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# Acaricides containing zein nanoparticles: A tool for a lower impact control of the cattle tick *Rhipicephalus microplus*

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ABSTRACT

Nanoformulations containing zein nanoparticles (ZN) can promote the stability and protection of molecules with acaricidal activity. The present study sought to develop nanoformulations with ZN associated with cypermethrin (CYPE) + chlorpyrifos (CHLO) + a plant compound (citral, menthol or limonene), characterize them, and verify their efficacy against *Rhipicephalus microplus* ticks. Additionally, we aimed to assess its safety in nontarget nematodes found in soil at a site subjected to contamination by acaricides. The nanoformulations were characterized by dynamic light scattering and nanoparticle tracking analysis. Nanoformulations 1 (ZN+CYPE+CHLO+citral), 2 (ZN+CYPE+CHLO+menthol), and 3 (ZN+CYPE+CHLO+limonene) were measured for diameter, polydispersion, zeta potential, concentration, and encapsulation efficiency. Nanoformulations 1, 2, and 3 were evaluated in a range from 0.004 to 0.466 mg/mL on *R. microplus* larvae and caused mortality > 80% at concentrations above 0.029 mg/mL. The commercial acaricide Colosso® (CYPE 15 g + CHLO 25 g + citronellal 1 g) was evaluated also from 0.004 to 0.512 mg/mL and resulted in 71.9% larval mortality at 0.064 mg/mL. Formulations 1, 2, and 3 at 0.466 mg/mL showed acaricidal efficacy of 50.2%, 40.5%, and 60.1% on engorged females, respectively, while Colosso® at 0.512 mg/mL obtained only 39.4%. The nanoformulations exhibited long residual period of activity and lower toxicity to nontarget nematodes. ZN was able to protect the active compounds against degradation during the storage period. Thus, ZN can be an alternative for the development of new acaricidal formulations using lower concentrations of active compounds.

## 1. Introduction

Parasitism by *Rhipicephalus microplus* ticks causes high economic losses that include decreased production efficiency, damage to leather, and transmission of hemiparasites that can cause diseases in cattle (Grisi et al., 2014; Andreotti et al., 2016; Cabezas-Cruz et al., 2016; Calvano et al., 2019).

The use of synthetic acaricides is currently the leading method of tick control. However, the indiscriminate use of these products has been causing selection of acaricide-resistant tick strains (Klafke et al., 2017; Rodriguez-Vivas et al., 2018; Vilela et al., 2020), and can also harm human and environmental health (Graf et al., 2004; Fagnani et al., 2011; Wang and Kannan, 2018). Many antiparasitics administered to animals present in feces and urine can contaminate the environment. Additionally, topically applied compounds can be washed by rain or even spilled into soil at the time of application, and inappropriate disposal of packaging, also is responsible for other important environmental entry points. Thus, beyond the negative impact on nontarget species in soil and feces, contamination can also affect other ecosystems, such as watercourses (Boxall et al., 2003; Sinclair et al., 2006).

Many plant species have biopesticide potential, and their compounds can replace synthetic pesticides or be part of new formulations of

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synthetic acaricides (Arafa et al., 2021). Substances such as citral, menthol, and limonene, which are the main compounds obtained from *Eucalyptus* spp, *Mentha* spp, and *Lippia* spp, respectively, can be used to improve the acaricidal activity of formulations containing synthetic compounds (Silva Lima et al., 2016). These compounds can also contribute to repellent activity against ticks and other arthropods of veterinary importance such as the horn fly (*Haematobia irritans*) and stable fly (*Stomoxys calcitrans*), other economically important pests of cattle worldwide (Silva Lima et al., 2016; Chauhan et al., 2018; Espinoza et al., 2021; Changbunjong et al., 2022). Furthermore, some plant compounds have low toxicity in nontarget organisms and contribute to the reduction of parasite resistance to chemicals due to multiple mechanisms of action on these parasites (Corrêa and Salgado, 2011; Chagas, 2015; Pavela and Benelli, 2016). However, there are limitations in the application of these products, due to their high volatility, low solubility in water, and low physicochemical stability, which cause rapid degradation and a short residual period (Chagas et al., 2014; Sousa et al., 2022).

Nanotechnology contributes to the physicochemical stability of formulations, and nanoencapsulation can improve the bioavailability and biological effects of active compounds, increase the apparent aqueous solubility, contribute to the slow release of active compounds, and protect them against premature degradation (Fricker et al., 2010; Kah and Hofmann, 2014). Zein, the main component of corn protein, is an important product in science and industry and has been reported as a good wall material for nanoencapsulation (Shukla and Cheryan, 2001; Sozer and Kokini, 2009).

We sought to develop nanoformulations with zein nanoparticles (ZN) associated with cypermethrin (CYPE) + chlorpyrifos (CHLO) + a plant compound (citral, menthol or limonene), characterize them, and verify their efficacy on larvae and engorged females of *R. microplus*. Additionally, we aimed to assess its safety in nontarget nematodes found in soil at a site subjected to contamination by acaricides.

## 2. Materials and methods

### 2.1. Location and experimental conditions

The nanoformulations were developed and characterized in the Environmental Nanotechnology Laboratory of Paulista State University (UNESP), Sorocaba Campus. Engorged *R. microplus* females were obtained through artificial infestations on cattle free from acaricidal treatment belonging to the Embrapa Southeast Livestock research unit (CPPSE), in São Carlos, São Paulo. Tick colony maintenance and acaricidal bioassays were carried out in the Laboratory of Veterinary Parasitology at CPPSE. The toxicological evaluation was carried out by the Research Group in Biochemistry and Toxicology in *Caenorhabditis elegans* (GBToxCE) of Federal University of Pampa, in Bagé , Rio Grande do Sul.

### 2.2. Reagents

The following products were used: cypermethrin (Sigma-Aldrich, Ref: 51991); chlorpyrifos (Sigma-Aldrich, Ref: NMIP1666A); citral (Sigma-Aldrich, Ref: C83007/Code: 5392405); menthol (Sigma-Aldrich, Ref: W266523/Code: 1002126617); limonene (Sigma-Aldrich, Ref: 183164/Code: 1001647352); zein (Sigma-Aldrich, Ref. 232–722–9); Pluronic F68 (Sigma-Aldrich, Ref.: P1300); ethanol (Sigma-Aldrich, Ref.: 493511); methanol (Sigma-Aldrich, Ref.: 1424109); chloroform (Sigma-Aldrich, Ref.: C2432); acetonitrile. HPLC grade, Sigma-Aldrich, Ref.: 34851); and cellulose filter membranes 30 kDa (Sigma-Millipore, Ref.: MRCF0R030).

### 2.3. Preparation of zein nanoparticles

ZN containing the active compounds was prepared using the anti-solvent precipitation method as described by Hu and McClements (2015). Briefly, zein (2% w/v) was dissolved in a hydroethanolic solution (85% v/v) and stirred overnight. The solution was then filtered through syringe filters (0.45 μm; Millipore, Burlington, USA) to remove insoluble particles. Then 56.25 mg of CYPE, 93.75 mg of CHLO, and 3.75 mg of the plant compound (menthol, citral or limonene) were added to the zein solution. An aqueous solution of Pluronic F68 (2% w/v) was also prepared, and the pH was adjusted to 4.0 using HCl 1 M. Next, the zein solution was rapidly added to 30 mL of the Pluronic solution using a syringe and the solution was kept under stirring. The solvent was removed at room temperature. After evaporation, the lost volume was compensated by adding water.

At the end of the preparation, three different nanoformulations had concentration of 5.125 mg/mL: Formulation 1 (1.875, 3.125, and 0.125, mg/mL of CYPE, CHLO, and citral, respectively); Formulation 2 (1.875, 3.125, and 0.125, mg/mL of CYPE, CHLO, and menthol, respectively); and Formulation 3 (1.875, 3.125, and 0.125, mg/mL of CYPE, CHLO, and limonene, respectively). ZN blank (nanoformulations without CYPE, CHLO or plant-based compound) was prepared following the same procedure (Supplementary Material – Table 1).

### 2.4. Quantification of active ingredients and determination of encapsulation efficiency

The active compounds were quantified using high-performance liquid chromatography according to the methods described by Figueiredo et al. (2022). The chromatographic column, mobile phase, and specific parameters for the analysis of each active compound can be seen in Supplementary Material - Table 2.

The encapsulation efficiency (EE) of the active ingredients was determined by the ultrafiltration/centrifugation method as described by Figueiredo et al. (2022).

### 2.5. Characterization of zein nanoparticles

The diameter, polydispersion, zeta potential, size distribution, concentration, and pH of all nanoformulations were monitored on days 0, 7, 15, 30, 60, 90 and 120 of storage.

#### 2.5.1. Diameter, polydispersion, and zeta potential

The nanoformulations were examined with a particle size analyzer (ZetaSizer Nano ZS90, Malvern®) for diameter, polydispersion, and zeta potential measurements as described by Figueiredo et al. (2022). The mean diameter distribution was obtained from the polydispersion index by dynamic light scattering (Govender et al., 1999; Venkatraman et al., 2005).

#### 2.5.2. Particle size distribution and concentration

Particle size distribution and concentration measurements were obtained by nanoparticle tracking as described by Anderson et al. (2013) and Figueiredo et al. (2022). Data were obtained using a NanoSight LM 10 cell (green laser, 532 nm) and an sCMOS camera using NanoSight (v. 3.1).

#### 2.5.3. Lipid stability (pH)

The pH of the nanoformulations was measured on days 0, 7, 15, 30, 60, 90 and 120 with a pHmeter (Jenway®, model 3510).

### 2.6. In vitro tests with nanoformulations on Rhipicephalus microplus

#### 2.6.1. Larval packet test (LPT)

The concentrations of 0.004, 0.007, 0.014, 0.029, 0.058, 0.116, 0.233 and 0.466 mg/mL of each nanoformulation diluted in distilled water were evaluated on *R. microplus* using the LPT protocol as described by FAO (1971). Briefly, filter papers (2 cm × 2 cm) were impregnated with 1 mL of each nanoformulation. About 100 larvae aged between 14 and 21 days were deposited on one of these papers, covered with another, and then placed in a filter paper envelope (7.5 ×8.5 cm), which was sealed with clips and kept in an incubator ( ± 27 °C and RH > 80%) for 24 h. The counts were performed using a vacuum pump with a barrier tip attached, counting the live and dead larvae. Results were expressed in triplicate for each concentration.

ZN blank (nanoformulations without, CYPE, CHLO or plant-based compound), negative control (distilled water), and positive control at 0.512 mg/mL were also evaluated. The commercial acaricide Colosso Pulverizaç ão® (Ourofino Saúde Animal, with each 100 mL containing CYPE 15 g, CHLO 25 g, and citronellal 1 g), was used as a positive control. Following the dose recommended by the manufacturer, the product was diluted in distilled water (1:800 (v/v)), obtaining a concentration of 0.512 mg/mL of the product (0.187, 0.312 and 0.012 μg/mL of CYPE, CHLO and citronellal, respectively).

#### 2.6.2. Residual activity period

The same procedures as in the previous section were performed for the highest concentration of the nanoformulations (0.466 mg/mL) and Colosso® (0.512 mg/mL), in triplicate. The filter paper was impregnated with the nanoformulation on day 0, and 100 tick larvae were deposited on one of these papers, covered with another, and then placed in a filter paper envelope. After 24 h, the number of live and dead tick larvae was counted. On day 3, the filter papers received 1 mL of distilled water and ± 100 new larvae, without adding any nanoformulations. After 24 h, the number of live and dead tick larvae was evaluated. The same procedure was performed on 7, 14, 42, and 49 days after the first paper impregnation with the nanoformulations on day 0.

#### 2.6.3. Adult immersion test (AIT)

*Rhipicephalus microplus* engorged females were collected from artificially infested cattle, washed with water, and dried with absorbent paper. Then they were divided into homogeneous groups (n = 10) that were weighed on an analytical scale (accuracy of 0.001 g) (model AUX220, Shimadzu, Kyoto, Japan) and immersed in the nanoformulations for 5 min at concentrations of 0.466 and 0.233 mg/mL, in triplicate. Similarly, ZN (blank), the negative control (distilled water), and the positive control (Colosso® at 0.512 mg/mL) were evaluated. After immersion, the excess product was removed with absorbent paper and the groups were placed in individual Petri dishes and kept in an incubator (27 °C; RH > 80%) for 18 days for oviposition (Drummond et al., 1973). After this period, the eggs were weighed, placed in syringes, and incubated again for another 15 days. Subsequently, the percentage of larvae hatched was determined by quantification by sampling as described by Figueiredo et al. (2018). The oviposition index, estimated hatching, reproductive efficiency index (REI), and efficacy of nanoformulations were evaluated according to Drummond et al. (1973).

### 2.7. Toxicological evaluation of the nanoformulations on Caenorhabditis elegans

#### 2.7.1. Caenorhabditis elegans strains

*Caenorhabditis elegans* of the Bristol N2 lineage (wild type) was obtained from the *Caenorhabditis* Genetics Center, kept in nematode growth medium (NGM) with *Escherichia coli* OP50 as a food source, under a constant temperature of ± 21 °C (Brenner, 1974). The cuticle of fertilized nematodes was removed by lysing them with a bleaching mixture (1% NaOCl; 0.25 M NaOH and distilled water) for the release of eggs. The eggs obtained were washed with M9 buffer (0.02 M $KH_2PO_4$, 0.04 M $Na_2HPO_4$, 0.08 M NaCl, and 0.001 M $MgSO_4$) and were kept in dishes containing M9 without bacteria for 14 h, until the larvae hatched (stage L1) (Stiernagle, 2006).

#### 2.7.2. Survival of Caenorhabditis elegans

A survival curve was plotted for the positive control Colosso®, through dilutions in distilled water, to obtain an adequate working range and determine the $LC_{50}$. Then, the survival curve was built at a concentration range of 0.005, 0.01, 0.03, 0.0, and 0.1 mg/mL. The concentration of 0.1 mg/mL, which showed highest toxicity against the nematode, was prioritized to compare with the nanoformulations. For the toxicity bioassay, 1500 larvae of *C. elegans* at the L1 stage were exposed to the nanoformulations, positive control (Colosso®), and negative control (distilled water) for 30 min in microtubes. Subsequently, the microtubes were centrifuged at 400 x*g* for 3 min to remove the solutions, and three washing steps with M9 buffer were performed. The supernatant was removed, and the larvae were placed in Petri dishes with NGM medium and *E. coli* OP50. After 48 h, live larvae in the L4 stage were counted and the percentage of the negative control was used to determine the survival rate. The assay was performed in duplicate and repeated at least three times.

#### 2.7.3. Brood size test

*Caenorhabditis elegans* exposed to the nanoformulations were evaluated throughout their reproductive period. One L4 from each treatment group was transferred to a new Petri dish containing the NGM medium and *E. coli* OP50. These same worms were transferred to new plates for four days, consecutively, until the end of the reproductive period. After 48 h, the number of hatched larvae was counted, and the treated groups were compared with the control. The assay was performed in duplicate and repeated at least three times.

#### 2.7.4. Body size test

The body size of the L4 was measured after 48 h of treatment. Ten worm samples from each group were placed on microscopic slides and anesthetized by freezing (−20 °C). Photographs were taken using a Nikon Eclipse 50i optical microscope (Tokyo, Japan) and their body size was measured using the ImageJ software (Madison, WI, USA). Means and percentages of the control group were used for the statistical analyses. This test was repeated at least three times.

### 2.8. Statistical analyses

The results of the characterization measures, stability, and pH of the nanoformulations, as well as the LPTs to verify the residual period, were submitted to analysis of variance (ANOVA) followed by the Tukey–Kramer test, using the GraphPad Prism v. 7® software. The results of LPT and AIT were submitted to one-way ANOVA and multiple comparisons by the Tukey test. The determination of the lethal concentrations ($LC_{50}$ and $LC_{90}$) of the nanoformulations was performed by probit linear regression, using the normal distribution, and the generalized linear model was used for binary data (logistic regression), with estimates of the parameters of these equations by maximum likelihood. Data were analyzed using the SAS® statistical package (version 9.1.3, 2010). The results obtained from the nanotoxicity tests were plotted and analyzed using GraphPad Prism v.7.04®. Data were expressed as mean ± the standard deviation of the mean and were statistically analyzed using one-way ANOVA followed by the Tukey post hoc test. Results with $p < 0.05$ were considered statistically significant after comparing the groups.

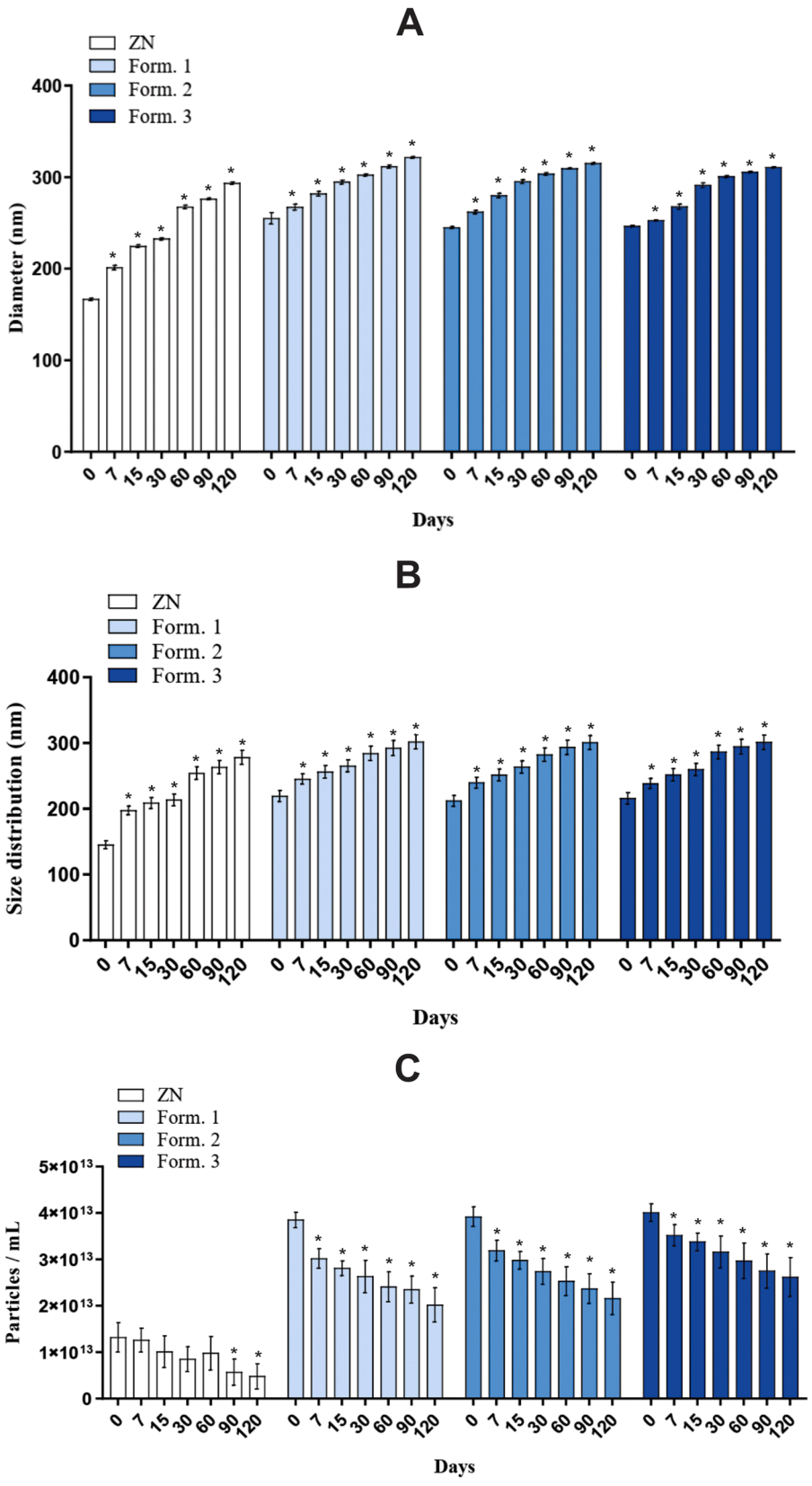


**Fig. 1.** Physicochemical characterization of nanoformulations. **A)** Mean particle diameter; **B)** Mean particle size distribution; **C)** Mean particle concentration. ZN = zein nanoparticles (blank). Form. 1 = ZN+CYPE+CHLO+citral, Form. 2 = ZN+CYPE+CHLO+menthol, Form. 3 = ZN+CYPE+CHLO+limonene. CYPE = cypermethrin (1.875 mg/mL); CHLO = chlorpyrifos (3.125 mg/mL); citral, menthol, limonene (0.125 mg/mL). * represents significant (Tukey test, $p < 0.05$) variations between ZN or the same formulation, as a function of time, compared to the initial time (0).

## 3. Results

### 3.1. Characterization and stability of zein nanoformulations

The LD values obtained through linear regression of the calibration curves were 1.05, 0.37, 12.58, 12.94, and 2.59 μg/mL for cypermethrin, chlorpyrifos, citral, menthol and limonene, respectively. For the same compounds, the limits of quantification (LQ) obtained were 3.49, 1.23, 41.95, 43.14, and 9.64 μg/mL, respectively. The analytical curves for each nanoformulation had a correlation coefficient (r) $> 0.99$. The encapsulation efficiency values (%) of all active ingredients were 96.48 $\pm$ 0.20%, 96.77 $\pm$ 0.21%, 97.44 $\pm$ 0.49%, 97.33 $\pm$ 0.48%, and 97.34 $\pm$ 0.50% for CYPE, CHLO, citral, menthol and limonene, respectively.

The average diameters obtained for the nanoparticles of each nanoformulation are represented in Fig. 1A. All particles had diameters below 300 nm at the starting time (0). The mean for ZN (blank) was 237.46 nm, while for nanoformulations 1, 2, and 3 the means were 290.64, 287.12, and 282.18 nm, respectively. A significant ($p < 0.05$) variation in the diameter of the nanoparticles were observed for nanoformulations after day 7.

At the initial time (0), the ZN (blank) showed polydispersion below 0.2 and varied between 0.2 and 0.3 during the evaluated period (Supplementary Material Fig. 1A). The mean for ZN (blank) was 0.24, while for nanoformulations 1, 2, and 3 the means were 0.25, 0.24, and 0.24, respectively (Supplementary Material Fig. 1A). Significant variations ($p < 0.05$) were observed for nanoformulations from day 7.

The average zeta potential of the particles increased over time, varying between $-$ 16.10 and $-$ 21.47 mV for ZN and between $-$ 1 and $-$ 9 mV for the formulations (Supplementary Material Fig. 1B). The average zeta potential for ZN was $-$ 18.9 mV, while for nanoformulations 1, 2, and 3 the means were $-$ 3.35, $-$ 4.28 and $-$ 6.10 mV, respectively (Supplementary Material Fig. 1B). Nanoformulations 1 and 3 showed significant variations ($p < 0.05$) from day 7, and formulation 2 from day 15 onwards.

The particle size distribution averages were below 300 nm at the initial time (0) (Fig. 1B). ZN (blank) had an average of 223 nm and nanoformulations 1, 2, and 3 had averages of 266, 263 and 264 nm, respectively (Fig. 1B). Significant variations ($p < 0.05$) were observed for nanoformulations from day 7.

The average concentration of ZN (blank) was $4.63 \pm 3.00 \times 10^{13}$ particles/mL, while for nanoformulations 1, 2 and 3 the means were $2.73 \pm 2.67 \times 10^{13}$, $2.84 \pm 2.68 \times 10^{13}$, and $3.20 \pm 3.03 \times 10^{13}$ particles/mL, respectively (Fig. 1C). The nanoformulations 1, 2, and 3 started to show significant variations ($p < 0.05$) from day 7, and in 120 days they had respective averages of $2.02 \pm 3.70 \times 10^{13}$, $2.16 \pm 3.50 \times 10^{13}$, and $2.62 \pm 4.20 \times 10^{13}$ particles/mL (Fig. 1C).

The pH remained below 5.0 during the period evaluated (Supplementary Material Fig. 1C). The average pH values obtained for nanoformulations 1, 2, and 3 were 4.3, 4.2 and 4.3, respectively, and significant variations ($p < 0.05$) were observed from day 7 (Supplementary Material Fig. 1C).

### 3.2. Tests on Rhipicephalus microplus

#### 3.2.1. Larval packet test (LPT)

The negative control (distilled water) and the ZN (blank) did not cause a deleterious effect on *R. microplus* larvae (Table 1). Colosso® did not cause mortality of larvae exposed at 0.004 and 0.008 mg/mL of the product. Mortality above 71.9% was observed when the larvae were exposed at 0.064 mg/mL. Total (100%) mortality was observed of larvae exposed to 0.256 and 0.512 mg/mL of Colosso®. Nanoformulations 1, 2, and 3 caused 100% mortality of larvae from concentrations of 0.233, 0.116, and 0.058 mg/mL, respectively, and caused mortality above 80% at 0.029 mg/mL. Even at lower concentrations, the nanoformulations had a larvicidal effect. At a concentration of 0.014 mg/mL, nanoformulations 1, 2, and 3 resulted in 66.1%, 47.1%, and 54.6% larval mortality, respectively, while the positive control (Colosso®) at 0.016 mg/mL caused 28.8% mortality. When the positive control no longer had a larvicidal effect, nanoformulations 1, 2, and 3, even at low concentrations, continued to cause larval mortality at 0.004 and 0.007 mg/mL, namely 5.7% and 13.3%; 13.7% and 38.2%; and 12.4% and 16.9%, respectively. In general, the nanoformulations produced better results than those obtained by the positive control, highlighting nanoformulation 3, which achieved such efficacy with the lowest concentration of active compounds.

The $LC_{50}$ and $LC_{90}$ values determined for each nanoformulation were: (a) Formulation 1: 0.0136 mg/mL (0.0108–0.0171 mg/mL) and 0.0352 mg/mL (0.0266–0.0538 mg/mL), respectively; (b) Formulation 2: 0.0115 mg/mL (0.0098–0.0133 mg/mL) and 0.0386 mg/mL (0.0314–0.0501 mg/mL), respectively; and (c) Formulation 3: 0.0117 mg/mL (0.0104–0.0132 mg/mL) and 0.0301 mg/mL (0.0256–0.0369 mg/mL), respectively. The $LC_{50}$ and $LC_{90}$ values obtained for Colosso® used as a positive control were 0.0319 mg/mL (0.0274–0.0373 mg/mL) and 0.1028 mg/mL (0.0827–0.1368 mg/mL), respectively.

#### 3.2.2. Residual activity period

The negative control (distilled water) and ZN (blank) did not cause larval mortality (data not shown). The nanoformulations presented high residual activity, although less than that shown by Colosso® at 0.512 mg/mL, which caused 100% larval mortality throughout the period evaluated (49 days) (Fig. 2). The nanoformulations were capable of killing 100% of larvae in 24 h, and values above 98.2%, 98.4%, and 97.8% in 2, 3, and 7 days, respectively (Fig. 2). The nanoformulations continued to have high efficacy of larval mortality on day 14, showing 93.7%, 97.0%, and 92.5% mortality, respectively, for nanoformulations

**Table 1**
Efficacy average of zein nanoparticles nanoformulations in relation to mortality (%) of *Rhipicephalus microplus* larvae.

| Concentration (mg/mL) | Form. 1 | Form. 2 | Form. 3 | Concentration (mg/mL) | Colosso® |
|---|---|---|---|---|---|
| 0.466 | $100 \pm 0.0^{Af}$ | $100 \pm 0.0^{Ac}$ | $100 \pm 0.0^{Ac}$ | 0.512 | $100 \pm 0.0^{Ae}$ |
| 0.233 | $100 \pm 0.0^{Af}$ | $100 \pm 0.0^{Ac}$ | $100 \pm 0.0^{Ac}$ | 0.256 | $100 \pm 0.0^{Ae}$ |
| 0.116 | $98.2 \pm 1.8^{Af}$ | $100 \pm 0.0^{Ac}$ | $100 \pm 0.0^{Ac}$ | 0.128 | $92.2 \pm 0.0^{Ae}$ |
| 0.058 | $99.6 \pm 0.4^{Af}$ | $98.3 \pm 1.7^{Ac}$ | $100 \pm 0.0^{Ac}$ | 0.064 | $71.9 \pm 0.0^{Bd}$ |
| 0.029 | $82.6 \pm 5.6^{Be}$ | $82.8 \pm 7.2^{Bc}$ | $91.8 \pm 0.4^{Ac}$ | 0.032 | $58 \pm 0.0^{Cc}$ |
| 0.014 | $66.1 \pm 16.2^{Cd}$ | $47.1 \pm 5.7^{Cb}$ | $54.6 \pm 8.8^{Bb}$ | 0.016 | $28.8 \pm 0.0^{Db}$ |
| 0.007 | $13.3 \pm 4.6^{Dc}$ | $38.2 \pm 5.4^{Db}$ | $16.9 \pm 2.8^{Ca}$ | 0.008 | $0.0 \pm 0.0^{Ea}$ |
| 0.004 | $5.7 \pm 2.5^{Eb}$ | $13.7 \pm 3.3^{Ea}$ | $12.4 \pm 2.0^{CDa}$ | 0.004 | $0.0 \pm 0.0^{Ea}$ |
| ZN (blank) | $0.0 \pm 0.0^{Fa}$ | $0.0 \pm 0.0^{Fa}$ | $0.0 \pm 0.0^{Da}$ | - | $0.0 \pm 0.0^{Ea}$ |
| Distilled water | $0.0 \pm 0.0^{Fa}$ | $0.0 \pm 0.0^{Fa}$ | $0.0 \pm 0.0^{Da}$ | - | $0.0 \pm 0.0^{Ea}$ |

Capital letters compare the average values in the columns (different concentrations) and lowercase letters compare the average values in the rows (different formulations). Different letters indicate significant differences by ANOVA and Tukey ($p < 0.05$). ZN = Zein Nanoparticles; CYPE = cypermethrin; CHLO = chlorpyrifos; Form. 1 = ZN+CYPE+CHLO+citral; Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene. ZN (blank) = zein nanoparticle without active compounds. Colosso® = commercial acaricide containing CYPE, CHLO, and citronellal.

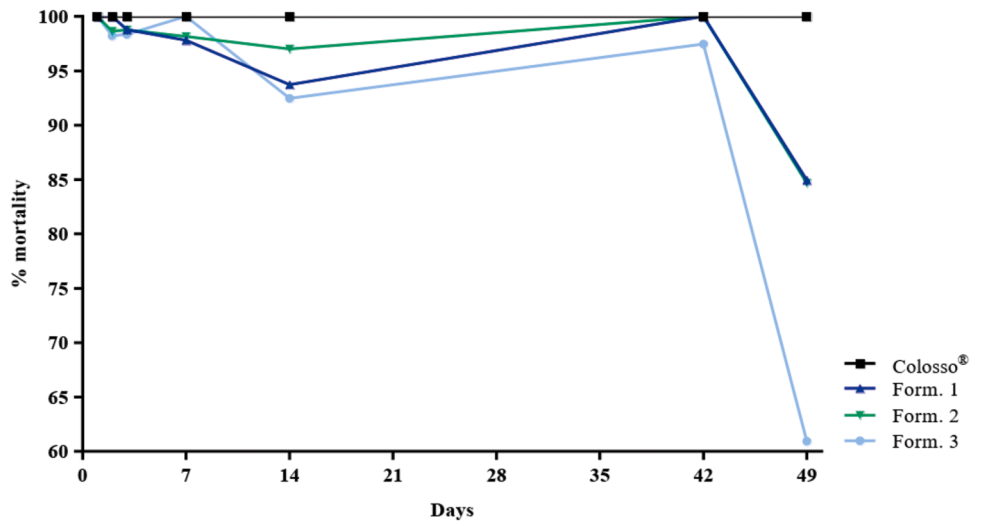


**Fig. 2.** Evaluation of the residual period of activity of the formulations on *Rhipicephalus microplus* larvae. Form. 1 = ZN+CYPE+CHLO+citral; Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene; Colosso® = commercial acaricide containing CYPE, CHLO, and citronellal. ZN = zein nanoparticles; CYPE = cypermethrin; CHLO = chlorpyrifos.

**Table 2**
Means of *Rhipicephalus microplus* engorged female weight (FW), eggs weight (EW), percentage (%) hatching of larvae, reproductive efficiency index (REI) and efficacy of zein nanoformulations on tick adults.

| Treatments (mg/mL) | | FW (g) | EW (g) | % Hatching | REI | Efficacy (%) |
|---|---|---|---|---|---|---|
| Distilled water | | $2.03 \pm 0.0^{a}$ | $1.02 \pm 0.0^{a}$ | $95 \pm 1.7^{a}$ | $95.3 \pm 8.1^{a}$ | - |
| ZN (blank) | | $2.03 \pm 0.0^{a}$ | $0.96 \pm 0.1^{ab}$ | $89.3 \pm 5.7^{ab}$ | $84.8 \pm 15.5^{ab}$ | $5.6 \pm 11^{ab}$ |
| Form. 1 | 0.466 mg/mL | $2.04 \pm 0.0^{a}$ | $0.68 \pm 0.1^{cd}$ | $70.7 \pm 8.3^{abc}$ | $47.4 \pm 16.6^{cd}$ | $50.2 \pm 13.3^{bc}$ |
| | 0.233 mg/mL | $2.01 \pm 0.0^{a}$ | $0.88 \pm 0.1^{abcd}$ | $89.7 \pm 2.9^{ab}$ | $78.4 \pm 9.7^{abc}$ | $17.8 \pm 7.8^{ab}$ |
| Form. 2 | 0.466 mg/mL | $2.02 \pm 0.0^{a}$ | $0.78 \pm 0.0^{abcd}$ | $73.7 \pm 10.5^{abc}$ | $56.7 \pm 11.3^{bcd}$ | $40.5 \pm 8^{abc}$ |
| | 0.233 mg/mL | $2.03 \pm 0.0^{a}$ | $0.89 \pm 0.1^{abcd}$ | $87.3 \pm 3.1^{ab}$ | $76.6 \pm 6.0^{abc}$ | $19.7 \pm 4.6^{ab}$ |
| Form. 3 | 0.466 mg/mL | $2.03 \pm 0.0^{a}$ | $0.63 \pm 0.0^{d}$ | $61 \pm 17.3^{c}$ | $38.1 \pm 11.5^{d}$ | $60.1 \pm 8.9^{c}$ |
| | 0.233 mg/mL | $2.03 \pm 0.0^{a}$ | $0.90 \pm 0.1^{abc}$ | $92.7 \pm 4.9^{ab}$ | $82.6 \pm 11.4^{abc}$ | $13.3 \pm 8.3^{ab}$ |
| Colosso® (0.512 mg/mL) | | $2.03 \pm 0.0^{a}$ | $0.71 \pm 0.1^{bcd}$ | $82.3 \pm 7.8^{abc}$ | $57.8 \pm 16.9^{bcd}$ | $39.4 \pm 12.54^{abc}$ |

*Different letters indicate significant differences by Tukey ($p < 0.05$). ZN = Zein Nanoparticles; CYPE = cypermethrin; CHLO = chlorpyrifos; Form. 1 = ZN+CYPE+CHLO+citral; Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene. ZN (blank) = zein nanoparticle without active compounds. Colosso® = commercial acaricide containing CYPE, CHLO, and citronellal.

1, 2, and 3 (Fig. 2). On day 42, they had higher efficacy: 100%, 100% and 97.5%, for nanoformulations 1, 2 and 3, respectively. There was a drop in larvicidal activity on day 49, resulting in 85%, 84.7%, and 60.9% for nanoformulations 1, 2 and 3, respectively.

### 3.2.3. Adult immersion test (AIT)

The results obtained by the AIT for the nanoformulations are summarized in Table 2. The negative control (distilled water) had no deleterious effect on engorged females, resulting in a high REI (95.31%). ZN (blank) had an REI of 84.79% and efficacy of 5.56%, with no difference ($p > 0.05$) when compared to the negative control group.

At the highest concentration evaluated (0.466 mg/mL), nanoformulations 1, 2 and 3 had acaricidal efficacies of 50.2%, 40.5% and 60.1%, respectively, while at 0.233 mg/mL the efficacies were 17.8%, 19.7% and 13.1%, respectively. The nanoformulations inhibited the egg laying by females also inhibited the larval hatching. Colosso® reached only 39.4% acaricidal efficacy on the engorged females at 0.512 mg/mL.

## 3.3. Toxicological evaluation of the nanoformulations

### 3.3.1. Survival of Caenorhabditis elegans

Survival curves were constructed from the evaluation of different concentrations of the positive control on the *C. elegans* larvae. Colosso® caused significant ($p < 0.05$) mortality at concentrations of 0.05 and 0.1 mg/mL (Fig. 3). The concentration of 0.1 mg/mL had the greatest

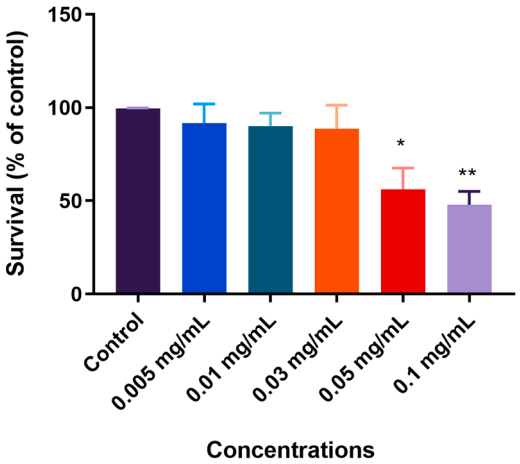


**Fig. 3.** Survival percentage of *Caenorhabditis elegans* exposed to different concentrations of Colosso® (commercial acaricide). * represents differences in relation to Control (distilled water) with * $p < 0.05$ and * * $p < 0.01$.

significance, so it was chosen for the performance of subsequent tests.

ZN (blank) did not interfere with the nematodes' survival (Fig. 4). Those exposed to the nanoformulations did not differ statistically ($p > 0.05$) from the control group, indicating attenuation of the toxicity

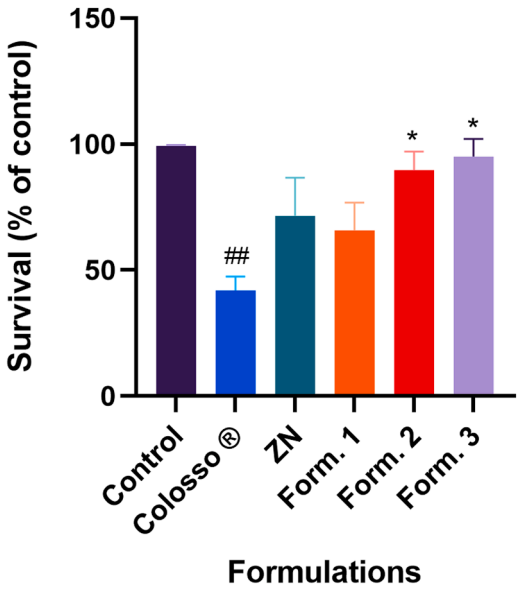


**Fig. 4.** Survival percentage of *Caenorhabditis elegans* exposed to Colosso® and nanoformulations. ZN = zein nanoparticles (blank); Form. 1 = ZN+CYPE+CHLO+citral, Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene. CYPE = cypermethrin; CHLO = chlorpyrifos. *represents differences in relation to Colosso® with $p < 0.05$, ## represents differences in relation to Control (distilled water) with $p < 0.01$.

of the CYPE, CHLO and plant compound association (Fig. 4). The survival percentages of nematodes exposed to formulations 1, 2 and 3 were 65.77%, 89.67% and 95%, respectively, higher than Colosso® (Fig. 4).

### 3.3.2. Brood size test

No significant changes ($p > 0.05$) were observed in the total *C. elegans* brood size in any tested group when the total number of larvae was evaluated in relation to the control (Fig. 5A). However, in the daily assessment of the brood size, there were oscillations in the number of larvae between the different formulations tested and especially over the days (Fig. 5B). Additionally, formulations 2 and 3 caused a decrease in the egg-laying of nematodes on the first day, but the same did not occur on the following days (Fig. 5B). Formulation 3 caused a decrease in the brood size (Fig. 5A), which suggests that this result might have been associated with a delay in the development of the nematodes.

### 3.3.3. Body size test

The nanoformulations 1 and 2 did not interfere with the growth of *C. elegans* larvae (Fig. 6). Colosso® and nanoformulations 1, 2, and 3 caused 20%, 11.59%, 18.43%, and 26.6% reductions in body size, respectively. Colosso® and formulation 3 were statistically different ($p < 0.05$) in comparison with the negative control (distilled water).

## 4. Discussion

This study demonstrated that it was possible to encapsulate three active compounds simultaneously in the nanoformulations. The possibility of encapsulating different active compounds in ZN has been reported previously (Bilenler et al., 2015; Chen and Zhong, 2015; Dai et al., 2018; Oliveira et al., 2018; Pascoli et al., 2018; Oliveira et al., 2019).

When using nanoparticles as a carrier system for bioactive compounds, it is important to consider possible interactions between the coating components and the active compounds (Pascoli et al., 2018). Thus, the characterization and stability measures applied to the nanoformulations provided important parameters for the evaluation of these interactions between the active compounds.

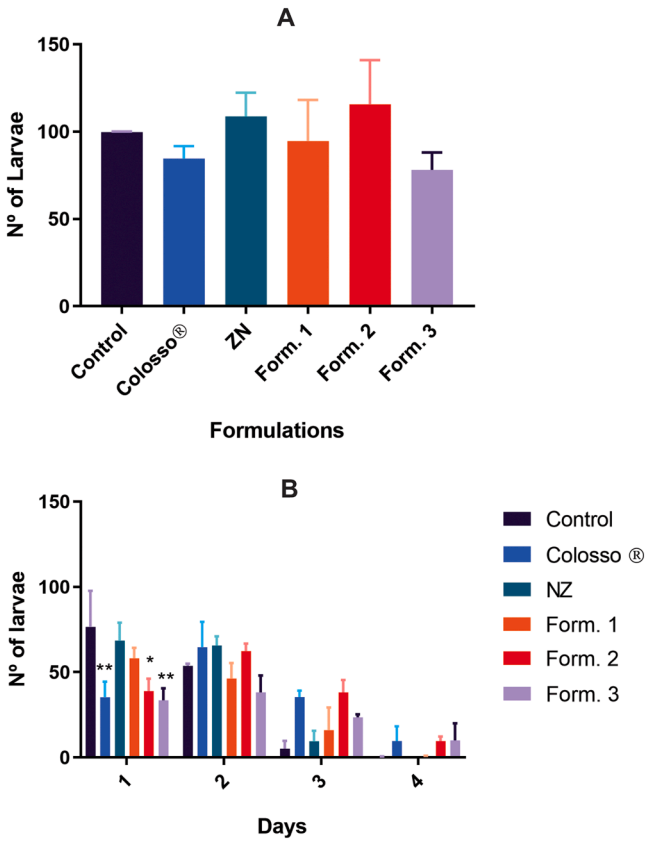


**Fig. 5.** Toxicology evaluation in *Caenorhabditis elegans* exposed to the nanoformulations. A) Total brood size and B) Daily brood size of *Caenorhabditis elegans* exposed to Colosso® and nanoformulations. ZN = zein nanoparticles (blank); Form. 1 = ZN+CYPE+CHLO+citral, Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene. Control = distilled water; CYPE = cypermethrin; CHLO = chlorpyrifos. * represents differences in relation to control with *$p < 0.05$ and * *$p < 0.01$.

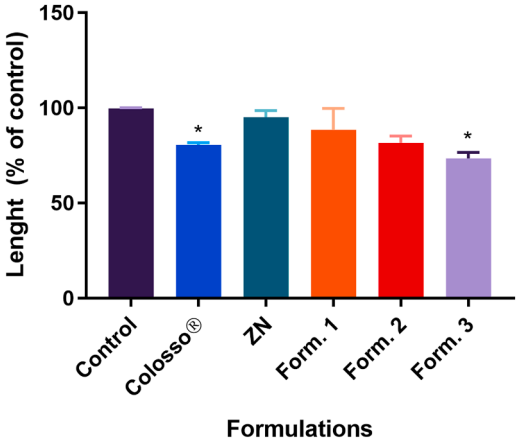


**Fig. 6.** Body size of *Caenorhabditis elegans* exposed to Colosso® and nanoformulations. ZN = zein nanoparticles (blank); Form. 1 = ZN+CYPE+CHLO+citral, Form. 2 = ZN+CYPE+CHLO+menthol; Form. 3 = ZN+CYPE+CHLO+limonene. Control = distilled water; CYPE = cypermethrin; CHLO = chlorpyrifos. *represents differences in relation to control with $p < 0.05$.

For each nanoformulation, the calibration curves obtained a correlation coefficient (r) greater than 0.99, demonstrating that the analytical methods used had high linearity, making it possible to estimate the

percentage of retention of nonencapsulated and free active compounds in the ultrafiltrate through the equation used. Furthermore, the nanoformulations presented EE above 96% for all active compounds, which corroborates the results reported by Oliveira et al. (2018, 2019), who obtained EE above 90% for nanoformulations containing encapsulated plant compounds, reinforcing the promising capacity of zein as a nanocarrier.

The mean diameters of ZN at the initial time (0), in the presence or absence of active compounds, were < 300 nm, but the mean diameters obtained by DLS (which evaluates particles together) were higher than the mean sizes obtained by NTA (which individually evaluates each of the particles), as also observed by Pascoli et al. (2020). A significant ($p < 0.05$) increase in mean values was observed for both the ZN blank and the nanoformulations, as a function of time (especially after 120 days), which indicates gradual aggregation of particles (Oliveira et al., 2019).

The ZN blank had lower averages for both diameter and particle size distribution, compared to the average values obtained by the nanoformulations containing the active compounds. These results agree with those of Parris et al. (2005), whose evaluation of the size distribution indicated that the zein nanospheres without active compounds had smaller mean diameters in relation to the diameters of the particles encapsulated with oregano essential oil (*Origanum vulgare*). This differs from the results of Oliveira et al. (2018, 2019), whose mean ZN values were higher compared to the nanoformulations, which may have been related to the quantity and characteristics of the encapsulated active compounds, since we encapsulated three active compounds simultaneously.

The polydispersion averages of the nanoformulations were between 0.2 and 0.3. These results were similar to those reported by Rosa et al. (2015) and close (between 0.3 and 0.5) to those obtained by Oliveira et al. (2018), demonstrating good stability of the nanoparticle system.

Oliveira et al. (2018) also obtained low averages of zeta potential (between −12 and −25 mV). Despite being low, these values do not necessarily indicate nanoformulation instability, since the surfactant Pluronic F68 (used in the preparation of zein nanoformulations) causes steric hindrance, being the main factor responsible for stabilizing the system. Thus, despite the low values found in the present study (between −1.25 and −21.47 mV), the surfactant Pluronic F68 was necessary to guarantee stabilization of the nanoformulations.

Mean concentrations (particles/mL) were lower for ZN blank compared to nanoformulations 1, 2 and 3. Oliveira et al. (2018) obtained similar results, attributing this to a possible lack of stability in relation to the nanoformulations. Our results seem to confirm this possibility, since a higher concentration of ZN was observed, which did not show significant ($p > 0.05$) variations for 90 days. Despite the consequent reduction in particles/mL during the entire storage period, the nanoformulations were able to protect the active compounds against degradation in solution, which is also a characteristic of stability aimed at acaricide formulations.

Solvents and surfactants in plant extracts, essential oils or acaricide formulations have the main purpose of solubilizing the active ingredient and promoting homogeneous distribution throughout the tick's cuticle. At the same time, they should act as excipients, having little or no effect, so that the results reflect the actual acaricidal activity of the substance evaluated (Chagas et al., 2003; Resende et al., 2012). Thus, since adjuvants can interfere with the efficacy of a particular active ingredient and generate unreliable results, we evaluated ZN and found that it did not act on *R. microplus* larvae and engorged females, as it was the negative control.

The nanoformulations' effect on *R. microplus* ticks was already expected, since CYPE and CHLO have been used in association to increase their activity on cattle ticks (Mendes et al., 2013; Machado et al., 2014). Rodrigues et al. (2018) evaluated Colosso® (CYPE+CHLO+citronellal) and Colosso FC-30® (CYPE+CHLO+fention) on larval stages of *R. microplus* at the concentration recommended by the manufacturer (0.512 mg/mL) and obtained > 95% acaricidal efficacy of both products. We observed a similar result for Colosso® at 0.512 mg/mL (containing 0.187, 0.312, and 0.012, mg/mL of CYPE, CHLO and citronellal, respectively), which was able to kill 100% of larvae. Colosso® did not cause mortality of larvae at concentrations 0.004 and 0.008 mg/mL but caused mortality of 71.9% when the ticks were exposed to 0.064 mg/mL. Nanoformulations 1, 2 and 3 caused 100% mortality when the larvae were exposed to concentrations of 0.233, 0.116 and 0.058 mg/mL, respectively, and caused mortality above 80% at 0.029 mg/mL. Even at lower concentrations, the nanoformulations had a larvicidal effect. At 0.014 mg/mL, nanoformulations 1, 2 and 3 resulted in 66.1%, 47.1% and 54.6% larval mortality, respectively, while the positive control (Colosso®) at 0.016 mg/mL caused only 28.8% mortality. When the positive control no longer had larvicidal activity, nanoformulations 1, 2 and 3, even at low concentrations, continued to cause mortality of the larvae exposed at 0.004 and 0.007 mg/mL, that is, 5.7 and 13.3; 13.7 and 38.2; and 12.4% and 16.9%, respectively. In general, the nanoformulations obtained better results than those obtained by the positive control, highlighting nanoformulation 3, which achieved such efficacy with the lowest concentration of active compounds. These results are similar to those reported by Figueiredo et al. (2022), who studied the acaricidal activity of formulations containing solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) associated with CYPE, CHLO and plant compounds (citral, menthol and limonene). The authors also reported that the addition of citral, menthol and limonene in the nanoformulations improved their acaricide action against cattle tick larvae, similar to the results obtained here for ZN.

Nanoformulations were also evaluated against *R. microplus* adults. At the highest concentration (0.466 mg/mL), nanoformulations 1, 2 and 3 showed acaricidal efficacies of 50.2%, 40.5% and 60.1%, respectively, and at 0.233 mg/mL, the respective efficacies were 17.8%, 19.7% and 13.1%. The nanoformulations influenced the inhibition of the laying egg by females, and the inhibition larval hatching. Colosso® reached only 39.4% acaricidal efficacy on the engorged females at 0.512 mg/mL, which indicated the acaricide resistance of the *R. microplus* strain to the compounds present in this product. These findings are supported by the results obtained by Rodrigues et al. (2018), who observed 100% mortality in *R. microplus* engorged females collected in 2018 in the municipality of Água Clara (Mato Grosso do Sul, Brazil) when these ticks were exposed to 0.512 mg/mL of Colosso® and Colosso FC-30®. Despite this, the nanoformulations had greater acaricidal efficacies than those obtained by Colosso®, so it is possible to suggest that ZN enhanced the acaricidal activity of the synthetic compounds, corroborating information related to the advantages of using nanocarriers, such as increased solubility and bioavailability (Fricker et al., 2010; Kah and Hofmann, 2014). Such aspects may be related to the combination of active compounds, greater absorption of the ingredients in the target organism, as well as greater protection against abiotic and biotic factors promoted by the carrier system. However, it should be noted that such hypotheses still need to be validated.

Plant compounds, even in small amounts, can synergistically contribute to increase the acaricidal and repellent potential of essential oils (Nerio et al., 2010; Silva Lima et al., 2016). Citral, menthol and limonene are present in several essential oils with proven efficacy on *R. microplus* larvae and engorged females (Cruz et al., 2013; Chagas et al., 2016; Silva Lima et al., 2016; Vinturelle et al., 2017). Thus, the acaricidal activity of the nanoformulations in the present study, promoted mainly by cypermethrin and chlorpyrifos, may also have received the contribution of plant compounds.

The nanoformulations demonstrated high residual period of activity, although it was lower than that observed for Colosso®. The fact that the nanoformulations showed higher activity after 42 days compared to that observed after 14 days may be related to the natural degradation of ZN. During the study period, nanoparticles might have degraded, releasing the active compounds that initially had action on the larvae, but

subsequently had lower action potential.

The toxicological evaluation of these nanoformulations in nontarget organisms such as *C. elegans* is an important factor to prevent the negative ecotoxicological impacts. Thus, we performed the toxicological evaluation through physiological parameters that are usually more evident in the nematode, such as body size, survival, and reproduction rate of *C. elegans* (Tuck, 2014; Woodruff et al., 2019). Colosso® caused significant ($p < 0.05$) mortality of the nematodes in relation to the control group when the worms were exposed to 0.1 mg/mL.

As mentioned above, Colosso® is composed of CYPE + CHLO + citronellal. Toxicological studies with these components on *C. elegans* were previously performed. XueFeng et al. (2019) observed that low doses of cypermethrin (0.005–3.2 mg/L) were able to cause damage to the reproductive biology of *C. elegans*, reducing the egg hatching rate and brood size. Furthermore, exposure to this active compound caused an increase in the expression of genes in the JNF and MAPK pathways, two important complexes related to apoptosis, inducing cell death. Shashikumar and Rajini (2010) also evaluated cypermethrin at concentrations of 5, 10 and 15 mM for 4, 12, 24 and 36 h on *C. elegans* and found that it caused increased expression of heat shock proteins, which are closely related to stress. Roh et al. (2016), in turn, demonstrated that exposure to chlorpyrifos for 4 h, by the passive dosage method in which the concentration was gradually increased (0.03, 0.06, 0.15 and 0.30 mg/L), caused damage to the cholinergic system and detoxification of *C. elegans*, effects that were stronger as the concentration of the active ingredient increased. For that evaluation, the authors analyzed the activity of the enzyme acetylcholinesterase (AChE) and the expression of genes related to the phase I detoxification system, cytochrome P540 (CYP), and thus there was a greater expression of CYPs, while AchE was inhibited depending on the concentration and time of exposure. Despite the toxicity of the cypermethrin and chlorpyrifos compounds used in the nanoformulations, our results demonstrate that ZN was able to reduce the effects of these active compounds on nematodes.

Some bioassays evaluating citral and menthol have shown only an antimicrobial effect of these compounds against the pathogenic bacteria analyzed, with no toxicity on the nematode *C. elegans* (Husain et al., 2015; Silva et al., 2015; Pedroso et al., 2019). On the other hand, a recent study evaluated the nematicidal effect of menthol and its dihydrophosphoric derivatives, and unlike these derivatives, it did not have a toxic effect (Nikitin et al., 2021). In the present study, formulation 3 (composed of limonene) caused the greatest changes in the body size and reproduction of the nematodes. This can be explained by the nematicidal effect of limonene, which interferes with the permeability of the worm's cuticle (Piao et al., 2020). However, this factor does not detract from the merit of formulation 3 from a toxicological point of view, since it had a positive effect against *R. microplus* larvae and adults at concentrations lower than that evaluated versus *C. elegans*.

## 5. Conclusions

It was possible to encapsulate the active compounds in ZN. Despite the consequent decrease in particles/mL during storage, the ZN was able to protect the active compounds against degradation in solution, which is also a characteristic of stability aimed for acaricidal formulations. The nanoformulations showed high larvicidal potential on *R. microplus*, resulting in high mortality rates. Nanoformulation 3 was the most prominent against larvae and had the greatest effect on engorged females of *R. microplus*. The three nanoformulations showed a high residual period of activity, with a drop in action potential over time due to the possible natural biodegradation of ZN. The evaluations of nanoformulations on *C. elegans* nematodes showed they had lower toxicity rates compared to the commercial acaricide Colosso®.

## Ethics statement

All protocols were evaluated and approved by the Embrapa Southeast Livestock Ethics Committee on Animal Experimentation (CEUA-CPPSE no. 01/2019).

## CRediT authorship contribution statement

**Amanda Figueiredo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Luís Adriano Anholeto:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Diego Faria Cola:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Rafaela Regina Fantatto:** Investigation, Methodology. **Yousmel Alemán Gainza:** Investigation, Methodology. **Isabella Barbosa dos Santos:** Investigation, Methodology. **Vicozzi Gabriel Pedroso Viçozzie:** Investigation, Methodology. **Avila Daiana Silva Ávilae:** Investigation, Methodology. **Leonardo Fernandes Fraceto:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing original draft. **Ana Carolina de Souza Chagas:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, review & editing.

All authors interpreted the results and substantively revised the manuscript, read, and approved the final manuscript.

## Declaration of Competing Interest

The authors warrant that there are no any conflicts of interests among authors and between authors and other people, institutions or organizations.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109918.

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