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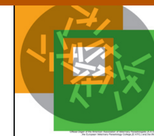

Efficacy of free and nanoencapsulated *Eucalyptus citriodora* essential oils on sheep gastrointestinal nematodes and toxicity for mice

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ABSTRACT

Herbal medicines with anthelmintic effects are alternatives for the sustainable control and prevention of disease caused by gastrointestinal parasites. The nanoencapsulation of essential oils has been proposed to enhance the absorption of their constituents and improve their efficacy. The present study aimed to evaluate the efficacy of free and nanoencapsulated *Eucalyptus citriodora* essential oil (EcEO) on the control of gastrointestinal nematodes of small ruminants *in vitro* and *in vivo*. Chitosan was used as a matrix for the formulation of a nanoemulsion. Chromatographic and physico-chemical analyses of EcEO were performed. Egg hatch (EHT) and larval development (LDT) tests were conducted to evaluate the effectiveness of nanoencapsulated and free EcEO on the eggs and larvae of *Haemonchus contortus*. Acute toxicity of free and nanoencapsulated EcEO was evaluated using mice. Finally, nanoencapsulated EcEO efficacy on the control of gastrointestinal nematodes was calculated by fecal egg count reduction test (FECRT) treating 30 sheep naturally infected with 250 mg/kg of free and nanoencapsulated EcEO. *In vitro* tests were analyzed by an analysis of variance (ANOVA) followed by comparison with the Tukey test. The efficacy of FECRT was calculated by the BootStreet program through arithmetic average, using the formula 100 (1 − XT/XC). To compare the differences between epg, the data were transformed to $\log(x+1)$ and subjected to an ANOVA to compare the significant differences between groups by Tukey's. The level of significance was $P < 0.05$. The free (4 mg/ml concentration) and nanoencapsulated (2 mg/ml concentration) EcEO inhibited larvae hatching by 97.2% and 92.8%, respectively. Free and nanoencapsulated EcEO at 8 mg/ml inhibited larval development by 99.8% and 98.1%, respectively. In the acute toxicity test, the LD10 and LD50 of free EcEO was 1999 and 2653 mg/kg, respectively, while the LD10 and LD50 of nanoencapsulated EcEO was 1121 and 1681 mg/kg, respectively. Nanoencapsulated and free EcEO reduced FEC similarly by 40.5% and 55.9%, respectively at 10 days post-treatment. Nanoencapsulated EcEO did not obtain the expected efficacy *in vivo*.

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1. Introduction

Diseases caused by gastrointestinal nematodes in small ruminants reduce animal production, causing serious economic losses worldwide (Molento, 2009). In northeastern Brazil, the following nematodes can be found in sheep: *Trichostrongylus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Trichuris* spp. and *Haemonchus contortus*, the latter is an abomasal nematode that is prevalent throughout the region (Melo et al., 2009). The inappropriate use of conventional anthelmintics led to the emergence of drug-resistant nematode populations (Jackson and Coop, 2000).

Currently, low cost and efficient methods have been studied to control and prevent infections with gastrointestinal parasites. Among these are pasture management, selection of nematode resistant animals and the development of herbal medicines with anthelmintic activity, which involves the formulation of herbal drugs and their metabolites (Vieira, 2008; Hoste and Torres-Acosta, 2011).

Eucalyptus spp., belonging to the family Myrtaceae, has several species of economic importance, and its essential oils have been used for various purposes (Vitti and Brito, 1999; Silva et al., 2006). The essential oil of *Eucalyptus citriodora* is well known for its use in the fragrance industry, and its major constituent is citronellal (Vitti and Brito, 2003). Studies have shown that *E. citriodora* oil functions as an antioxidant (Singh et al., 2012), antifungal (Brito et al., 2012), antibacterial (Cimanga et al., 2002) anti-inflammatory, analgesic (Gbenou et al., 2013), insect repellent (Seyoum et al., 2003), insecticide (Maciel et al., 2010) and acaricide (Clemente et al., 2010).

Eucalyptus citriodora essential oil presented activity against goat gastrointestinal nematodes, but the efficacy did not reach the therapeutically required level (Macedo et al., 2011). Therefore, the encapsulation of essential oils of *E. citriodora* was used to improve their efficacy against gastrointestinal nematodes of small ruminants (Underwood and Eps, 2012). Encapsulation is the incorporation of a bioactive substance into a material called an encapsulating matrix, coating material or carrier (Paramera et al., 2011). This incorporation protects the drug from degradation, improves its absorption and facilitates its diffusion through the epithelium, thus promoting tissue and intracellular distribution more efficiently (Couvreur and Vauthier, 2006).

Chitosan is a natural polymer (Sahoo et al., 2010) obtained by the deacetylation of chitin, the main component of the exoskeleton of crustaceans and the cell wall of some fungi (Sinha et al., 2004). It has been used as an encapsulating matrix for drug delivery because it is biodegradable, biocompatible, renewable, non-toxic and aqueous, and it circumvents the need for organic solvents (Senel et al., 2000; Abreu et al., 2012). The chitosan microsphere formulation for the controlled release of drugs improves their dissolution and bioavailability (Hejazi and Amiji, 2003; Sinha et al., 2004). Thus, the aim of this study was to evaluate the action of the essential oil of *E. citriodora* on the gastrointestinal nematodes of small ruminants by comparing both free and nanoencapsulated forms.

2. Materials and methods

This study was approved by the Ethics Committee for the Use of Animals of State University of Ceará under protocol number 12641984-1 and followed the standards of animal welfare recommended by law.

2.1. Chemical analysis of E. citriodora essential oil

Eucalyptus citriodora essential oil (EcEO) was purchased from FERQUIMA® (Vargem Grande Paulista, São Paulo, Brazil). The chemical composition of the EcEO used in this study was determined by gas chromatography (GC) and mass spectrometry (MS). The oil was analyzed in a Hewlett-Packard 5971 instrument using the following experimental conditions: DB-1 coated fused silica capillary column (30 m × 0.25 mm); helium carrier gas; injector temperature of 250 °C; detector temperature of 200 °C; column temperature program: 35–180 °C at 48 °C/min and then 180–250 °C at 10 °C/min. For MS, the electron impact was 70 eV.

Compounds of EcEO were identified according to their GC retention time expressed through Kovat's index, which was calculated by the Van den Dool and Kratz equation using a hydrocarbon homologous series (Adams, 2001). Additionally, the test compound mass spectra were compared to spectra in the National Institute for Standard Technology computer database (NIST; 62,235 compounds) and published spectra (Adams, 2001).

2.2. Nanoencapsulation of E. citriodora essential oil

Chitosan powder (POLYMAR®) was used as encapsulant biopolymer. Chitosan was solubilized in acetic acid (1%) under constant stirring for 24 h to obtain a solution of 1% chitosan. At the end of this period, vacuum filtration was conducted for the remaining particles still in suspension. In parallel, Tween 80 was added to EcEO at a ratio of 1:4 under constant stirring for 10 min. The organic phase (EcEO + Tween 80) was added to the 1% chitosan solution under mechanical stirring for 5 min at 188.49 rad seg^{-1}, thereby obtaining a nanoemulsion of chitosan. We tested various proportions of the organic phase in relation to the chitosan solution to obtain a stable product containing higher oil content. The macroscopic characteristics of EcEO stability were observed for 72 h after the preparation of the nanoemulsion to monitor for a possible phase separation.

2.3. Physicochemical characterization of the nanoemulsion

The nanoemulsion of EcEO was characterized by infrared spectroscopy (FTIR) using the model 8300 (Shimadzu Corporation, Japan). The particle size and distribution were determined by photon correlation spectroscopy (PCS) on a Malvern Zetasizer Zen model 3500.

2.4. Egg hatch test (EHT)

Feces were collected directly from the rectum of sheep kept in metabolic cages and harboring a monospecific

infection of *H. contortus*. *H. contortus* eggs were recovered according to Hubert and Kerboeuf (1992). The egg hatch test (EHT) was performed based on the methodology described by Coles et al. (2006). Briefly, 250 ml of an egg suspension, containing approximately 100 fresh eggs, was incubated with 250 ml of EcEO at concentrations of 4, 2, 1, 0.5, 0.25 and 0.125 mg/ml in test tubes for 48 h at 25 °C. After this time, drops of Lugol iodine were added to the tubes. The eggs and first stage larvae (L1) were counted under a microscope. Five groups were included: G1: nanoencapsulated EcEO; G2: free EcEO; G3: negative control containing 3% Tween 80 and 1% chitosan; G4: negative control containing 3% Tween 80 and G5: positive control containing 0.025 mg/ml thiabendazole. Three repetitions with five replicates for each concentration and for each control were performed.

2.5. 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 37 °C to obtain L1. The LDT was performed as described by Camurça-Vasconcelos et al. (2007). A 1 ml aliquot of solution containing approximately 250 L1 *H. contortus* was incubated with 2 g of feces collected from a sheep free of gastrointestinal nematodes, and 1 ml of oils solution was added. After six days, third stage larvae (L3) were recovered, and drops of Lugol were added. The L3 were counted under an dissecting microscope. In this test, five groups were included: G1: nanoencapsulated EcEO at concentrations of 8, 4, 2, 1 and 0.5 mg/ml; G2: free EcEO at the same concentrations; G3: negative control consisting of 3% Tween 80 and 1% chitosan; G4: negative control consisting of 3% Tween 80; G5: positive control with 0.008 mg/ml ivermectin. Three repetitions with five replicates for each concentration and for each control were performed.

2.6. Acute toxicity test

A total of 90 female Swiss albino mice (*Mus musculus*) were used, and they weighed between 25.8 and 32.6 g. The mice were kept in polypropylene cages and given commercial feed and water *ad libitum*. The animals were randomly divided into groups ($n = 10$) based on treatment: G1 to G4: 500, 1000, 1500 and 2000 mg/kg of nanoencapsulated EcEO, G5 to G8: 2000, 2500, 3000 and 3500 mg/kg of free EcEO and G9: 4000 mg/kg of matrix containing 1% chitosan solution and 3% Tween 80.

A single dose of the oils and chitosan solution (control) were administered by esophageal gavage. The doses were established from the results of Macedo et al. (2011) assuming the nanoencapsulated oil could increase toxicity. The animals were observed for 15 days, and behavioral changes and mortality were recorded. The total number of dead animals was recorded to calculate the lethal dose for 10% (LD10) and 50% (LD50) of animals.

2.7. Fecal egg count reduction test (FECRT)

A total of 30 crossbred sheep of both sexes was used, and they weighed 30 kg on average and were aged between 6 and 18 months. The sheep lived in semi-intensive systems, were fed with commercial feed and hay and received mineral salt and water *ad libitum*.

The sheep were selected according to the number of eggs in feces (epg) over 500 using the McMaster technique (Ueno and Gonçalves, 1998). The sheep were divided into 3 groups ($n = 10$) and randomly assigned to treatment for FEC, G1: 250 mg/kg of nanoencapsulated EcEO; G2: 250 mg/kg of free EcEO; and G3: 250 mg/kg of 1% chitosan and 3% Tween 80 (control). Feces were collected directly from the rectum of the animals before and after 10 and 17 days of treatment.

The dose was chosen based on the work of Macedo et al. (2011) who used 500 mg/kg of free *E. citriodora* oil for 3 consecutive days and the results of the toxicity test with nanoencapsulated oil.

Treatment efficacy was determined by the reduction of epg, compared with control. Fecal cultures were performed according to the method of Roberts and O'Sullivan for the identification of larvae.

2.8. Statistical analysis

The results of *in vitro* tests are presented as percentages and were analyzed by an analysis of variance (ANOVA) followed by comparison with the Tukey test using Graph Pad Prism 5.0 software. The significance level was $P < 0.05$.

For EHT, the efficacy of each treatment was calculated based on the percentage of hatching using the following formula: (number of hatched larvae/number of hatched larvae + number of eggs) × 100. For the LDT, the efficacy of each treatment was calculated using the formula: [(L3 control group − L3 treated group)/L3 control group] × 100. The effective concentration to inhibit 50% (EC50) of hatching and larval development was calculated by linear regression between the dose and efficacy using probit analysis with the SPSS 8.0 software.

The doses required to kill 50% (LD50) and 10% (DL10) of mice were calculated from the acute toxicity by probit analysis with the SPSS 8.0 software.

The efficacy of FECRT was calculated by the BootStreet program through arithmetic average, using the formula $100\,(1 - XT/XC)$, where XT and XC are the average epg in treatment and control groups, respectively (Coles et al., 1992). The model used was based on the pre-selection of 150 animals available in commercial farm. The ovines were of both genders, with ages ranging from 6 to 18 months. Opg of these animals was done and 30 individuals with opg above 500 were selected and distributed so that there was no statistical difference between the opg of the groups ($n = 10$). In this model, only opg and treatments were analyzed. To compare the differences between epg, the data were transformed to $\log(x + 1)$ and subjected to an ANOVA to compare the significant differences between groups by Tukey's test using the Graph Pad Prism 5.0 software. The significance level was $P < 0.05$.

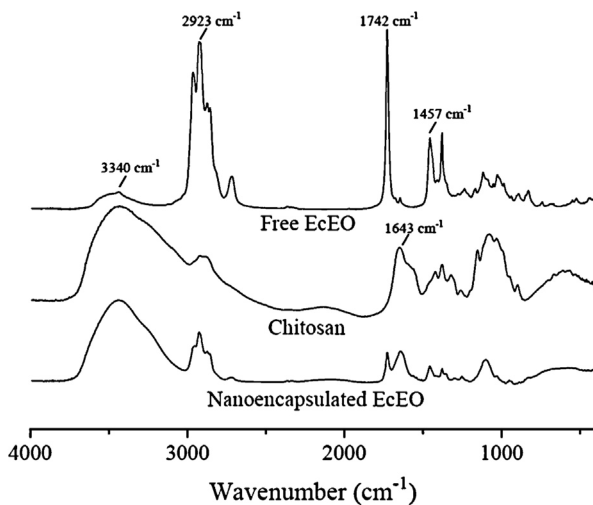

Fig. 1. Infrared spectra of the sample of *Eucalyptus citriodora* free essential oil, chitosan (matrix) and nanoencapsulated *E. citriodora* essential oil.

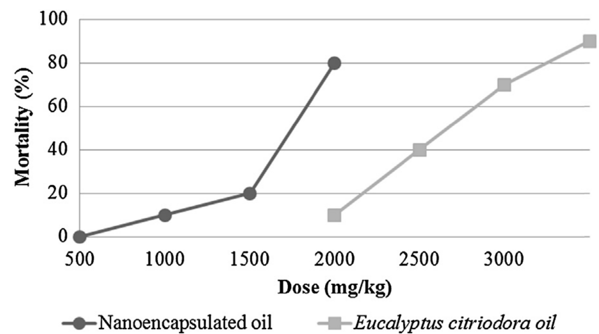

Fig. 2. Evaluation of acute toxicity of *Eucalyptus citriodora* free and nanoencapsulated essential oil for mice.

3. Results

Chromatographic analysis revealed that the major EcEO components were citronellal (67.5%), citronellol (6.9%) and menthol (6.1%).

To obtain a stable nanoemulsion (no phase separation), 36.4% (v/v) of the EcEO content was used. Analysis of the resulting nanoemulsion particles demonstrated a mean size of 232 nm with unimodal distribution and polydispersity. By infrared spectroscopy, chitosan displayed absorption bands appearing at 1643, 1457, 1375 and 1099 cm^{-1} (Fig. 1). The characteristic absorption bands of citronellal can be observed at 2923 and 1742 cm^{-1} and are assigned to CH and C=O aldehyde groups, respectively. Vibrational modes of (CH) methyls and methylenes of the essential oil components were observed between 3000 and 2800 cm^{-1}. The absorption bands of the hydroxyl groups (OH) of chitosan and the essential oil components was observed between 3500 and 3340 cm^{-1}.

In the acute toxicity test, the LD10 and LD50 of free EcEO were 1999.0 (1365.5–2286.1) mg/kg and 2653.0 (2337.9–2967.5) mg/kg, respectively, and the LD10 and LD50 of nanoencapsulated EcEO was 1120.9 (551.7–1355.5) mg/kg and 1680.7 (1404.0–2269.4) mg/kg, respectively (Fig. 2). In the acute toxicity test, no changes were observed in the behavior of mice.

The inhibition of egg hatching was dose-dependent, and the results are shown in Table 1. The dose of 4 mg/ml of free EcEO inhibited 97.2% of eggs hatching, while the nanoencapsulated EcEO inhibited 92.8% at a dose of 2 mg/ml. The effective doses to inhibit 50% of hatching eggs (EC50) were 1.3 (0.5–8.7) and 0.4 (0.4–0.5) mg/ml for free and nanoencapsulated EcEO, respectively.

The effectiveness of the larval development test was also dose-dependent, and the results are shown in Table 2. The most effective dose was 8 mg/ml for both the free and nanoencapsulated EcEO, which inhibited larval development in 99.8% and 98.1% of cases, respectively. The EC50 for the development of larvae and 95% confidence intervals were 1.7 (1.0–2.6) and 1.7 (1.0–2.6) mg/ml for free and nanoencapsulated EcEO, respectively.

The FECRT of free and nanoencapsulated EcEO are expressed as the mean epg on days 0, 10 and 17 post-treatment (Table 3). Nanoencapsulated and free EcEO reduced FEC by 40.5% and 55.9% at 10 days post-treatment, but these results did not significantly differ from the control.

The larvae recovered in fecal cultures of sheep before treatment were identified as *Haemonchus* spp. (94%), *Trichostrongylus* spp. (5%) and *Oesophagostumum* spp. (1%), while those recovered 10 days after treatment were *Haemonchus* spp. (90%), *Trichostrongylus* spp. (7%) and *Oesophagostumum* spp. (3%).

Table 1
Efficacy (±standard error) of the free and nanoencapsulated *Eucalyptus citriodora* essential oil on hatching of eggs of *Haemonchus contortus*.

Concentrations (mg/ml)	Efficacy (%)	
	Free EcEO	Nanoencapsulated EcEO
4	97.1 ± 0.2^{a}	–
2	58.5 ± 3.2^{b}	92.8 ± 1.5^{a}
1	23.5 ± 1.8^{c}	85.5 ± 2.3^{b}
0.5	16.0 ± 1.3^{d}	56.8 ± 2.6^{c}
0.25	12.5 ± 0.9^{de}	25.9 ± 1.3^{d}
0.125	–	11.9 ± 0.9^{e}
Control	7.4 ± 0.7^{e}	9.1 ± 0.5^{e}
TBZ (0.025 mg/ml)	93.5 ± 1.1^{a}	93.5 ± 1.1^{a}

Different letters mean statistically different values ($P < 0.05$) in the same column.

Table 2
Efficacy (±standard error) of the free and nanoencapsulated *Eucalyptus citriodora* essential oil on L1 development of *Haemonchus contortus*.

Concentrations (mg/ml)	Efficacy (%)	
	Free EcEO	Nanoencapsulated EcEO
8	99.7 ± 0.1^{Aa}	98.0 ± 0.5^{Aa}
4	83.8 ± 1.7^{Bb}	75.8 ± 2.5^{Bb}
2	47.5 ± 3.1^{Cc}	49.4 ± 2.3^{Cc}
1	25.1 ± 1.3^{Dd}	39.1 ± 3.2^{Cc}
0.5	14.4 ± 2.7^{Ee}	10.0 ± 2.9^{De}
Control	7.7 ± 2.5^{Ee}	7.7 ± 1.6^{De}
IVM (0.008 mg/ml)	98.9 ± 0.2^{Aa}	98.7 ± 0.2^{Aa}

Capital letters compare the means between the lines. Lowercase letters compare the means between the columns. Results show different letters differ significantly ($P < 0.05$).

Table 3
Reduction in egg counts per gram of feces (epg) and efficacy of nanoencapsulated and free *Eucalyptus citriodora* essential oil (EcEO).

Treatments	Day 0	Day 10	Day 17
Free EcEO			
Mean epg	1920 ± 414.1[Aa]	915 ± 279.4[Aab]	615 ± 231.7[Ab]
Efficacy (%)	–	55.9	34.5
Nanoencapsulated EcEO			
Mean epg	1930 ± 407.9[Aa]	1235 ± 282.2[Aab]	680 ± 174.5[Ab]
Efficacy (%)	–	40.5	27.6
Tween 80 (3%) + chitosan			
Mean epg	1890 ± 378.8[Aa]	2075 ± 444.2[Aa]	940 ± 205.9[Aa]

Capital letters compare the means between the lines. Lower case letters compare the means between the columns. Results show different letters differ significantly ($P < 0.05$).

4. Discussion

Anthelmintic resistance and the appreciation of organic animals have boosted the development of herbal medicines with anthelmintic activity, as this treatment is beneficial in small ruminant husbandry (Athanasiadou et al., 2007). Among the studied products are essential oils, which are volatile substances. The use of nanoemulsions is intended to prevent oil degradation for oral administration, improving absorption in the animal body (Donsì et al., 2011). This can lead to increased efficiency of the product with a consequent reduction in the frequency of treatment (Cui et al., 2006). The process of nanoemulsion in this study was satisfactory because the formulated particles ranged from 1–1000 nm (Irache et al., 2011). The percentage of encapsulation obtained was similar to other studies that used hydrogel formulations, which were obtained at a concentration of 36.5% (v/v) the EcEO (Mesquita et al., 2013; Ribeiro et al., 2013).

The use of chitosan as a matrix to encapsulate essential oils or their compounds has been successful in several studies using various encapsulation techniques (Keawchaoon and Yoksan, 2011). Chitosan has a positive charge, which in turn draws lipids of a negative charge (Azevedo et al., 2007). Consequently, the compounds bind strongly. This was the reason for the choice of chitosan as a matrix to encapsulate EcEO. Furthermore, the hydrophilic–lipophilic balance of this substance is high, which allows it to stabilize oil–water emulsions (Schulz, 1998).

Infrared radiation causes atoms and groups of atoms in organic compounds to vibrate with increased amplitude around the covalent bonds that connect them. The vibrational spectrum usually appears as a series of absorption bands. The positions of the absorption bands in the spectrum can be displayed as a wave number using reverse drive centimeters (400–400 cm^{-1}) or micrometers (2.5–16 μm) (Lopes and Fascio, 2004). The absorption bands for chitosan and citronellal are characteristic of these compounds (Paula et al., 2011). However, the absorption bands at 1565 cm^{-1}, which corresponds to the group II amide of chitosan, disappeared. This was most likely due to a joining of the group II amide with the aldehyde citronellal groups (Abreu et al., 2012), demonstrating encapsulation of EcEO by chitosan.

EcEO was also effective against gastrointestinal nematodes *in vitro*, inhibiting *H. contortus* egg hatching by 98.8% at a dose of 5.3 mg/ml (Macedo et al., 2011). The results from this study differed, as we obtained a similar efficiency using only 4 mg/ml of free EcEO and 2 mg/ml of nanoencapsulated EcEO. Although chitosan is insoluble in water, organic solvents and bases (Damian et al., 2005), the prior solubilization of chitosan in acetic acid in the present study made it water-soluble, explaining the improved effectiveness of the nanoencapsulated oil.

For the LDT, the results obtained with the free or nanoencapsulated oil were statistically similar, however free EcEO results were similar to Macedo et al. (2011). This is likely due to the use of feces in the culture medium, which hindered the solubility of chitosan and consequently, hindered the effect on the larvae.

Previous studies have shown that the LD10 and LD50, conducted through acute toxicity tests of EcEO, were 2609 (689.7–3466.4) mg/kg and 4153.2 (2861.8–5849.2) mg/kg, respectively (Macedo et al., 2011). These results do not corroborate the current study because the lethal doses were lower for the free and nanoencapsulated EcEO. Furthermore, the nanoencapsuled oil toxicity increased, but chitosan is not known to be toxic, so this process probably resulted in increased oil toxicity.

The effectiveness of EcEO at 500 mg/kg over three consecutive days in goats was 66.2% and 60.3% at 8 and 15 days post-treatment, respectively (Macedo et al., 2011). In the present study, we observed increased efficacy of free and nanoencapsulated oil, as similar results were obtained with only a single dose of 250 mg/kg. Nevertheless, the FEC did not differ statistically between the control and the treatments. This may be because the test was performed at the beginning of the dry season in the semi-arid region of northeastern Brazil. At this time, the epg and worm burdens suffer drastic reductions because the free-living stages cannot survive on pasture (Arosemena et al., 1999).

Since one of the properties of chitosan is mucoadhesivity, we expected that the nanoencapsulated oil with chitosan would extend the residence time in the abomasum, thus optimizing the effect. However as the results of the nanoencapsulated oil were not higher than the free oil, yet clarifications about the oil release mechanism in abomasal pH are needed (Ribeiro et al., 2013). However, the use of this oil for the control of gastrointestinal nematodes in small ruminants should not be disregarded because it is possible to develop other encapsulating matrices that are more efficient than chitosan. Thus, plant products can be an alternative for the control of these parasites and may be used in combination with synthetic products to enhance their effectiveness.

Conflict of interest

The authors declare that they have no conflicting interests.

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