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Original Article

Anthelmintic activity of nanoencapsulated carvacryl acetate against gastrointestinal nematodes of sheep and its toxicity in rodents

Atividade anti-helmíntica do acetato de carvacrila nanoencapsulado sobre nematoides gastrintestinais de ovinos e toxicidade em roedores

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Abstract

The objective of this study was to evaluate the efficacy of carvacryl acetate (CVA) and nanoencapsulated CVA (nCVA) on gastrointestinal nematodes of sheep. The CVA was nanoencapsulated with chitosan/gum arabic and the efficacy of nanoencapsulation (EE), yield, zeta potential, nanoparticle morphology and release kinetics at pH 3 and 8 were analyzed. Acute and subchronic toxicity were evaluated in rodents and reduction of egg counts in the faeces (FECRT) of sheep. The sheep were divided into four groups (n = 10): G1, 250 mg/kg CVA; G2, 250 mg/kg nCVA; G3, polymer matrix and G4: 2.5 mg/kg monepantel. EE and nCVA yield were 65% and 57%, respectively. The morphology of the nanoparticles was spherical, size (810.6±286.7 nm), zeta potential in pH 3.2 (+18.3 mV) and the 50% release of CVA at pHs 3 and 8 occurred at 200 and 10 h, respectively. nCVA showed LD_{50} of 2,609 mg/kg. CVA, nCVA and monepantel reduced the number of eggs per gram of faeces (epg) by 57.7%, 51.1% and 97.7%, respectively. The epg of sheep treated with CVA and nCVA did not differ from the negative control (P>0.05). Nanoencapsulation reduced the toxicity of CVA; however, nCVA and CVA presented similar results in the FECRT.

Keywords: Chitosan, gum arabic, biocompounds, acetylation, toxicological safety, *Haemonchus contortus.*

Resumo

O objetivo deste trabalho foi avaliar a eficácia do acetato de carvacrila (ACV) e do ACV nanoencapsulado (nACV) sobre nematóides gastrintestinais de ovinos. O ACV foi nanoencapsulado com quitosana/goma arábica e foi analisada a eficácia de nanoencapsulamento (EE), o rendimento,

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potencial zeta, morfologia das nanopartículas e cinética de liberação em pH 3 e 8. Foram avaliadas as toxicidades aguda e subcrônica em roedores e a redução da contagem de ovos nas fezes (RCOF) de ovinos. Os ovinos foram divididos em quatro grupos (n = 10): G1, 250 mg/kg ACV; G2, 250 mg/kg de nACV; G3, matriz polimérica e G4: 2,5 mg/kg de monepantel. A EE e o rendimento de nACV foram de 65% e 57%, respectivamente. A morfologia das nanopartículas foi esférica, tamanho (810,6±286,7 nm), potencial zeta no pH 3,2 (+18,3 mV) e a liberação de 50% de CVA nos pHs 3 e 8 ocorreu às 200 e 10 h, respectivamente. nACV apresentou DL_{50} de 2.609 mg/kg. ACV, nACV e o monepantel reduziram a contagem de ovos por grama de fezes (opg) em 57,7%, 51,1% e 97,7%, respectivamente. A contagem de opg de ovelhas tratadas com ACV e nCVA não diferiu do controle negativo (P>0,05). O nanoencapsulamento reduziu a toxicidade do AVC; no entanto, nACV e ACV apresentaram resultados semelhantes na RCOF.

Palavras-chave: Quitosana, goma arábica, biocompostos, acetilação, segurança toxicológica, *Haemonchus contortus.*

Introduction

Gastrointestinal nematode parasitism, especially *Haemonchus contortus,* is one of the main limiting factors of small ruminant production worldwide because it endangers the health, welfare and productivity of sheep and goats (Araújo-Filho et al., 2018; Elmahalawy et al., 2018). The control of these nematodes is performed with the administration of anthelmintics. However, the continuous use of these drugs has inevitably led to the development of resistant and/or multiresistant nematode populations worldwide (Kotze & Prichard, 2016; Santos et al., 2017), including in northeastern Brazil (Silva et al., 2018).

New alternatives for the control of gastrointestinal nematodes are being studied, with an emphasis on plant bioactives and their isolated compounds (Kearney et al., 2016). Essential oils are complex mixtures of volatile constituents produced by aromatic plants that present several biological activities, mainly anthelmintic activity (Macedo et al., 2010; Raut & Karuppayil, 2014).

Carvacrol is a phenolic compound found mainly in essential oils of plants of the family Lamiaceae (Besier et al., 2016) and has antiparasitic activity (Zhu et al., 2013; Shang et al., 2016; Fabbri et al., 2016). This monoterpene presents high toxicity, and acetylation is an alternative that potentiates its biological activity and increases its toxicological safety (Morais et al., 2014; Andre et al., 2016). Carvacryl acetate (CVA) showed anthelmintic activity against *Schistosoma mansoni* (Moraes et al., 2013), *H. contortus* and other sheep gastrointestinal nematodes in *in vitro* and *in vivo* assays, but the efficacy did not reach the desired therapeutic level (Andre et al., 2016).

The nanoencapsulation of bioactive compounds has been used as an alternative to protect drugs and promote a sustained release in animals, increasing bioavailability and maximizing the nematicidal effect (Zhao et al., 2010; Ribeiro et al., 2014; Ribeiro et al., 2017). The polyelectrolyte complex is a technique used for the nanoencapsulation of bioactive compounds by mixing oppositely charged polymers in solutions (Minkal et al., 2018). Among these polymers, we highlight chitosan, a polysaccharide that is obtained by the deacetylation of chitin. This biopolymer presents properties such as biocompatibility, biodegradability and nontoxicity and is used in pharmaceutical applications for controlled drug release (Shariatinia & Mazloom-Jalali, 2019). The positively charged amino groups of chitosan react with an anionic group of polysaccharides, such as gum arabic, leading to the formation of a polyelectrolyte complex (Tan et al., 2016; Minkal et al., 2018). Gum arabic is a biopolymer obtained from *Acacia senegal* or *Acacia seyal* and is widely used in the food and pharmaceutical industries (Guan & Zhong, 2015; Sanchez et al., 2018).

Thus, the aim of this study was to evaluate the anthelmintic activity of CVA and nCVA against gastrointestinal nematodes of sheep and the toxicity of CVA and nCVA in rodents.

Materials and Methods

Carvacrol acetylation

A solution containing 1 g of carvacrol (purity ≥ 98%; Sigma–Aldrich®, St. Louis, USA), 15 mL of acetic anhydride P.A. (Dinâmica®, São Paulo, Brazil) and 1.5 g of sodium acetate P.A. (Dinâmica®, São Paulo, Brazil) was refluxed for 1 h at room temperature. Cold water was added (20 mL), and the solution was neutralized to pH 7.0 with 5% sodium bicarbonate P.A. (Dinâmica®, São Paulo, Brazil). The solution was transferred to a separating funnel and washed three times with chloroform P.A. (100 mL) (Dinâmica®, São Paulo, Brazil). The chloroform phase, containing the acetylated material, was washed with water and dried with sodium sulfate P.A. (Dinâmica®, São Paulo, Brazil). The solvent was rotoevaporated (Matos, 1997). CVA was subjected to thin layer chromatography and characterized by gas chromatography mass spectrometry (GC-MS). The yield of CVA was 83%.

GC- MS analysis

The chemical analysis of CVA was performed with a Shimadzu QP-2010 Ultra instrument employing the following conditions: column: Rtx-5MS (Crossbond 5%, diphenyl/95% dimethyl polysiloxane) with 30 m x 0.25 mm x 0.25 µm df; carrier gas: He (24.2 mL/min, in constant linear velocity mode); mass spectra: electron impact 70 eV; injector temperature: 250 °C in split mode (1:100); detector temperature: 250 °C; and the column temperature was programmed 35-180 °C at 4 °C/min, 180-280 °C at 17 °C/min and at 280 °C for 10 min. Compounds were identified by their retention times relative in GC to known compounds and by comparison of their mass spectra with those present in the computer data bank (NIST) and published literature (Adams, 2007).

Nanoencapsulation of carvacryl acetate

The nanoparticles were produced from a polyelectrolytic complexation system according to Abreu et al. (2008). The chitosan (Polymar®, Ceará, Brazil) were purified as described by Abreu et al. (2013), with a viscosity molar mass (Mz) 41.26 x 10^4 g/mol and 72% degree of deacetylation. A solution of 1% chitosan and Tween 80 was subjected to mechanical stirring. Subsequently, 300 µL of CVA was added to the solution and submitted to an ultrasonic bath (Ultra 800, Ciencor Scientific Ltd., São Paulo, Brazil) for 15 min. For formation of the prenucleus of the nanoparticles, 0.1% sodium tripolyphosphate P.A. (STP) (Dinâmica®, São Paulo, Brazil) was added dropwise at the chitosan:STP ratio of 50:1, and the solution was subjected to magnetic stirring for 30 min. Then, 1% gum arabic (w/v) (Dinâmica®, São Paulo, Brazil) was added to the solution at a chitosan:gum arabic ratio of 10:1, and then the solution was subjected to magnetic stirring for another 30 min. For the production of particles in bilayers, the monolayer particles were suspended in 20 mL of distilled water and subjected to magnetic stirring. Chitosan (1%) was added dropwise and was stirred continuously for an additional 30 min. Subsequently, 1% gum arabic (w/v) was added and stirred continuously for an additional 30 min. The solution was centrifuged at 4,000 rpm for 20 min, the supernatant was removed and the bilayer nanoparticles were subjected to the lyophilization process.

Encapsulation efficiency

The encapsulation efficiency (EE) of CVA was determined by absorption spectroscopy with a Genesys 10S UV-Vis (Thermo Scientific, Massachusetts, USA) at a wavelength of 271 nm. A solution of 10 mL of 95% ethanol with 10 mg of nanoparticles was stirred constantly for 48 h, and the concentration of CVA was determined according to the

calibration curve using equation 1, with the absorbance (*abs*) and the CVA concentration (*conc*) in mg/mL.

$$abs = 0.0028conc - 0.0368; R^2 = 0.994 \quad (1)$$

Physicochemical characterization of nanoparticles

nCVA was characterized by Fourier transform infrared spectroscopy (FTIR) using a Nicolet iS5 spectrophotometer (Thermo Scientific). The samples were prepared as potassium bromide pellets (KBr) at a ratio of 1:20 (m/m) (sample:KBr). The morphology of the nanoparticles was evaluated with scanning electron microscopy (Quanta FEG 450 Electronic Microscope, environmental FEI). The samples were fixed on carbon strips, dried in a desiccator and covered with a thin layer of gold, using a 20 kV acceleration voltage.

Particle size, surface charge at different pH values (3 - 8) and the point of zero charge was determined through zeta potential measurements using a Nano ZetaSizer analyzer (Malvern 3600, Worcestershire, UK) using a laser wavelength of 632.8 nm and a fixed dispersion angle of 173°.

Release kinetics

The kinetics of nCVA release were obtained using a dialysis system. The sample (60 mg nanoparticles) was introduced into cellulose acetate membranes (14 KDa pores) and dialyzed against 60 mL of 0.001 M HCL (pH 3) or borate buffer (pH 8) solution and 1% (v/v) Tween 80. Three aliquots were taken at regular time intervals and analyzed by spectrophotometry in Genesys 10S UV–vis (Thermo Fisher Scientific, USA). Thus, the concentration of CVA present in the medium was calculated using a calibration curve and equation 1.

The mechanisms of release of CVA from the nanoparticles were evaluated using the zero order, first order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas mathematical models (Costa, 2002). Linear regression was used to calculate the values of the release constants (k) and the correlation coefficients (r).

Ethics committee on animal welfare

This study was approved by the Ethics Committee of the Universidade Estadual do Ceará (Protocol Number: 6511846/2016).

Acute toxicity

Acute toxicity was performed according to OECD (2008a) (Up-and-down procedure). Female Swiss mice (n=12) weighing 28.4±2.5 g were allowed to acclimatize to the laboratory conditions (luminosity: 12 h/12 h, light/dark; temperature: 22±2 °C; relative humidity: 60%) for seven days and were provided commercial feed (Labina®, Purina, São Paulo, Brazil) and water *ad libitum*. The animals were divided into 2 groups and received the following treatments: G1 (n = 8), nCVA at doses of 175, 440, 1,110 and 2,800 mg/kg and G2 (n = 3), polymer matrix (negative control). The dose correction factor was 3.2-fold, and dose increase or reduction was performed according to the survival or mortality of the animals, respectively. Each animal was carefully evaluated for up to 48 h prior to the decision of the dose to be administered to the next animal. The dosage was discontinued when five reversals occurred in six consecutive nCVA-treated animals. All decisions regarding the doses administered and the estimation of the lethal dose for 50% (LD_{50}) were performed using the AOT425StatPgm software.

Subchronic toxicity in rats

Subchronic toxicity was performed according to OECD (2008b). Female Wistar albino rats (n=24) weighing 159.8±11.9 g were allowed to acclimatize to the laboratory conditions for seven days and were provided commercial feed (Labina®, Purina, São Paulo, Brazil) and potable water *ad libitum*. The animals were randomly divided into 3 groups (n=8) and received the following treatments: G1: 250 mg/kg CVA; G2: 250 mg/kg nCVA; and G3: polymer matrix (negative control). The treatments were administered daily by gavage for 28 days. Toxic manifestations and mortality were monitored daily. The animals were weighed on days 0, 15 and 30 to measure weight gain. On day 15, doses were adjusted for the average body weight of the animals.

After 28 days, the animals were anesthetized with xylazine (16 mg/kg) and ketamine (120 mg/kg), and blood samples were collected from the abdominal aorta for complete blood count and biochemical analysis. Subsequently, the animals were euthanized by anesthetic overdose. The hematological parameters analyzed were the red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (Plt) and white blood cell count (WBC) using species-specific smart cards for rats in the BC-2800Vet Auto Hematology Analyzer (Mindray®, China).

The biochemical parameters alanine aminotransferase (ALT), aminotransferase (AST), urea, serum creatinine, total protein and total bilirubin were determined using Labmax Plenno (Labtest®, Minas Gerais, Brazil).

For histopathological analysis, fragments of organs (liver, stomach, lung and kidney) were fixed in 10% buffered formaldehyde, processed, infiltrated and embedded in paraffin, and 5 µm sections were laminated and stained with hematoxylin-eosin for microscopic examination.

Molecular characterization of the anthelmintic resistance of the Haemonchus contortus isolate

Coprocultures were performed using the method described by Roberts & O'sullivan (1950) for all animals used in the FECRT. Third-stage larvae (L3) of *H. contortus* were recovered from coprocultures and characterized as resistant to benzimidazoles and levamisole by qPCR. The L3 DNA extraction protocol was performed according to Santos et al. (2014). The extracted DNA was resuspended in 50 µL of TE buffer, quantified by spectrophotometry, analyzed by agarose gel electrophoresis to determine its integrity and stored at -20 °C until use.

Real-time PCR for resistance to benzimidazoles

Real-time PCR assays (qPCR), using primers previously described by Santos et al. (2014), were performed in triplicate. Each reaction consisted of a total volume of 25 µL, containing 12.5 µL 2X Fast Start Universal SYBR Green Master Mix, 0.3 µmol/µL of the primers (sense and antisense) and 25 ng of the pooled DNA sample. Reactions were performed with a Mastercycler ep Realplex (Eppendorf®, Hamburg, Germany) and consisted of initial incubation at 95 °C for 10 min, followed by 35 two-step cycles for SNPs F200Y and F167Y and 34 two-step cycles for SNP E198A: denaturation at 95 °C for 15 s and annealing and extension at 58°C for 30 s. The qPCR products were differentiated from primer dimers by melting curve analysis. The negative control was performed without sample DNA.

Real-time PCR for resistance to levamisole

Real-time PCR assays using primers previously described by Santos et al. (2019) were performed in triplicate. Reactions were performed containing 12.5 µL SYBR Green (Roche, West Sussex, UK), 0.3 pmol/µL of each primer (Table 1), 50 ng DNA and water for a total

volume of 25 µL. Water was used as a negative control instead of DNA. The amplification conditions for both alleles were 95 °C for 10 min and 35 cycles of 95 °C for 15 s and 56 °C for 30 s. The melting curve analysis was performed to detect primer dimers.

Table 1. Kinetics of release of nanoencapsulated carvacryl acetate at acidic pH (3) and basic pH (8).

pH	Correlation coefficient (R_2)						
	Zero order	First order	Hixson-Crowell	Higuchi	Korsmeyer Peppas	*k*	*n*
pH 3	0.801	0.950	0.915	0.648	0.612	0.045	0.41
pH 8	0.851	0.748	0.784	0.926	0.909	0.127	0.55

Fecal Egg Count Reduction Test (FECRT)

We used forty sheep of both sexes ranging from 6 to 18 months of mixed breed, weighing an average of 30 kg and kept in semi-extensive management, fed on native pastures in a semiarid region of northeastern Brazil. The selected animals were naturally infected with gastrointestinal nematodes and had over 500 eggs per gram of feces (epg) using the McMaster technique (Ueno & Gonçalves, 1998).

A value lower than the LD_{50} value established in the acute toxicity test was used to ensure that the CVA (Andre et al., 2016) and nCVA would not cause any toxic effects on sheep. Sheep were divided into 4 groups (n = 10) according to epg and randomly assigned to the following treatments: G1: 250 mg/kg CVA; G2: nCVA; G3: polymer matrix (negative control) and G4: 2.5 mg/kg monepantel (Zolvix®, Novartis, New Zealand) (positive control). Sheep received a single treatment. Fecal samples from each animal were collected on days 0, 8 and 16 posttreatment to determine the epg. Coprocultures were performed using the method described by Roberts & O'sullivan (1950) and 100 larvae were identified according to Van Wyk & Mayhew (2013).

Statistical analysis

Data of hematological and biochemical variables of rats were submitted to ANOVA and means compared by Tukey´s test using GraphPad Prism® 5.0 software (GraphPad Software Inc., USA). The significance level was $p < 0.05$.

The efficacy of FECRT was calculated by the BootStreet program through arithmetic average, using the formula:

$$\mathbf{FECRT} = 100 \, x \left(1 - \frac{\mathbf{T}2}{\mathbf{T}1} \, x \frac{\mathbf{C}1}{\mathbf{C}2} \right).$$

In this formula, the arithmetic FECRT means in controls (C) and treated (T) animals before (T1 and C1) and 8 or 16 days after (T2 and C2) deworming were compared (Dash et al., 1988; Cabaret, 2014). The epg values were log transformed (log10[x + 1]), submitted to ANOVA and compared using the Tukey's test with GraphPad Prism® 5.0 software. The significance level was $p<0.05$. The confidence interval of the percentage of L3 recovered in coprocultures was calculated according to the following formula: $1.96 \sqrt{\frac{p \, x \, q}{n}}$ Where p is the total percentage of larvae of a nematode genus, q is the total percentage of the other genera of nematodes and n is the total percentage larvae of nematodes identified.

Results

GC-MS analysis identified two compounds corresponding to CVA (99.3%) and carvacrol (0.7%) (Supplementary Material Figure S1) (supplementary material). The EE and yield of nCVA were 65% and 57%, respectively.

The FTIR spectra of CVA, chitosan, gum arabic and nCVA are shown in Figure 1. CVA presented the following bands: 1590 cm^{-1} (C-H flexion), 1765 cm^{-1} (acetyl group) and 816 cm^{-1} (aromatic ring) (Andre et al., 2016). The 1086 cm^{-1} (C–O–C stretch) and 890 cm^{-1} (pyranose ring) bands correspond to chitosan (Keawchaoon & Yoksan, 2011). The gum arabic presented 1420 cm^{-1} (C=O symmetric stretching) and 1366 cm^{-1} (-OH bending) bands, indicating the presence of glucuronic acid (Daoub et al., 2018). The spectra of nCVA presented a 1418 cm^{-1} (C=O symmetric stretching) band, indicating the presence of glucuronic acid in the gum arabic; 1092 cm^{-1} (C–O–C stretch) and 894 cm^{-1} (pyranose ring) bands were present in chitosan (Keawchaoon & Yoksan, 2011). CVA was not detected, possibly due to overlap with other bands of the biopolymer matrix.

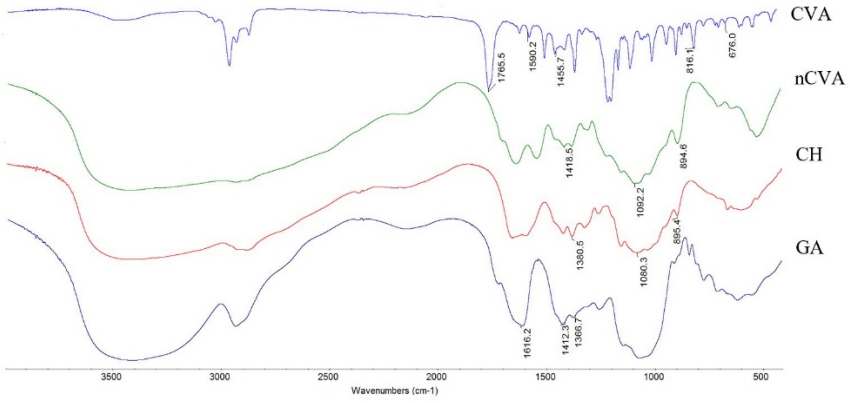

Figure 1. FTIR spectra of carvacryl acetate (CVA), nanoencapsulated carvacryl acetate (nCVA), chitosan (CH) and gum arabic (GA).

Scanning electron microscopy demonstrated that the nCVA nanoparticles had a spherical morphology and agglomeration of the nanoparticles was observed due to the centrifugation process performed prior to lyophilization (Figure 2). The average size of the nanoparticles was 810.6±286.7 nm and unimodal distribution (Figure 3). The nanoparticles showed a decrease in the zeta potential with increasing pH. At pH 3.2, the zeta potential was +18.3 mV, and the point of zero charge was situated at approximately pH 5.1 (Figure 4).

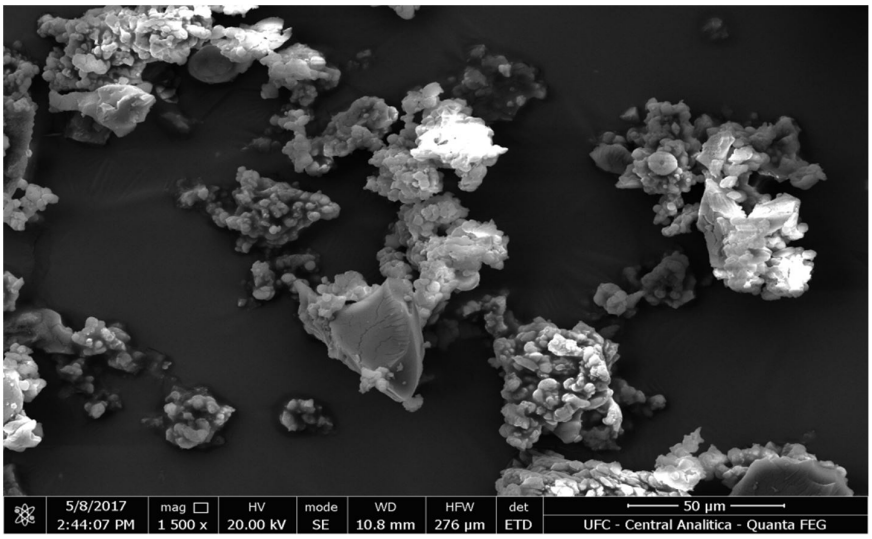

Figure 2. Scanning electron microscopy (SEM) analysis of nanoparticles of carvacryl acetate.

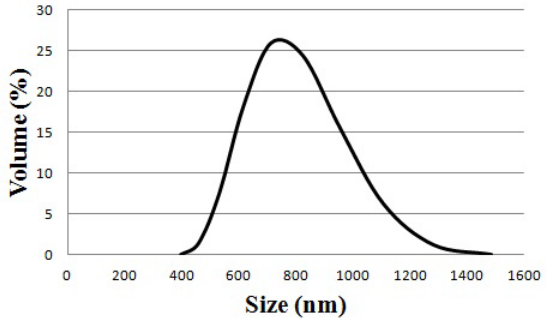

Figure 3. Particle size distribution of the nanoparticles of carvacryl acetate.

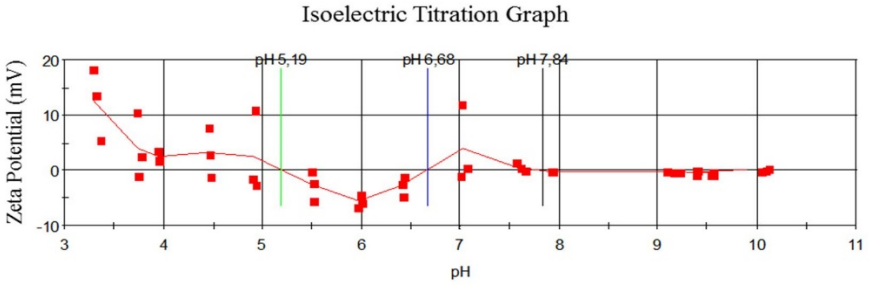

Figure 4. Zeta potential variation with pH for nanoencapsulated carvacryl acetate. Zeta potential ( ), weighted mean zeta potential (—), pH 5.1 (—), pH 6.6 (—) and pH 7.8 (—).

The kinetics of nCVA release at pH 3 and pH 8 are shown in Figure 5. At pH 3, there was a release of approximately 50% of CVA after 200 h. The equilibrium was achieved after 240 h, with approximately 93% of the CVA released into the medium. However, at pH 8, the release was faster, and approximately 50% of CVA was released after 10 h. Equilibrium was achieved after 48 h, with approximately 97% of the CVA released.

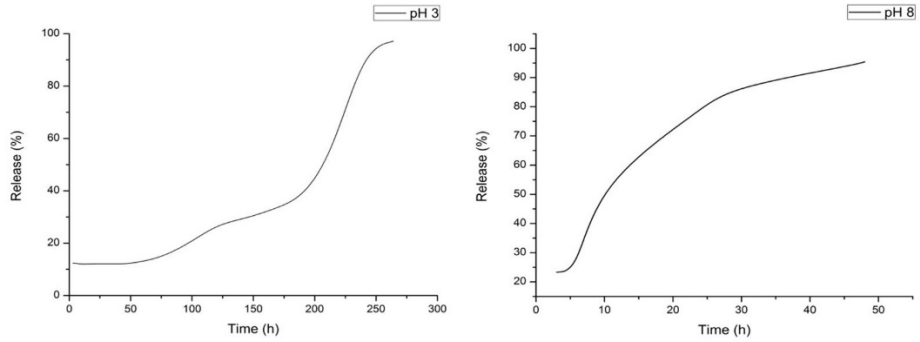

Figure 5. Kinetics of *in vitro* release of nanoencapsulated carvacryl acetate at acidic pH (3) and basic pH (8).

The best correlation coefficient of nCVA released at pH 3 was first-order model. Higuchi and Korsmeyer-Peppas are the models that represent the release of nCVA in pH 8. The Korsmeyer-Peppas kinetic parameters were calculated and coefficient n was 0.55 (Table 1).

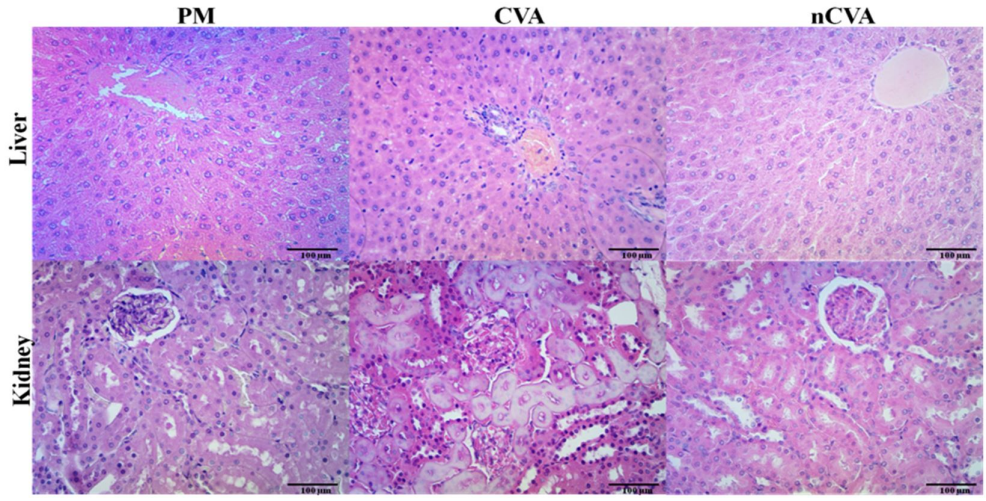

Figure 6. Histological analysis of *Rattus novergicus* kidney and liver treated for 28 days with 250 mg/kg polymer matrix (chitosan/gum arabic) (PM), carvacryl acetate (CVA), nanoencapsulated carvacryl acetate (nCVA).

In the acute toxicity test, nCVA presented an LD_{50} value of 2,609 mg/kg. The encapsulating matrix showed no toxicity. In the subchronic toxicity test, the results of the biochemical and hematological parameters of rats treated with CVA, nCVA and the polymer matrix showed that the parameters did not differ significantly among the studied groups (Table 2). Hepatic changes were observed (hepatocyte cell swelling, Kupffer cell hyperplasia, portal and centrilobular vein congestion, and the presence of inflammatory cells) in animals treated with CVA and nCVA. In the kidneys of all treated animals, moderate swelling of the tubular epithelium, sections with isomeric vacuolization of tubular epithelium and hyaline cylinders were observed. In the spleen, ectasia was observed with inflammatory cell visualization inside the vessels, in addition to brownish and/or blackish pigments suggestive of lipofuscin and hemosiderin, respectively. Lung, stomach and intestine samples did not present morphological alterations (Figure 6).

Table 2. Hematological and biochemical parameters (±standard deviation) of *Albino Wistar* rats treated with carvacryl acetate (250 mg/kg), nanoencapsulated carvacryl acetate (250 mg/kg) and polymer matrix (negative control).

Parameters	Carvacryl acetate	Nanoencapsulated carvacryl acetate	Polymer matrix (negative control	Reference[a]
WCB (10^3/µL)	4 ± 0.9^A	5.1 ± 1.5^A	4.6 ± 0.6^A	4.7 – 12.9
RBC (10^6/µL)	8 ± 0.4^A	7.5 ± 0.3^A	7.3 ± 0.6^A	7.3 – 8.6
Hb (g/dL)	15.4 ± 1.0^A	14.7 ± 0.9^A	14.1 ± 0.8^A	13.2 – 15.1
Ht (%)	48.7 ± 3.2^A	46.6 ± 2.9^A	44.7 ± 2.3^A	39.1 – 48.5
Plt (10^3/µl)	830.1 ± 123.9^A	939 ± 128.5^A	1,128 ± 118.7^B	757 – 1476
MCV (µm^3)	60.8 ± 0.8^A	61.4 ± 2.0^A	60.3 ± 1.2^A	49.1 – 62.5
MCHC (%)	31.7 ± 0.4^A	31.5 ± 0.3^A	31.5 ± 0.5^A	29.9 – 34.9
ALT (UI/L)	44.2 ± 9.6^A	58.7 ± 31.4^A	54 ± 17.8^A	26 – 60
AST (UI/L)	72.8 ± 15^A	126.5 ± 24.6^B	87.1 ± 29.8^B	83 – 184
CR (mg/dL)	0.7 ± 21.5^A	0.96 ± 0.48^A	0.71 ± 0.3^A	0.4 – 0.7
UR (mg/dL)	56 ± 6.32^A	69.3 ± 34.9^A	56.5 ± 4.5^A	30 – 57
ALB (g/dL)	3.41 ± 0.35^A	3.95 ± 1.2^A	3.3 ± 0.4^A	2.6 – 3.4

Different letters indicate significantly different averages in the rows (P<0.05). The polymer matrix (chitosan/arabic gum) was a negative control. The animals were randomly divided into 3 groups (n=8). The hematological parameters analyzed were the white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCHC), mean corpuscular hemoglobin concentration (MCHC), and platelets (Plt) and biochemical parameters were alanine aminotransferase (ALT), aminotransferase (AST), creatinine (CR), UR (urea) and albumin (ALB). [a] The reference values established by Melo et al. (2012).

The molecular characterization of *H. contortus* demonstrated that the isolate has a higher frequency of SNP F167Y resistant alleles. For levamisole, frequencies of 64% resistant alleles of the 63 bp indel of exon 3 of the *Hco-acr-8* gene were identified (Table 3).

Table 3. Sensitive and resistant allele frequencies for SNPs 200Y, 167Y, 198A and Hco-acr-8 gene em *Haemonchus contortus* isolate from farm.

Anthelmintics	SNPs	Allele	Frequency of each allele (%)
Benzimidazoles	F200Y	Sensitive	33.3%
		Resistant	66.7%
	F167Y	Sensitive	28%
		Resistant	72%
	E198A	Sensitive	100%
		Resistant	-
Levamisole	*Hco-acr-8* gene	Sensitive	36%
		Resistant	64%

The FECRT results of CVA, nCVA and monepantel are expressed as the mean epg on days 0, 8 and 16 posttreatment (Table 4). CVA, nCVA and monepantel reduced the epg by 57.7%, 51.1% and 97.7%, respectively, by 16 days posttreatment. The results of CVA and nCVA were not significantly different from the negative control (p>0.05). The prevalence of nematode genera in FECRT is presented in Table 5. After treatment, the frequency of *Trichostrongylus* spp. increased in relation to *Haemonchus* spp. in the groups treated with CVA and nCVA, indicating that this biocomposite is effective against *Haemonchus* spp.

Table 4. Mean efficacy and egg counts per gram of faeces (epg ± standard deviation) of sheep treated with of carvacryl acetate (CVA), nanoencapsulated carvacryl acetate (nCVA) or monepantel.

Treatments	Day 0	Day 8	Day 16
CVA			
Mean epg	1915±1131^{Aa}	1140±1074^{Aa}	585±788.9^{Aa}
Efficacy (%)	-	9.6	57.7
nCVA			
Mean epg	1885± 1039^{Aa}	695± 185^{Ba}	665±261^{Ca}
Efficacy (%)	-	44	51.1
Monepantel			
Mean epg	1865±748.1^{Aa}	125±182.7^{Bb}	30±44.4^{Cb}
Efficacy (%)		89.7	97.7
Polymer matrix (negative control)			
Mean epg	1875±1175^{Aa}	1235±562^{Aa}	1355±455^{Aa}

Different capital letters in the rows and small letters in the columns indicate significantly different means ($P < 0.05$). The animals were randomly divided into 4 groups (n=10). The efficacy was calculated using the formula 100 × (1 − [T2/T1] [C1/C2]), in which the arithmetic fecal egg count means in controls (C) and treated (T) animals before (T1 and C1) and 8 or 16 days after (T2 and C2) deworming were compared (Dash et al., 1988).

Table 5. Frequency (%) and 95% confidence interval of third stage larvae identified in coprocultures on days 0, 8 and 16 posttreatment.

Groups	Day 0	Day 8	Day 16
CVA			
Haemonchus spp.	65 (55.4 – 74.5)	23 (14.5 – 31.4)	27 (18.1 – 35.8)
Trichostrongylus spp.	32 (22.6 – 41.3)	73 (63.8 – 81.8)	70 (60.8 – 79.1)
Oesophagostomum spp.	3 (-0.4 – 6.4)	4 (0.5 – 7.9)	3 (-0.4 – 6.4)
nCVA			
Haemonchus spp.	60 (50.2 – 69.7)	27 (18.1 – 35.8)	31 (21.7 – 40.2)
Trichostrongylus spp.	38 (28.2 – 47.7)	70 (60.8 – 79.1)	65 (55.4 – 74.5)
Oesophagostomum spp.	2 (-0.2 – 4.2)	3 (1.1 – 4.8)	4 (1.6 – 6.3)
Negative control			
Haemonchus spp.	65 (55.4 – 74.5)	58 (43 – 67.8)	53 (43 – 62.9)
Trichostrongylus spp.	35 (28 – 42)	38 (30 – 45.9)	44 (34.9 – 53)
Oesophagostomum spp.	0	4 (1.4 – 6.5)	3 (0.6 – 5.3)
Positive control			
Haemonchus spp.	70 (60.8 – 79.1)	47 (37 – 56.9)	50 (40 – 60)
Trichostrongylus spp.	26 (20.4 – 31.5)	44 (34.3 – 53.6)	38 (29.2 – 46.6)
Oesophagostomum spp.	4 (1.8 – 6.1)	9 (4.6 – 13.3)	12 (7.1 – 16.9)

Groups were treated with 250 mg/kg CVA (carvacryl acetate) or nCVA (nanoencapsulated carvacryl acetate). The positive control group was treated with 2.5 mg/kg monepantel (Zolvix®), and the negative control group received polymer matrix.

Discussion

The control of gastrointestinal nematodes that are resistant and/or multiresistant to synthetic anthelmintics is one of the great challenges of sheep and goat breeding worldwide. The use of nanostructured bioactive compounds has been highlighted as a promising alternative to be used in the integrated management of these nematodes (Mesquita et al., 2013; Ribeiro et al., 2013, 2015, 2017).

In the evaluation of the zeta potential of nCVA at different pH values (3 and 8), the nanoparticles were more stable at pH 3.2, where they had a zeta potential of +18.3 mV. The positive zeta potential of nCVA at acidic pH occurred due to the protonation of the amine groups of chitosan, increasing the positive charges on the surface of the nanoparticles. These positively charged nanoparticles can interact electrostatically with the negative charges of components present in the mucus that protect the mucosa of the stomach of monogastric animals, resulting in the mucoadhesion of nanoparticles (Ways et al., 2018). The acidic pH of ovine abomasum may promote the deprotonation of chitosan, adhesion of nanoparticles to the abomasal mucosa and prolonged release of CVA. However, for nanoparticles that do not adhere to the abomasal mucosa and reach the small intestine where the pH is basic, deprotonation of the amine groups of chitosan occurs and the carboxyl groups of gum arabic are ionized, which reduces the zeta potential of the nanoparticles and approaches the isoelectric point, where charge neutralization occurs (Abreu et al., 2008), and consequently, the nanoencapsulated biocomposite is rapidly released.

The *in vitro* profile of the sustained release of nCVA was evaluated at pH 3 and 8 to simulate the release of this biocomposite in the gastrointestinal tract of the small ruminants. In the present study, at pH 3, the system followed first-order kinetics, where the CVA was released in a constant proportion per unit time and the rate of elimination was proportional to the amount of CVA within the particle. In this sense, the total amount of drug released decreased with time (Costa, 2002). However, at pH 8, nanoparticles followed the Higuchi and Korsmeyer-Peppas model. The Higuchi model states that the system follows the diffusional behavior; Korsmeyer-Peppas aims to identify the type of diffusion through coefficient n. The coefficient n was 0.55, presenting a release with non-Fickian behavior (anomalous transport), where the release occurs by mechanisms of diffusion and relaxation of the polymer chain (Costa, 2002). The release of nCVA in the *in vitro* tests at abomasal and small intestine pH values allowed us to verify that the nanoparticles at acidic pH slowly released CVA, which is related to the protonation of chitosan amino groups. In the basic pH of the intestine, the amine groups of chitosan were deprotonated, leading to a faster release.

The LD_{50} of nCVA was 2,609 mg/kg, while the LD_{50} of CVA was 1,544.5 mg/kg obtained from the acute toxicity test in mice (Andre et al., 2016). The nanoencapsulation of CVA reduced toxicity and increased the toxicology safety of this biocomposite. The reduction in toxicity of a nanoencapsulated essential oil was also verified when evaluating the acute toxicity of *Eucalyptus stageriana* essential oil (LD_{50} = 1,603.9 mg/kg) and its nanoemulsion (LD_{50} = 3,495.9 mg/kg) (Ribeiro et al., 2015). The reduction in toxicity may be associated with the sustained release of these biocomposites, maintaining their therapeutic effect and reducing the occurrence of plasma peaks that may trigger toxic effects on animal cells. However, in the subchronic toxicity analysis, CVA and nCVA did not cause changes in hepatic and renal biochemical parameters. However, histopathological alterations were observed in these organs. Hepatic and renal cell hyperplasia was the mechanism of adaptation of these organs to the stress of metabolizing and excreting CVA daily for 28 days. However, these changes were reversible (Vasconcelos et al., 2007).

The anthelmintics most frequently used in the control of gastrointestinal nematodes in the Brazilian Northeast are benzimidazol, ivermectin and levamisole. The use of these drugs has promoted the selection of multiresistant nematode populations (Silva et al., 2018). The molecular characterization of *H. contortus* anthelmintic resistance isolated from experimental animals demonstrated that the population was resistant to benzimidazol and levamisole. A study carried out in Ceará state, Northeast Brazil found that the anthelmintic used by 89% of the farms was LEV (Santos et al. 2017).

Thus, the control of these populations of multiresistant nematodes is one of the obstacles to maintaining animal health, and the use of nanostructured bioactive compounds may be an alternative control. The efficacy of 250 mg/kg CVA on ovine gastrointestinal nematodes was 65.9% 16 days posttreatment (Andre et al., 2016). In the

present study, there was a reduction in the efficacy of CVA and there was no statistical difference ($p > 0.05$) between the value of epg in the treated and the control groups. Similar results were verified when evaluating the efficacy of the essential oil of free *Eucalyptus citriodora* and its nanoemulsion in that same period (Ribeiro et al., 2014). The efficacy of nCVA was similar to that of CVA, and there was a reduction in *Haemonchus* spp. L3 percentage and a resulting increase in *Trichostrongylus* spp. L3 in posttreatment coprocultures. The activity of CVA against *Haemonchus* spp. was likely improved by the encapsulation process with chitosan, which is a bioadhesive polymer that is used in drug delivery systems, by promoting a sustained release in acidic pH, increasing the bioavailability of CVA and hence potentiating anthelmintic efficacy. Similar results were found when evaluating the anthelmintic activity of chitosan-nanoencapsulated *E. staigeriana* essential oil on the parasite load of sheep infected with *Haemonchus* spp., *Trichostrongylus* spp. and *Oesophagostomum* spp., where only the parasite load of *Haemonchus* spp. was reduced (Mesquita et al., 2013). In addition, efficacy has been shown to vary according to nematode species, particularly when they inhabit separate digestive organs (Hoste et al., 2008).

The nanoencapsulation system was effective, presenting a nanometric size, zeta potential and sustained release in acidic pH, besides to reducing the toxicity of the biocomposite. Thus, studies evaluating the anthelmintic action of CVA in experimentally infected animals with gastrointestinal nematodes should be performed. In addition, the bioavailability assessment should be evaluated to analyze the sustained release of CVA.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Chromatograms of carvacryl acetate obtained by gas chromatography coupled with mass spectrometry. A. The detection of compounds at retention times of 20,472 - carvacrol (0.55%) and 22,799 - carvacryl acetate (99.45%); B. and C. mass spectra corresponding to carvacrol and carvacryl acetate, respectively.

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