Veterinary Parasitology 204 (2014) 243–248

Contents lists available at ScienceDirect

Veterinary Parasitology

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

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

J.C. Ribeiro[a], W.L.C. Ribeiro[a], A.L.F. Camurça-Vasconcelos[a], I.T.F. Macedo[a], J.M.L. Santos[a], H.C.B. Paula[b], J.V. Araújo Filho[a], R.D. Magalhães[a], C.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*

ARTICLE INFO

Article history:
Received 20 November 2013
Received in revised form 5 May 2014
Accepted 10 May 2014

Keywords:
Chitosan
Nanoemulsion
Nematodes
Haemonchus contortus
Sheep
Eucalyptus citriodora

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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* Corresponding author at: Programa de Pós-graduação em Ciências Veterinárias/FAVET/UECE, Av. Silas Munguba, 1700, Campus do Itaperi, CEP 60714-903 Fortaleza, Ceará, Brazil. Tel.: +55 85 31019853; fax: +55 85 31019840.
E-mail addresses: bevilaqua.uece@gmail.com, claudiamlb@yahoo.com.br, claudia.bevilaqua@pq.cnpq.br (C.M.L. Bevilaqua).

http://dx.doi.org/10.1016/j.vetpar.2014.05.026
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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

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