The use of *Eucalyptus staigeriana* nanoemulsion for control of sheep haemonchosis


Sustainable control of gastrointestinal nematodes (GIN) in small ruminants has been based on the use of alternative methods, including targeted selective treatment, such as FAMACHA. Another GIN control alternative is the use of herbal medicines, although in many cases their use is based on empirical knowledge. Biopolymer nanoformulations has been investigated to maximize the essential oil effects against sheep gastrointestinal nematodes. The aim of the present study was to combine a *Eucalyptus staigeriana* essential oil nanoemulsion (EsNano) with FAMACHA as an alternative control for sheep haemonchosis. The study was performed over six months at a commercial sheep farm located in a semi-arid region of Northeast Brazil. Initially, a fecal egg count reduction test (FECRT) in sheep with levamisole, ivermectin and oxendazole in sheep was performed used to determine the most effective anthelmintic to use as the positive control. Levamisole has been selected because it showed efficacy superior to 95%. EsNano was obtained and then its physicochemical properties were characterized. The average (±SE) size of the particles in the nanoemulsion was 276.8 (±12.3) nm with bimodal distribution and polydispersity. Nine visits were performed, from April to September 2013, with an interval of 17 days. One hundred sixty-two male and female sheep were divided into three groups (n=54 each) and were treated when FAMACHA score was 3, 4, or 5: G-EsNano 250mg kg⁻¹ EsNano; G-Lev 7.5mg kg⁻¹ levamisole (positive control), and G-Neg was not treated (negative control). Feces from sheep were collected to quantify the number of eggs per gram of feces (epg) and to identify nematode genera. Sheep weight gain was monitored. The epg data for each group and the average sheep weight gains were analyzed by variance analysis and compared with the Tukey’s test (P<0.05). Significant difference between the number of animals treated with EsNano and levamisole was not observed in any visit (P>0.05). The epg variation was similar in the G-EsNano and G-Lev groups on visits (P>0.05), except the second and fifth evaluation in the epg groups were significantly different (P<0.05). *Haemonchus* spp. was the most prevalent nematode. There was no significant weight gain in any of the treated groups (P<0.05). The combination of phytotherapy and FAMACHA can be an alternative to minimize the use of synthetic anthelmintics to control resistant GIN populations of small ruminants.

RESUMO.- [Uso da nanoemulsão de Eucalyptus staige-
rian a no controle da hemoncose em ovinos.] O controle
sustentável de nematoides gastrintestinais (NGI) em pe-
quenos ruminantes tem sido baseado na utilização de mé-
todos alternativos, incluindo o tratamento alvo-seletivo, tal
como o FAMACHA. Outra alternativa de controle de NGI é
o uso de plantas medicinais, embora, em muitos casos, a
sua utilização seja baseada no conhecimento empírico. Na-
noformulações biopoliméricas-tem sido investigadas para
maximizar os efeitos de óleos essenciais sobre nematoides
gastrointestinais em ovinos. O objetivo do presente estudo
foi combinar a nanoemulsão do óleo essencial de Eucalyp-
tus staigeriana (EsNano) com o método FAMACHA como
uma alternativa para o controle da hemoncose em ovinos.
Este estudo foi realizado ao longo de seis meses em uma fa-
zena comercial de ovinos localizada em uma região semi-
árida do Nordeste do Brasil. Inicialmente, um teste de redu-
ção da contagem de ovos nas fezes (FECRT) em ovinos com
levamisol, ivermectina e oxendazole foi realizado para de-
terminar o anti-helmíntico mais eficaz, para posterior uso
como controle positivo. Levamisol foi selecionado porque
mostrou eficácia superior a 95%. EsNano foi obtido e, em
seguida, as suas propriedades físico-químicas foram carac-
terizadas. O tamanho médio (±SE) das partículas na nano-
emulsão foi 276,8 (±12,3) nm, com distribuição bimodal e
polidispersividade. Foram realizadas nove visitas, de abril
da setembro de 2013, com um intervalo de 17 dias. Cento
e sessenta e dois ovinos machos e fêmeas foram divididos
em três grupos (n=54 cada) e foram tratados quando o FA-
MACHA foi 3, 4 ou 5: G-EsNano 250 mg kg⁻¹ EsNano; G-Lev
7,5 mg kg⁻¹ de levamisol (controlo positivo), e G-Neg não foi
tratado (controlo negativo). As fezes dos ovinos foram co-
letadas para quantificar o número de ovos por grama de
fezes (opg) e identificar os géneros de nematóides. O ganho
de peso de ovinos foi monitorado. Os dados do opg de cada
grupo e os ganhos de peso médio dos ovinos foram analisa-
dos por análise de variância e comparadas com o teste de
Tukey (P<0,05). Diferença significativa entre o número de
animais tratados com EsNano e levamisol não foi observada
em nenhuma visita (P>0,05). A variação opg foi semelhan-
te para os grupos G-EsNano e G-Lev nas vistas (P>0,05),
excetuando a segunda e quinta avaliação em que os opg
dos grupos foram significativamente diferentes (P>0,05).
O nematoide Haemonchus spp. foi o mais prevalente. Não
houve aumento significativo de peso em qualquer um dos
grupos tratados (P>0,05). Assim, a combinação de fitotera-
pia e FAMACHA pode ser uma alternativa para minimizar o
uso anti-helmínticos sintéticos para controlar populações
resistentes NGI em pequenos ruminantes.

TERMOS DE INDEXAÇÃO: Pequenos ruminantes, Haemonchus
tortus, FAMACHA, óleo essencial, quitosana.

INTRODUCTION
Gastrointestinal nematodes (GIN) of small ruminants cause
severe pathology and major economic losses in sheep and
goat farming, particularly in tropical and subtropical are-
as worldwide (Akkari et al. 2013). GIN control programs
primarily rely on a combination of animal management
practices and the use of anti-parasitic drugs (Lifschitz et al.
2014). However, indiscriminate use of anthelmintics (AH)
is considered to be inefficient, costly and harmful to herds,
as it favors rapid selection of GIN-resistant populations to
all available AH classes (Molento et al. 2004). Anthelm-
tic resistance has already been reported for monepantel,
the latest anthelmintic released on the market (Scott et al.
2013, Van den Brom et al. 2015). Therefore, the develop-
ment of sustainable, environmentally acceptable methods
of nematode control is crucial.

Targeted selective treatment (TST) was proposed to
reduce the use of AH and thereby help maintain GIN po-
pulations in refugia, i.e., larvae and/or adults that remain
without treatment continue to harbor sensitivity alleles
(Cabaret 2008). FAMACHA is considered a TST approa-
ch for reducing parasite chemical exposure (Kenyon et
al. 2009). The method is based on the correlation between eye
mucous color and hematocrit values, which is used to iden-
tify animals that are able to withstand infections by Haem-
onchus contortus (Vilela et al. 2012).

Research on plants to obtain new bioactive compounds
has also been encouraged (Acharya et al. 2014). Elucida-
ting the mechanisms governing the anthelmintic activity of
plants against parasites of small ruminants is important for
the development of sustainable strategies of helmint control
(Sandoval-Castro et al. 2012). Eucalyptus spp. (Myr-
taceae) is native to Australia and is mainly cultivated for use
by the paper, pharmaceutical and cosmetic industries (Ha-
segawa et al. 2008). The nematicide action of Eucalyptus
staigeriana essential oils (EsEO) was described previously
(Macedo et al. 2010, Mesquita et al. 2013).

To protect the chemical constituents and maximize the
nematicidal effect of EsEO, nanoencapsulation techniques
employing chitosan have been investigated (Ribeiro et al.
2013, 2015). The emulsion technology is generally applied
for the encapsulation of bioactive compounds in aqueous
solutions through the production of nanoemulsions (Sha-
havi et al. 2015). Chitosan is a widely used biopolymer in
the biomedical area and offers several advantages, and the-
ese include its ability to control the release of active com-
pounds, low toxicity and high biodegradability (Dash et al.
2011).

The study aimed to assess the ability of E. staigeriana
essential oil nanoemulsion (EsNano) combined with FA-
MACHA to control haemonchosis in a sheep management
system.

MATERIALS AND METHODS
Eucalyptus staigeriana essential oil nanoemulsion (EsNa-
no). The EsEO was purchased from Avondale Essências (Braga-
ney, Brazil) and the sample used in the present study was similar
to that used by Ribeiro et al. (2015). The main chemical consti-
tuents were geraniol (16.60%), geraniol (14.83%), methyl geranate
(11.0%), geranyl acetate (9.2%) and limonene (7%) (Ribeiro et
al., 2015).

Chitosan powder, with 92% deacetylation was purchased
from Polymar S/A (Portaleza, Brazil). The EsNano was obtained
according to the methodology described by Ribeiro et al. (2015)
and the macroscopic characteristics of EsNano stability were ob-
served over 72 h.
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The EsNano, EsEO and 1% chitosan solution were subjected to thin layer chromatography and characterized by infrared spectroscopy (FTIR) using the model 8300 (Shimadzu Corporation, Japan).

The size and distribution of nanoparticles in solution were determined using a beam of red light with a wavelength of 633 nm and an angle of 175° (ZetaSizer 3600, Malvern, United Kingdom). For this purpose, EsNano samples were dissolved in deionized water to a final concentration of 0.1% (w/v) and were left to stir for 24 h. These analyses were performed in triplicate.

EsNano samples were prepared up to 72 h prior to each administration. The size distribution of the nanoparticles and macrosopic characteristics of all samples were evaluated to standardize the EsNano physicochemical properties.

**Study area.** The study was conducted on a sheep farm in the municipality of Solonópole (5°46.003’S and 38°51.000’W), a semi-arid region of the Ceará State, Brazil.

The climate is typically hot, semi-arid tropical with average temperatures ranging from 26 to 28°C. The annual rainfall is 717.1 mm with rains concentrated between January and April. The vegetation is predominantly formed by open shrubby Caatinga and dense shrubby Caatinga (Ceará 2011).

The mean rainfall at the Solonópole Rainfall Measuring Station during the study period was provided by Fundação Cearense de Meteorologia e Recursos Hídricos.

**Ethics committee on animal welfare.** The protocol was approved by the ethics committee for animal use of Universidade Estadual do Ceará (number: 10461354-B/65).

**Fecal egg count reduction test.** The sheep were originated from crosses between Doper, Santa Inês and Somalis races. The animals were kept under semi-extensive rearing management protocols, fed on native pastures and supplemented with mineral salt (Ovinofós, Tortuga, São Gonçalo do Amarante, Brazil).

A fecal egg count reduction test (FERCT) was used to determine the most effective anthelmintic to use as the positive control. Therefore, thirty sheep with egg counts per gram of feces (epg) greater than 250 were selected and randomly divided into three groups (n=10 each) for treatment with the anthelmintic classes that are most widely used by producers from the region: G1: 200µg kg⁻¹ ivermectin (Ivomec, Merial Saúde Animal, Paulínia, Brazil); G2: 7.5mg kg⁻¹ levamisole (Ripercol, Fort Dodge Saúde Animal LTDA, Campinas, Brazil) and G3: 5mg kg⁻¹ oxendazole (Systamex, Schering-Plough Animal Health, Guarulhos, Brazil).

The treatments were administered orally in a single dose. The fecal samples were collected directly from the rectums of animals treated with levamisole and EsNano for each visit using the GraphPad Prism 5.0 program. The mean epgs for each group at different visits were presented as the arithmetic mean ± SE.

**RESULTS**

The infrared spectroscopy has demonstrated that the chitosan main bands (Fig.1) appeared at 1643cm⁻¹ (carbonyl groups of partially acetylated groups), 1425 cm⁻¹ (angular deformation of CH₂), 1370cm⁻¹ (C-N axial deformation) and 1084cm⁻¹ (C-O-C glycosidic bonds) (Robles et al. 2013, Herculano et al. 2015). The main characteristic bands of EsEO can be observed at 2922, 1742, 1670, 1438, 1377, 1227 and 1146cm⁻¹. Most signals are referred to CH and C=O aldehyde groups, as well to methyl and methylene groups of limonene and citronelol. The nanoemulsion exhibits overlapped bands of both chitosan and EsEO, providing evidence of a successful encapsulation.

The nanoemulsion was formed almost immediately after the organic (chitosan solution) and inorganic (EsEO) phase come into contact. The EsEO concentration in the nanoemulsion was 36.4% (v/v). Physicochemical analyses of the resulting nanoemulsion particles revealed a mean size (±SE) of 276.8 (±12.3) nm with bimodal distribution and polydispersity in all samples. The nanoemulsion was white, with a milky consistency and could flow through an oral dosing pistol. No phase separation was visually observed after 72h.

The efficacy (±CI) of ivermectin, levamisole and oxendazole in the FERC was 55% (7–89), 97% (88–101) and 11% (66–55), respectively. Based on these results, levamisole was selected as the positive control. The most prevalent helminth in all of the larval cultures pre- and post-treatment was Haemonchus spp. (85.5%) followed by Trichostrongylus spp. (88%), Oesophagostomum spp. (4.2%) and Cooperia spp. (0.6%).

The percentage of animals treated with levamisole and EsNano based on FAMACHA is presented in Figure 2. There...
was no significant difference in the number of animals treated in the groups at all visits ($P>0.05$).

The curves of the mean epg variations of the G-EsNano, G-Lev and G-Neg groups are presented in Figure 3. The tendency of epg variation was similar for G-EsNano and G-Lev, except on the second (April 22th) and fifth (June 12th) visits, wherein the mean epg in G-EsNano was significantly higher ($P<0.05$). The mean epg of G-EsNano and G-Neg was significantly different except in the first (April 5th), second (April 22th), fourth (May 26th) and eighth (August 29th) visits ($P<0.05$).

A similar percentage of nematode genera were recovered from sheep in all of the groups after nine evaluation visits. *Haemonchus* spp. was the most prevalent genus with a mean of 79%, 80.1% and 82.3% for G-EsNano, G-Lev and G-Neg, respectively. The prevalence of *Trichostrongylus* spp. and *Oesophagostomum* spp. was not significantly different among the three groups. *Cooperia* spp. was the less prevalent genus.

The sheep weight gains in the groups are presented in Table 1. There were no significant differences in weight gain among the ages of animals in the same treatment group before and after treatment ($P<0.05$).

**DISCUSSION**

The adoption of alternative methods for GIN control must be based on the implementation of strategies to address anthelmintic resistance control while considering not only the parasite biology but also farm decisions and whole management decisions (Morgan & Van Dijk 2012). Therefore, new alternatives for controlling helminths in small ruminants have been widely tested, such as using phytotherapy and FAMACHA (Vieira et al. 2014).

The EsEO with limonene as major oil component (28.82%) showed effect of 76.57% against goat gastroin-
intestinal nematode parasitism is higher in sheep and Northeast Brazil (Vilela et al. 2008). In these months, gas-
in refugia (Kenyon et al. 2009). Therefore, chitosan was selected as the encapsulating matrix for EsNano to be evaluated against she-
ep haemonchosis.

The preparation of chitosan-based nanoemulsions for encapsulating volatile compounds has been proposed to promote active protection and to maximize the biological effects of essential oils (Paula et al. 2011). Nanoemulsions are versatile and can be prepared via numerous different aqueous solutions, surfactants and oil constitutes (Underwood & Van Eps 2012). In this study, the physicoche-
chemical characteristics of EsNano were similar to the nano-
emulsion used in in vitro tests on Haemonchus contortus (Ribeiro et al. 2015).

Although nanostructured essential oils have been de-
veloped for use against GIN small ruminants (Ribeiro et al. 2014, Grando et al. 2015), validation of these products requires an assessment of AH effects for a prolonged pe-
riod in sheep. The present study validated the anthelmintic effect of EsNano when combined with FAMACHA in the dry
season.

FAMACHA is the TST most used by sheep and goat far-
ners in Northeast Brazil. Furthermore, Haemonchus spp.
was the most prevalent nematode in larval cultures, suppor-
ting the application of FAMACHA (Bath & Van Wyk 2009). Although H. contortus infection persisted in the herd, the selective treatment strategy significantly reduced the use of AH, yielding significant health and economic benefits (Mo-
lento et al. 2009). Additionally, the FAMACHA method is able to identify sheep that are at risk of reducing the selection pressure for anthelmintic resistance. In this study, using FA-
MACHA, the highest percentage of animals treated per visit was 26% for the G-Lev, which is important for maintaining nematode populations in refugia (Cabaret, 2008). Further-
more, FAMACHA is particularly important in areas with a prolonged dry season, when the survival of free-living sta-
ges in the pasture is low or null, thus decreasing the propor-
tion of nematodes in refugia (Kenyon et al. 2009).

The study period (April to September 2013) correspond-
ds to the end of the rainy season and the early dry season in Northeast Brazil (Vilela et al. 2008). In these months, gas-
trointestinal nematode parasitism is higher in sheep and H. contortus represents more than 80% of the nematode po-
ulation in the flock (Souza et al. 2013). This condition was decisive for the choice of the experiment execution period.

The volume and regularity of rainfall this year were atypical, where a low rainfall occurred in every month of the study. The low rainfall may have influenced the decline of the first epg (5th April to 26th May). A more profound decline in G-EsNano and G-Lev epg curves was apparent and differed significantly from the negative control. The epg peak in the sixth visit (June 29th) can be explained by the final rainy period, when the epg is high (Costa et al. 2009), but this increase was significantly more pronoun-
ced in the negative control group. Moreover, EsNano was able to maintain the epg of G-EsNano similar to the epg in the positive control group at all visits, except on April 22th and June 12th. The EsNano AH effect was also demonstra-
ted for Eucalyptus staigeriana encapsulated oil, which was used against sheep GIN in a controlled test (Mesquita et al. 2013).

Although there was no significant difference in the number of animals treated between groups (P>0.05), on June 29th and July 16th, the number of animals treated in G-EsNano was twice the number of animals treated in G-
-Lev; however the percentage of treated animals declined with subsequent visits while the G-Lev group maintained the same number of treated sheep.

There was no significant weight gain for any of the age groups treated with EsNano or levamisole or that were untreated (P>0.05). This result may be attributed to the low food supply in the pasture, which is typical for periods with lower rainfall than average in semi-arid Northeastern Brazil. Even when sheep are exposed to supplementary fe-
eding, Leask et al. (2013), in a study conducted in South Africa, did not observe a significant difference in weight gain among sheep treated with FAMACHA versus a conven-
tional treatment with levamisole for twelve weeks during the summer and autumn seasons.

The combination of herbal medicines and FAMACHA mini-
imizes the use of synthetic anthelmintics and main-
tains GIN populations in refugia. However, the survival of GIN populations at higher than acceptable levels requires a balance between the population in refugia, established by the new approach, and the potential adverse consequen-
ces of excessive parasitism (Besier 2012). This study de-
monstrated that levamisole may be replaced by EsNano, as they demonstrated equivalent anthelmintic effects. Further studies evaluating phytotherapy and FAMACHA in the rainy season are required to confirm the powerful effects of this combination in times of high pasture infestation.

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Conflicts of interest.- The authors declare that they have no conflicts of interest.

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