and toxicological studies when the substance dose and toxicity are unknown.

The aims of this work were to evaluate the preventive or curative anthelmintic activity of encapsulated anethole and carvone in lambs experimentally and naturally infected with *H. contortus* and maintained in both confined and in semi-confinement systems.

2. Material and methods

Experimental site: Instituto de Zootecnia, São Paulo State farming system, located in Southeastern Brazil at latitude 22º46′39″ S and longitude 47º17′45″ W, at 570 m altitude. The local climate is Tropical with dry winters and wet summers, with annual temperatures ranging from 10 °C to 35 °C, and average humidity of 76%, classified as Cfa (Humid Subtropical Climate) according to Alvares et al., (2013).

Encapsulated essential oils: Anethole and carvone were kindly supplied and encapsulated by GRASP Ind. e Com. LTDA (Curitiba-PR, Brazil). The product formulated for this study contained 10% anethole, 10% carvone and 80% lipid matrix. The encapsulated oils (EO) were administered to animals at 250 mg/kg body weight (bw) (Experiment 1) and 100 mg/kg bw (Experiment 2), corresponding to 50 mg/kg bw and 20 mg/kg bw of the anethole/carvone mixture, and provided to animals mixed in their feed concentrate.

Animals: The study was conducted according to ethical principles in animal experimentation, approved by the Ethics Committees (CEUA-IZ), protocol number: 2012/153. Thirty two 'Santa Inês' lambs were used in Experiment 1 and 34 'Morada Nova' lambs were used in Experiment 2.

Experiment 1: In this experiment, 32 lambs approximately 5 months old were housed in individual cages, measuring 1.0 m × 2.0 m, equipped with individual feeders. Before the experiment, animals were cleaned of their natural parasite infections with the anthelmintic Monepantel (Zolvix® - dose 0.1 mL/kg) and with oxytetracycline (Terramycin LA® - dose 0.1 mL/kg) to treat any bacterial infection. The animals were ranked according to their body weight and equally distributed to balance each treatment:

Treatments: The animals were divided in 4 treatments: 2 parasitic conditions (infected or non-infected) and 2 treatments (with or without encapsulated oil – EO at 50 mg/kg bw). The experimental period lasted 45 days.

1 **CTL = (non-infected) (n = 6)**
2 **CTL + EO = non-infected/with EO (n = 6)**
3 **INF = artificially infected with *H. contortus* (n = 10)**
4 **INF + EO = artificially infected with *H. contortus*/with EO (n = 10)**

All lambs were fed a basal diet with 11% of crude protein with a 50:50 ratio of concentrate: roughage in individual feeders. The diet had high energy with total digestible nutrients around 70% and with the concentrate composed of ground corn and soybean meal, mineral salt, and vitamins. The forage used as roughage was grass hay (*Cynodon* spp.). Food and water were offered *ad libitum*. Diets were given in two daily meals, one at 8 a.m. and another at 4 p.m. The oils were administered only in the morning, mixed with the concentrate ration. After the animals were dosed with the EO, they were offered *Cynodon* hay.

Intake and weight gain: Feed intake was determined as the differences between feed offered and rejected daily. To ensure *ad libitum* supply, diets were calculated to allow 10%–15% leftover and when it was inferior to 10% or superior to 15%; the amount of hay offered was adjusted. To adjust the quantity of food from diets and obtain the average daily weight gain, the animals were weighed individually before the morning meal at day zero; then, every 15 days until the end of the experiment (45 days).

H. contortus infection: Lambs were infected with a single dose of 4000 L3 larvae of *H. contortus* multidrug-resistant strain with resistance to moxidectin, closantel, trichlorfon, levamisole phosphate, albendazole and ivermectin (Almeida et al., 2010). The treatments started 15 days before animals were infected with *H. contortus* so that animals would adapt and the preventive effect of EO against the L3 establishment in the abomasum of infected animals could be tested.

2.1. Parasitological assessments

Fecal egg count (FEC): Feces were collected directly from the

rectum on days 35; 37; 40; 43 and 45 for FEC according to Ueno and Gonçalves (1998).

Total worm count: Animals were euthanized at day 45. Abomasum was removed for total worm counting and worms were separated in flasks by sex with a microscope stereoscope.

Measurements of worms: One hundred males and 100 female adult worms (10 worms chosen at random from each animal) from each treatment were measured with a micrometric ruler.

Fecundity of female worms: The fecundity was also verified in 100 females of each treatment (10 worms chosen at random from each animal), which had their internal eggs quantified. For this, each female was placed in a plastic tube containing 950 µL of a sodium hypochlorite solution with 0.25% active chlorine. The tube was vortexed until the fragments of the parasite disappeared. At that time, 50 µL of 1% sodium thiosulfate (a chlorine neutralizer) solution was added to the tube to prevent egg degradation. The total number of eggs in each tube was estimated by counting 10 drops of 10 µL each, which allowed the calculation of the number of eggs per female (Kloosterman et al., 1978).

Toxicity (renal and hepatic profile): Animal blood sera were evaluated for renal profile (urea and creatinine) and hepatic profile (Aspartate Amino Transferase - AST and Gamma Glutamyl Transferase - GGT) through serum biochemical analyzer (Bio200, BIOPLUS®, São Paulo, Brazil) semiautomatic equipment with specific kits (Labtest®).

Hematologic profile: complete blood count of animals was performed with a blood analyzer (POOCH-VET, Sysmex®) calibrated for ovine species. Leukocytes were differentiated by counting 100 cells from blood smears stained with panoptic stain.

Experimental design and Statistical analysis: The blocks were randomized (by weight of animals) in factorial 2 (with and without EO) x 2 (with and without infection). The hematological and parasitological (FEC) variables were analyzed by repeated measures over time by statistical program SAS- PROC MIXED. The overall average for feed consumption, body weight gain and worm counts were analyzed by statistical program SAS- PROC GLM. The data relative to FEC and worm burden was transformed onto log 10 (x+1). Significant differences between the group means were determined by Tukey's test. Significant differences were established for values of $P \leq 0.05$.

Experiment 2: In this experiment, 34 male 'Morada Nova' lambs, approximately 3 months old, free of parasitic infection, were divided in two collective pens and were evaluated during 5 months in order to verify the EO activity at a lower dose (20 mg/kg bw) and for a longer time than experiment 1, and the acquisition of parasites in natural condition (pastures). Animals received a basal diet containing 15% of crude protein, composed of corn silage and concentrate enough to supply nutrient requirements for growing lambs (NRC, 2007), balanced by weight/hematocrit, and allocated to two treatment groups:

1 **CTL (n = 17):** Before the silage supply, the trays were completely cleaned and the concentrate without EO was offered.
2 **EO (n = 17):** Animals received 100 mg of encapsulated EO/kg bw daily (20 mg of anethole/carvone oil mix/kg bw) mixed with the concentrate in a clean tray before the silage supply.

Infection: During the first 2.5 months, EO and CTL animals were kept separated by treatment in collective pens. In the last 2.5 months, animals from EO and CTL were sent to the same pastures in alternated days, one group at a time, in order to graze and acquire natural infection. Animals were kept in pastures of *Panicum maximum* cv. Aruanã for 8 h. After this period, they returned to confinement (in each respective pen) in order to receive the above-described basal diet. The encapsulated EO was offered during the whole experimental period (5 months) mixed in the concentrate. Both treatments received mineral salt and water *ad libitum*.

Clinical evaluation: For five months, animals from EO and CTL groups were evaluated every 15 days until the end of the experiment through weight (to adjust doses of EO), and monthly for FEC. Animals with FEC over 3000 were treated with an efficient commercial anthelmintic. Coprocultures were performed in order to determine which helminth genera were present in experimental animals, according to Ueno and Gonçalves (1998).

Hematologic profile: complete blood count of animals from Experiment 2 were performed with a blood analyzer (POOCH-VET, Sysmex®) calibrated for ovine species. Leukocytes were differentiated by counting 100 cells from blood smears stained with panoptic stain.

2.2. Experimental design and Statistical analysis

The experiment was set up in a complete randomized design with two treatments (with and without EO) and challenge infection in both treatments. Data were analyzed by using a one-way analysis of variance. The data relative to FEC was transformed onto log 10 (x+1). Significant differences between the group means were determined by Tukey's test. Significant differences were established for values of $P \leq 0.05$.

3. Results

3.1. Experiment 1

The dose 50 mg/kg EO reduced FEC on Days 43 and 45 (Fig. 2) and reduced male length indicating an underdevelopment that may have affected the reproductive capacity and lower number of eggs per female worm (Table 1). Infected sheep, both INF and INF + EO had lower hemoglobin and hematocrit values on Day 45 than control animals (Fig. 3). 50 mg/kg EO had no effect on kidney and liver function (Fig. 4), as blood creatinine, urea and AST were in the reference range and GGT below the reference range. Daily body weight gain and DM intake were reduced by 50 mg/kg EO administration (Table 2).

3.2. Experiment 2

The dose of 20 mg/kg EO did not affect acquisition of parasites after pasture access as FEC raised while body weight gain decreased in both treatments (Fig. 5) with animals infected mostly with *Haemonchus*. In the last observation, FEC from most of the animals from the EO treatment increased over 3000 eggs per gram. Both treatment presented similar hemogram results as a consequence of *Haemonchus* infection (see Fig. 6). The experiment was finalized after 5 months of EO administration, and the experimental animals were treated with a commercial anthelmintic.

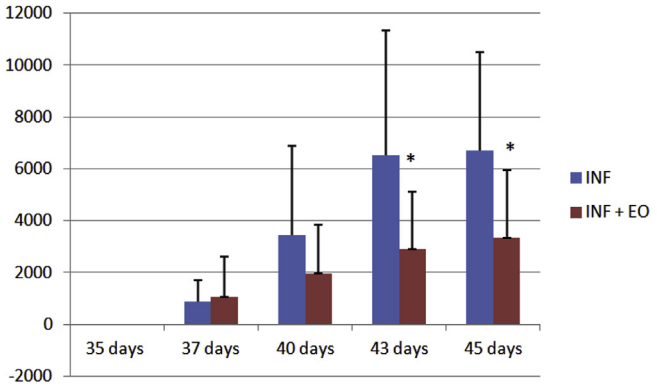

Fig. 2. Mean of fecal egg counts at 35, 37, 40, 43, and 45 days from animals infected with *H. contortus* multidrug resistant strain on day 15 and treated with the EO anethole and carvone daily at 50 mg/kg. Bars: standard deviations. * indicates significant effect $P < 0.05$.

Table 1
Mean ± S.D. *Haemonchus contortus* worm count, worm body length (cm) and fecundity (eggs/female) of parasites recovered from INF (infected) and INF + EO (infected + EO) lambs from Experiment 1.

Worm characteristics	INF	INF + EO	*P*
Female	739.7 ± 197.5	721.5 ± 173.9	0.82
Male	669.9 ± 235	707.3 ± 210.3	0.71
Total	1409.6 ± 378.1	1428.8 ± 285.1	0.89
Female length	1.7 ± 0.11	1.76 ± 0.05	0.1
Male length	1.29 ± 0.09[a]	1.21 ± 0.04[b]	0.0005
Eggs per female	475.3 ± 108.75[a]	394.2 ± 70.14[b]	0.004

Different letters in the lines indicate significant difference ($P < 0.05$).

4. Discussion

In the present study, the EO component mix at 50 mg/kg mixed in food for 45 days was tested to evaluate: 1- the acceptability of the oil; 2- the prevention of L3 establishment in the abomasum, 3- the mortality of adult *H. contortus*. At 50 mg/kg bw, the encapsulated product was effective in reducing FEC, which occurred due to the reduction in the adult worm fertility and not due to adult mortality, as no difference was found in worm counts between treated or non-treated groups. EO at 50 mg/kg bw had a negative effect on both feed intake and weight gain in control animals. Thus, in Experiment 2 (5 months), the 20 mg/kg dose was chosen. However, 20 mg/kg dose was not able to prevent or treat the natural infection with GIN. The doses chosen for Experiments 1 and 2 were based on reports of Marinov and Valcheva-Kuzmanova (2015), who reported that 120 mg/kg bw/day of trans-anethole in mice's diet for 90 days led to a severe loss of body weight and dehydration, associated with poor palatability of feed and reduced food intake. Thus, for the Experiment 1, we chose 50 mg/kg. The same report mentions that male mice that received 30 mg/kg/day for 90 days had enlarged livers, then we chose to use 20 mg/kg bw/day for Experiment 2, which lasted for 5 months.

Anethole and carvone negatively affected the parasite life cycle. As observed in other studies (Paolini et al., 2003), it is difficult to determine whether the decreased fecundity is related to lesions induced in the female reproductive system or whether this is due to the stimulation of the local immune response, as observed by González et al. (2011) who found similar results when they compared fecundity, adult worm length, and FEC in resistant versus susceptible sheep to *H. contortus*, indicating that the immune response was directed against the adult parasitic stage, in particular to the parasite's ability to produce eggs. This effect on parasite reproduction, in the long run, could have epidemiological benefits due to reduction in pasture contamination over time.

Significant reductions in nematode burden or in FEC when using crude essential oils or purified compounds were obtained with very high doses and for a few days as follows: Squires et al. (2010) treated lambs with 600 mg/kg (single dose) of an orange oil (60% terpenes) emulsion and obtained 97.4% reductions in *H. contortus* FEC. Macedo et al. (2010) administered *Eucalyptus staigeriana* essential oil at 500 mg/kg to goats and observed a 76% reduction in FEC of *Trychostrongylus* spp. André et al. (2017) tested thymol acetate at 250 mg/kg in sheep infected with *H. contortus* and observed a 60% efficacy on *H. contortus* on day 14. All of these essential oils and isolated compounds caused a significant reduction in FEC similar to the one we observed in Experiment 1. However, none of these essential oils had their efficacy evaluated through parasitological necropsy to obtain a total worm count as done in our work.

Treating infected animals with anethole and carvone at 50 mg/kg for 45 days resulted in a significant reduction in FEC, but no reduction in total worm count. Azando et al. (2017) reported a significant reduction in FEC (89%) and total worm count with mixed infection of *H. contortus* and *T. colubriformis* with 1 mL/kg of 0.42% *Zanthoxylum zanthoxyloïdes* essential oil solution for 3 days. The total worm count of *H. contortus* was reduced in 96% and *T. colubriformis* in 93%. The female reproduction was also impacted as *H. contortus* produced 68% less eggs. The major essential oil component was ɣ-terpinene. Although some studies with EO have reported positive effects, the given doses were very high (as single dose or as three daily doses) which represent at least 10 times more than the dose used in this experiment. The clinical symptoms of intoxication after their administration were not mild. Animals treated with high doses of essential oils presented apathy, anorexia, and lethargy, as observed by Squires et al. (2010) and Katiki et al. (2011).

Infected animals treated with anethole and carvone at 50 mg/kg had the benefit of keeping their body weight gain similar to that of animals not infected with *H. contortus*. The same did not occur when non-infected (healthy) animals received EO (CTL + EO) at 50 mg/kg. At this dose, the EO caused reduction in feed intake and lowered body weight gain, suggesting that this product either induces satiety or affects both the palatability of the feed and animal feed intake, and thus should not be offered to healthy animals (CTL + EO) to prevent parasite infections at the detriment of reducing their weight gain. In contrast, the infected animals that received EO (INF + EO) were benefited with anethole and carvone in the diet because it stimulated the DM intake, being similar to the group of uninfected/untreated animals (CTL). The main evidence of hepatic activity in ruminants can be determined by quantification of AST and GGT. Increased values of AST and GGT indicate liver lesions (Meyer and Harvey, 1998). Toxic agents may cause hepatotoxicity or nephrotoxicity due to the biotransformation capacity of these products by these organs. Lipophilic compounds tend to be more hepatotoxic than hydrophilic compounds because the latter are eliminated faster and in greater quantities by the kidneys (Kaneko et al., 1997). No toxicity was observed by 50 mg/kg as kidney and liver function were evaluated, and mean values of all treatments were below the mean

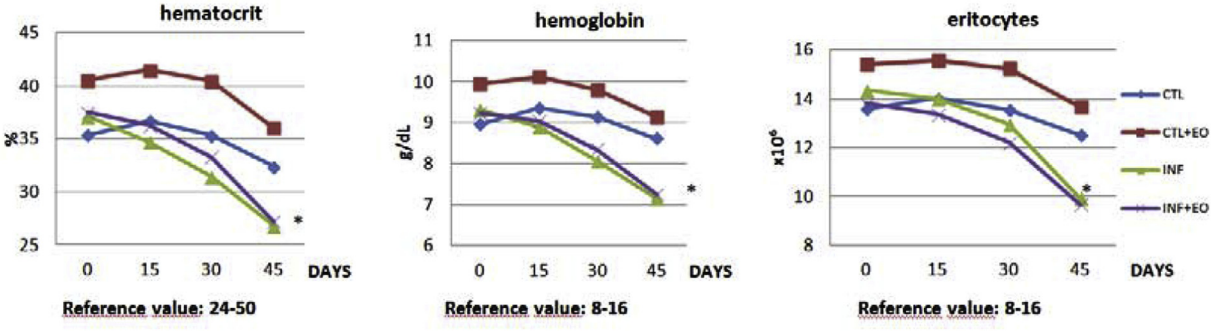

Fig. 3. Mean red blood count (hematocrit, hemoglobin and erythrocytes) at 0, 15, 30 and 45 days from control (CTL) animals, CTL + encapsulated oil (EO), infected animals (INF) and INF + EO. * INF and INF + EO are different ($P < 0.05$) compared to CTL and CTL + EO at day 45. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

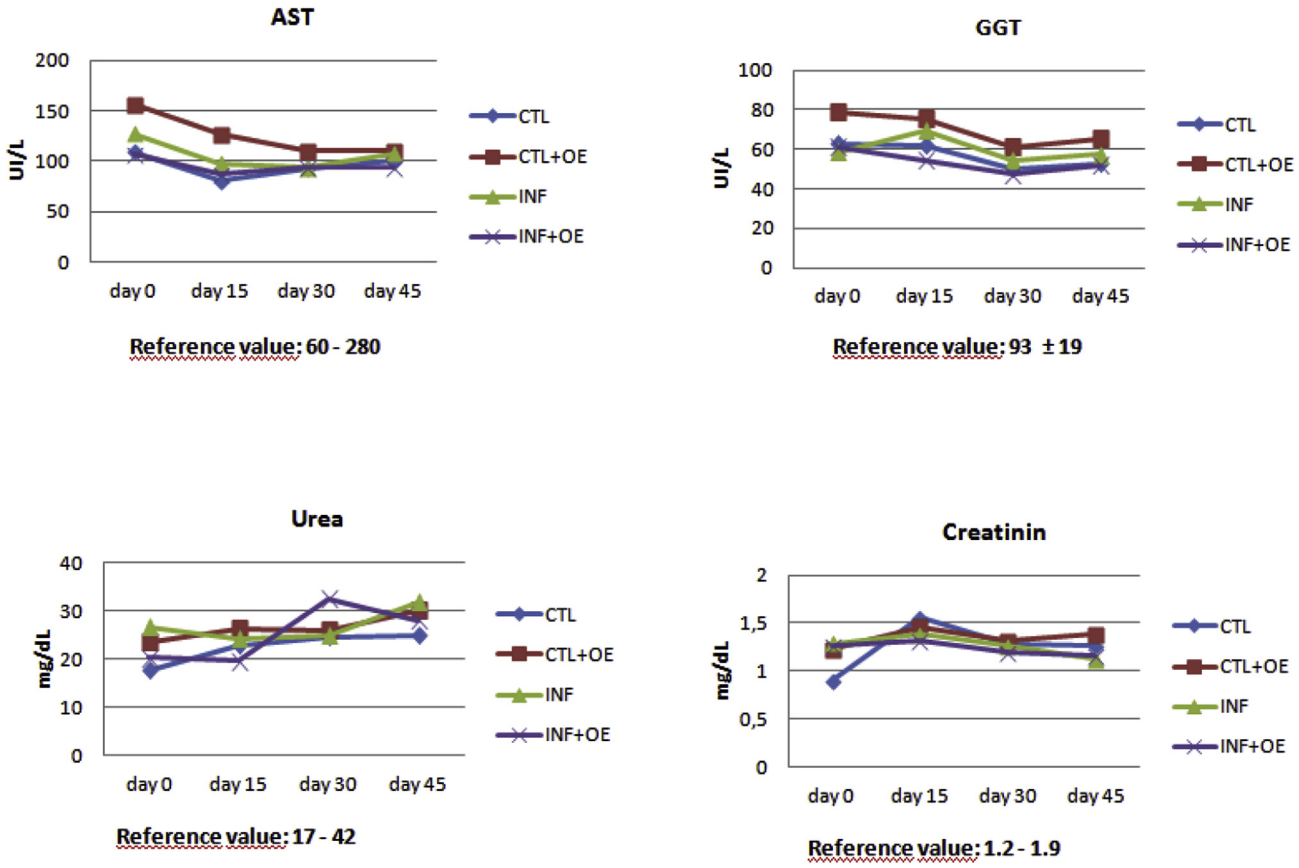

Fig. 4. Mean values of seric AST (aspartate aminotransferase) and GGT (gamma-glutamyl transferase) (liver profile), urea and creatinine (kidney profile) at 0, 15, 30 and 45 days from control (CTL) animals, CTL + encapsulated oil (EO), infected animals (INF) and INF + EO. No difference was found between treatments ($P > 0.05$).

Table 2
Mean ± SD of daily body-weight (BW) gain, dry matter (DM) intake expressed as percentage of BW and as metabolic weight of CTL (non-infected), CTL + EO, INF(infected with *Haemonchus contortus*) and INF + EO from Experiment 1.

	Daily BW gain	DM intake	
	(kg)	% Body weight	(g/kg.BW^{0,75})
CTL	126.6 ± 17.6 ^{ab}	3.18 ± 0.13 ^{a}	64.71 ± 3.12 ^{a}
CTL + EO	76.2 ± 11.9 ^{b}	2.46 ± 0.13 ^{c}	48.7 ± 13.72 ^{c}
INF	145.5 ± 5.35 ^{a}	2.89 ± 0.1 ^{b}	57.05 ± 4.48 ^{b}
INF + EO	131.2 ± 27.2 ^{ab}	3.03 ± 0.1 ^{ab}	60.00 ± 3.12 ^{ab}

Different letters in the column indicate significant difference ($P < 0.05$).

reference values of the species. The toxicity to the essential oils depends on the dose, and toxic reactions are often found only in high doses, and can trigger allergic reactions, especially in sensitive individuals. However, since the toxicity of these oils are dose-dependent, most adverse effects could be avoided by using lower concentrations (Bakkali et al., 2008).

Essential oils have previously been indicated as a food additive to improve feed intake, daily weight gain and feed conversion rate. The doses previously used in animal production were 125 ppm in ration (Hong et al., 2012) or 100–150 g/ton of ration (Li et al., 2012). These daily doses were a fraction of the doses used in our experiment, although none of these reference values are related to the use of essential oils for parasite control.

In Experiment 2, Morada Nova breed was used. Although this breed is different from that used in Experiment 1, the parasitological, productive, and reproductive performance of 'Morada Nova' are similar to those of 'Santa Ines', as described by Issakowicz et al. (2016). Lambs from EO group received 20 mg/kg EO and had their development and body weight gain, FEC, and blood parameters similar to those of animals in the CTL group. This 20 mg/kg dose was not able to prevent or treat the natural infection with GIN. In Experiment 2, because of the long experimental period, we observed the hematological variation of animals exposed to parasites, and the negative effects caused by *H. contortus* (90% in coprocultures), including smaller numbers of red blood cells, hematocrit, and hemoglobin. A decrease in red blood cells was observed at the beginning of Experiment 2 because lambs were stressed by the sudden change in their diet (weaning). The white blood cells were also affected by parasite infection. After animals went to pastures to acquire natural infection, there was an increase in leucocytes due to the increase in neutrophils and eosinophils. Eosinophils are recognized as important multifaceted immune cells releasing multiple cytokines and mediators (González et al., 2011). *H. contortus* perforates abomasum mucosa and an immunological and systemic reaction ensues, as observed in the hematological graphics. No differences were observed between CTL and EO treatment for these parameters and the clinical events were within the standard of normality for *H. contortus* infection.

5. Conclusions

Lambs artificially infected with a multi-drug resistant *H. contortus* strain and treated with anethole and carvone at 50 mg/kg bw/day, for 45 days, had significantly lower FEC. This effect was probably due to the EO effect on *Haemonchus* reproductive functions (female fecundity).

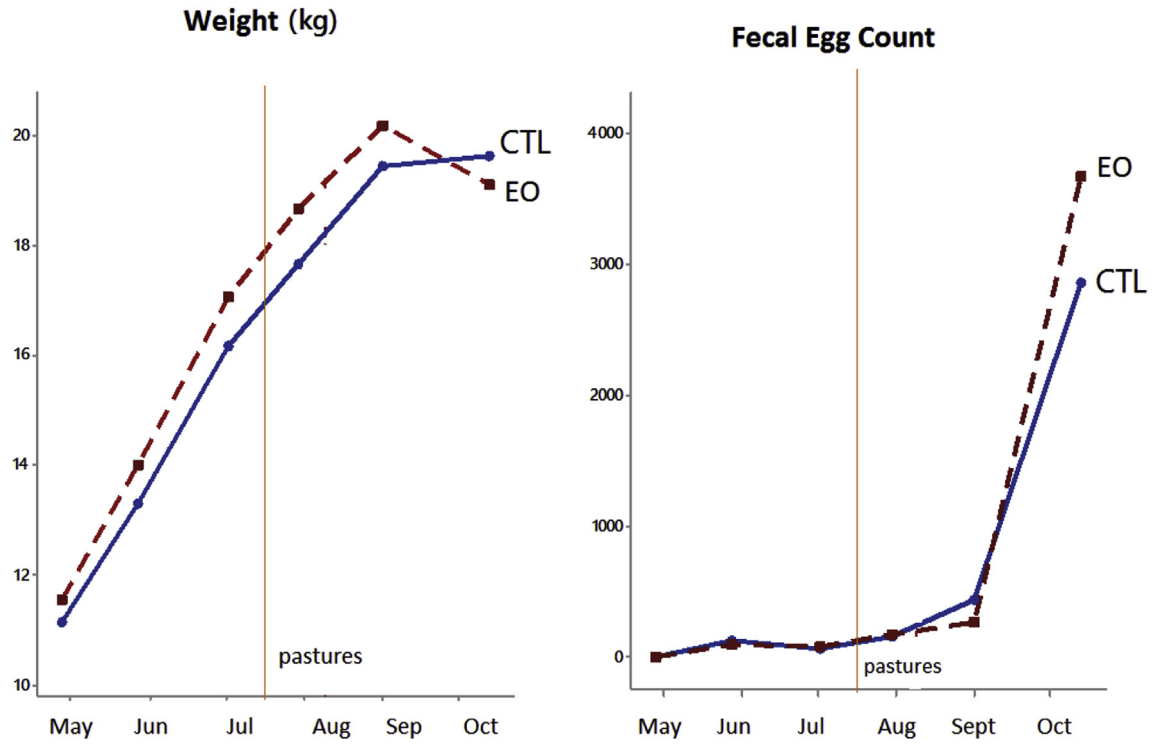

Fig. 5. Body weight and fecal egg count from lambs treated with the combination anethole + carvone (EO) at 20 mg/kg and Control (CTL) both in confinement (May –mid July) and after access to pastures (Mid July - end September) in Experiment 2. No differences were found between treatments ($P > 0.05$).

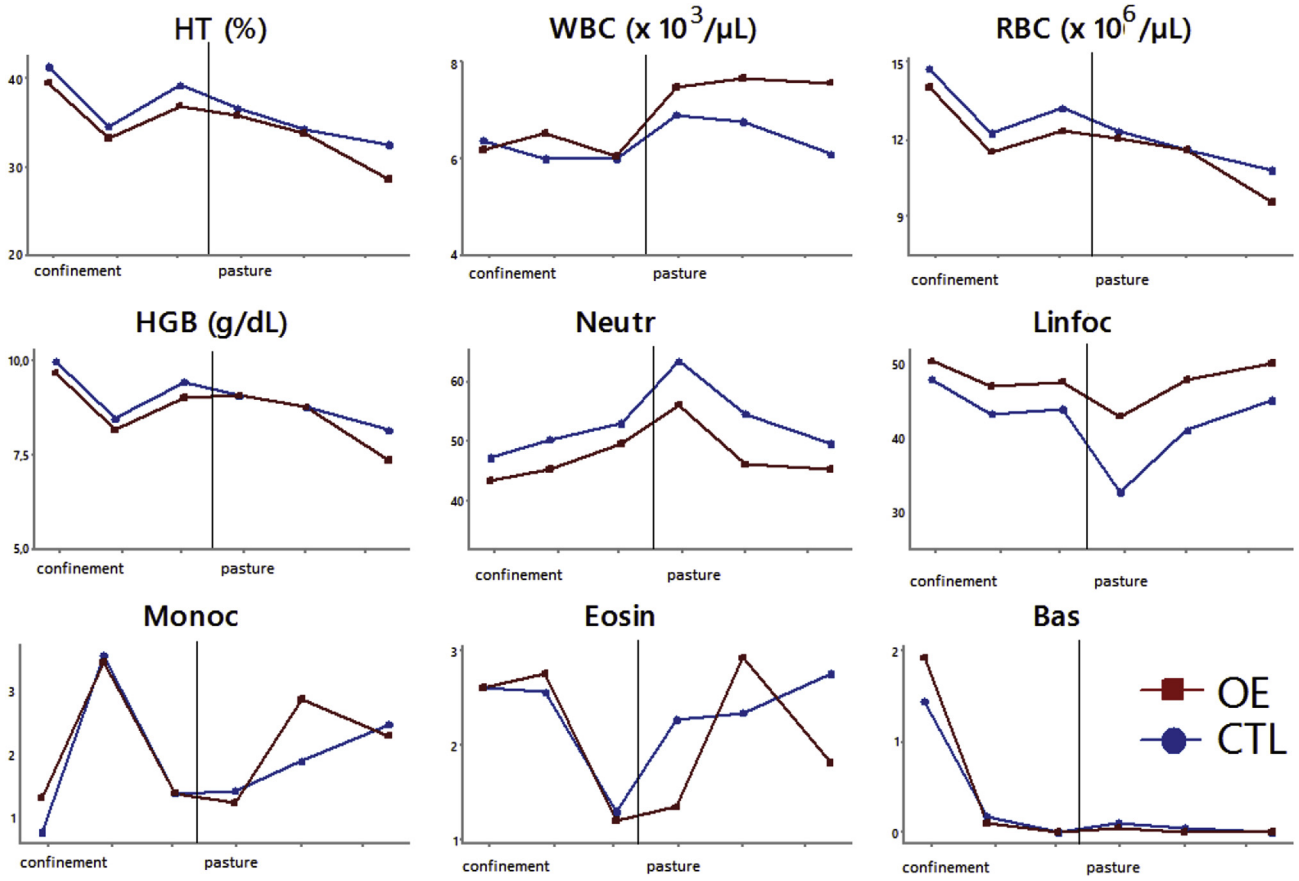

Fig. 6. Hemogram (hematocrit –HT; Red blood cells – RBC; hemoglobin – HGB) and leucogram (white blood cells – WBC; neutrophils, lymphocytes, monocytes, eosinophils and basophils) from lambs treated with encapsulated oil (EO at 20 mg/kg) and control (CTL). Exclusive confinement from May-mid July. Access to pastures from mid of July until end of September in Experiment 2. No difference was found between treatments ($P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

EO at 50 mg/kg bw caused a reduction in feed consumption and low weight gain, but the smaller daily dose of 20 mg/kg bw for 5 months had no undesirable side effect. EO at 20 mg/kg given to sheep naturally infected with *H. contortus* did not promote parasite control. Although continuous treatment of infected animals with essential oils have yet to become an effective parasite control measure, finding an essential oil with an ideal dose that can reduce FEC when animals are highly infected with GIN can lead to a reduction in the infection of pastures and may reduce animal reinfection. Based on the low toxicities to mammals of both anethole and carvone, future studies should be performed at higher doses to try to establish an ideal dose, other vehicles, other routes of administration (e.g., injection or rectal administration), and targeting other gastrointestinal parasites besides *Haemonchus contortus*.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Fundação de Apoio à Pesquisa do Estado de São Paulo - FAPESP (grant number 2012/50587-5) and Ricardo L. D. Costa and Roberto Colacioppo for the experimental support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2019.01.002.

References

Almeida, F.A., Garcia, K.C.O.D., Torgerson, P.R., Amarante, A.F.T., 2010. Multiple resistance to anthelmintics by *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Brazil. Parasitol. Int. 59, 622–625. https://doi.org/10.1016/j.parint.2010.09.006.

Alvares, C.A., Stape, J.L., Sentelhas, P.C., De Moraes Gonçalves, J.L., Sparovek, G., 2013. Köppen's climate classification map for Brazil. Meteorol. Z. 22, 711–728. https://doi.org/10.1127/0941-2948/2013/0507.

André, W.P.P., Cavalcante, G.S., Ribeiro, W.L.C., Santos, J.M.L.D., Macedo, I.T.F., Paula, H.C.B.D., Morais, S.M.D., Melo, J.V.D., Bevilaqua, C.M.L., 2017. Anthelmintic effect of thymol and thymol acetate on sheep gastrointestinal nematodes and their toxicity in mice. Rev. Bras. Parasitol. Vet. 26, 323–330. https://doi.org/10.1590/s1984-29612017056.

Andre, W.P.P., Ribeiro, W.L.C., Cavalcante, G.S., Santos, J.M.L.D., Macedo, I.T.F., Paula, H.C.B.D., De Freitas, R.M., De Morais, S.M., Melo, J.V.D., Bevilaqua, C.M.L., 2016. Comparative efficacy and toxic effects of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes and mice. Vet. Parasitol. 218, 52–58. https://doi.org/10.1016/j.vetpar.2016.01.001.

Azando, E.V.B., Olounlade, A.P., Houzangbe-Adote, M.S., Tam Há, T.B., Fabre, N., Valentin, A., 2017. Contrôle des parasitoses gastro-intestinales ovines par l'huile essentielle de *Zantholylum zantholyloïdes* (*Fagara zanthoxyloïdes*). Rev. Med. Vet. (Bogota) 168, 205–212.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – a review. Food Chem. Toxicol. 46, 446–475. https://doi.org/10.1016/j.fct.2007.09.106.

Camurça-Vasconcelos, A.L.F., Morais, S.M., Santos, L.F.L., Rocha, M.F.G., Bevilaqua, C.M.L., 2005. Validação de plantas medicinais com atividade anti-helmíntica. Rev. Bras. Plantas Med. 7 (3), 97–106.

Carvalho, C.O., Chagas, A.C.S., Contiguiba, F., Furlan, M., Brito, L.G., Chaves, F.C.M., Stephan M, Bizzo, H.R., Amarante, A.F.T., 2012. The anthelmintic effect of plant extracts on *Haemonchus contortus* and *Strongyloides venezuelensis*. Vet. Parasitol. 183, 260–268. https://doi.org/10.1016/j.vetpar.2011.07.051.

Edris, A., 2007. Pharmaceutical and therapeutical potentials of essential oils and their individual volatile constituents: a review. Phytother Res. 21, 308–323. https://doi.org/10.1002/ptr.2072.

González, J.F., Hernández, A., Meeusen, E.N.T., Rodríguez, F., Molina, J.M., Jaber, J.R., Raadsma, H.W., Piedrafita, D., 2011. Fecundity in adult *Haemonchus contortus* parasites is correlated with abomasal tissue eosinophils and T cells in resistant Canaria Hair Breed sheep. Vet. Parasitol. 178, 286–292. https://doi.org/10.1016/j.vetpar.2011.01.005.

Hong, J., Steiner, T., Aufy, A., Lien, T., 2012. Effects of supplemental essential oil on growth performance, lipid metabolites and immunity, intestinal characteristics, microbiota and carcass traits in broilers. Livest. Sci. 144, 253–262. https://doi.org/10.1016/j.livsci.2011.12.008.

Issakowicz, J., Issakowicz, A.C.K.S., Bueno, M.S., Costa, R.L.D., Katiki, L.M., Geraldo, G.T., Abdalla, A.L., McManus, C., Louvandini, H., 2016. Parasitic infection, reproductive and productive performance from Santa Ines and Morada Nova ewes. Small Rum.Res. 136, 96–103. https://doi.org/10.1016/j.smallrumres.2016.01.015.

Kaneko, J.J., Harvey, J., Bruss, M., 1997. Clinical biochemistry of domestic animal, 5 ed. Academic Press, New York.

Katiki, L.M., Chagas, A.C.S., Bizzo, H.R., Ferreira, J.F.S., Amarante, A.F.T., 2011. Anthelmintic activity of *Cymbopogon martinii, Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests. Vet. Parasitol. 183, 103–108. https://doi.org/10.1016/j.vetpar.2011.07.001.

Katiki, L.M., Chagas, A.C.S., Takahira, R.K., Juliani, H.R., Ferreira, J.F.S., Amarante, A.F.T., 2012. Evaluation of *Cymbopogon schoenanthus* essential oil in lambs experimentally infected with *Haemonchus contortus*. Vet. Parasitol. 186, 312–318. https://doi.org/10.1016/j.vetpar.2011.12.003.

Katiki, L.M., Barbieri, A.M.E., Araújo, R.C., Veríssimo, C.J., Louvandini, H., Ferreira, J.F.S., 2017. Synergistic interaction of 10 essential oils against *Haemonchus contortus in vitro*. Vet. Parasitol. 243, 47–51. https://doi.org/10.1016/j.vetpar.2017.06.008.

Kloosterman, A., Albers, G.A.A., Van Den Brink, R., 1978. Genetic variation among calves in resistance to nematode parasites. Vet. Parasitol. 4, 353–368. https://doi.org/10.1016/0304-4017(78)90021-3.

Li, S.Y., Liu, M., Xu, B., Péron, A., Shi, X.G., 2012. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. Livest. Sci. 145, 119–123. https://doi.org/10.1016/j.livsci.2012.01.005.

Macedo, I.T.F., Bevilaqua, C.M.L., Oliveira, L.M.B., Camurça-Vasconcelos, A.L.F., Vieira, L.S., Oliveira, F.R., Queiroz-Junior, E.M., Tomé, A.D.R., 2010. Anthelmintic effect of *Eucalyptus staigeriana* essential oil against goat gastrointestinal nematodes. Vet. Parasitol. 173, 93–98. https://doi.org/10.1016/j.vetpar.2010.06.004.

Marinov, V., Valcheva-Kuzmanova, S., 2015. Review on the pharmacological activities of anethole. Scripta Scientifica Pharmaceutica 2 (2), 14–19.

Meyer, D.J., Harvey, J.W., 1998. Veterinary laboratory medicine: interpretation & diagnosis. W.B. Saunders, Philadelphia.

National Library of Medicine HSDB Database (2018) Access 07/01/2018. https://toxnet.nlm.nih.gov/cgi-bin_/sis/search/a?dbs+hsdb:@term+@ DOCNO+707.

National Research Council, 2007. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. The National Academies Press, Washington, DC. https://doi.org/10.17226/11654.

Paolini, V., Frayssines, A., Farge, F.D.L., Dorchies, P., Hoste, H., 2003. Effect of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. Vet. Res. 34, 331–339. https://doi.org/10.1051/vetres:2003008.

Ribeiro, W.L.C., Macedo, I.T.F., Santos, J.M.L., Oliveira, E.F., Camurça-Vasconcelos, A.L.F., 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. https://doi.org/10.1016/j.exppara.2013.05.014.

Squires, J.M., Foster, J.G., Lindsay, D.S., Caudell, D.L., Zajac, A.M., 2010. Efficacy of an orange oil emulsion as an anthelmintic against *Haemonchus contortus* in gerbils (*Meriones uniguiculatus*) and in sheep. Vet. Parasitol. 172, 95–99. https://doi.org/10.1016/j.vetpar.2010.04.017.

Ueno, H., Gonçalves, P.C., 1998. Manual para diagnóstico das helmintoses de ruminantes, 4 ed. JICA.