Association of zein nanoparticles with botanical compounds for effective pest control systems

Short running title: Botanical compound-loaded zein nanoparticles: a promising system aiming pest control

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Abstract

BACKGROUND: Botanical compounds from plant species are known to have pesticidal activity and have been used in integrated pest management programs. The varied spectrum of pesticidal action of these compounds can also avoid selection of resistance in pest populations. In this study, mixtures of the botanical compounds geraniol, eugenol and cinnamaldehyde were encapsulated in zein nanoparticles to improve their stability and efficiency. Biological effects of the nano-scale formulations of the botanical compounds were evaluated against two agricultural pests - the two-spotted spider mite (*Tetranychus urticae*) and the soybean looper (*Chrysodeixis includes*). RESULTS: The formulations were stable over time (120 days) with a high encapsulation efficiency (>90%). Nanoencapsulation also provided protection against degradation of the compounds during storage and led to a decrease in toxicity to non-target organisms. The release of the compounds (especially eugenol and cinnamaldehyde) from the nanoparticles was directly influenced by temperature, and the main mechanism of release through diffusion-based process. Nanoencapsulated compounds also showed superior efficiency than the emulsified compounds in terms of repellency and insecticidal activity. CONCLUSION: The findings of this study indicate that the convergence of botanical compounds with nano-scale formulation has the promise to improve efficacy for their sustainable use in integrated pest management in agriculture.

Keywords: botanical pesticides, nano-scale, environmentally friendly formulations

1. Introduction

There have been enormous scientific and technological changes (use of pesticides and fertilizers, mechanization of production, transgenesis) in agriculture since the 40's. As a result, food production has increased significantly [1]. Despite the great advancements, such practices have also brought several health and environmental impacts (soil and water contamination, toxicity to non-target organisms) [2]. In this context, there is an increasing emphasis on development of practices, methods and technologies that can contribute to increasing safety and sustainability of agriculture [3].

Botanical pesticides have been sought as an important tool for sustainable agriculture. These compounds are produced in the secondary metabolism of various plant species to form a defense against pests and diseases [4]. Being degradable, these compounds generally present minimal adverse effects on human and animal health and the environment. With few exceptions, they can therefore be considered safer than most synthetic pesticides [5].

Geraniol is a compound derived from different essential oils (citronella, palmrose, among others), and is classified as an acyclic alcohol having a vapor pressure of 2.21 x 10^{-2} mm Hg at 25ºC, water solubility of 100 mg.L^{-1} and boiling point at 230° C. Several applications of geraniol are reported in literature, including in the control of agricultural pests [6–9]. Eugenol, the main component of clove essential oil, belongs to the chemical class of phenylpropanoids. It has a vapor pressure of 2.89 x 10^{-2} mm Hg at 25°C, solubility in water of 1.460 mg.L^{-1} and a boiling point of 225°C. Due to its anesthetic properties, it is used as pain relief agent in dental applications[10]. In addition to bactericidal and antifungal properties [11], it is also known for pest control properties [12,13]. Cinnamaldehyde (vapor pressure 3.2 x 10^{-2} mm Hg at 25°C, solubility in water of 1,420 mg.L^{-1} and boiling point of 246°C) is also a phenylpropanoid found in essential oil of cinnamon bark (*Cinnamomum zeylanicum* J.Presl) and other *Cinnamomum* spp. It is known for antifungal as well as pest control properties [14–16].

The use of a combination of different compounds from plants is an important strategy to enhance biological activity and to develop novel formulations that have a mixture of active principles that are not normally present together in one plant [17]. For example, a mixture of geraniol and cinnamaldehyde in equivalent proportions does not occur naturally, since the compounds originate from different plants. Therefore, this strategy may also contribute towards increased effectiveness (because of a combined efficacy underpinned by different modes of action) and retarding the development of

resistance by pests [18]. Despite such potentials of natural compounds, the existing agricultural applications also face certain limitations. Natural substances are generally sensitive to degradation by light, humidity and temperature in the field [19].

In this context, innovative formulations based on nanoencapsulation have been shown to improve stability and efficacy of natural compounds [20]. Protection against premature degradation coupled with sustained release and increased solubility of active compounds have been reported in numerous studies for nanoencapsuled botanical pesticides [9,21–23]. Such nanostructured systems can be produced from different matrices (natural and synthetic). A particular example is zein, which belongs to a class of prolamins, and is the main storage protein of maize that makes up around 50% of the total protein content. Among its desirable properties are: high coating capacity, biodegradability and biocompatibility. Zein is extensively investigated in the production of biodegradable nanoparticles, including encapsulates of botanical pesticides [24].

In view of this context, the objective of the present study was to develop nanopesticide formulations that contain mixtures of botanical compounds that are known to be active against insect pests (geraniol+eugenol and geraniol+cinnamaldehyde). The study used zein as the biodegradable matrix for nano-scale encapsulation. The nanostructured carrier systems were prepared and characterized for stability and rate of release of the compounds. Biological efficacy was evaluated in terms of cytotoxicity against two cell lines, and two species of agricultural pests: the two-spotted spider mite (*Tetranychus urticae* Koch) and the soybean looper [*Chrysodeixis includes* (Walker, 1858)]. The approach adopted in this study is likely to contribute towards the development of safe and sustainable pest control systems for use in agriculture.

2. Materials and Methods

2.1. Materials

Geraniol (GRL), Eugenol (EGL), Trans-cinnamaldehyde (CND), Zein and Pluronic F-68 were obtained from Sigma-Aldrich, EUA. Ethanol was purchased from Labsynth (Brazil). Acetonitrile (Grade HPLC) was obtained from J.T. Baker (USA). Other reagents (analytical or higher) were purchased from local vendors.

2.2. Preparation of Zein nanoparticles

Zein nanoparticles containing the actives were prepared according to the anti-solvent precipitation method described by (Hu e McClements, 2014) [25], with certain modifications. Initially, a solution of zein (2% w/v) was prepared in hydroethanolic solution (85% v/v) and stirred overnight. The zein solution was then centrifuged for 30 minutes at 4,500 rpm and subsequently subjected to a heat treatment (15 minutes at 75°C). Finally, the solution was filtered through syringe filters (0.45 μm - Milipore). To prepare the particles containing the active compounds 600 mg of each active were added to 10 mL of the zein solution. Formulations containing the geraniol mixture with eugenol (NP_GRL + EGL) and the mixture of geraniol and cinnamaldehyde (NP_GRL + CND), both formulations containing 2% (w/v) of each active compound, were prepared. An aqueous solution of 1% Pluronic F68 (w/v) (Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) surfactant was prepared and pH was adjusted to 4. With the aid of a syringe the zein solution (10 mL) was rapidly injected into the solution of Pluronic F68 under magnetic stirring. The colloidal dispersion was stirred for ethanol evaporation (room temperature). Control nanoparticles were also prepared without the addition of the active compounds in zein solution. The loss of active compound(s) during the preparation process was investigated by high performance liquid chromatography (HPLC).

2.3. Physicochemical characterization of zein nanoparticles

Analysis of size distribution and polydispersity was performed using the dynamic light scattering (DLS) technique. The zeta potential was determined by the microelectrophoresis method. For both techniques a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) was used at a fixed angle of 90° and 25°C, the samples were diluted about 100 to 500 times. In addition, the nanoparticle tracking analysis technique was used to measure size distribution and nanoparticle concentration. For this, NanoSight

LM 10 cell (green laser, 532 nm) and a sCMOS camera controlled by NanoSight v. 3.1 were used. The results were expressed as the average of three determinations. The formulations were stored in amber bottles at room temperature and their stability was investigated as a function of time (after 0, 15, 30, 60, 90 and 120 days).

The morphology of the nanoparticles was investigated by atomic force microscopy (AFM). For this, the nanoparticles were diluted (2,000 times) and deposited on silicon plates that were dried in a desiccator. The analysis were performed using an atomic force microscope Easy Scan 2 Basic BT02217 (Nanosurf, Switzerland), operated in contactless mode with the TapAl-G (BudgetSensors, Bulgaria) cantilevers and a scan rate of 90 Hz. The images (256x256 pixels, TIFF format) were captured in time mode and analyzed using Gwyddion software.

2.4. Quantification of botanical compounds and determination of encapsulation efficiency (EE)

The quantification of the botanical compounds was carried out using high performance liquid chromatography (HPLC). For geraniol, a Phenomenex Gemini C18 reverse phase column (150 mm×4.6 mm, 5.0 µm) maintained at 30°C was used, the mobile phase was composed of acetonitrile: water (60:40, v/v) and flow rate of 1 mL.min^{-1}. The injection volume was 100 µL and the wavelength of the detector was set at 210 nm. For eugenol and cinnamaldehyde compounds, Phenomenex Kinetex C18 reverse phase column (150 mm×4.6 mm, 3.0 µm) was used. For eugenol, the mobile phase was composed of acetonitrile: water (50:50 v/v), whereas for cinnamaldehyde it was methanol: water (65:35 v: v), at a flow rate of 1mL.min^{-1} for both. The wavelength for the detection of the compounds was set at 210 nm and the injection volume was 100 µL.

It is noteworthy that all chromatographic analysis were performed in a UltiMate 3000 system (Thermo Scientific), operated by Chromeleon 7.2 software, which was used for the acquisition and analysis of the chromatograms. All analytical curves showed correlation coefficients (r^2) higher than 0.99.

The ultrafiltration/centrifugation method was used to quantify botanical compounds encapsulated in zein nanoparticles [9]. The technique is based on the use of Microcon 10 kDa regenerated cellulose ultrafilters (Millipore), which allows the passage of only the non-encapsulated substances. Thus, the difference between the quantity initially added and the quantity not encapsulated gives the encapsulation efficiency (EE). It should be

noted that the total amounts of botanical compounds (100%) present in the formulations were calculated considering the total amount added minus any losses during the preparation process.

2.5. Release assays and assessment of release mechanisms

The *in vitro* release assay was performed according to Chang et al. (2017)[26] with some modifications. The nanoparticle suspension (2 mL) containing the botanicals was placed in dialysis membrane bags (1 kDa exclusion pore, SpectraPore) and immersed in 100 mL solution of 3% Pluronic F68 (w/v). Over time, aliquots were collected and subjected to HPLC quantification. The containers were kept closed to avoid losses by evaporation and were only opened during sampling (in triplicate). In order to investigate the influence of temperature on the release of the actives from the nanoparticles, the tests were performed at three different temperatures (20, 25 and 30°C). The release data were submitted to mathematical modeling using the order zero, first order, Higuchi and Korsmeyer-Peppas models to investigate the mechanism of release of the active substances through the nanoparticles [27].

2.6. Cytotoxicity assays

Cytotoxicity assays were conducted according to the cell viability method, measured in terms of reduction of tetrazolium dye (MTT test) [28]. For this, two cell lines were used: pulmonary fibroblast permanent cell line (v79) and a fibroblast cell line (3T3). Cells were maintained in continuous culture using DMEM medium and 10% fetal bovine serum. A supplementation with 100 IU mL^{-1} of penicillin and 100 µg.mL of streptomycin sulphate was added and cells were maintained at pH 7.4, 37 °C, under humidified atmosphere with 5% CO_2. To perform the assays, the plates containing $1x10^4$ viable cells were incubated (37 ºC) for 48h until semiconduction, and the cells were then exposed (for 24h) to the following solutions: zein nanoparticles (NP), zein nanoparticles containing geraniol and eugenol (NP_GRL+EGL), geraniol and eugenol emulsified with Pluronic (EM_GRL+EGL), geraniol and cinnamaldehyde emulsified with Pluronic (EM_GRL+CND) and zein nanoparticles containing geraniol and cinnamaldehyde (NP_GRL+CND). The absorbance was measured using a plate reader at 570 nm, and cell viability was determined in triplicate and results expressed in terms of percentage means and standard deviation.

2.7. Biological activity assays

2.7.1. Repellency against the two-spotted spider mite (*Tetranychus urticae*)

The bioassays with *T. urticae* were carried out in the Laboratory of Acarology (UNESP/FCAV, Jaboticabal Campus). First stage of the experiment was performed in a greenhouse (mean temperature 25.3°C, 79.3% relative humidity). Initially, seeds of *Canavalia ensiformes* (L) DC. were planted in pots of 5 L capacity, containing soil, sand and bovine manure (1:1:1 w/w/w) as substrate. After germination of the seeds, only one plant per pot was kept. Thirty (30) days after germination, the formulations (treatments) were applied to the plants. For each treatment, three (3) plants were distributed in a completely randomized design in the greenhouse. The treatments comprising 5 $mg.mL^{-1}$ of active compound were applied with manual sprayer (500 mL capacity) until complete coverage of the plants. The products were carefully applied so that all top and bottom surfaces of the plants were covered with the product. On average 15 mL of each formulation per plant was used. After 12, 24, 72, 120 and 168 h, leaflets were removed from the plants, placed in plastic trays, and sent to the laboratory. Circular leaf arenas (2.5 cm diameter) were removed. The leaflets were placed in Petri dishes 9.0 cm in diameter x 2.0 cm in height on a layer of moist foam and hydrophilic cotton. For each treatment, eight (8) arenas were used, corresponding to eight (8) repetitions. In the sequence, 10 adults female of the two-spotted spider mite were transferred to each arena with the aid of a brush and a stereoscope microscope (Zeiss® Stemi DV4). Evaluation of the live and dead mites was carried out as well as of those trapped in the glue barrier 24 hours after the transfer of mites to the arenas.

2.8. Soybean looper (*Chrysodeixis includes*) assays

Bioassays with *C. includes* were carried out at the Laboratory of Microbial Control of Arthropods-Pest (UNESP/FCAV, Jaboticabal campus). Aliquots of 800 µL (sufficient to wet the whole diet surface) of the nanoparticle formulations and of the emulsified compounds (GRL, EGL and CND) were applied to the artificial diet discs (4.8 cm^3), and packed in clear acrylic plates (10 cm x 1.2 cm). The control diet disc was treated with the same volume of sterilized water. After complete drying, ten (10) second instar larvae were transferred to the plates and ten replicates were performed. The plates were incubated in a BOD (biological oxygen demand) incubator at 25 ± 1 ° C and 70 ± 10% relative

humidity, with photoperiod of 12 hours. Larval mortality was assessed on the seventh day. In addition, sublethal effects of the formulations were evaluated by weighing the larvae 15 days after the end of the mortality evaluation. The evaluation of oviposition was performed in PVC cages.

3. Results and Discussion

3.1. Characterization and physicochemical stability

The results of characterization of nano-formulations in terms of mean diameter (MD, nm), polydispersity index (PDI), zeta potential (ZP, mV), nanoparticle concentration (CT, particles/mL) and encapsulation efficiency (EE, %) are presented in Table 1. Data on physicochemical stability are presented in Figure S1 (supplementary material).

Table 1: Characterization of zein nanoparticles containing the botanical compounds (geraniol, eugenol and cinnamaldehyde). Values are expressed as mean of three determinations.

Formulations	MD (nm)		PDI	ZP (mV)	CT (x10^{12} particles/mL)	EE (%)
	DLS	NTA				
NP	302 ± 8	232 ± 9	0.52 ± 0.09	-15 ± 1	0.8 ± 0.1	-
NP_GRL+CND	234 ± 5	156 ± 6	0.38 ± 0.02	43 ± 2	3.3 ± 0.8	GRL 99 ± 1 CND 97 ± 2
NP_GRL+EGL	282 ± 3	160 ± 8	0.34 ± 0.05	41 ± 2	3.2 ± 0.7	GRL 99 ± 1 EGL 98 ± 1

MD – Mean diameter; PDI - Polydispersity index; ZP - zeta potential; CT - Nanoparticle concentration; EE - Encapsulation efficiency

The control nanoparticles had a larger mean diameter compared to the other formulations and a high value polydispersity index and a relatively low zeta potential (Table 1). This indicates a low stability of these formulations, which prevented the continuation of the analysis after 15 days of storage due to precipitation and phase separation, and were therefore not included in the extended stability analysis (Figure S1). According to Da Rosa et al. (2015b), the presence of active compounds in the dispersion can play a stabilizing role that can prevent aggregation and consequent increase of particle size.

With the two techniques used for analysis of average diameter, a significant increase was observed in the values for both formulations of nanoparticles as a function of time, especially with 120 days, indicating gradual particle aggregation (Figure S1). However, no significant changes were observed in both the polydispersity index and the nanoparticle concentration. The zeta potential decreased within 15 days but remained

299 stable until the final analysis. Furthermore, the prepared nanoparticle formulations
300 showed few changes in size distribution as a function of time (Figure 1 A and B). The
301 AFM micrographs (Figure 1 - Ab and Bb), show spherical morphology and smooth
302 surface of the nanoparticles. They also show high polydispersity of the formulations, with
303 particle size distribution between 90 and 550 nm. This corroborates with the
304 polydispersity index (Figure S1-C) that shows values higher than 0.3. This indicates good
305 physicochemical stability of the prepared nanoparticle formulations, which is extremely
306 important for commercial applications that require storage over long periods. These
307 results also corroborate a previous study by our research group [9] that prepared zein
308 nanoparticles for encapsulation of geraniol and R-citronellal separately. The results of
309 that study had also shown that the prepared nanoparticle formulations were stable over
310 time, with mean size of 200 nm, polydispersion index of 0.3, and zeta potential of -20
311 mV.
312 The encapsulation efficiency was also investigated in the current study (Figure 1-
313 Ac and Bc). It was observed that, as shown in the previous work by Oliveira et al., (2018)[9],
314 the encapsulation index of the botanical compounds was high (>98%) in zein
315 nanoparticles. This is likely to be due to the strong interaction between the studied
316 compounds and the hydrophobic part of the zein. For the encapsulated compounds, it was
317 observed that there was a significant decrease in encapsulation efficiency only at the
318 extended storage times (90 and 120 days). This is most likely because of the loss of
319 compounds due to volatilization, and/or degradation of the particles and release of the
320 compounds. This is still a major improvement in stability as there was a much greater
321 degradation when preparations were made only by emulsifying the substances with a
322 surfactant. For encapsulated GRL and CND, 92± 2 and 90 ± 2 % of the active substance
323 were available after 120 days, while emulsions has 65 ± 3 and 44 ± 2 % of the active
324 substances, respectively. Similar results were obtained for encapsulated formulations
325 containing GRL and EGL, that had 94 ± 1 and 92 ± 2 % of the substances, while
326 emulsified forms had 68 ± 3 and 61 ± 4 % of the compounds respectively. It appears that
327 when the compounds are not encapsulated, they are more prone to loss due to
328 volatilization and degradation than when they are encapsulated in nanoparticles. The
329 results of this study therefore provide further evidence that encapsulation can protect
330 active ingredients against rapid volatilization and degradation [9,30,31]. Scremin et al.,
331 (2018)[32] also observed that encapsulation of eugenol in rice-bran protein based

microcapsules provided protection to the active substance against degradation (around 30% compared to non-encapsulated compound).

[Figure 1]

The formulations containing mixture of active compounds were more stable as a function of time (Table 1, Figure 1 and Figure S1). In addition, the formulations were able to protect the active substance against degradation in solution. Thus, the nanoparticle formulations containing mixtures of geraniol with eugenol, and geraniol with cinnamaldehyde, were stable from physicochemical point of view. These findings are very important for the formulations to be useable in agricultural applications since a stable shelf life of active substance is essential to maintain efficacy.

3.2. *In vitro* release and release mechanism

Figure 2 shows release data for the zein nanoparticle formulations containing mixtures of the active substances: geraniol with eugenol and geraniol with cinnamaldehyde at different temperatures (25, 30 and 35°C). Geraniol, both when encapsulated with eugenol (Fig. 2-A) and encapsulated with cinnamaldehyde (Fig. 2-C), exhibited the same release profile, averaging at 48 ± 3 % within 1440 minutes. In addition, no differences were observed with increasing temperature. On the other hand, eugenol (Fig. 2-C) showed a greater release compared to geraniol, and differences as a function of temperature increases. At 1440 minutes, the release of eugenol was 55 ± 1 %, 58 ± 2 % and 68 ± 3 % at temperatures of 25, 30 and 35ºC, respectively. The highest release was observed for cinnamaldehyde, which under the same experimental time released 52 ± 2 %, 76 ± 1 % and 88 ± 2 % at temperatures of 25, 30 and 35°C, respectively. The increase in the release of active compounds with increasing temperature reflects the differences in physicochemical characteristics, such as volatility and solubility. The tendency of a substances to evaporate is depicted in terms of vapor pressure, and a higher vapor pressure indicates the substance to be more volatile [33]. Among the substances studied, cinnamaldehyde has the highest vapor pressure ($3.2x10^{-2}$ mm Hg at 25ºC), followed by eugenol ($2.89x10^{-2}$ mm Hg at 25ºC) and geraniol ($2.21x10^{-2}$ mm Hg at 25°C). Differences in the release of active eugenol and cinnamaldehyde have also been observed by Gomes

et al., (2011) [34] for poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing eugenol and cinnamaldehyde. The release assays showed differences in the release profile of the substances from nanoparticles, with around 80% of cinnamaldehyde released after 5 hours, compared to 45% of eugenol. According to the authors, the steric conformation of eugenol and greater lipophilicity than trans-cinnamaldehyde probably makes it more difficult for eugenol to diffuse from inside the nanoparticles to the external medium.

[Figure 2]

Mathematical models are widely used to predict the time release patterns of the encapsulated molecules to understand the mechanisms of release and assist in the design of formulations [35]. This study used different mathematical models to evaluate the mechanism of release of the active compounds through zein nanoparticles (Table 2).

Table 2: Constants (k) and correlation coefficients (r^2) for different mathematical models applied to evaluate the release of active compounds from zein nanoparticles at different temperatures.

	Mathematical model								
	Zero order		**First order**		**Higuchi**		**Korsmeyer-Peppas**		
	k (h^{-1})	r^2	k (h^{-1})	r^2	k ($h^{-1/2}$)	r^2	k (h^{-1})	n	r^2
					25 ºC				
NP_GRL+EGL									
GRL	0.0081	0.4594	$2.82x10^{-4}$	0.3414	1.166	0.7123	1.372	0.3897	**0.8441**
EGL	0.0011	0.2751	$1.61x10^{-4}$	0.1872	1.031	0.5185	1.695	0.2612	**0.6799**
NP_GRL+CND									
GRL	0.0084	0.4439	$3.71x10^{-4}$	0.2783	1.285	0.6888	0.4261	0.5493	**0.7899**
CND	0.0096	0.4482	$3.87x10^{-4}$	0.2672	1.446	0.6904	2.1551	0.5805	**0.7762**
					30 ºC				
NP_GRL+EGL									
GRL	0.0105	0.5072	$3.58x10^{-4}$	0.3228	1.287	0.7493	0.6727	0.4045	**0.8288**
EGL	0.0025	0.3081	$1.46x10^{-4}$	0.2957	1.059	0.5613	1.709	0.2107	**0.7937**
NP_GRL+CND									
GRL	0.0082	0.4329	$3.46x10^{-4}$	0.2688	1.321	0.6812	0.6825	0.5184	**0.7781**
CND	0.3631	0.8971	$5.95x10^{-4}$	0.7196	6.206	0.9117	2.8282	0.7321	**0.9633**
					35 ºC				
NP_GRL+EGL									
GRL	0.0103	0.4952	$3.45x10^{-4}$	0.2502	1.308	0.7375	0.7175	0.4138	**0.8187**
EGL	0.0842	0.6865	$1.19x10^{-4}$	0.7252	3.311	0.9192	1.3512	0.3183	**0.9791**
NP_GRL+CND									
GRL	0.0069	0.3979	$2.99x10^{-4}$	0.2967	1.322	0.6433	1.262	0.5273	**0.7825**
CND	0.3821	0.8971	$6.33x10^{-4}$	0.7441	6.474	0.9152	3.132	0.6849	**0.9654**

According to the data presented in Table 2, it is possible to observe that the mathematical model that best fits for all active compounds was the Korsmeyer-Peppas model. Through use of this model, it is possible to determine if the release of the active substances followed Fick's law of diffusion, or a different mechanism such as

swelling/relaxation phenomena (Case-II transport). It can be seen that for zein nanoparticles containing geraniol and eugenol, the value of n was <0.45, which indicates that the diffusion is the main mechanism that controls release of the active substance in the system. For zein nanoparticles containing geraniol and cinnamaldehyde, the value of n was between 0.45 and 0.89, indicating an anomalous transport kinetics, which indicates a combination of two mechanisms (diffusion and transport of Case II)[27]. However, diffusion is the main form of release in both systems, which leads to the compound passing through the zein protein chain matrix to the external environment. In such a type, the rate of release usually decreases with time, since more internalized compound has a greater distance to cross, which requires more time. This is supported by the results shown in Figure 2, which show a faster release of the active substances within the first 60 minutes. This is due to diffusion of the most superficial layers of the encapsulates as well as any adsorbed substances on the outer surface of the nanoparticles. After this period, the internalized active compounds diffuse into the nanoparticle matrix. These results also corroborate previous work described in the literature. For example, diffusion has been suggested as the main mechanism of release of geraniol from chitosan/gum arabic nanoparticles [36]. Campos et al., (2018)[37] evaluated the mechanism of release of carvacrol and linalool through chitosan nanoparticles functionalized with β-cyclodextrin. The authors also found that diffusion, was the main mechanism for the release of the active substances, along with relaxation of the polymer chains (Case Transport II). This shows that mathematical models can be important tools in the study of the release of active compounds from nanoparticle based formulations.

3.3. Cytotoxicity

Toxicity tests are important in order to assess the safety of these systems for non-target organisms In this study, two cell lines (V79-4 and 3T3) were used (cytotoxicity assays performed to determine cell viability). Both the surfactant used (Pluronic F-68) and the control nanoparticles (without addition of the active compounds) did not cause any significant decrease in cell viability (Figure 3). The emulsions, as well as the active compounds encapsulated in the nanoparticles, showed a decrease in cellular viability with increasing concentration. According to A. Al-Tamimi et al., (2016)[38] essential oils and their active components can have cytotoxic effects amongst other biological activities.

Indeed, cytotoxicity has been reported in literature for geraniol [39,40], eugenol [41,42] and cinnamaldehyde, especially in tumor cell lines.

In this study for 3T3 cell line (Figure 3-A) and for V79 (Figure 3-B), the encapsulation of the active compounds in zein nanoparticles decreased IC_{50} values. For the 3T3 line, the emulsion containing geraniol and eugenol showed IC_{50} (obtained through the probit analysis) of 0.0362 ± 0.0012 $mg.mL^{-1}$, whereas the emulsion containing geraniol and cinnamaldehyde showed IC_{50} of 0.0348 ± 0.0042 $mg.mL^{-1}$. When the compounds were encapsulated in nanoparticles, the IC_{50} values were 0.0780 ± 0.0114 and 0.0661 ± 0.0135 $mg.mL^{-1}$, respectively. For the V79 line, the emulsion containing geraniol and eugenol, showed IC_{50} of 0.0361±0.0110 $mg.mL^{-1}$, while the emulsion containing geraniol and cinnamaldehyde showed a value of 0.0266±0.0094 $mg.mL^{-1}$. For 3T3 cell line, the IC_{50} values were higher for the compounds when they were in encapsulated form (0.0841±0.0185 and 0.0640±0.0121 $mg.mL^{-1}$ respectively). This indicates that encapsulation of the substances in nanoparticles not only had a protective effect, but also reduced their cytotoxicity. This is likely to be due to that the compounds are encapsulated in the protein matrix, which reduces the amount available freely to cause immediate toxic effects. Similar results have also been observed in previous studies of our research group. The encapsulation of geraniol and R-citronelal in zein nanoparticles caused a decrease in cytotoxic activity [9]. Campos and co-workers (2018)[35] have also shown that the encapsulation of carvacrol and linalool compounds in chitosan nanoparticles functionalized with β-cyclodextrin significantly increased IC_{50} values. Chen et al. (2009) [25] also observed a reduction in the cytotoxic activity of eugenol when encapsulated in chitosan nanoparticles. According to the authors the fibroblasts exhibited >80% viability when treated with the encapsulated compound, whereas for the free compound the viability values were < 20%.

[Figure 3]

3.4. Biological activity assays

3.4.1. Two-spotted spider mite (*Tetranychus urticae)*

Figure 4 shows results of the repellency assays of the formulations containing blends of the botanical compounds against the two-spotted spider mite (*T. urticae*). The formulations were tested at a concentration of 5 mg/mL (0.5%) of each botanical

repellent, based on previous work of our research group (Oliveira et al., 2018)[9], that showed no toxic effects at this concentration. From the repellency curves, an adjustment was applied to the area under the curve (Fig. 4), and data are presented in Table 3.

The emulsions showed a significantly higher repellency against the mite than the encapsulated compounds two hours after application of the products (Figure 4). However, whilst repellency of the formulation decreased as a function of time, the repellent effect of the encapsulated compounds increased significantly. This is likely to be due to a sustained release of the encapsulated compounds, and protection of the compounds from premature degradation. This is evident in the area under curve (AUC) values (Table 3). Geraniol and eugenol showed an AUC of 19.9 ± 1.4 repellency x time when emulsified, and 24.2 ± 1.0 when they were encapsulated. Geraniol and cinnamaldehyde showed an AUC of 16.1 ± 1.1 repellency x time when emulsified, and 25.5 ± 0.9 when they were encapsulated. The higher AUC of the encapsulated botanicals than the emulsified compounds indicates an increase in overall effectiveness. The control, as well as the nanoparticles in the absence of the botanical compounds (Figure 4 - inset) did not present repellent effect, and no significant differences were found between them.

In a previous study, Tak e Isman, (2017) [43] evaluated acaricidal and repellent activity of different terpenes derived from plant essential oils, in addition to the effect of binary mixtures against *T. urticae.* The authors tested twice the concentration used in the present study (10 mg mL^{-1}) and obtained repellency value of 66.7 ± 6.7% for trans-cinnamaldehyde, 62.4 ± 10.5% for eugenol and 74.3 ± 6.1% for geraniol. Also, in the binary mix effect tests, the authors studied synergistic effects between the eugenol, trans-cinnamaldehyde and geraniol compounds, and reported that only vanillin had any significant synergistic effect. The authors however noted a significant increase in the acaricidal effects of the compound mixtures. Other studies have also described the repellent activity of botanical compounds [7,44,45]. In a previous study, our research group (Oliveira et al., 2018)[9] also observed repellent effects of geraniol and R-citronellal compounds against *T. urticae* at the same concentrations (5 $mg.mL^{-1}$) used in the present study. A repellent effect of about 60% was observed for R-citronellal and 35% for geraniol, with the repellency of the encapsulated compound superior to that of the emulsified compound. It needs to be emphasized that, as in the previous study, the repellent activity of these compounds was evaluated under controlled conditions. Other studies have carried out evaluation under semi-field conditions, where formulations were applied to plants in greenhouse under the action of light, humidity and uncontrolled

temperatures, to demonstrate that the processes are also dependent on environmental factors. Furthermore, it has been reported that other factors, such as vapor pressure and interaction with treated surfaces, also have a significant influence on the repellent effect [46–48]. In the present study, for example, the speed and degree of metabolism in addition to the penetration of the compounds into the leaf structure may have played a major role in the repellent effect, since the leaf arenas were only removed from the plants for the tests.

Overall the results of this study showed a promising enhancement of efficacy of the nanoparticle formulations containing the botanicals, which still had a repellent effect of ~15% after 7 days, as compared to emulsified formulations.

Table 3: Area values on the curve for the repellent activity assays of the formulations containing the mixture of the active compounds, as well as the respective controls. Nanoparticles of zein (NP); geraniol and eugenol emulsified with surfactant (EM_GRL+EGL); geraniol and cinnamaldehyde emulsified with surfactant (EM_GRL+CND); zein nanoparticles loaded with geraniol and eugenol (NP_GRL+EGL); zein nanoparticles loaded with geraniol and cinnamaldehyde (NP_GRL+CND). Significance level of $p < 0.05$ (OneWay ANOVA) for the differences between groups, where in a * there is a significant difference in relation to the control; in b * a significant difference in relation to geraniol and eugenol emulsified and c * a significant difference in relation to geraniol and cinnamaldehyde emulsified.

Formulation	Area under the curve (repellency x time)
Control	1.7 ± 0.3
NP	2.8 ± 0.4
EM_GRL+EGL	19.9 ± 1.4 [a]
NP_GRL+EGL	24.2 ± 1.1 [a,b]
EM_GRL+CND	16.2 ± 1.1 [a]
NP_GRL+CND	25.5 ± 0.9 [a,c]

[Figure 4]

3.4.2. Soybean looper (*Chrysodeixis includes)*

The effects of the formulations on the larvae were evaluated considering mortality rates as well as sublethal effects determined in terms of larval and pupal weight (Table 3). It is noteworthy that for the bioassays mortality was assessed after 7 days and the sublethal effects 15 days after the end of the mortality assessment.

It was observed (Table 4) that all the treatments showed mortality rate significantly higher than the control. Except for the formulation of nanoparticles containing the mixture

of geraniol and eugenol, the other treatments presented mortality above 80% (index recommended as satisfactory). However, when evaluated for sublethal effects, the larval and pupal weights treated with the nanoparticle formulations containing the active compound mixtures was significantly lower than the emulsified compounds. Except for the control and for the emulsion containing geraniol and eugenol, all the other treatments prevented adult oviposition. Thus, the results indicate that the effects of the nanoparticle formulations are longer term, most likely due to the sustained release of the active compounds. For example, nanoparticle encapsulated formulations containing geraniol and eugenol caused mortality rates lower than the emulsified formulations. However, it manifested not only in higher sublethal effects but also prevention of oviposition, whereas adult oviposition was observed for the emulsions.

These results show that the nanoencapsulation improved efficacy of the botanical compounds. Such improvements have also been reported by other researchers. Campos et al. (2018)[35] studied sublethal effects of the chitosan nanoparticle formulations containing the carvacrol and linalool mixture against *H. armigera*. The encapsulated compounds also had a greater sublethal effect than the emulsified compounds, as demonstrated in this study. A. Al-Tamimi et al., (2016) [49] evaluated the effect of nanoparticles of chitosan containing botanical pesticide Ponneem® (neem oil and karanj oil) against *H. armigera*. The formulations produced growth and developmental abnormalities in *H. armigera* larvae. However, the nanoformulations showed more effectiveness, and a lower concentration of 0.3% caused 9.1% of defective pupae, compared to 7.8% of the free compound. The mean weight of the pupae was also significantly reduced in the treatment with the nanoformulations containing the botanical pesticides compared to other treatments, and for the control group.

The greater effects of nanoformulations under larvae development may be a result of higher uptake and accumulation in the larvae after feeding. Koo et al., (2015)[50] investigated biomagnification of quantum dot functionalized polymer nanoparticles (QD). For this, they used *Arabidopsis thaliana* (L.) Heynh. ingestion by cabbage looper [*Trichoplusia ni* (Hübner)]. After feeding the larvae for 7 days, the authors observed a high level of fluorescence in the tissues of the larvae fed with the leaves treated with the nanoparticles compared to those fed with the control plants. This showed accumulation of nanoparticles in the larvae that led to a weight reduction of about 1.5 time in comparison to control.

555 **Table 4:** Biological effects on mortality and mass of larvae and pupae of *Chrysodeixis includes* fed
556 with artificial diets treated with emulsified and nanoencapsulated botanicals. Laboratory evaluation at 25 ±
557 2 °C, 70 ± 10% relative humidity and 12-hour photoperiod. Nanoparticles of zein (NP); geraniol and
558 eugenol emulsified with surfactant (EM_GRL+EGL); geraniol and cinnamaldehyde emulsified with
559 surfactant (EM_GRL+CND); zein nanoparticles loaded with geraniol and eugenol (NP_GRL+EGL); zein
560 nanoparticles loaded with geraniol and cinnamaldehyde (NP_GRL+CND). Significance level of $p < 0.05$
561 (OneWay ANOVA) for the differences between groups, where in a * there is a significant difference in
562 relation to the control; in b * a significant difference in relation to geraniol and eugenol emulsified and c *
563 a significant difference in relation to geraniol and cinnamaldehyde emulsified.
564

Formulations	Mortality (%)	Larvae mass (mg)	Pupae mass (mg)	Oviposition
Control	0	198.4 ± 4.6	212.4 ± 3.2	YES
NP	47.6 ± 3.1 [a]	172.1 ± 3.1 [a]	191.3 ± 2.1 [a]	NO
EM_GRL+EGL	81.8 ± 3.5 [a]	163.4 ± 2.3 [a]	178.4 ± 1.9 [a]	YES
NP_GRL+EGL	76.4 ± 2.2 [a,b]	151.1 ± 2.1 [a,b]	167.1 ± 2.5 [a,b]	NO
EM_GRL+CND	88.4 ± 1.5 [a]	160.8 ± 2.2 [a]	174.1 ± 3.3 [a]	NO
NP_GRL+CND	82.2 ± 1.9 [a,b]	147.8 ± 4.3 [a,b]	165.1 ± 1.5 [a,c]	NO

4. Conclusions

Our studies have shown that both nanoparticle formulations containing blends of the botanic compounds - geraniol, eugenol and cinnamaldehyde - had physicochemical properties suitable for the colloidal stability over 120 days. The encapsulation of the compounds not only offered protection against degradation but also enabled a sustained release of the actives over time. The nanoencapsulation also led to a decrease in IC_{50} values for the cell viability indicating that the nanoparticles lowered the acute toxic effect of the botanical compounds. Testing of the systems demonstrated effectiveness in the control of two species of agricultural pests: the two-spotted spider mite and the soybean looper. For both organisms, significant efficacy improvements were observed for the nanoencapsulated formulation compared to the emulsified compounds. Thus, zein based nanoparticles enabled effective encapsulation of the blends of botanicals, provided protection against their rapid degradation, decreased acute toxic effect, and increased longer-term effectiveness to the target organisms. The use mixtures of active compounds from different plants may also aid in the prevention of resistance selection in pest species. Thus, a convergence between nanotechnology based formulations and botanical control agents offers a promising new approach to the sustainable management of pests in agriculture and reduce negative impacts on the human health and the environment.

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6. Author contribution

JLO, EVRM and LFF performed experimental delineation for carrier systems preparation and characterization. TG and RL performed the cytotoxicity assays. JFDV, STS and DJA performed evaluation of biological activity against *Tetranychus urticae*. KGC, JN and RAP performed the evaluation of biological activity against *Chrysodeixis includes*. JLO led the writing of the manuscript. All authors contributed critically to the drafts and gave approval for the fnal version.

7. Compliance with ethical standards

Conflict of interest: The authors declare that they have no confict of interest.

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