

Full Length Research Paper

Application of polymeric nanoparticles for controlled release of ethanolic extract of guapeva leaves (*Pouteria gardneriana* Radlk) against *Rhipicephalus (Boophilus) microplus* through *in vitro* studies

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The study was designed to evaluate the ethanolic extract acaricide action from *Pouteria gardneriana* Radlk specie adsorbed in chitosan nanoparticles for controlled release against *Rhipicephalus (Boophilus) microplus*. Leaves of *P. gardneriana* Radlk were collected, air-dried, ground and extracted with ethanol. Chitosan nanospheres were obtained by the phase inversion method. Conductometric titration, UV-Vis and FTIR analysis were conducted with the materials to evaluate the chitosan anchoring ability. *In vitro* test was performed using engorged females for each treatment, which consisted of the control, raw extract and three treatments diluted, with increasing concentrations of 0.2, 0.4 and 1%. The most effectiveness extract was for 0.4% concentration by observing reduction in the eggs mass compared to control group. The nanomaterial proved to be able to anchor and release the insecticide gradually in pH between 5 and 6 regions, which makes it feasible for use in cattle, prolonging the exposure time between the tick and acaricide.

Key words: Extracts, nanoparticle, controlled release, tick.

INTRODUCTION

Tick *Rhipicephalus (Boophilus) microplus* is a livestock parasite that is responsible for decrease in production and this has led to losses estimated to 2 billion dollars per year (Junior and Oliveira, 2005). Besides tick

parasitism affect meat and milk quality through inoculation of toxins to the host, it can transmit viral and bacterial diseases and protozoan infections (Massard and Fonseca, 2004). The chemical acaricides is the

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principal tool for tick control. In Brazil there are 6 principal acaricidal classes, but improper use of them has generated resistant strains. It can be explained by the selection and recombination of resistance genes in tick populations exposed to acaricides selection pressures (Hocayen and Pimenta, 2013).

Consumer demand for meat and milk free of chemical residues and the search for new molecules that present adequate toxicity for tick control are reasons for increased interest in plant species that have bioactive potential. In this context plant extracts of some species of Brazilian cerrado are targets for research (Rocha et al., 2011).

Guapeva is a large tree belonging to the family Sapotaceae and genus *Pouteria*. The most abundant secondary metabolites in this species are phenolic compounds, especially tannins (Rocha et al., 2011). The tannins present toxicological activity for some insect species, making guapeva interesting for studying and verification if its leaves extract is effective in *R. (B.)microplus* control (Monteiro et al., 2005; Silva et al., 2009).

Botanical acaricides have many advantages, they have low toxicity, their source is renewable depending of specie, besides being considered biodegradable (Agnolin et al., 2014). It is possible to prolong the action of the herbal products by using a material that can adsorb and release them in a controlled way. In this context the use of polymeric materials that have the capacity mentioned above can increase the product effectiveness (Saha and Ray, 2013).

Chitosan is a polymer material obtained from chitin deacetylation reaction, the second most abundant natural polymer in nature. This polymer has biochemical and physicochemical properties, considered biodegradable, biocompatible, non-toxic besides being a low-cost product. It presents various applications such as adsorption of ions, in the pharmaceutical and food industries, for functionalized precursor molecules, removal of heavy metals and dyes tributaries, among other applications (Witt et al., 2010).

Considering the bioactive potential of some species of Brazilian cerrado and their toxicological activity of tannins group as described by Scalbert (1991). The presence of tannins in the *Pouteria gardneriana* Radlk species makes this plant useful for use in control of *R. (Boophilus) microplus* tick. Hence, the present study aimed at verifying the efficacy of the leaves ethanol extract along with the controlled release technology by the chitosan nanoparticle surface.

MATERIALS AND METHODS

Collection and preparation of plant material

Plant leaves collection

The *Pouteria gardneriana* Radlk Guapeva leaves were collected

from Goiano Federal Institute (Instituto Federal Goiano) on the Rio Verde campus, Brazil (17° 48' 16" S, 50° 54' 19" W, 749 m altitude). After collecting them, they were separated from their stems and weighed. The collected plant materials were dried in an oven with forced air (CIENLAB, CE-220) at a temperature of 40°C and ground to fine powder by use of an electrical grinder (SPLABOR, SP-31) to facilitate the extraction.

Extraction

The fine ground powder of *P. gardneriana* Radlk leaves were weighed, extracted with ethanol and residues were filtered using Whatman filter paper no 2. The extract was concentrated using rotary evaporator (LABORGAS, LGI-52CS-1), obtaining the DCE (dried crude extract).

Nanospheres production

The chitosan solution was prepared with 5% (v/v) acetic acid. The mixture formed was under stirring until complete homogenization was achieved. Chitosan solution was introduced into a "spray dryer" system in which the sample passed through a nebulizer to form an aerosol. Other solution was prepared, NaOH at 10% in 100 ml of distilled water. The solution was standardized until getting a 0.995 correction factor. The aerosol dropped down in the NaOH solution mentioned above, which aerosol was gelled in the chitosan nanospheres form. The nanospheres, obtained were washed until reaching pH 7. Subsequently the nanospheres were exposed at room temperature for drying. All this method is known as phase inversion (Dias et al., 2008).

Anchoring and conductimetric titration

For raw extract anchoring on the chitosan nanosphere surface, 1 g of nanosphere and 0.5 g of raw extract were used. It was prepared 5% (v/v) hydrochloric acid (Sigma Aldrich) solution in 10 ml. 60 mL of distilled water in the solution was added. The solution was stirred for 24 h.

The conductivity and pH meter was used to check the solution conductivity and pH before and during the titration. The titration started at pH 2, then added gradually amounts (μL) of NaOH in the system. As pH and conductivity suffered variations by changing their values, aliquots were removed of each variation in conductivity for subsequent analysis in the UV-vis and FTIR. All procedures were performed in triplicate.

Fourier transform infrared (FTIR)

Analyses were performed by FTIR-ATR-NIRA- Frontier PerkinElmer simultaneous with the collection of aliquots during titration. It was observed the relationship between transmittance and wavelength in the spectra. For comparative purposes, were analyzed as aliquots as extract and chitosan solution at 5% and was observed and the shift of the energy band.

Ultraviolet in the visible region (UV-Vis)

During the conductimetric titrations, the collected aliquots at the points of greatest conductivity variation were analyzed separately in the UV-Vis Lambda 750 PerkinElmer, which did full scan spectrum (200 to 1000 nm) to obtain the samples gradients absorbance. For this analysis, we used 200 μl of each sample diluted in 4 ml of ethanol.

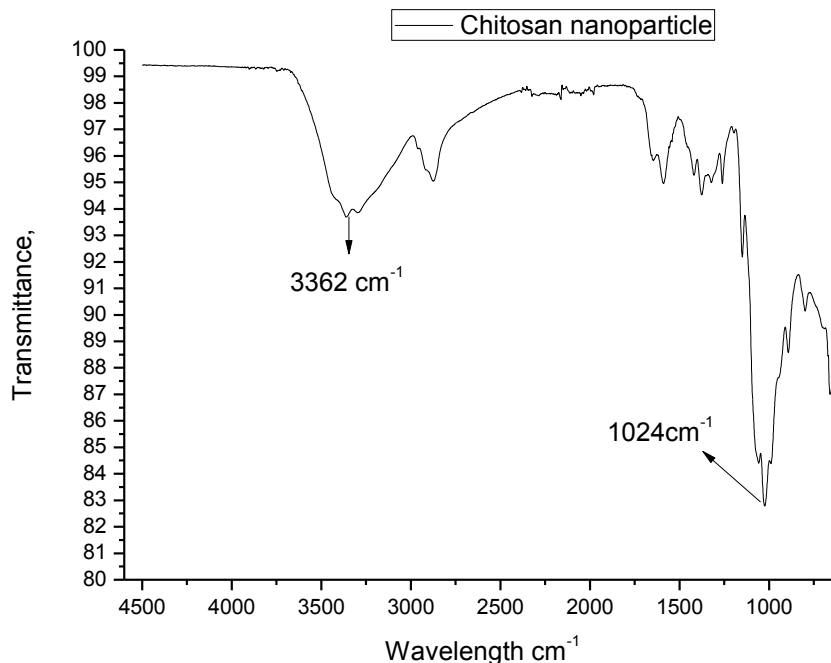


Figure 1. Chitosan nanoparticle spectrum in the FTIR, showing the principal bands and deformation peaks.

Analyses by scanning electron microscopy (SEM)

Nanoparticles were analyzed by Scanning Electron Microscope (SEM), JEOL JSM - 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging, using metallizer BALTEC SCD 050 for nanoparticles morphological determination, as described by Costa et al. (2006).

In vitro test

The *in vitro* test was performed according to Drummond et al. (1973). *R. (B.) microplus* engorged females were collected in naturally infested cattle from a farm near Rio Verde city, Goiás, Brazil. In the laboratory, they were washed with distilled water, dried and separated into 5 groups. The study consisted of five treatments, group I was control group (ethanol), group II corresponded to the raw extract and the groups III until V were the groups in which the raw extract was diluted in ethanol at concentrations of 0.2, 0.4 and 1%, respectively. In treatments III, IV and V were added 0.5 g of chitosan nanosphere and solutions were stirred for 24 h before the start of the tests.

The engorged female ticks were immersed in 20 ml of the solutions corresponding to each treatment for 5 min; this time was established by Leite (1988). After immersion, the engorged females were dried on paper towels and fixed by adhesive on Petri plates, previously identified. 6 engorged females for all groups were performed in triplicate. Next, these were taken to BOD incubator (28 ± 1°C, 80% humidity) for 14 days. After the laying period, the eggs of each tick female for all treatments were transferred to syringes without tips, sealed with cotton, and again sent to the BOD incubator (28 ± 1°C, 80% humidity) where they were kept for 26 days to evaluate for egg hatchability. The treatments were performed in triplicate and the results were obtained from averages. Data were submitted to analysis of variance and means compared by Tukey test at 5% error probability.

RESULTS AND DISCUSSION

The infrared radiation when absorbed by molecules is converted into molecular vibration energy. The spectrum reflects the vibration movement and is usually represented in bands form. The band intensity is measured by transmittance, which is the ratio for the electricity fed into the incident energy. When the molecules suffer changes in their structure, in this case, the adsorption of neighbor molecules by active sites, the new spectra has different characteristics. The bands displacement, increase electron density in the groups, increase the stretch peaks to the emergence of new bands (Aragão and Messaddeq, 2010).

Figures 1 and 2 show the nanoparticle, chitosan and guapeva extract spectra. It is observed bands and stretch peaks for chitosan in the 3362 and 1024 cm⁻¹ regions, such regions correspond to angular deformation of N-H, O-H bond and C-O stretching. In Figure 2, bands and deformation peaks in the 3338 and 1046 cm⁻¹ regions are observed, including some organic functions, for example, aromatic groups, alcohols, phenols, alkylphenols and carboxylic acid. Those regions found in Figure 2 can be explained by *P. gardneriana* Radlk's secondary metabolites, tannins, which there are aromatic rings with their substituents, especially hydroxyl.

In comparison with the figures mentioned, Figure 3 shows the extract spectrum anchored in the chitosan nanoparticle. The bands in the 3362 and 3338 cm⁻¹ regions of the chitosan and the extract, respectively, suffered displacement, as evidenced by the new band

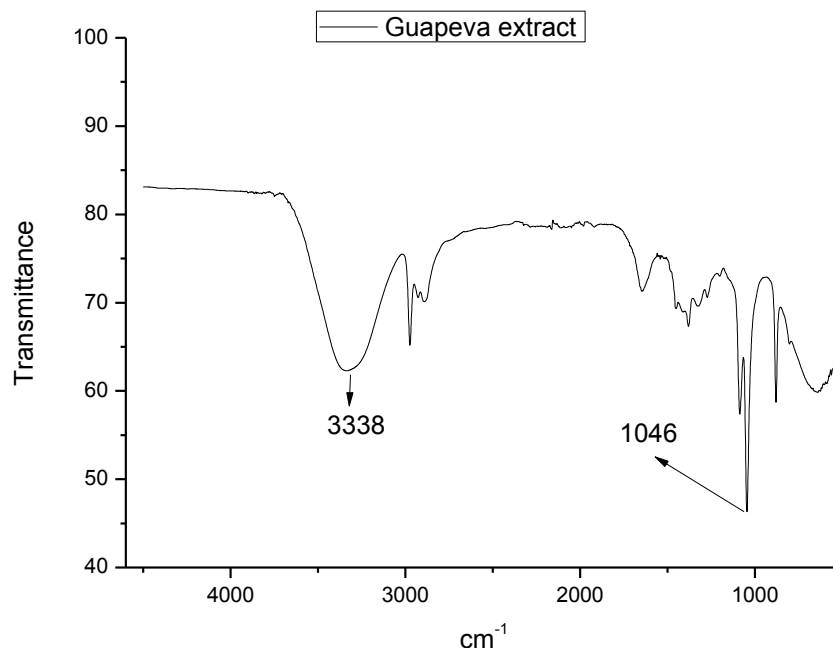


Figure 2. Guapeva extract spectrum in the FTIR, showing the principal bands and deformation peaks.

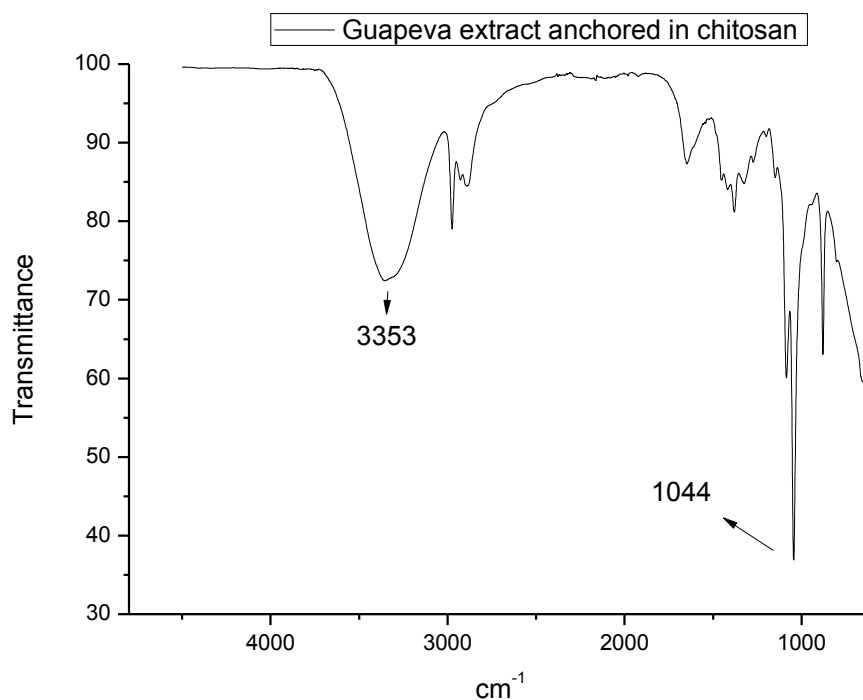


Figure 3. Extract spectrum anchored in chitosan in the FTIR, showing the principals bands and deformation peaks.

appearance in the 3353 cm^{-1} region. Regarding the stretch peak, it is observed in Figure 3 that 1044 cm^{-1} region there was an electron density increase, an

increase in stretch peak and consequently a decrease in the transmittance intensity due to the interactions occurring between polymer surface and the molecules

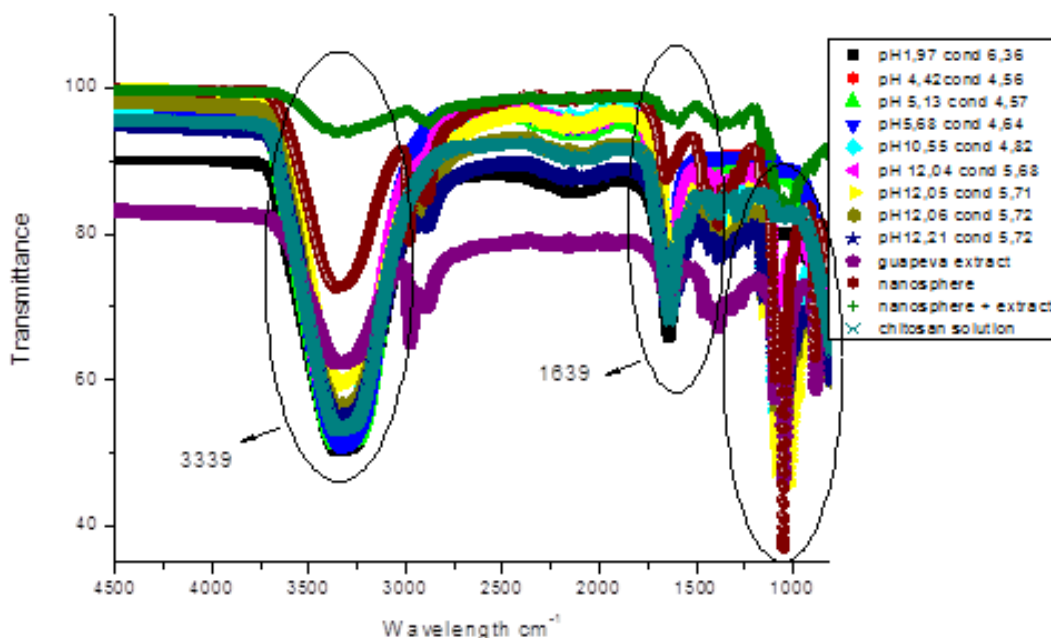


Figure 4. Infrared spectrum of the conductivity variation points obtained during conductimetric titration, the bands displacement according to pH solution.

present in the extract.

In addition, from infrared analyzes with the aliquots collected during conductimetric titration, it is clear that the infrared bands are displaced. Characteristic peaks in the 3500, 1600 and 1000 regions suffered increase and decrease in the transmittance intensity due to the interactions occurring on the polymer surface and the extract molecules, as shown in Figure 4. A higher electron density for spectra that were closer to neutral pH was observed, for example at pH 5.13 and 5.68. The electron density is linked to organic compound release by the polymer matrix. The FTIR analysis justify the appearance of other peaks found in the UV-Vis analyzes, because of interactions occurring during the release process promoting the formation of different spectra of those initial before conductimetric titration process.

Studies performed by Baroni et al. (2007) showed the same behavior of chitosan infrared spectra modified with glutaraldehyde and epichlorohydrin reagents adsorbing chromium ions. The authors demonstrated changes in the spectrum after ions adsorption, concluding that the ions adsorption affects amino group bonds, and the nitrogen atoms are the main adsorption sites for the ions (Baroni et al., 2007).

During the titration process, the solution conductivity changed according to the volume of NaOH added. For greater changes, aliquots were collected and analyzed in the UV-Vis. Depending on the pH, the behavior of the spectra analyzed took another form. The appearance of peaks is shown in Figure 5, besides an intensity increase

in the UV absorbance bands according to the pH of the solution. From the figure, it can be observed that the absorbance peaks reached 0.80 and 0.79 at 331 cm^{-1} for pH 2.07 and 2.08, respectively. This shows that in acidic medium chitosan is more protonated and through the largest absorbance peaks observed in the graph, it is stated that the adsorbed extract concentration is higher. The amino group protonation and deprotonation by the chitosan molecule are responsible for solution electronic conductivity. Moreover, higher or lower extract concentrations in the medium are shown by the conductivity difference.

In contrast, there was a peak intensity decrease at 300 cm^{-1} , when the solution was in pH 6.70 and 7.95, while the absorbance values found were 0.50 and 0.47, respectively. Another noted peak is in pH 6.25, at this point there is an increase in absorbance peak, so inferring for the solution ionic equilibrium. There was higher extract concentration released for the medium at this pH. As the solution becomes more alkaline, the solution tendency is occurring deprotonation of the amine group, thereby releasing the adsorbed material. Higher solution ion exchange is, higher will be the solution conductivity, it naturally occurs by neutralization reaction with acid-base, but with the extract adsorption by the polymer surface, the medium conductivity increases and decreases, as a result of adsorption and release effects of the active ingredient.

The relationship between conductivity and pH is demonstrated as shown in Figure 6. The solution

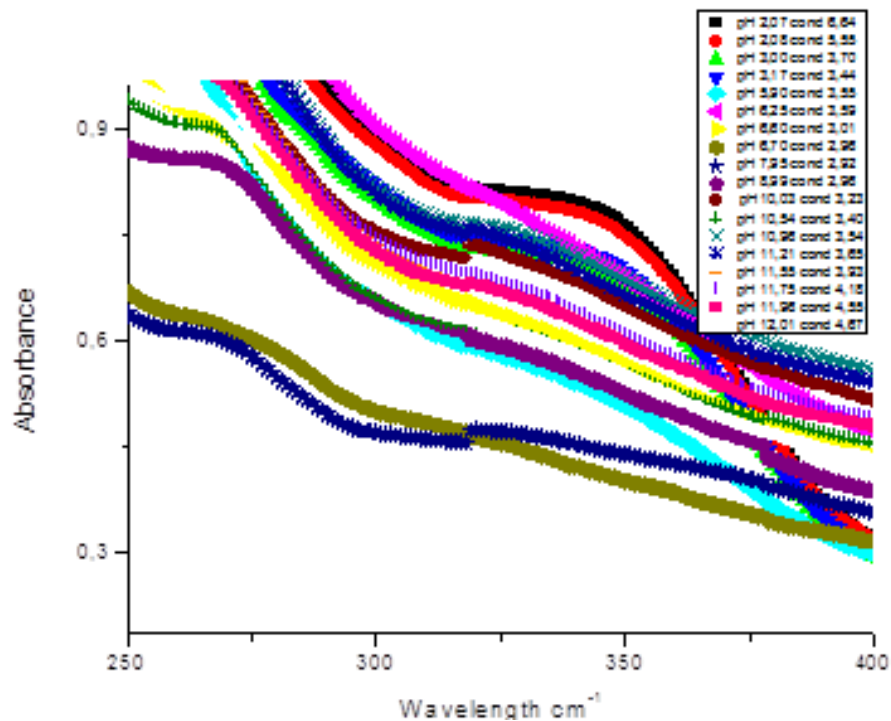


Figure 5. UV-Vis spectra represented by conductometric titration curves, shown the peaks intensity increase for same spectrums close to neutral pH.

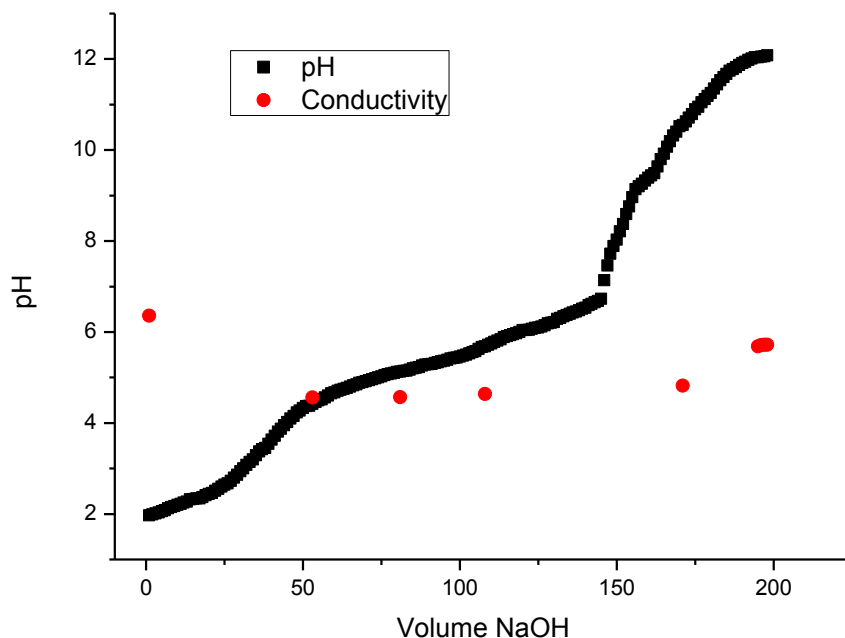


Figure 6. Solution pH and conductivity during the conductimetric titration.

conductivity decreases due to the fact that H^+ from the solution still in acid pH, is being consumed by the OH^- base, that is being added, but the pH ranged between 4.5 and 5, there was a significant increase in conductivity,

which could be explained by the extract molecule release from the biopolymer surface.

The images taken by SEM shows that the methodology used to produce nanoparticles was efficient, displaying

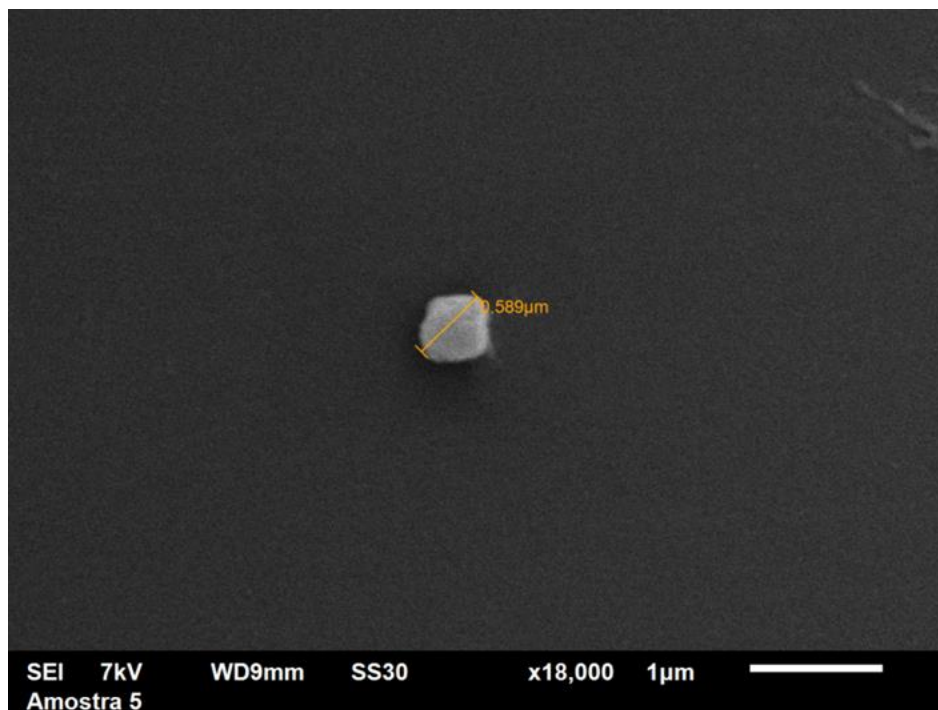


Figure 7. SEM image of nanoparticles produced from spray-dry system by gelation method in NaOH.

uniformity in particle size, about 589 nm, as shown in Figure 7.

The extracts toxicity determination associated with controlled release performed by the nanoparticles was determined by counting dead ticks in different concentrations of the extract anchored in the nanoparticles.

It was observed that for the raw extract treatment, 100% of extract concentration, occurred 100% of death for all individuals, so in higher concentration, the *P. gardneriana* Radlk extract acts as an effective acaricide.

After 14 days from beginning of bioassay, the oviposition eggs were collected and weighed separately. Eggs mass reduction is associated with the concentration used for treatments preparation. The graph below (Figure 8) relates the eggs mass reduction by the treatments used, using Except for treatment 2 (raw extract), the treatment IV, 0.4%, had the highest percentage of eggs mass reduction. Treatments A, B and C correspond to same the treatments, but done three times. Different treatments are expressed by roman letters.

Figure 9 represented by the control, the raw extract and 0.4% concentration treatments oviposition, numbered 1, 2 and 3, respectively. It is notable the eggs mass reduction in the treatment 4 compared with the control, this confirms the results of statistical tests performed, in which pointed to extract effectiveness. In addition, death for all individuals is represented in Figure 9-2, it is understood that at this concentration the extract active

molecules completely inhibit the parasite's cycle; however the use of this becomes unfeasible due to the amount of material used in its production.

Through the obtained results, it is possible to determine that the extract concentration of 0.4%, the eggs mass reduction, together with the number of deaths and non oviposition was significant at 5% Tukey test, which proves the efficiency of controlled release technique of *P. gardneriana* Radlk extract.

Conclusion

The spray-drying methodology is efficient in the nanoparticles production. The chitosan applicability as an anchoring material and in the controlled release study was effective. There was higher release extract between pH 5 and 6, promoting the active ingredient slow release, increasing the exposure time between the tick and acaricide. When raw extract treatment (treatment II) was used, all individuals died before spawning occur. For diluted treatments, as the concentration increased the egg mass decreased, thus the concentration (treatment IV) of 0.4%, the results were considered significant at the 5% Tukey test.

Conflicts of Interests

The authors have not declared any conflict of interests.

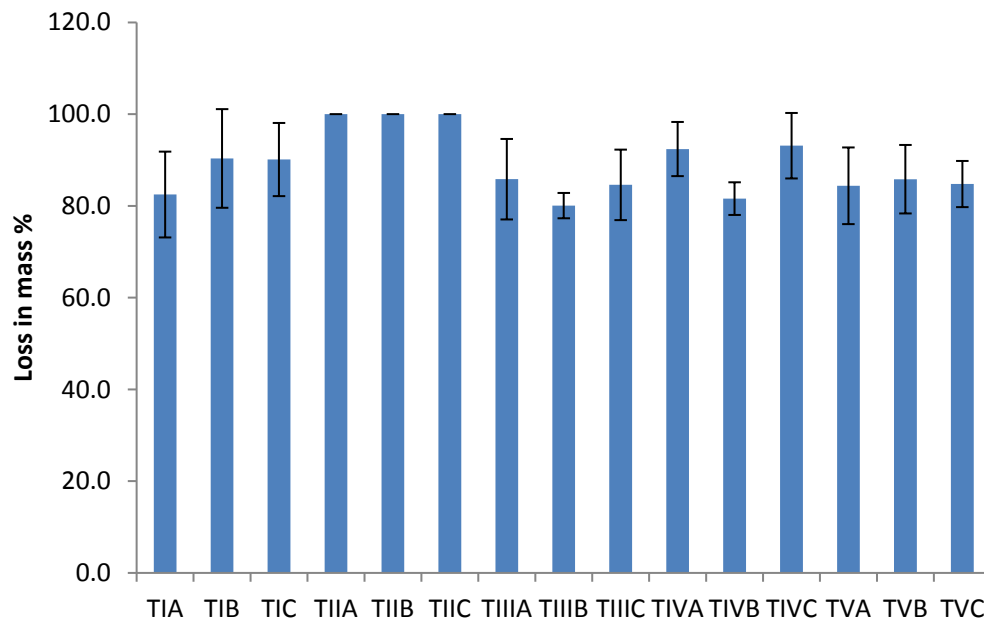


Figure 8. Reduced rate representation in percent of the eggs mass after 14 days from the beginning the treatment.

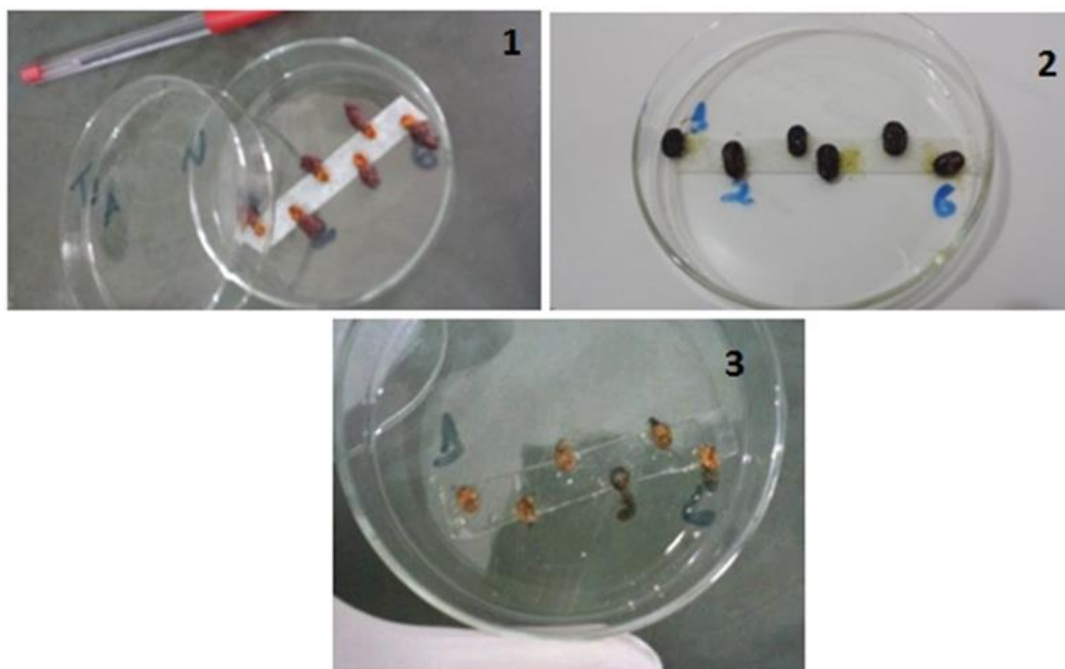


Figure 9. Control treatment, raw extract and concentration of 0.4% oviposition after 14 days from the beginning of the bioassay.

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