UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Sorocaba

PROGRAMA DE PÓS-GRADUAÇÃO em
Ciências Ambientais

JHONES LUIZ DE OLIVEIRA

DESENVOLVIMENTO DE SISTEMAS DE LIBERAÇÃO MODIFICADA A BASE DE ZEÍNA E QUITOSANA PARA REPELENTES BOTÂNICOS VISANDO O CONTROLE DE MOSCA-BRANCA (*Bemisia tabaci*) EM DIFERENTES CULTURAS

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Orientador: Prof. Dr. Leonardo Fernandes Fraceto

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2 Pedro 1:5-7

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ABSTRACT

Arthropods have been a major cause of agricultural losses worldwide. Only in Brazil can these losses reach 7.7% per year. The extensive use of synthetic pesticides has been the main form of control of this type of pest, however, the adverse effects of these compounds for both the environment and human health have motivated the search for less impactful alternatives. In this context, several mechanisms are being studied in order to minimize these damages, such as the development of modified release systems using biodegradable polymers and proteins. In addition to this, the use of botanical pesticides has also shown potential for combating these pests, due to the lower impacts caused by these products of natural origin. Therefore, the present work showed the development (preparation and characterization) of nanocarrier systems produced from chitosan and gum arabic polymers and zein protein for the botanical compounds (geraniol, citronellal, eugenol and cinnamaldehyde), as well as the potential effects and phytotoxic systems. In addition, tests of the biological activity of these systems on agricultural pests (whitefly, two-spotted spider mite and soybean looper) were also carried out. The botanical compounds showed high encapsulation efficiency in both carrier systems, and the systems were able to protect the compounds against premature degradation and also against ultraviolet radiation. The phyto and cytotoxicity results showed that encapsulation decreased the toxic effect of the active compounds. The systems also showed biological activity against the agricultural pests tested. The zein nanoparticles containing the botanicals showed a repellent effect against the brindle two-spotted spider mite (*Tetranychus urticae*) under laboratory and semi-field conditions, as well as a sublethal effect on soybean looper (*Chrysodeixis includes*). The chitosan/gum arabic nanoparticles containing geraniol showed attractive effect for whitefly, being promising for applications in trap systems. The importance of this thesis lies in the dimension that the agricultural sector represents for the Brazilian and world economy, in addition to that the market for agricultural pesticides grows annually, in special as Brazil is one of the leaders in the consumption of these products. In this way, the development of new technologies to pest control in agriculture is promising since more efficient pest control systems can be produced, less impacting the environment and consequently to human health.

Keywords: botanical pesticides; zein nanoparticles; chitosan/gum arabic nanoparticles; sustainable agriculture

RESUMO

Os artrópodes tem sido uma das principais causas de perdas agrícolas em todo o mundo. Só no Brasil essas perdas podem chegar a 7,7 % ao ano. A utilização extensiva de pesticidas sintéticos tem sido a principal forma de controle deste tipo de praga, no entanto, os efeitos adversos destes compostos tanto para o meio ambiente quanto para a saúde humana tem motivado a busca por alternativas menos impactantes. Neste contexto, diversos mecanismos estão sendo estudados a fim de minimizar estes danos, como por exemplo, o desenvolvimento de sistemas de liberação modificada, utilizando polímeros biodegradáveis e proteínas. Aliado a isto, a utilização de pesticidas botânicos também tem demostrado potencialidade para o combate a essas pragas, devido aos menores impactos causados por esses produtos de origem natural. Portanto, o presente trabalho apresenta o desenvolvimento (preparo e caracterização) de sistemas nanocarreadores produzidos a partir dos polímeros quitosana e goma arábica e da proteína zeína para os compostos botânicos (geraniol, citronelal, eugenol e cinamaldeído), bem como, os potenciais efeitos cito e fitotóxicos destes sistemas. Ademais foram também realizados ensaios de atividade biológica destes sistemas em pragas agrícolas (mosca-branca, ácaro-rajado e lagarta falsa-medideira). Os compostos botânicos apresentaram elevada eficiência de encapsulação em ambos os sistemas carreadores, sendo que os sistemas foram capazes de proteger os compostos contra uma degradação prematura e também contra a radiação ultravioleta. Os resultados de fito e citotoxicidade mostraram que a encapsulação diminuiu o efeito tóxico dos ativos. Os sistemas também apresentaram atividade biológica contra as pragas agrícolas testadas. As nanopartículas de zeína contendo os compostos botânicos mostraram efeito repelente contra o ácaro-rajado (*Tetranychus urticae*) em condições laboratoriais e de semi-campo, além de efeito subletal em largarta falsa-medideira (*Chrysodeixis includes*). Já as nanopartículas de quitosana/goma arábica contendo geraniol mostraram efeito atrativo para mosca-branca, sendo promissora para aplicações em sistemas de armadilha. A importância desta tese reside na dimensão que o setor agrícola representa para a economia brasileira e mundial, aliado a isto o mercado de defensivos agrícolas cresce anualmente, sendo o Brasil um dos líderes no consumo destes produtos. Desta forma, o desenvolvimento de tecnologia com elevado valor agregado à área de controle de pragas em agricultura é promissora uma vez que podem ser produzidos sistemas mais eficientes no controle de pragas, menos impactantes ao ambiente e consequentemente à saúde humana.

Palavras-chave: pesticidas botânicos; nanopartículas de zeína; nanopartículas de quitosana/goma arábica; agricultura sustentável.

Sumário

INTRODUÇÃO GERAL

A busca pelo aumento da produção agrícola tem ocasionado profundas mudanças no manejo agrícola ao longo dos tempos. Destaca-se a utilização dos pesticidas, adubos orgânicos, controle biológico e também a mecanização da produção. Além disso, podemos destacar a utilização de plantas geneticamente modificadas, as quais apresentam variedades mais produtivas e/ou mais resistentes a pragas e patógenos (PELLEGRINI; FERNÁNDEZ, 2018). No entanto, devido às inúmeras dificuldades enfrentadas por agricultores ao redor do mundo, estes procuram métodos que sejam mais simples, baratos e acessíveis. Sendo assim, a utilização dos pesticidas, em especial os sintéticos, tem sido a principal método de manejo utilizado (SHIVA, 2016a). Porém o uso excessivo desses compostos, acompanhado da falta de conhecimento ou acompanhamento técnico sobre o seu manejo adequado, tem trazido inúmeros impactos negativos para o meio-ambiente e à saúde humana, tais como o surgimento de pragas resistentes, surgimento de pragas secundárias em função dos efeitos tóxicos sobre os inimigos naturais dessas pragas, intoxicação dos produtores rurais, contaminação da água e do solo, impactos negativos sobre os organismos não-alvo e presença de resíduos tóxicos nos alimentos (SANKOH et al., 2016).

Neste contexto, a utilização de compostos de origem natural (pesticidas botânicos) se mostram como um alternativa mais ambientalmente favorável em relação aos pesticidas sintéticos. Além das substâncias que participam de seu metabolismo primário (essencial para sua sobrevivência), as plantas desenvolveram ao longo do tempo vias metabólicas secundárias, as quais produzem uma grande variedade de substâncias (alcalóides, terpenóides, fenilpropanóides, entre outras). Estas susbtâncias por sua vez, apresentam inúmeras atividades biológicas, incluindo controle de pragas agrícolas (CAMPOS et al., 2018a). Sendo assim, quando se pensa em aplicação agrícola, essas formulações apresentam algumas vantagens como: baixa à moderada toxicidade para organismos não-alvo; mecanismo de ação bastante variados, podendo agir sobre os insetos provocando: inibição de oviposição e da alimentação, distúrbios no desenvolvimento, deformações, infertilidade, mortalidade e também repelência (ISMAN; GRIENEISEN, 2014). No entanto, a rápida degradação desses compostos pela luz solar, por processos oxidaditos e por enzimas desintoxicantes presentes nos organismos alvos representam um importante fator limitante para o uso destes compostos. Sendo assim, cada vez mais se faz necessário a busca por formulações que sejam eficazes na aplicação desses compostos botânicos, melhorando a sua estabilidade e eficácia (ISMAN, 2017).

A nanotecnologia tem se mostrado promisora para o desenvolvimento de formulações inteligentes, que podem ser utilizadas em agricultura para o uma melhor eficácia desses compostos naturais (DE OLIVEIRA et al., 2014a). Basicamente estas formulações tem por objetivo: i) aumentar a solubilidade desses compostos ativos; ii) liberá-los de uma forma lenta/sustentada e/ou iii) protegê-los contra a degradação prematura. Desta maneira, é possivel que o composto bioativo o alvo com melhor eficiência, associado à diminuição da toxicidade e impactos ambientais (SINHA; GHOSH; SIL, 2017). Estes sistemas nanoestruturados podem ser produzidos a partir de diversas matrizes, a exemplo dos polímeros biodegradáveis, proteínas entre outros, sendo encontrado na literatura diversas formas de preparo destas formulações. Alguns polímeros como por exemplo, a quitosana e zeína podem ser utilizados como matriz para obtenção desses sistemas. A grande vantagem na utilização desses materiais esta justamente no baixo custo e também na disponibilidade, o que facilitaria a produção em larga escala dessas formulações (MERINO; CASALONGUÉ; ALVAREZ, 2018).

A utilização de produtos e formulações a base de nanotecnologia, já é uma realidade, sendo ao longos dos últimos anos diversos produtos disponibilizados no mercado. Desta maneira, com crescente utilização desses compostos e consequente disponibilização no ambiente, é necessário a compreensão do comportamento destes, assim como seus efeitos em organismos alvos e não-alvos (TSANG et al., 2017). De acordo com Kah et al. (2018) é de extrema importância que estudos se aprofundem no estudo das interações entre os nano sistemas e o meio ambiente, inclusive concentrações ambientais desses materiais. Além disso os autores destacam o escasso números de trabalhos que avaliam a eficácia e impacto ambiental desses materiais em condições de campo. Ademais, os autores também discutem a complexidade das amostras ambientais e a consequente caracterização desses nanomateriais.

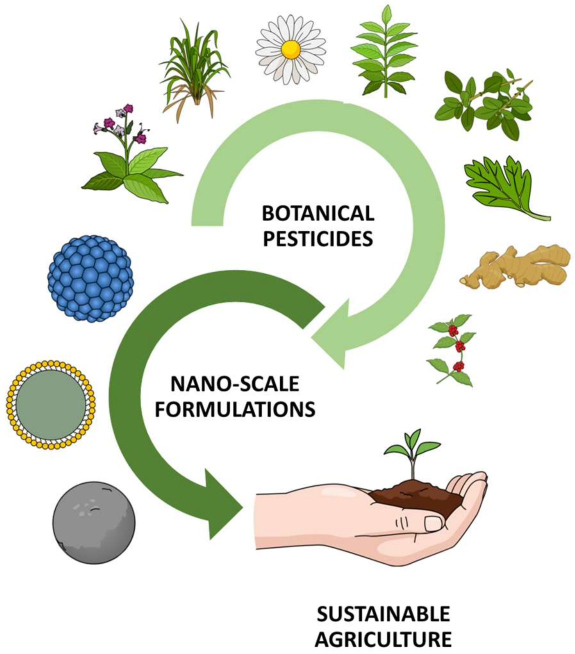
Neste contexto, o objetivo principal da presente tese foi preparar e caracterizar sistemas carreadores baseados nas matrizes naturais de zeína e quitosana para a liberação sustentada dos compostos botânicos, geraniol, R-citronelal, eugenol e cinamaldeído. Foram também investigados os efeitos das formulações à organismos não-alvo (testes de citotoxicidade e fitotoxicidade), além da avaliação da eficácia biológica dos sistemas contra diferentes pragas agrícolas. A tese está estruturada em capítulos, os quais são compostos por artigos publicados e/ou submetidos à periódicos científicos internacionais.

No ***primeiro capítulo*** é apresentada uma revisão de literatura onde são abordados os recentes desenvolvimentos e também os desafios das formulações nanotecnológicas para pesticidas botânicos visando o uso em agricultura sustentável. O artigo apresenta uma avaliação da pesquisa científica recente, juntamente com um levantamento dos produtos que já chegaram ao mercado. Embora reconheça o grande potencial das formulações de pesticidas botânicos derivados da nanotecnologia para aumentar a produtividade agrícola e reduzir danos a saúde e os impactos ambientais, também são destacados os desafios tecnológicos que devem ser enfrentados para permitir a adoção da tecnologia para uso mais amplo na produção agroalimentar. Já o ***segundo capítulo*** mostra o desenvolvimento de nanopartículas de zeína para o carreamento dos compostos botânicos geraniol e R-citronelal, visando aplicações em agricultura sustentável. Neste capítulo são apresentados o preparo e caracterização das nanopartículas, além do ensaios de fitotoxicidade e citotoxicidade e também atividade biológica contra organismos alvo. Os resultados mostraram que os compostos botânicos apresentaram alta eficiência de encapsulação (> 90%) nas nanopartículas, boa estabilidade físico-química e efetiva proteção dos repelentes contra a degradação por UV. Ensaios de citotoxicidade e fitotoxicidade mostraram que o encapsulamento dos repelentes botânicos diminuiu sua toxicidade. Testes de atividade repelente mostraram que as nanopartículas contendo os repelentes botânicos eram altamente repelentes contra o ácaro *Tetranychus urticae* Koch. No ***terceiro capítulo*** são apresentados o resultados do desenvolvimento de nanopartículas de quitosana/goma arábica para encapsulação de geraniol. Além do preparo e caracterização das nanopartículas, foi avaliada a atividade biológica do geraniol encapsulado contra à mosca branca (*Bemisia tabaci*). Os resultados mostram que a formulação otimizada apresentou alta eficiência de encapsulação (> 90%) e permaneceu estável por cerca de 120 dias. A formulação protegeu o geraniol contra a degradação por radiação UV, e a liberação *in vitro* foi influenciada pela temperatura. Devido a liberação sustentada através das nanopartículas, um efeito de atração foi observado para mosca-branca, indicando o potencial deste tipo de sistema para uso no manejo de pragas, especialmente em armadilhas. No ***quarto capítulo*** se observa os resultados do desenvolvimento de formulações a base de nanopartículas de zeía contendo a mistura de compostos botânicos (geraniol, eugenol e cinamaldeído). A mistura de compostos botânicos é importane a fim de aumentar o espectro de ação e também reduzir a criação de resistência pelas populações de pragas. As formulações foram preparadas e caracterizadas e os efeitos biológicos das formulações foram avaliados contra duas pragas agrícolas - o ácaro rajado

(*Tetranychus urticae*) e a largarta falsa-medideira (*Chrysodeixis includes*). As formulações foram estáveis ao longo do tempo (120 dias) com uma alta eficiência de encapsulação (> 90%). A nanoencapsulação permitiu uma diminuição da degradação e também da toxicidade a organismos não-alvo. A liberação dos compostos ativos (especialmente eugenol e cinamaldeído) foi diretamente influenciada pela temperatura. Os compostos encapsulados apresentaram eficiência superior ao compostos emulsionados tanto no efeito repelente contra o ácaro-rajado como também no efeito subletal para a largarta falsa-medideira. Por fim, a tese é seguida de uma ***conclusão geral e perspectivas***.

CHAPTER I

Recent developments and challenges for nano-scale formulation of botanical pesticides for use in sustainable agriculture



OLIVEIRA., J. L.; CAMPOS., E. V. R.; FRACETO., L. F. Recent developments and challenges for nano-scale formulation of botanical pesticides for use in sustainable agriculture.Aceito no **Journal of Agricultural and Food Chemistry** (in press)

Abstract

In recent years, the use of substances of natural origin, such as botanical pesticides, has emerged as a preferred alternative to the use of synthetic pesticides, the excessive use of which has raised a lot of concern over safety to human/animal health and the environment. Recent developments in nanotechnology have opened up a new avenue for the development of more efficient formulations that can overcome many of the obstacles generally faced in their use in the field, such as loss of activity because of degradation, instability, volatilization, etc. This review discusses the key developments in this area, as well as the challenges in relation to nano-scale formulation of botanical pesticides. It presents an appraisal of the recent scientific research, along with an account of the products that have already reached the market. Whilst it acknowledges the great potential of nanotechnology-derived formulations of botanical pesticides for increasing agricultural productivity and reducing health and the environmental impacts, it also highlights the technological challenges that must be addressed to enable adoption of the technology for wider use in agri-food production.

Keywords: Nanotechnology, Botanical Pesticide, Sustainable agriculture, Environmentally friendly

1. Introduction

Global agricultural production suffers considerable damage every year due to pathogens, insects, and other pest species. Losses occur during production as well as during storage of agri-food products, with associated deterioration of food quality. The widespread use of synthetic pesticides belonging to several classes is currently employed for pest control in crops and stored food commodities (SHIVA, 2016a). However, the excessive use of pesticides also has negative impacts on human health and the environment, and leads to development of resistance in pest species (LAMICHHANE et al., 2015).

The use of compounds of natural origin, such as botanical pesticides, has emerged as an alternative to synthetic pesticides (PAVELA, 2016). Growing interest in these compounds for use in agricultural management has led to a number of advances in R&D into ways to develop effective formulations that can overcome the current challenges and enable their effective use in the field. As shown in Table 1, the use of innovative techniques and tools, such as nanotechnology, are essential for the sustainable growth of agri-food production (BENELLI et al., 2017a).

Table 1. Ideal characteristics of a pesticide formulation, advantages and limitations of botanical pesticides, and potential use of nanotechnology to addressing the limitations.

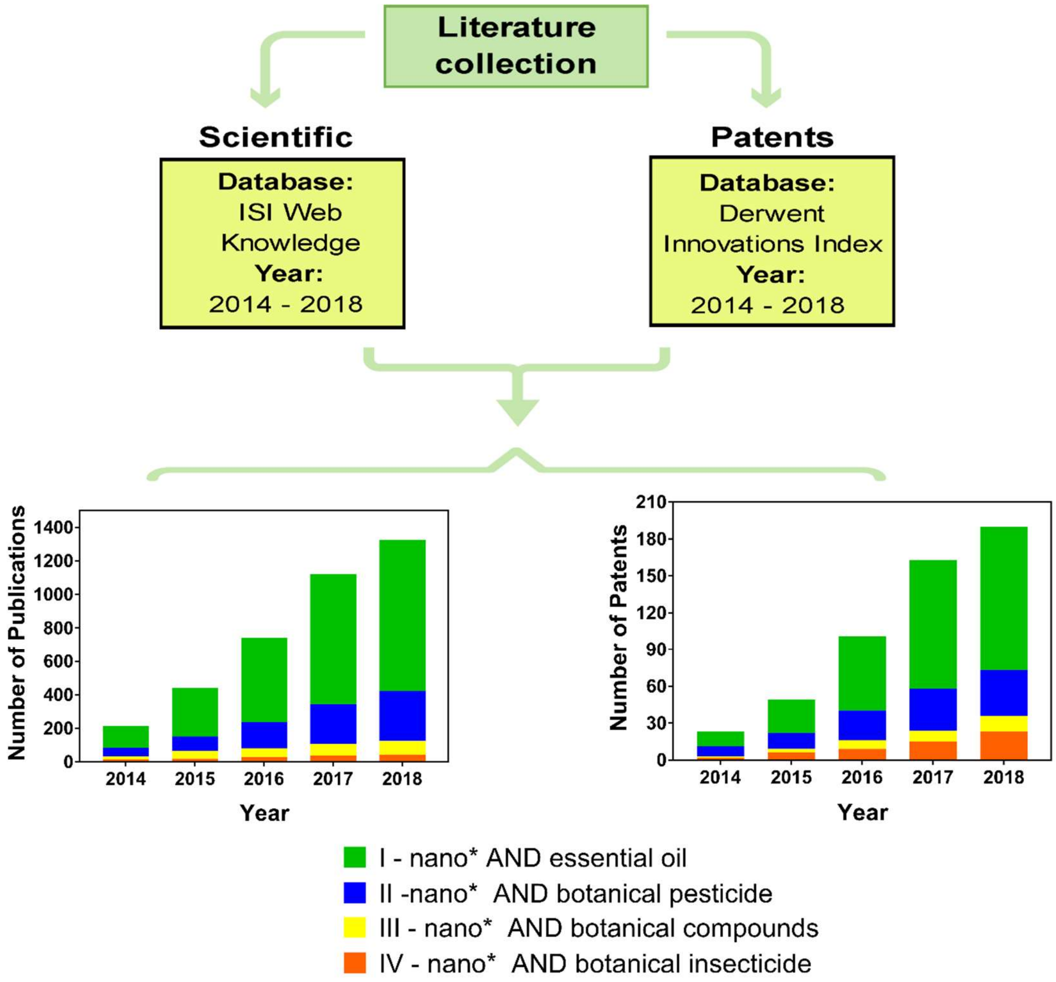
Ideal characteristics of pesticide formulations	**Botanical pesticides - advantages**	**Botanical pesticides - limitations**	**Potential use of nanotechnology**
Increased efficiency.	➢ Mixtures of active compounds (e.g. essential oils) can control pests and diseases via different modes of action. ➢ Many compounds act quickly, and thus inhibit feeding/damage by pests.	➢ Natural compounds may be rapidly degraded by environmental factors, such as sunlight, moisture, temperature, making the residual effects only short-lived.	➢ Formulation in nanocarriers can aid in increasing bioavailability, stability, and protection against rapid degradation. ➢ Formulations may offer intelligent controlled-release systems for active compounds that are triggered by changes in pH, temperature, or due to enzymatic activity.
Low toxicity towards non-target organisms.	➢ Due to natural origin, and rapid degradation, they generally have a comparatively lower toxicity to mammals.	➢ Some natural compounds (e.g. nicotine and rotenone) may have toxicities similar to those of synthetic compounds.	➢ Nanoencapsulation can improve efficiency while reducing the amount of the active compound. ➢ Nano-scale encapsulation can enable sustained release of the active substances that can also reduce toxic effects to non-target species by limiting their exposure.
No contamination of soils and water resources.	➢ Botanical pesticides are mostly biodegradable, non-persistent in the environment, and have low phytotoxicity.		➢ Development of formulations based on biodegradable matrices can further improve effectiveness.
Minimal development of pest resistance.	➢ Generally a mixture of active compounds (such as essential oils), and therefore the pesticidal action may be	➢ Amounts and qualities of active compounds may vary according to region and abiotic factors.	➢ Nanotechnology can enable development of targeted formulations for specific pest

	derived from several mechanism(s) of action.	➢ Claims and recommendations by producers may not have been scientifically verified.	species through functionalization of the carrier systems. ➢ Formulations may release active compounds only when triggered by a specific stimulus such as pH, temperature, moisture, enzymes, etc.
Easy to store.		➢ Low stability and short shelf life of formulations may make them difficult to store.	➢ Nano-encapsulation can increase the stability of active compound(s) during storage, and may improve dispersion/ solubility.
Low cost and economically viable.	➢ Abundance of promising plants and metabolites. ➢ Plant materials or extracts may be known to farmers and may already be used in local areas. ➢ The same plants may also have other uses, such as flavorings or medicinal applications.	➢ Extraction processes are generally expensive. ➢ Active components may not have been standardized.	➢ Formulation of botanical pesticides using nanotechnology is likely to improve effectiveness, demand and commercial availability.

In 2014, we published a review concerning the applications of nanotechnology for encapsulation of botanical pesticides for use in sustainable agriculture (DE OLIVEIRA et al., 2014a). Other groups have since published on this topic (PRASAD; BHATTACHARYYA; NGUYEN, 2017a; SINHA; GHOSH; SIL, 2017). Sahayaraj *et al.* (2014)(SAHAYARAJ, 2014) published a review outlining the potential of using nanotechnology for formulation of botanical pesticides. The authors highlighted the importance of reducing the use of synthetic pesticides and encouraged farmers to use botanicals. In addition to discussing potential use of nano-carrier systems, the authors emphasized the importance of field trials to elucidate the true potential of such systems. More recently, there has been a substantial increase in the numbers of articles and patents published (Figure 1) and around 238 articles and 34 patents have been published in this field by 2017.

To bring the information up-to-date, we carried out a detailed bibliographic search following the workflow shown in Figure 1. The search for scientific articles was carried out using the ISI Web of Knowledge database, while the search of patents used Derwent Innovations Index database. All relevant publications from 2014 onwards were catalogued. The literature searches were carried out using a combination of keywords (I to IV), with the articles as a function of time. All the terms were searched in English, and the searches were included published records until April 2018. The outcome of literature and patents searches showed a steep increase in the number of articles over recent years, which indicates increasing interest in the subject and the potential for market introduction of the formulations. In addition, the articles seem to be focusing on improvements in the technology to address the problems faced in the area. In this context, the present study reviewed the main advances in the area of nanotechnology and botanical pesticides, and assessed the main advantages and limitations in relation to their use in sustainable agriculture.

Figure 1. Scheme for selection and categorization of publications and patents.

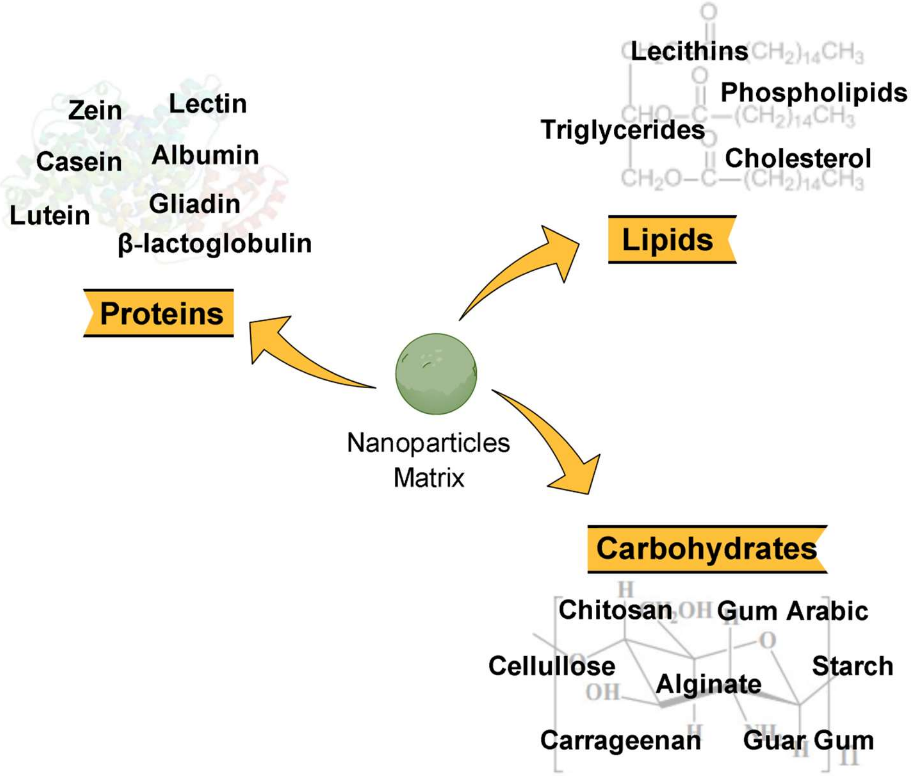


1.1 Advances in the field

1.1.1 Ecofriendly formulations

The use of botanical pesticides for agricultural applications, instead of synthetic pesticides, is generally considered an environmentally friendly option. This is largely due to the inherent characteristics of botanical materials, which generally degrade and therefore have a lower potential for long-term negative impacts on the environment and nontarget organisms (DE OLIVEIRA et al., 2014a). Encapsulation of these substances using nanostructured systems offers the further possibility for developing novel formulations with potentially enhanced properties and field performance. Such systems can be produced using different types of matrices (Figure 2), which may be of synthetic or natural origins and have desirable properties, such as biocompatibility and bioavailability (CALDERÓN; SOSNIK, 2015; KHANDELWAL et al., 2016).

Figure 2. Examples of matrices used for the production of nano-carrier systems.



In recent years, efforts have also been made for the development of green nanotechnology, i.e. seeking processes that can enable reduction in energy consumption, waste generation, and emissions of greenhouse gases. The use of materials from renewable sources for the production of nano-carrier systems can further contribute towards the development and implementation of green nanotechnology (SIVARAJ et al., 2015). Various matrices of natural origin have recently gained prominence, for example proteins, polymers, and gums. Many publications and patents have described the use of these materials for the production of nano-carrier systems for botanical pesticides (summarized in Table 2). .

One of the most prominent materials is zein, which is the main storage protein of maize, and represents about 50% of the total protein content of the plant seed. Zein belongs to the class of prolamins, with α-zein being the most abundant. Due to its low solubility in water, and high coating capacity, biodegradability and biocompatibility, this protein has been investigated for the production of nanoparticles and the encapsulation systems for botanical pesticides (PALIWAL; PALAKURTHI, 2014a). Veneranda *et al.*

(2018) used zein/sodium caseinate/pectin for the encapsulation of eugenol, using a complexation process induced by heat and pH. The authors investigated the influence of the concentration of each component used in the preparation process. The formulations obtained were stable, with good physicochemical properties and high dispersibility, and showed potential for use in agriculture and food industry. Our research group prepared and characterized zein nanoparticles containing the botanical compounds geraniol and R-citronellal. The nanoparticles presented good physicochemical properties and protected the active agents against UV degradation. The formulation was also shown to provide effective control of spider mite (*Tetranychus urticae* Koch), an important agricultural pest (OLIVEIRA et al., 2018). Although not registered for any specific compound, a number of patents have claimed the effective use of zein-based systems for nano-scale formulation. For example, patent CA2805581A1 describes the preparation and characterization of zein nanoparticles used for the encapsulation of biologically active compounds - both water-soluble and fat soluble. High encapsulation efficiencies were achieved for the compounds studied, and the system was recommended for potential applications in the agricultural, food, and pharmaceutical sectors.

Another useful natural polymer is alginate, which is a biopolymer extracted from algae by alkaline hydrolysis. Due to its biocompatibility and gelation properties, alginate has been extensively investigated for the production of various nano/micro particles including those containing botanical pesticides (PAQUES et al., 2014). Alboofetileh Mehdi *et al.* (2018) prepared alginate/clay nanocomposites for the encapsulation of marjoram essential oil. The material showed good physicochemical properties and the formulation provided effective microbial control, offering a promising formulation for the control of pathogens. Kavoosi *et al*. (2018) studied the encapsulation of Zataria oil in alginate particles. The formulation comprised mean particle diameter of 597 ± 22 nm, polydispersity index of 0.42, and zeta potential of -16.3 ± 4.2 mV. Encapsulation resulted in reduced volatility and increased stability of the active agent, and the system showed promise for use in different areas including agriculture.

Natural gums can be obtained from various sources, such as the tree exudates (an example is gum arabic, from *Acacia senegal*). Natural gums are often preferred over synthetic materials because they are generally nontoxic and low-cost. They are therefore considered promising materials for use in the preparation of nano/micro formulations. Khoshakhlagh *et al.* (2017) studied electrospray method to derived nanoparticles made of gum extracted from *Alyssum homolocarpum* seeds. The spherical particles ranging in size from 35 to 90 nm were loaded with 10 to 20% of D-limonene, achieving an encapsulation efficiency of 87-93%.

Chitosan is another biopolymer that has been widely investigated for the production of nano/micro particulate systems. Chitosan is a hydrophilic cationic polyelectrolyte obtained by the alkaline deacetylation of chitin, which is found in crustacean exoskeletons (AGNIHOTRI; MALLIKARJUNA; AMINABHAVI, 2004). Ahmadi *et al*. (2018) evaluated the acaricidal effect of chitosan nanoparticles containing *Achillea millefolium* essential oil against *Tetranychus urticae*. The nanoparticles presented spherical morphology and average diameters between 85 and 145 nm. In repellency and contact toxicity tests, the nanoparticles showed slow release and persistence of the oil on the leaf, with better results than the non-encapsulated oil. These findings demonstrated that encapsulation improved the efficiency of the essential oil in pest control. Campos *et al*. (2018) prepared nanoparticles of chitosan functionalized with β-cyclodextrin for the encapsulation of carvacrol and linalool. The nanoparticles containing carvacrol and linalool had mean diameters of 175.2 and 245.8 nm, respectively, and presented high encapsulation efficiency (around 90%). Biological assays with the mites *Tetranychus urticae* showed that the nanoparticles had repellent, acaricidal, and anti-oviposition activities, and were more effective than the non-encapsulated compounds. The patent, US9480656B1 describes a method for preparation of chitosan nanoparticles for encapsulation of rosemary essential oil. The nanoparticles produced had an average sizes from 69 to 99 nm and high encapsulation efficiency (>90%), showing promise for use in agriculture.

Table 2. Natural matrices for the production of micro/nano formulations of biopesticides.

Matrix	Carrier system	Carrier properties	Botanical compound	Main results	Reference
Alginate (ALG) and cashew gum (CG)	Nanoparticles	The spherical nanoparticles showed sizes in the range 233-399 nm and zeta potential from -30 to -36 mV.	*Lippia sidoides* essential oil	The essential oil was encapsulated in ALG/CG nanoparticles and the release was found to be dependent on the ALG:CG ratio. Faster release was observed with nanoparticles produced with a higher concentration of CG, which could be explained by higher hydrophilicity, allowing greater solute diffusion through the polymeric chains.	(OLIVEIRA; PAULA; PAULA, 2014)
Alginate (ALG)	Microparticles	The microparticles were spherical in shape, with sizes in the range 450-530 µM (dry state) and polydispersity index from 0.4 to 1.4.	Thyme essential oil	The microsphere size increased proportionally to the thyme essential oil concentration used in the preparation. An increase in the oil concentration led to loss of the spherical shape of the nanoparticles. The encapsulation efficiency decreased with increase of the thyme essential oil concentration, while the loading capacity increased. Encapsulation maintained antibacterial activity of the	(BENAVIDES et al., 2016)

					essential oil. The best performing formulation was produced with 2% of thyme essential oil.	
Alginate (ALG) and/or Galbanum gum (GG)	Film	_		*Ziziphora persica* essential oil (ZEO)	ALG gel, GG gel, and composite CAG gels improved the chemical and microbial properties of chicken fillet and increased its shelf life. The combination of these coatings with ZEO showed significantly higher in vitro antioxidant and antimicrobial activities. ALG had no significant effect on decreasing the microbial load of aerobic mesophilic and psychrotrophic bacteria, lactic acid bacteria, *Pseudomonas* spp., *Enterobacteriaceae*, or *L. monocytogenes* ($p > 0.05$), while the coating of chicken fillet with GG or CAG (alone or in combination with ZEO) showed significant differences to the other treatments during 12 days of storage.	(HAMEDI et al., 2017)

Alginate (ALG)	Microcapsules	The microcapsules had diameters close to 600 μm.	Cedarwood essential oil (CWO)	The results showed that a concentration of 3% of alginate provided good microcapsule viscosity. The addition of $CaCl_2$ at a concentration of 0.50 M resulted in the highest amount of CWO (177.2 mg per gram of microcapsules). The addition of crosslinking agent (glutaraldehyde) decreased the amount of CWO encapsulated. The encapsulation efficiency was 65% and the microcapsules did not show cytotoxic effects towards the L929 cell line.	(FERRANDIZ MARCELA et al., 2017)
Alginate (ALG) and montmorillonite nanoclay (MMT)	Nanocomposite films	The films were opaque, and provided a barrier against visible light.	*Origanum manjerona* essential oil (MO)	There was a reduction of around 47% in the water solubility of alginate films when MMT and MO were included in the films, while the tensile strength tended to decrease. However, the inclusion of MO in ALG/MMT films resulted in increased water vapor permeability of the films. ALG/MMT films containing 1.5% of MO showed inhibition of the growth	(ALBOOFETILEH MEHDI et al., 2018)

				of *E. coli*, *L. monocytogenes*, *B. cereus*, and *S. aureus*.	
Alginate or agar or carrageenan	Dispersion	The particles in the dispersions showed zeta potentials of -8.8, -16, and -8 mV for agar, alginate, and carrageenan, respectively. For all the systems produced, the polydispersity index was around 0.4. The dispersions showed a particle size range of 359-634 nm.	Zataria essential oil (ZEO)	Incorporation of ZEO in alginate, agar, or carrageenan resulted in decreases of pH, zeta potential, and particle size, and increases of the viscosity and antioxidant capacity of the dispersion. Encapsulation resulted in reduced volatility and increased stability and bioavailability of ZEO. The most effective polymer was carrageenan.	(KAVOOSI et al., 2018)
Gum arabic (GA); modified starch (MS); modified starch and maltodextrin (MD); modified starch and inulin (IN); gum arabic	Microcapsules	The microcapsules generally showed spherical shapes. Microcapsules produced with starch and gum arabic showed larger diameters. Addition of maltodextrin or inulin led to decreased diameters in both	Rosemary essential oil	The moisture of the microcapsules ranged from 1.40 to 3.56, with the highest values observed for the particles prepared with IN. The nanoparticles produced with IN required less time to become fully wet. There were no differences among the microcapsules in terms of oil retention, with the exception of the microcapsules produced with GA:IN. The type	(FERNANDES; BORGES; BOTREL, 2014)

and maltodextrin; gum arabic and inulin		cases, without significant difference between them.		of wall material significantly affected the size of the microcapsules, with the largest sizes observed using only GA or MS.	
Cashew gum (CG)	Nanoparticles	The nanoparticles presented a size range from 27.70 to 432.67 nm and had negatively charged surfaces (from -10.45 to -24.50 mV).	*Eucalyptus staigeriana* essential oil (ESO)	The formulations were produced varying the ratio of CG to ESO and also varying the ratio of ESO to Tween 80. An increase in the proportion of the matrix increased the encapsulation efficiency, while the oil loading decreased. Nanoparticles produced with both CG:ESO and ESO:TW80 (2:1) showed the greatest capacity to prolong ESO release. Increase of the CG concentration led to rapid oil release.	(HERCULANO et al., 2015)
Cashew gum (CG) and inulin (IN)	Microcapsules	The microcapsules had spherical shapes and sizes from 13.64 to 17.70 µm. No significant differences were observed in the polydispersion index values	*Ginger essential oil*	Increased inulin concentration coupled with decreased cashew gum concentration resulted in the formation of larger microcapsules. These nanoparticles presented cracks in the polymer network, which resulted in lower encapsulation efficiency, due to volatilization and loss of the active compound, compared to	(FERNANDES et al., 2016)

		of the different microcapsules produced.		microparticles with higher concentrations of cashew gum.	
Starch	Nanoparticles	The nanoparticles showed spherical shape, regardless of the temperature employed in the synthesis process. The nanoparticles loaded with menthone showed a particle size range from 93 to 112 nm, with a narrow polydispersity index range from 0.156 to 0.262.	*Menthone*	Higher nanoparticle synthesis temperature increased the nanoparticles yield, encapsulation efficiency, and loading capacity. All the nanoparticles showed slow and sustained release of menthone. However, nanoparticles produced using higher temperature showed improved sustained release. Both free and nanoencapsulated menthone showed antibacterial action against *E. coli* and *S. aureus*. However, the nanoencapsulation of menthone resulted in sustained antibacterial activity during the test period.	(QIU et al., 2017)
Alyssum homolocarpum seed gum (AHSG)	Nanocapsules	The morphology of the AHSG nanostructure was dependent on the D-limonene concentration, varying from a heterogeneous structure to	*D-limonene*	Addition of limonene (10 and 20%) and Tween 20 increased the viscosity of the solution and decreased the conductivity, which resulted in the formation of round and smooth nanocapsules. The encapsulation efficiency	(KHOSHAKHLAGH et al., 2017)

		ultrafine fibers. The size of the particles ranged from 35 to 90 nm and increased with increase of the D-limonene content.		was around 87-93% and decreased with increase of the limonene concentration. Encapsulation increased the thermal stability of limonene.	
Chitosan-benzoic acid (CS-BA)	Nanogel	The nanogel particles had a spherical shape and mean diameter below 100 nm.	*Rosmarinus officinalis essential oil (REO)*	The CS-BA nanogel loaded with REO and coated onto beef cutlets inoculated with *Salmonella typhimurium* caused a more pronounced reduction in the population of this bacteria, under refrigerated storage, compared to the use of free REO. Nanoencapsulation led to reduced pH increase of the beef cutlet samples.	(HADIAN et al., 2017)
Zein with sodium caseinate (SC) and chitosan hydrochloride (CHC)	Nanoparticles	Zein nanoparticles stabilized with SC and loaded with thymol showed a particle size range from 176.85 to 204.75 nm, polydispersity index below 0.2, and pH around 7. The nanoparticles stabilized	*Thymol*	Zein nanoparticles loaded with thymol were prepared using SC and CHC as electrostatic stabilizers. After lyophilization, the particles showed good dispersibility in an aqueous system at neutral pH. Double-stabilized zein nanoparticles showed higher thymol encapsulation (>80%), compared to	(ZHANG et al., 2014a)

		with CHC and SC had a size range from 199.40 to 741.55 nm, with polydispersity index around 0.2, and high positive zeta potential. The nanoparticles had a spherical shape and a smooth surface.		nanoparticles stabilized only with SC (65%). Nanoencapsulated thymol showed greater antibacterial activity against *S. aureus*.	
Zein and gum arabic (GA)	Nanoparticles	The zein nanoparticles presented a spherical structure and diameter between 100 and 165 nm. The zeta potential did not present any differences at pH 4-8. However, a smaller negative zeta potential (-26.6 mV) was obtained at pH 3 for the loaded nanoparticles.	*Peppermint oil*	A new method for the synthesis of zein nanoparticles employed propylene glycol and low intensity mixing. The nanoparticles produced had a mean diameter below 170 nm and the addition of GA resulted in stabilization of the nanoparticles in the pH range 3-8 and improved protein solubility at pH 4-8. The release of peppermint oil into PBS at pH 2, 4, and 8 showed that increase in pH resulted in faster release of the oil.	(CHEN; ZHONG, 2015)
Zein	Nanoparticles	Zein nanoparticles loaded with both carvacrol and thymol showed sizes in the	*Thymol and carvacrol*	The nanoparticles produced did not show any variation in colloidal stability (average diameter, polydispersity index, zeta potential,	(DA ROSA et al., 2015a)

		range from 108 to 122 nm for two storage temperatures (6 and 20 °C). The polydispersity index (below 0.3) showed a narrow size distribution. The zeta potential was in the range between +9 and +30 mV. The nanoparticles were spherical, with smooth surfaces.		and encapsulation efficiency), as a function of storage time (90 days), at both temperatures tested (6 and 20 °C). The nanoencapsulated thymol and carvacrol showed antimicrobial activity. However, higher antimicrobial activity was against Gram-positive bacteria, compared to Gram-negative organisms. The nanoparticles showed sustained release of both compounds, with around 50% release after 72 h.	
Zein and β-lactoglobulin	Nanoparticles	Nanoparticles loaded with tangeretin had a mean diameter of 263 ± 13 nm and a well-defined spherical shape.	*Tangeretin*	Concentrations >0.4% (w/v) of tangeretin in the initial organic phase resulted in formation of crystals, due to supersaturation of the compound in the nanoformulation. The nanoparticles were stable at low salt concentrations below 50 mM, while aggregation occurred at higher concentrations. The nanoparticles were stable at pH <4.5 and >5.5, but were unstable at pH between these values. The nanoparticles	(CHEN et al., 2014a)

				tended to aggregate at temperatures higher than 60 °C.	
Zein	Microparticles	The unloaded microparticles showed a diameter of around 1.21 μm, while the addition of essential oil resulted in an increase to 4.23 μm. The zein microparticles presented an irregular shape.	*Thyme essential oil (TEO)*	The encapsulation efficiency of TEO varied from 65.72 to 97.02% for non-centrifuged and centrifuged microparticles, respectively. The antimicrobial activity of the free essential oil was greater than that of the nanoencapsulated essential oil for all six bacteria and two yeasts tested. The highest activity was observed for Gram-positive bacteria, followed by Gram-negative organisms and yeasts. The thyme essential oil was released rapidly from zein nanoparticles to the medium containing triacylglycerols, with 85.3% release after 45 min.	(BILENLER TUGCA et al., 2015)
Zein	Films	The average thickness of the films, unloaded or loaded with ZEO, was 33 ± 2 μm. The films showed high transparency and no	*Zataria multiflora Boiss. essential oil (ZEO)*	Zein films were loaded with 5 or 10% ZEO. In terms of antimicrobial activity, the zein film with 5% ZEO showed reductions of 1.18 log and 1.14 log against *L. monocytogenes* and *E. coli*, respectively. The reductions increased to 2.16 log and 2.65 log when films with 10% ZEO were used. The zein films were also	(KASHIRI et al., 2017)

		significant differences were observed after addition of ZEO.		effective in controlling the *L. monocytogenes* population, after coating propylene bags with this film for the preservation of pasteurized cow's milk.	
Zein-caseinate-pectin	Nanoparticles	Nanoparticles produced without caseinate showed a size range from 409 to 786 nm, polydispersity index below 0.3, and zeta potential above -40 mV. The addition of caseinate resulted in smaller nanoparticles with sizes ranging from 186 to 331 nm, while there was no change in the polydispersity index, and the zeta potential became slightly more negative.	*Eugenol*	The concentrations of zein and sodium caseinate, as well as heating and pH conditions, played crucial roles in the synthesis of nanoformulations with small and homogeneous particle size. The nanoformulations produced with and without caseinate showed good dispersibility in water. The presence of caseinate helped to maintain the original nanoscale dimensions of the particles during dispersion.	(VENERANDA et al., 2018)
Zein	Nanoparticles	Nanoparticles loaded with geraniol had a mean diameter of 172.3 ± 3.8, polydispersion index of 0.351 ± 0.032, and	Geraniol and R-citronellal	The botanical formulations showed high encapsulation efficiency (>90%), good physicochemical stability, and effective	(OLIVEIRA et al., 2018)

		zeta potential of −18.8 ± 1.02 mV. Nanoparticles loaded with R-citronellal had a mean diameter of 142.5 ± 9.3, polydispersion index of 0.330 ± 0.052, and zeta potential of −12.8 ± 0.98 mV.		protection of the repellents against UV degradation. Cytotoxicity and phytotoxicity assays showed that encapsulation of the botanical repellents decreased their toxicity. Repellency activity tests showed that nanoparticles containing the botanical repellents were highly repellent against the *Tetranychus urticae* Koch mite.	
Chitosan	Nanocapsules	Nanoparticles loaded with AEO had sizes ranging from 82 to 165 nm, depending on the pH used. All the nanoparticles were spherical.	*Achillea millefolium* L. essential oil (AEO)	Higher pH during the synthesis resulted in a greater mean diameter of the nanoparticles, while the encapsulation efficiency decreased. In terms of fumigant activity against *Tetranychus urticae*, the LC_{50} for the free essential oil (5.05 µL/L) was 3.74 times lower than observed for the nanoencapsulated oil (18.90 µL/L), at pH 3.5. However, increase of the pH value resulted in decreased effectiveness of the formulation, as demonstrated by increases of the LC_{50} values. For contact toxicity against *T. urticae*, the LC_{50}	(AHMADI et al., 2018)

				values were 5.51, 4.23, 1.88, and 1.56 µL/cm for free AEO and the encapsulated oil prepared at pH 3.5, 4.5, and 5.5, respectively. Both assays with mites showed that the sustained release of AEO increased the effectiveness of this oil.	
Chitosan	Nanoparticles	The nanoparticles showed mean sizes ranging from 30 to 80 nm and were spherical in shape.	*Carum copticum* essential oil (CEO)	The best formulation was achieved with a ratio of chitosan to CEO of 1:1, using 0.5% (w/v) TPP as crosslinking agent. The nanoparticles released the CEO faster at acid pH, followed by basic and neutral conditions. In all cases, there was an initial burst release, followed by sustained release over time. The encapsulation of CEO could maintain the antioxidant activity, protect the phenolic compounds of CEO, and improve the antimicrobial activity.	(ESMAEILI; ASGARI, 2015)
Chitosan and alginate	Nanocapsules	The nanoparticles had a spherical surface structure, sizes in the range from 226.4 to 786.6 nm, and zeta	Turmeric and lemongrass essential oils	The best nanoparticles for both turmeric and lemongrass essential oils were obtained using 0.3 mg/mL alginate and 0.6 mg/mL chitosan, which resulted in the smallest particle sizes.	(NATRAJAN et al., 2015)

		potentials in the range from -21.3 to + 45.5 mV.		The encapsulation efficiencies for turmeric and lemongrass essential oils were 71.1 and 86.9%, respectively. The sustained release was evaluated at pH 1.5 and 7.4. For turmeric oil, there was release of approximately 90% and less than 70% of the oil, at pH 7.4 and 1.5, respectively, after 48 h. For lemongrass oil, there was release of 42% and less than 38%, at pH 7.4 and 1.5, respectively. High cell viability was observed in the presence of the carrier containing essential oils, indicating that it was nontoxic.	
Chitosan	Nanoparticles	The nanoparticles presented a size range from 100 to 190 nm, loading capacity below 4%, and encapsulation efficiency below 17%.	*Cinnamomum zeylanicum* essential oil (CEO)	A higher CEO concentration resulted in greater mean nanoparticle diameter, while the encapsulation and drug loading efficiencies decreased. The nanoparticles showed an initial burst release, followed by sustained release over time. After 40 days, only 31.65% of the oil was released from the nanoparticles. CEO nanoencapsulated at 1.5 g/L significantly	(MOHAMMADI; HASHEMI; HOSSEINI, 2015)

				decreased disease incidence and severity in cucumbers inoculated with *Phytophthora drechsleri*, during storage at 4 °C.	
Chitosan	Nanoparticles	The chitosan nanoparticles had spherical shapes and sizes in the range from 135.52 to 237.40 nm. The zeta potential was between -7.54 and -21.12 mV.	*Satureja hortensis* L. essential oil	Average nanoparticle diameter increased significantly at higher pH, due to aggregation. The encapsulation efficiency increased proportionally to the oil concentration. However, use of concentrations higher than 1.4% resulted in decreased encapsulation efficiency, due to supersaturation. The nanoencapsulated oil exhibited strong concentration-dependent antibacterial activity and DPPH scavenging activity.	(FEYZIOGLU; TORNUK, 2016)
Chitosan	Nanoparticles	The nanoparticles had spherical shapes and sizes in the range 50-100 nm. The zeta potential was higher than +50 mV.	Cardamom essential oil (CEO)	The nanoparticles loaded with CEO were able to control the growth of multidrug-resistant *S. aureus* and *E. coli* for up to 7 days, while no control was observed using empty nanoparticles or free CEO. The nanoencapsulated CEO did not show hemolytic or cytotoxic behavior in human	(JAMIL et al., 2016)

				corneal epithelial cells and the HepG2 cell line.	
Chitosan-cinnamic acid (CS-CI)	Microgel	Microencapsulated GPEO showed spherical particle morphology and particle sizes in the range from 7 to 90 µm.	*Gaultheria procumbens* essential oil (GPEO)	Microencapsulation of GPEO enhanced its antimicrobial and anti-aflatoxigenic activities, as well as stability, compared to free GPEO. The microencapsulated GPEO negatively affected the cell membrane and intracellular components of target *A. flavus* species, causing cell death and decreased aflatoxin secretion.	(KUJUR et al., 2017)
Chitosan	Nanoparticles and nanocapsules	The average size of the nanoparticles was 6.4 ± 0.5 nm, while the nanocapsules were larger (9.1 ± 1.6 nm). Both were spherical in shape.	Thyme essential oil	The encapsulation efficiency was higher for carvacrol than for thymol, in both the nanoparticles and the nanocapsules. For the nanoparticles, 100% release of carvacrol and thymol was observed in 390 and 360 min, respectively. The release from the nanocapsules was slower for both compounds, with 100% release of carvacrol and thymol in 540 and 630 min, respectively. The nanoparticles loaded with thyme essential oil showed greater antimicrobial activity against	(SOTELO-BOYÁS et al., 2017a)

				S. aureus, *L. monocytogenes*, *B. cereus*, *S, typhi*, *S.dysenteriae*, and *E.coli*, compared to the nanocapsules.	
Chitosan	Nanoparticles	The nanoparticles were spherical in shape and had a size of 235.6 nm, zeta potential of around 25.1 mV, and polydispersity index of 0.33.	Cinnamon essential oil (CEO)	The encapsulation efficiency of CEO was 37.87%. Sustained release tests were performed at pH 5.0 (the pH of beef patties). There was an initial burst release, followed by a more gradual increase in the cumulative release, finally reaching a plateau without additional CEO release after 56 h, when 61.7% of the oil had been released. Both free and encapsulated CEO decreased the microbial population of the patties, compared to the control ($p < 0.05$), throughout the experiment. However, free CEO had an unfavorable impact on color and odor, while the incorporation of encapsulated CEO improved consumer acceptance.	(GHADERI-GHAHFAROKHI et al., 2017)

Chitosan-β-cyclodextrin and TPP	Nanoparticles	The nanoparticles loaded with carvacrol had a mean diameter of 175.2 ± 2.97 nm. For linalool, the mean diameter was 245.8 ± 29.5 nm. The zeta potential was positive and the polydispersity index was below 0.3. The nanoparticles were spherical in shape.	Carvacrol and linalool	The nanoparticles showed mean diameters that differed according to the compound nanoencapsulated. However, both carvacrol and linalool nanoformulations were stable over time during storage. Biological assays with mites (*Tetranychus urticae*) showed that the nanoparticles possessed repellency, acaricidal, and anti-oviposition activities against this organism. Nanoencapsulated carvacrol and linalool were significantly more effective in terms of acaricidal and anti-oviposition activities, while the unencapsulated compounds showed better repellency activity.	CAMPOS et al., 2018[a]
Chitosan-β-cyclodextrin and gum arabic	Nanoparticles	The nanoparticles co-loaded carvacrol and linalool had a mean diameter of 225.9 ± 5.23 nm. The zeta potential was positive 19.3 ± 0.61 mV and the polydispersity index	Carvacrol and linalool co-loaded	The nanoparticles were effective in control of the two species used in this study, presenting both repellent and insecticidal activity in both cases, while decreased oviposition was also observed for *T. urticae*. An important feature was that in both cases, the combined	(CAMPOS et al., 2018c)

		was 0.185 ± 0.02. The nanoparticles were spherical in shape and remained stable for 60 days.		encapsulation of the monoterpenes (carvacrol and linalool) provided greater effectiveness, compared to encapsulation of only one of the compounds (data not shown).	
Chitosan and gum arabic	Nanoparticles	The nanoparticles loaded geraniol had a mean around 200 nm. The zeta potential was positive 32.1 ± 2.1 mV and the polydispersity index was around 0.4. The nanoparticles remained stable for 90 days and presented spherical in shape.	Geraniol	The chitosan/gum arabic nanoparticles containing the active agent geraniol showed good colloidal properties and were also able to protect the active compound against degradation by UV radiation. The biological results with whitefly showed that the nanoparticle formulation containing geraniol presented significant attraction activity and could be used in trap systems.	(DE OLIVEIRA et al., 2018)
Chitosan	Microcapsules	The microcapsule diameters were 289.3, 3893.2, and 8843.2 nm for particles produced using Tween 60, Span 80, and Tween 20/Span 80 as emulsifiers. The	Citrus essential oil	The microcapsules produced with Tween 60 presented the smallest particle size and the best encapsulation efficiency (68.1%). Use of the emulsifiers with higher HLB resulted in smaller microcapsules with higher stability. The surface energy increased with the	(LI et al., 2018)

		particles had spherical shapes and fairly uniform structures.		reduction of particle size, so less internal energy was needed for melting.	
Chitosan linked to ferulic acid	Nanogels	The nanogels showed a mean particle size range between 1021 and 5060 nm, with zeta potential from +31.27 to +55.34 mV. The nanogels possessed spherical cavities in their structures.	*Lippia origanoides* Kunth essential oil	The nanogels synthesized with high amounts of ferulic acid and loaded with the essential oil showed pores inside the nanogel, suggesting that a high concentration of essential oil was stably encapsulated in this material. The number and size of the microporous holes inside the materials increased with increase of the ferulic acid concentration used in the synthesis, and was associated with a greater presence of the essential oil.	(ALMEIDA et al., 2018)
Chitosan	Nanoparticles	The nanoparticles had a size range from 154.9 to 340.8 nm, depending on the CO concentration used. The polydispersity index was below 0.3 and the zeta potential was higher than 30 mV.	Clove oil (CO)	The best performing formulation was produced using 2.50 mg/mL of CO. The nanoparticles showed high antibacterial activity against *E. coli* biofilms, with a reduction of 99.98% in the bacterial population after 8 h. Incorporation of the nanoparticles into gelatin nanofibers reduced the *E. coli* population by 99%.	(CUI et al., 2018)

Zein or polylactic acid (PLA)	Fibers	Zein fibers with or without carvacrol showed a ribbon-like morphology. PLA fibers without carvacrol presented smooth surfaces, while fibers containing carvacrol showed a fused morphology.	Carvacrol	The zein fibers showed an increase in diameter after incorporation of 5% carvacrol. However, further increase in the oil concentration led to a decrease in the mean diameter of the fibers. In contrast, for PLA fibers, an increase in the carvacrol content resulted in an increase of the mean fiber diameter. The antioxidant activities of carvacrol loaded into zein and PLA fibers were in the ranges 62-75% and 53-65%, for carvacrol contents between 5 and 20%.	(ALTAN; AYTAC; UYAR, 2018)

As indicated above, a lot of R&D has recently been focused on finding environmentally friendly formulations for sustainable agriculture. An important point is that despite the efforts to use materials of natural origin for the production of nanostructured systems, toxicological analyses and mechanistic studies are essentially needed to understand any risk of these materials (TAN et al., 2018). The encapsulation of botanical pesticides represents an attractive technological advancement, from the perspective of both increasing the effectiveness of the compounds, as well as safety to the environment and human health. However, as numerous factors and processes can affect the environmental behavior and effects of nano-scale formulated pesticides, this requires a new and refined approach to risk assessment (KOOKANA et al., 2014).

1.1.2 Botanical pesticide products

The botanical pesticide market has grown in recent years. This seems to have been driven mainly by increased concerns over contamination of foodstuffs with residues of synthetic pesticides. This together with more stringent regulatory and import/export controls has changed to focus to new 'safer' alternatives (ISMAN MURRAY B, 2015). The use of botanical pesticides in pest control dates back many years, with records of the use of plant extracts by the Egyptians for the protection of stored products and for the control of disease causing microorganisms (ISMAN; MIRESMAILLI; MACHIAL, 2011; ATANASOV et al., 2015). The traditional use of plant extracts for crop protection is still continued, with many of these being produced by farmers according to the traditional recipes passed on from generation to generation, often using plant species that are abundant in the particular region. Although previous work has highlighted their importance, it is difficult to assess the quantities used for such traditional purposes due to the great diversity of plant species (MKENDA et al., 2015).

A number of products derived from plants and used traditionally in agriculture are also marketed now as botanical pesticides. As research has progressed, it has enabled the identification and isolation of specific active substances, some of which have also been used for the production of botanical pesticides (MIRESMAILLI; ISMAN, 2014). An example is the oil of neem (*Azadirachta indica* Juss., Meliaceae), which is extensively used in Indian subcontinent for agricultural pest control applications and is currently one of the most widely sold botanical pesticides at commercial scales. The oil from seed and other parts of neem tree contain a number of bioactive metabolites, including azadirachtin (A-G) that are used in several botanical pest-control products (CAMPOS et al., 2016). Table 3 shows examples of commercially produced botanical pesticides.

Table 3. Examples of commercially produced botanical pesticides, classified according to active compound, with trade names, main applications, and manufacturer.

Active compounds	Commercial products	Applications	Manufacturers
Neem oil *Azadirachta indica* Juss. (Meleaceae)	BioNeem®	Broad spectrum of action against over 300 species of insects. • Anti-feeding • Insect growth regulator • Reproduction inhibitor • Insect repellent • Other physiological effects	Woodstream Corporation (USA)
	DalNeem®		Dalquim Ltda. (Brazil)
	OzoNeem Oil®		Ozone Biotech (India)
	Margasom®		Agri Life (India)
	Triact70EC®		Certis Company (USA)
	Molt-X®		BioWorks, Inc. (USA)
	Neemix 4.5®		Certis (USA)
Azadirachtin	Fortune Aza3%EC®		Fortune Biotech (USA)
	Azamax®		UPL Ltda. (Brazil)
Garlic oil *Allium sativum* L.	EcoA-Z®, L'EcoMix®, CapsiAlil®	Natural repellent of nematodes, mites, and various insect pests.	EcofloraAgro (Colombia)
	Garlic Barrier®		Garlic Research Labs, Inc. (Canada)

(Liliaceae)	Biorepel®		SaferGro Laboratories Inc. (USA)
Chenopodium ambrosioides essential oil	Requiem EC®	Foliar-applied insecticide for use on a range of field-grown fruit and vegetable crops.	Bayer AG (Germany)
Rotenone	5% Rotenone ME®	Broad-spectrum insecticide with nonselective action.	Beijing Kingbo Biotech Co. Ltd. (China)
	Rotenone Dust®		Bonide Products, Inc. (USA)
Thyme essential oil	EcoVia WD®	Broad-spectrum insecticide against ants, beetles, fleas, flies, ticks, spiders, and other insects. Internal and external application.	Rockwell Labs Ltd. (USA)
Clove oil	Ant Out®	Ant killer. It wipes out all types of ants and controls all life stages (egg, larva, pupa, and adult). It is also effective against spiders, silverfish, crickets, and other common insect pests.	SaferGro Laboratories Inc. (USA)

		Applications in homes, parks, schools, yards, gardens, and organic food production.	
Jojoba oil	Eco E-Rase®	Effective against powdery mildew and whitefly.	IJO Products (USA)
Limonene	Demize EC®	Insecticidal, fungicidal, and acaricidal activity in a wide range of crops.	Paragon Professional Pest Control Products (USA)
	Prev-Am®		Oro Agri SA (Pty) Ltd. (South Africa)
Mixtures of active agents			
Rosemary, thyme, and clove oils	Sporatec®	Mite and insect control.	Brandt (USA)
Clove, rosemary, and peppermint oils	EF400®	Broad-spectrum fungicidal activity against powdery & downy mildew, white and gray molds, fruit rots, rusts, leaf spots, and blights.	Anjon AG, Inc. (Canada)
Thyme, clove, cinnamon, peppermint, and garlic oils	BacStop®	Broad spectrum bactericide/fungicide.	

Peppermint, cinnamon, and sesame oils	Flying Insect Killer®	Effective against flies, gnats, mosquitoes, moths, and other listed pests.	EcoSMART (USA)
Rosemary oil, peppermint oil, and geraniol	Mosquito Fogger®		
Rosemary, cinnamon leaf, lemongrass, and geraniol	Insect Repellent®		
Clove, rosemary, peppermint, thyme	Home Pest Control®		
Rosemary oil, peppermint oil, and geraniol	Essentria® IC-3	Effective against ants, bed bugs, cockroaches, fleas, flies, mosquitoes, occasional invaders, spiders, ticks, wasps, and 30+ other pests.	Zoëcon Professional Products (USA)
2-Phenethyl propionate and pyrethrin	EcoPCO®		
Lavandin oil and geraniol	Swine Clear®	Highly effective deterrent against all flying, biting, and bloodsucking pests, including mosquitoes, flies, lice, ticks, and all types of mite.	Barrier Animal Healthcare (USA)

As emphasized above, the encapsulation of botanical pesticides in nano- and microstructured systems has emerged as an important tool to improve quality of the formulations, increase persistence of the active agent(s) in the field and also improve their stability (DE OLIVEIRA et al., 2014a; SAHAYARAJ, 2014). Consequently, commercial companies have been seeking to market nanotechnology-based formulations of botanical pesticides. However, the number of formulations based on these technologies is still lower than those that use the botanical substance(s). Therefore, it is necessary to strengthen the collaboration between commercial companies, university and public research institutions, to advances this field further (SINGH, 2014). Table 4 presents some commercial products based on nano- and microencapsulation systems for botanical pesticides. The marketing of products based on botanical pesticides is already a commercial reality, although they have not yet emerged as a complete replacement for synthetic pesticides. Nonetheless, the agricultural sector is already benefiting from coexistence of these products, although botanical products are produced on a relatively much smaller scale (DIMETRY, 2014). The development of nanotechnology has the potential to contribute to the more promising formulations in terms of increased efficiency and cost-effectiveness of botanical materials/compounds. It can be reasonably expected that further R&D into practical applications will increase the number of products that are commercially available. Another aspect in this regard to consider is that many synthetic pesticides have been banned from use due to regulatory and phytosanitary issues and development of any new lead molecules is also stagnant. This adds to the need for increased emphasis on the use of botanical pesticides (DAMALAS; KOUTROUBAS, 2018). However, a number of challenges need to be addressed before botanical pesticides will be able to gain a substantial share of the pesticide market. These factors are further explored in the following sections.

Table 4. Commercially produced botanical pesticides based on nano/micro encapsulation technology.

Active compounds	**Commercial products**	**Formulation characteristics**	**Applications**	**Manufacturer's claims**	**Manufacturers**
Eugenol, geraniol, and thymol	Melavone CS® 3Logy® Hawk™	Yeast cells as matrix Neutral pH Diameter < 500 nm Active release on contact with water	Botrytis in grapes, field and greenhouse aubergines, kiwis, pomegranates, and fresh onions.	Eden's encapsulation technology platform provides slow release of active ingredients. This environmentally friendly solution enables terpenes to be used as effective low-risk agrochemicals.	Eden Research (UK)
Botanical compounds	eShield™ Technology	No description	Microencapsulation of botanical compounds as biopesticides for pest control.	EcoPesticides offers a technology that protects biopesticides from harmful solar UV radiation, preserving potency and substantially increasing lethality.	Ecopesticide International (USA)
Pyrethrin	Microcare 3%®	Liquid capsule suspension Polymer Matrix Diameter > 1µm	Ants, Asian lady beetles, box elder bugs, clover mites, cockroaches, confused flour beetles, crickets, dermestid beetles, dried fruit beetles,	Microcare 3% is the only microencapsulated pyrethrum product that provides fast knockdown and residual control. The technology creates a shell	Whitmire Micro-Gen Research Laboratories Inc. (USA)

			drugstore beetles, firebrats, fleas, flies, and gnats, moths, scorpions, silverfish, sowbugs, spiders, ticks, trogoderma, wasps, weevils, and yellowjackets.	around the active ingredient that increases residual control by protecting it from ultraviolet rays, weather, porous and greasy surfaces, and absorption into soil or organic matter.	
Botanical extracts	Tag Folder® Tag Combo® Tag Poly® Gama Flo® Eco® Agro Clean®	Nanoemulsions Better penetration	Recommended for all crops. Broad-spectrum action against a wide range of sucking insect pests, mites, and early instar leaf folder caterpillars.	Nanoscale size allows better penetration and effect of the active compounds.	Tropical Agrosystem Ltda. (India)
Extracts of *Cinnamomum verum*, *Syzygium aromaticum*, and	NanoGuard®	Nanoemulsion Diameter < 200nm Stabilization by surfactants	Protectant bactericide and fungicide.	The nanotechnological formulation improves the stability of the active compound and protects against premature degradation.	Vision Mark Biotech (India)

Cinnamomum camphor					
Botanical extract of *Pongamia Pinnata* Shakti	Shakti Falcon®	Nanoemulsion Stabilized with non-ionic surfactant	All kinds of mites; sucking insects such as thrips, jassids, and aphids; whitefly; others.	Slow release of the active compound and long-lasting effects on pests.	Nivshakti Bionergy Ltda. (India)
Botanical compounds	BotanoFresh™ OrGranular™ PotatoFresh™ Organocide™ DeccoTab™ NanoHMO® Mealynok®	Polymer Matrix Controlled difusion BotanoCap's technology is protected by patents covering the composition of compounds, the process of preparation and applications.	BotanoCap produces biopesticides for crop protection during growth. These products include insecticides and repellants, fungicides, bactericides, and nematicide.	BotanoCap’s technology consists of proprietary nano/micro-encapsulation that enables the slow release of volatile compounds, such as essential oils. BotanoCap has full control over the particle size and the release profile of the encapsulated compound.	BotanoCap (Israel)

1.2 Limitations

As already pointed out, the search for new alternatives to synthetic pesticides has become an important field of scientific research. The advances achieved have also contributed to improving food security, based on sustainable agriculture (DE OLIVEIRA et al., 2014a). Although botanical pesticides cannot entirely replace the use of synthetic ones at present, they are already contributing significantly to reducing the problems caused by the excessive use of synthetic pesticides. Nanotechnology-derived formulations provide further improvements in bio-efficacy, stability of the active agent(s) against early degradation by external factors such as light, humidity, temperature, and others (FERNANDES et al., 2016; PRAKASH et al., 2018). However, despite such advances, there are a few limiting factors that may undermine or restrict the wider use of botanical pesticides.

1.2.1 Botanical pesticides composition

The effectiveness of botanical pesticides in controlling pests and pathogens is directly related to their composition. These products may comprise plant extracts, which contain a mixture of active compounds of variable concentrations, or isolated phytochemicals (PAVELA; BENELLI, 2016). The main limitations for wide scale use of these materials/compounds include high cost of the raw materials and extraction/purification and formulation steps, and difficulties in optimizing the quantity and quality of the active agents (ISMAN, 2017). The extraction process is a critical determinant of the bioactivity of phytochemicals because plants may contain complex mixtures of numerous compounds, such as alkaloids, terpenoids, flavonoids, etc. The quality and quantity of the active compounds are influenced by the extraction technique, the part(s) of the plant used, the solvents used, and the type of equipment used (PAVELA, 2014).

The concentration and application rates of botanical substances can also be limiting factors for their use as pesticides. The concentration of a compound is a critical factor that determines biological effects (NOLLET; RATHORE, 2017). Several studies have reported the repellency activity of the essential oil of rosemary (*Rosmarinus officinalis* L.) against different pest species (S.; PRAKASH, 2015; TAK JUN-HYUNG; JOVEL EDUARDO; ISMAN MURRAY B, 2015; DAYARAM; KHAN, 2016). In contrast, Sadeh *et al*. (2017)(SADEH et al., 2017a) recently reported that whitefly

(*Bemisia tabaci*) is attracted to rosemary essential oil. This effect seems to be due to the composition and concentration of the volatile compounds present in the plant. The authors noted that compound concentrations play a key role in the biological effect, and that attraction/repellency effects may also be influenced by environmental and geographic variables, as well as by stress induced by abiotic factors.

In order to achieve large-scale adoption of botanical pesticides in agriculture, the plant material needs to be available in sufficient quantities to ensure continuity of supply. For this reason, botanical pesticides are often more expensive, compared to the synthetic chemicals, due to the limited cultivation, limited yields of the materials of required quality. An additional problem is that the amounts of secondary plant metabolites can differ according to location or season, making it difficult to standardize the pesticides in terms of quality (ISMAN; MIRESMAILLI; MACHIAL, 2011; PAVELA; BENELLI, 2016; BENELLI et al., 2017a). A possible solution could be to encourage research aimed at synthesizing bioactive compounds using techniques such as tissue culture in bioreactors. In addition, techniques including polyploidization and plant micropropagation are also important tools that could help to increase the production of biologically active substances (DZIGGEL CLARISSA; SCHÄFER HOLGER; WINK MICHAEL, 2017; MOSES et al., 2017).

In the development of more effective and standardized products, it is necessary to study the isolated compounds and their formulation to reduce the variables to more precisely investigate the effects of active compound(s) present in a given plant. This approach can also enable the development of novel formulations comprising mixtures of various active agents that are not normally found together in nature. For example, the essential oil of citronella contains geraniol, citronellal, citronellol, and limonene. In contrast, cinnamaldehyde, eugenol, and alpha-pinene are the main components of cinnamon essential oil. Consequently, a mixture of geraniol and cinnamaldehyde is not found in natural sources. Therefore, the production of formulations based on mixtures of active compounds could not only provide better pesticidal action but may also help to reduce the chances for the development of resistance in pests because of the diversity of the mechanisms of action.

1.2.2 Regulatory barriers

Amongst the main barriers affecting the commercialization and marketing of new botanical pesticide products are regulatory controls and insufficient government support (CHANDLER et al., 2011). Pesticides being heavily regulated substances, have to go through lengthy and expensive testing and documentation procedure for the registration as new products. This has largely been caused by increasing negative perceptions of the consumers towards pesticide residues in foods (SOLA et al., 2014), and no distinction is made between a synthetic pesticide or a biopesticide in most countries. The high costs associated with overcoming regulatory barriers is a major difficulty for botanical pesticides, since the market for these products is still limited, and any profits made are not able to compensate the expense of regulatory compliance (LOW TENG YONG et al., 2017). It is however clear that botanical pesticides cannot be excluded from testing and regulatory procedures, because they may also present risks and cannot not be assumed to be totally safe (examples are rotenone and nicotine). Nonetheless, vast majority of the botanicals, including essential oils and their main constituents, do not present substantial toxicity to mammals and non-target organisms - not at least the level of some synthetic ones. The historical use of some of these substances as food and beverage flavorings, as well as culinary spices, needs to be taken into consideration in their registration and should be an important point to be considered in the development of new products (PAVELA, 2016).

Differences in regulatory frameworks in various jurisdictions also pose a trade barrier. In many countries, agricultural trade is based on commodities, which has contributed to the increased restrictions on pesticides, notably in the case of exports to developed countries (YEUNG et al., 2017). Countries that export to markets such as the European Union and the United States have to comply increasingly stringent regulations, and only approved pesticides can be used if agri-food products are to be exported. Therefore, even where countries have abundant botanical material available for the development of botanical pesticides, they may be restricted to the use of other compounds, including for the domestic market (JENNINGS; LI, 2017).

It can be seen from the discussion presented here that nanopesticides represent a major technological advancement in terms of improved effectiveness and contributions towards protection of the environment and human health. In terms of regulations, it is necessary to take into account that the factors and processes that influence the environmental behavior and toxic effects of nanopesticides may be different from those that apply to conventional pesticides. Consequently, it is necessary to develop new or more refined risk assessment techniques (KOOKANA et al., 2014). Unlike conventional (synthetic) pesticides, the properties of nanopesticides (such as toxicity, absorption, bioavailability) depend on many variables including the chemical composition of encapsulating materials, size distribution, nanoparticle concentration, zeta potential, etc. Therefore, methodological adaptations or additional techniques for the characterization of these systems are necessary (KAH, 2015a). The risk assessments currently used for nanopesticides, such as those implemented in the European Union, provide a useful framework, but (as shown above) adaptations are necessary in order to obtain a clearer understanding of the real factors that may drive safety concerns. Technological advances in this area, including the development of new tools and techniques, will certainly contribute to improving our understanding of the effects of the materials (JOSÉ VILLAVERDE et al., 2017).

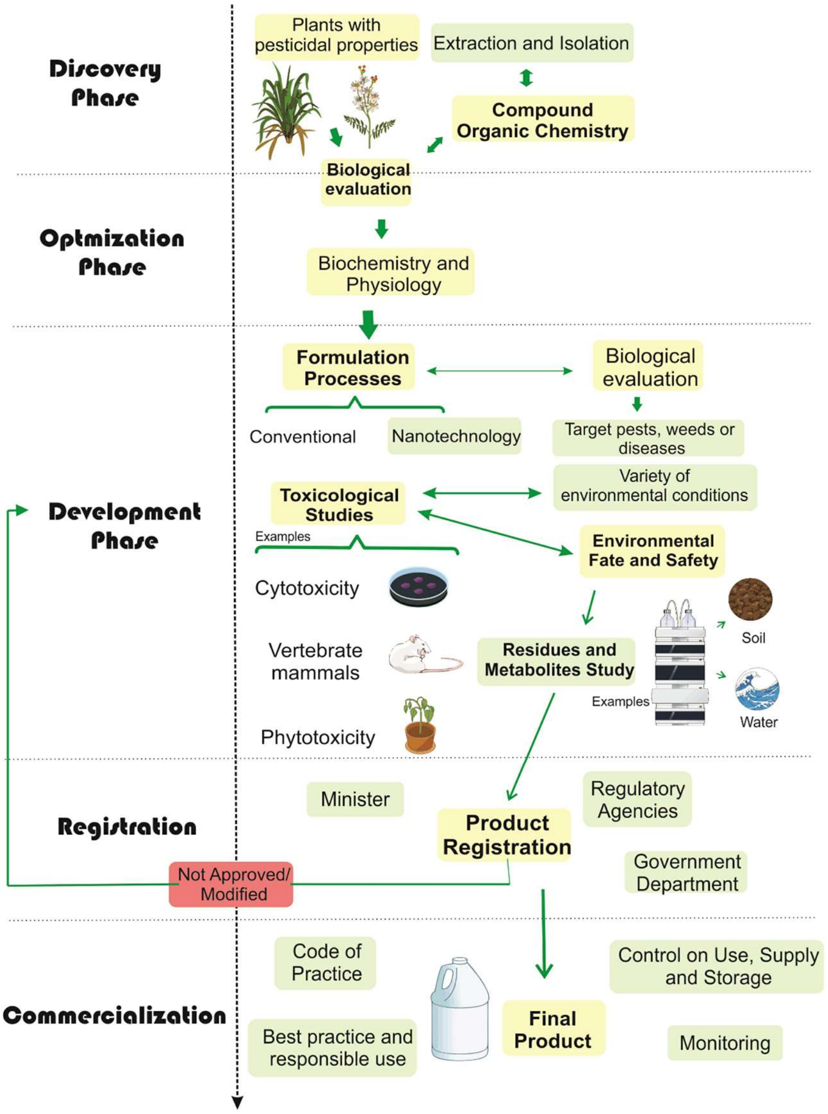
2. Concluding remarks

Nanotechnology has the potential to contribute significantly to the advancement of botanical pesticide industry in terms of more effective and stable formulations. The low efficacy of botanical pesticides, compared to some synthetic pesticides, has always been highlighted as one of the main problems faced by the sector. As described in this review, numerous advancements have been made using nanotechnology to develop new formulations, which also address some of the limitations that could help expand of the use of such systems in sustainable agriculture.

Growing concern about environmental contamination and degradation of soils and water resources is the key factor that can increase demand for environmentally friendly alternatives. Botanical pesticides offer a better alternative to synthetic pesticides. When nanotechnology is used to produce new products, consideration also need to be given to potential impacts that this technology may have on the environment. For this reason, there is increasing interest in the development of green nanotechnology, with the production of botanical pesticide formulations employing matrices based on natural polymers (biodegradable and biocompatible) in efficient production processes that reduce the use of solvents and energy. It is essential that the development of green nanotechnology should be combined with constant improvements in toxicological analysis techniques, to ensure safety of the new formulations. It is worth noting that there are few studies that discuss and specifically address the environmental fate of botanical pesticides, and this approach is extremely important to understand the true impact of these compounds on the environment. In a recent publication, Liu *et al.* (2017)[82], studied the environmental fate of thymol in soil and water after agricultural applications in a tropical climate. The authors observed that thymol sorption occurred in the three types of tropical soils studied, with sorption increasing in the order sandy soil <loamy soil <clay soil (being dependent on the organic carbon content of the soil). However, the authors observed that the degradation of the compound in both matrices (soil and water) was rapid. Being that the photodegradation (50%) in water through the action of sunlight occurred within 28 h. As for thymol in soil, the same percentage with the same conditions was achieved with 8.4 days. The authors conclude that the application of thymol in crop production presents a low environmental risk. Such studies of environmental fate are also extreme importance for nano-pesticides field. Recently, Kah *et al.* (2018), makes a critical assessment of the impacts of these nanopesticides. For the authors, nanopesticides increase on average 30% the efficacy in comparison with conventional compounds. In addition, such an approach allows changes in the environmental fate of these compounds, however, such an amendment does not necessarily translate into a reduction of the environmental impact. Also, they pointed that many papers in literature are vague in the study of interactions between nano systems and the environment. For the authors, there is currently no comprehensive study in the literature that evaluates the efficacy and environmental impact of these nanopesticides under field conditions. This is an important knowledge gap and it is necessary to develop more scientific work in the field.

The growing market for botanical pesticides is directly linked to the consumer trends towards safer technologies that reduce impacts in the environment and on human health. As shown schematically in Figure 3, there are many steps and barriers that still need to be overcome in order to enable these products to become commercially competitive for use on a wider global scale.

Figure 3. Schematic representation of the development and marketing of products based on botanical pesticides.



At present, the production and marketing of botanical products is mainly driven by university research and partnerships with small farmers and producers. This is because large companies are mainly focused on their patented synthetic products and see the botanical alternatives as unfamiliar and nonstandard products for which there are added uncertainties because of the current regulatory requirements. It is clear that botanical pesticides will not be able to completely replace the use of synthetic chemicals in the short term. However, they will continue to contribute in management practices aiming at sustainable agriculture, as well as reducing concerns over safety to health and the environment. The agricultural sector has a wide array of tools and products available that can assist in more sustainable management of crops, such as the use of botanical pesticides, biological control, plasticulture, and precision agriculture. At the same time, it is important that awareness of the producers and farmers is raised for such choices. Such actions could contribute towards medium to long-term replacement of conventional pesticides by new low risk products such as those based on botanicals.

Close collaboration among universities, research centers, commercial companies, and governmental agencies will be fundamental in facilitating the development, manufacture, and adoption of these products. Through large-scale production and standardization, producers of botanical pesticides will be able to address regulatory issues. Every year numerous studies report new botanicals with pesticidal activity but the in the vast majority of cases findings are not translated into new products. However, despite the current shortcomings and challenges, new developments such as the use of nanotechnology to produce more effective botanical pesticide formulations, is already knocking our door as a real opportunity. As demonstrated throughout this study, the convergence of nanotechnology with botanical pesticides presents numerous advantages, including the increased effectiveness of these natural compounds. However, it is worth mentioning that the sector still faces some problems and challenges. The scalability combined with the cost of producing nanotechnology-based systems is one of the main obstacles. In addition, toxicity studies of these materials need to be further developed. Because nano-scale properties these materials can cause different levels of toxicity. Therefore, a study of the interaction of these materials with different biological matrices is a key point for the advancement of the sector. We emphasize that continued research, and collaboration amongst the key players, will be needed to take turn this opportunity into a sustainable commercial reality.

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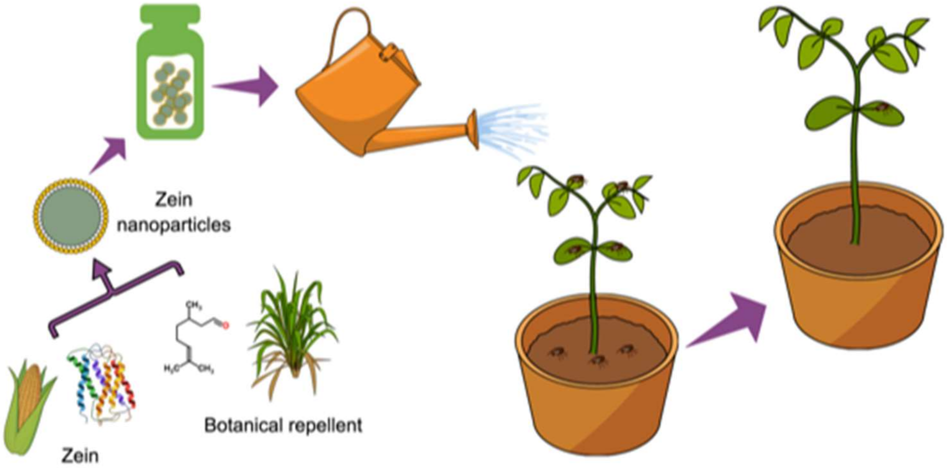
CHAPTER II

Zein nanoparticles as eco-friendly carrier systems for botanical repellents aiming sustainable agriculture



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Abstract

Botanical repellents represent one of the main ways of reducing the use of synthetic pesticides and the contamination of soil and hydric resources. However, the poor stability and rapid degradation of these compounds in the environment hinders their effective application in the field. Zein nanoparticles can be used as eco-friendly carrier systems to protect these substances against premature degradation, provide desirable release characteristics, and reduce toxicity in the environment and to humans. In this study, we describe the preparation and characterization of zein nanoparticles loaded with the main constituents of the essential oil of citronella (geraniol and R-citronellal). The phytotoxicity, cytotoxicity, and insect activity of the nanoparticles towards target and nontarget organisms were also evaluated. The botanical formulations showed high encapsulation efficiency (> 90 %) in the nanoparticles, good physicochemical stability, and effective protection of the repellents against UV degradation. Cytotoxicity and phytotoxicity assays showed that encapsulation of the botanical repellents decreased their toxicity. Repellent activity tests showed that nanoparticles containing the botanical repellents were highly repellent against the *Tetranychus urticae* Koch mite. This nanotechnological formulation offers a new option for the effective use of botanical repellents in agriculture, reducing toxicity, protecting against premature degradation, and providing effective pest control.

Keywords: Botanical repellents; zein nanoparticles; nanopesticides; sustainable agriculture; insect control.

1. Introduction

Pests including insects and mites are among the main factors that decrease agricultural productivity worldwide, due to losses in the field (pre-harvest) and during storage (post-harvest) (SPENCER; HUGHSON; LEVINE, 2014). The damage caused by these organisms depends on a series of factors including the cultivated plant species, the technology employed in crop production, and pest management. Pests can cause damage directly, when they feed on plants (phytophagous), or indirectly, when they act as vectors for phytopathogens (ISHAAYA; HOROWITZ, 2016). According to Oliveira et al. (OLIVEIRA et al., 2014), damage caused by insect pests is one of the main factors leading to reduced production in large-scale cultivations. As an example, in Brazil, average annual losses can reach around 7.7 % of production, representing a reduction of approximately 25 million tons of food, fiber, and biofuels.

The two-spotted spider mite (*Tetranychus urticae* Koch) is among the most important agricultural pests worldwide and is one of the most polyphagous mite species. Its host plants (over 1,000 plant species) include vegetables, fruits, maize, cotton, and a wide range of ornamentals (ATTIA et al., 2013).

Chemical control using pesticides and repellents (organophosphates, carbamates, and thiadiazines) is generally the main way of combating this insect pest (NARANJO; ELLSWORTH, 2009). However, misuse of these pesticides can lead to adverse effects including pest resistance, toxic effects in humans and other animals, and contamination of soils, surface waters, and groundwater (BOLLAZZI et al., 2013). Therefore, strategies are needed to reduce environmental impacts and costs related to the control of these organisms (SHWETA AGRAWAL; PRAGYA RATHORE, 2014). Currently, one option is to use botanical repellents, insecticides, and acaricides. These substances (alkaloids, terpenoids, and phenylpropanoids, among others) are produced by plants in secondary metabolic routes, and they have many biological activities. Examples include geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) and citronellal (3,7-dimethyloct-6-en-1-al), both of which are constituents of the essential oil of citronella (*Cymbopogon winterianus* Jowitt and *Cymbopogon nardus* (L.)) (NAKAHARA et al., 2013).

Geraniol is an acyclic alcohol, widely used as a base ingredient in cosmetic fragrances and for home use (RASTOGI et al., 2001). The literature reports numerous applications of geraniol as a repellent/insecticide for pest control, as well as its chemopreventive activity and its antimicrobial, antioxidant, and anti-inflammatory properties (CARNESECCHI et al., 2001b, 2004; AHMAD et al., 2011; A; T, 2015).

Citronellal has a stereochemical center and is found in the chiral forms (R)-(+)-citronellal and (S)-(+)-citronellal (SEIXAS et al., 2013). Both forms present arthropod repellent activities (SOLOMON et al., 2012; LICCIARDELLO et al., 2013; SRITABUTRA; SOONWERA, 2013), as well as bactericidal (DIMITRIJEVIĆ et al., 2007) and antifungal properties (DE OLIVEIRA LIMA et al., 2006).

In agriculture, advantages of botanical repellents include lower production costs, reduced chemical contamination of the environment, and food preservation, contributing to improvements in product quality and human health (ISMAN, 2006). However, despite their potential benefits, the use of natural compounds in agriculture can be limited, due to their sensitivity to light and high temperatures, as well as their degradation by microorganisms (REHMAN; ALI; KHAN, 2014). Nanotechnology has been shown to be a potential tool for the development of formulations capable of increasing the stability and efficacy of active agents (CHENG et al., 2016; FRACETO et al., 2016; PRASAD; BHATTACHARYYA; NGUYEN, 2017b). These formulations, also known as nanopesticides, essentially aim to enhance the performance of active ingredients (AIs) by: i) increasing their apparent solubility; ii) controlling their release in the field; and/or iii) protecting them against premature degradation (KAH et al., 2013; MATTOS et al., 2017).

Nanopesticides can be produced using a variety of different matrices, including biodegradable polymers, lipids, and proteins, among others (KAH et al., 2013; KAH, 2015b; GRILLO; ABHILASH; FRACETO, 2016; KAH; WENIGER; HOFMANN, 2016). Zein is the main storage protein in maize and is found in four main fractions (α-, β-, γ-, and δ-zein), with the α-zein fraction corresponding to about 75-85% of the total (GIRIJA ASWATHY et al., 2012). Zein is generally recognized as safe by the Food and Drug Administration (FDA) and presents promising characteristics such as biocompatibility, biodegradability, low toxicity, and competitive production costs (PALIWAL; PALAKURTHI, 2014b). Its hydrophobic nature and variable solubility under different conditions enable simple manufacture of micro/nanoparticles (LUO; WANG, 2014). Several previous studies have used zein nanoparticles for the encapsulation of natural compounds (LUO et al., 2011; WU; LUO; WANG, 2012; CHEN et al., 2014b; ZHANG et al., 2014b; CHEN; ZHANG; ZHONG, 2015; DA ROSA et al., 2015b; CHUACHAROEN; SABLIOV, 2016a), although none of these are used as nanopesticides.

Given this background, the aim of the present study was to develop a biodegradable nanopesticide, employing zein as a carrier system for the botanical repellents geraniol and R-citronellal, as well as to demonstrate the effectiveness of the repellent against *T. urticae*. The photostability of the AIs was evaluated, as well as the cytotoxicity and phytotoxicity of the nanoformulations. These nanopesticides based on zein nanoparticles could be used to control arthropod pests in crops, especially by organic farmers, since only natural chemicals are used in their composition, hence contributing to the goal of sustainable agriculture.

2. Materials and Methods

2.1 Materials

Geraniol (GRL), R-citronellal (R-CTL), zein, and Pluronic F-68 were obtained from Sigma-Aldrich. Ethanol was acquired from Labsynth. Acetonitrile (HPLC grade) was obtained from J. T. Baker. Seeds of *Phaseolus vulgaris* was purchased from ISLA Sementes, and substrate was obtained from Hortaliça Mix. Other reagents (analytical grade or better) were purchased from local suppliers.

2.2 Preparation of the zein nanoparticles

Zein nanoparticles were obtained using the antisolvent precipitation method described by Hu et al. (2014), with slight modifications. Firstly, a zein solution (1% w/v) was prepared in a hydroethanolic solution (85%, v/v), with stirring overnight. The pH of the zein solution was adjusted to pH 5.8 with 1.0 mol/L HCl and the solution was filtered through a 0.45 μm membrane (Millipore). For loading of the zein nanoparticles with the botanical repellents, 100 mg of each compound (GRL and R-CTL) was added to the zein solution separately. An aqueous 1% (w/v) solution of Pluronic F-68 was prepared and adjusted to pH 4. The zein solution was rapidly injected into the surfactant solution, under magnetic stirring. The final concentration of each botanical repellent in the nanoformulations was 5 mg/mL. The resulting colloidal dispersion containing geraniol (NP_GRL) and R-citronellal (NP_R-CTL) was stirred for a further 30 min and the ethanol was evaporated at room temperature (the volume lost was compensated with water at pH 4). As a nanoparticle control, zein nanoparticles were prepared without botanical repellents. The botanical repellents were also prepared using surfactant (Pluronic F-68), as a pesticide control.

2.3 Physicochemical characterization of the zein nanoparticles

The nanoparticle size distribution and polydispersity index were measured by the dynamic light scattering technique. The zeta potential was determined by the microelectrophoresis method. For both techniques, the nanoparticles formulation was diluted 1000-fold in deionized water and analyzed using a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) at a fixed angle of 90° and 25 °C. Each result was expressed as the average of three determinations. The stabilities of the formulations were evaluated using measurements at predetermined time intervals (after 0, 15, 30, 60, 90, and 120 days). Particle concentrations and size distributions were also determined by nanoparticle tracking analysis, using a NanoSight LM 10 cell (green laser, 532 nm) and a sCMOS camera, controlled by NanoSight v. 3.1 software. The nanoparticle suspensions were diluted 1500-fold and the analyses were performed in triplicate.

The morphologies of the zein nanoparticles were analyzed using atomic force microscopy (AFM). For the AFM analyses, diluted nanoparticles were deposited onto silicon plates. The analyses were performed using an Easy Scan 2 Basic BT02217 atomic force microscope (Nanosurf, Switzerland), operated in non-contact mode with TapAl-G cantilevers (BudgetSensors, Bulgaria) and a scan rate of 90 Hz. The images (256 x 256 pixels) were captured in time mode and were analyzed using Gwyddion software.

Fourier Transform Infrared Spectroscopy (FTIR) analyses were performed to investigate interactions between the botanical repellents and the zein nanoparticles. An infrared spectrophotometer (Agilent) was used in the range from 400 to 4000 cm^{-1}, with 128 scans per sample and 8 cm^{-1} resolution. The components of the suspensions and the formulations were analyzed using an attenuated total reflectance (ATR) accessory (WU; LUO; WANG, 2012).

2.3.1 Determination of encapsulation efficiency

The ultrafiltration/centrifugation method was used to quantify the botanical repellents encapsulated in the zein nanoparticles. The use of Microcon 10 kDa regenerated cellulose ultrafiltration units (Millipore) enabled quantification of the non-associated fraction of each repellent, so the encapsulation efficiency (EE) could be determined by difference, considering the total amount initially added. Several studies have used this technique to determine the EE of AIs in nanocarriers (PEREIRA et al., 2014; ZHANG et al., 2014c). The total amounts of the botanical repellents (100%) present in the formulations were calculated taking into account the total amount added as well as the losses during the preparation process. Subsequently, the AIs were quantified by high performance liquid chromatography (HPLC), using a validated method, as detailed in the Supporting Information.

2.4 Photostability assays

Suspensions of the nanopesticides, as well as solutions of the botanical repellents with only surfactant, were submitted to ultraviolet light ($\lambda = 365$ nm) using a darkroom box as described by Patel et al. (2010). Aliquots were periodically collected for quantification of the AIs, which were extracted from the nanoparticle suspensions using acetonitrile. Two successive extractions were carried out, each with duration of 30 min, and the AIs were quantified by HPLC. Also, different kinetic models were applied (zero order, first order, pseudo-first order, and pseudo-second order).

2.5 Phytotoxicity assays

The effects of the nanopesticides on plants were evaluated using bean (*P. vulgaris*) seeds. The seeds were first sterilized by immersion in 10% sodium hypochlorite solution for 10 min, followed by washing with deionized water in triplicate. The seeds were then treated with the nanoparticle suspensions: zein nanoparticles (NP), zein nanoparticles loaded with geraniol (NP_GRL), zein nanoparticles loaded with R-citronellal (NP_R-CTL), geraniol emulsified with Pluronic (GRL + Pluronic 1%), R-citronellal emulsified with Pluronic (R-CTL + Pluronic 1%), and Pluronic solution (Pluronic 1%). Ten seeds were placed in Erlenmeyer flasks containing 15 mL of the suspensions or solutions, followed by stirring at 160 rpm for 1 h. The seeds were then transferred to Petri dishes (100 mm x 15 mm) containing an agar layer (1.5%, m/v). The assays were performed using 0.05, 0.5, and 5 mg/mL concentrations of the botanical repellents. The Petri dishes were covered, sealed with adhesive tape, and placed in a germination chamber in the dark at 25 °C. After 5 days, the germination process was stopped and measurements were made of the root and shoot lengths and the fresh and dried masses of the seedlings. The assays were performed in triplicate and the results were expressed as means and standard deviations.

2.6 Cytotoxicity assays

The cytotoxicity assays were performed using a permanent lung fibroblast cell line (v79) and a fibroblast cell line (3T3), which were kept in continuous culture (in

DMEM medium with 10% fetal bovine serum). The culture was supplemented with 100 IU/mL penicillin and 100 µL streptomycin sulfate, and was kept at pH 7.4, 37 °C, under a moist atmosphere with 5% CO_2. The plates ($1x10^4$ viable cells) were incubated for 48 h until semi-confluence was reached. The cells were then exposed (for 24 h) to the same suspensions as described in Section 2.5. A plate reader was used to measure the absorbance of each well (at 570 nm). Cell viability was determined (in triplicate) by the reduction of tetrazolium dye (MTT test). The percentage values were expressed as means and standard deviations.

2.7 Mite repellent activity

The repellent activity experiments were conducted in the Acarology Laboratory (UNESP/FCAV, Jaboticabal campus), using the mites species *T. urticae*, kept in greenhouse cultivations on bean plants (*P. vulgaris*). The mites were collected in areas without application of agricultural chemicals, in order to avoid problems of mite resistance to pesticides. For evaluation of the repellent activities of the formulations (the same as those described in Sections 2.5), disks (diameter of 2.5 cm) were cut from the leaves of the host plant (*P. vulgaris*). The disks (arenas) were placed in 9 x 2 cm Petri dishes containing moistened foam (height of 1.0 cm) and a thin layer of hydrophilic cotton on the foam. In order to evaluate the repellent activity and avoid escape of the mites, the arenas were surrounded with entomological glue. The formulations were applied with a Potter spray tower calibrated at 4 $lbf.in^{-2}$, using 2 mL of the formulation per arena, corresponding to 1.56 mg cm^{-2}. After drying the products on the leaves (approximately 2 hours), 10 adult female *T. urticae* mites were transferred to each arena. Each treatment was repeated 8 times and each repetition was composed of one arena, totaling 80 mites per treatment. The arenas were conditioned in a climate chamber at a temperature of 25 ± 1 ºC, relative humidity of 60 ± 10%, and photoperiod of 12 hours. Quantifications of dead and live mites, and mites adhered to the glue barrier, were performed 8, 24, and 48 hours after transfer of the mites. The percentage of mite repellency (% repellency) was calculated considering the total number of mites and the number adhered to the entomological glue barrier.

2.8 Data analysis

All the experiments were performed in triplicate and the results were presented as means and standard deviations. Statistical analyses were performed with GraphPad Prism 5.0 software, using two-way ANOVA (significance level of $p < 0.05$).

3. Results and Discussion

3.1 Preparation and Characterization

The zein nanoparticles containing geraniol (NP_GRL) and R-citronellal (NP_R-CTL) were characterized and the colloidal stability was evaluated during 120 days. Table 1 shows the initial characteristics of the formulations in terms of particle mean diameter (MD, nm), polydispersity index (PDI), zeta potential (ZP, mV), nanoparticle concentration (CT, particles/mL), and encapsulation efficiency (EE, %). Also, nanoparticles without AIs (NP) were prepared as controls in order to evaluate the effect of addition of the compounds on the nanoparticle properties.

Table 1. Characterization of the zein nanoparticles containing the botanical repellents: Mean diameter (MD) using the dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) techniques; polydispersity index (PDI); zeta potential (ZP), concentration (CT); and encapsulation efficiency (EE). The values are expressed as the average of three determinations.

Samples	**MD (nm)**		**PDI**	**ZP (mV)**	**CT**	**EE (%)**
	DLS	**NTA**			**(10^{12} particles/mL)**	
NP	205.2 ± 6.3	211.0 ± 8.2	0.442 ± 0.011	-20.2 ± 0.97	0.66 ± 0.021	-
NP_GRL	172.3 ± 3.8	145.6 ± 4.5	0.351 ± 0.032	-18.8 ± 1.02	1.97 ± 0.330	94.4 ± 1.20
NP_R-CTL	142.5 ± 9.3	159.1 ± 7.5	0.330 ± 0.052	-12.8 ± 0.98	1.57 ± 0.536	99.2 ± 0.32

The results of the stability assays revealed significant changes in the parameters for the nanopesticides, within the first 30 days. After this period, the formulations showed no further significant changes up to 120 days of storage, demonstrating the importance of the stabilizing effect of the Pluronic F-68 surfactant (CHUACHAROEN; SABLIOV, 2016b). Nanoparticles without botanical repellents (NP) presented a larger average diameter and lower physicochemical stability, compared to the nanoparticles loaded with the botanical repellents (Table 1). This could have been due to a stabilizing effect of the presence of the AIs, which prevented the formation of aggregates. It should be noted that no significant differences were observed between the size distribution values obtained using the DLS and NTA techniques. Similar results were reported by Rosa et al. (2015), who prepared zein nanoparticles for the encapsulation of thymol and carvacrol. The formulation without the AIs showed a larger average diameter than the other nanoformulations, with the former precipitating within 60 days of storage, while the latter could be analyzed during a longer period.

The NP_GRL and NP_R-CTL nanopesticides presented average diameters smaller than 200 nm, in agreement with previous studies (CHEN et al., 2014b; CHEN; ZHANG; ZHONG, 2015). Moreover, all the nanoformulations (with and without AIs) showed monomodal size distributions, with no major changes observed as a function of time (Figure 1). This is a desirable feature for commercial applications, since it shows that the system did not lose its properties, enabling storage over longer periods.

The nanoparticles presented PDI values in the range from 0.3 to 0.5, similar to those reported in the literature for matrices of natural origin such as zein, chitosan, and others (ABREU et al., 2012c; BILIA et al., 2014; DA ROSA et al., 2015b). The formulations showed good homogeneity (Figure 1) and zeta potentials ranging from -12 to -25 mV, in agreement with the results of Podaralla and Perumal (2012), who prepared zein nanoparticles stabilized with Pluronic F-68 and lectin.

Despite the low zeta potentials, the formulations remained stable over time, mainly due to the steric stabilization provided by the surfactant. The hydrophobic polyoxypropylene moieties present in Pluronic F-68 interacted (by London dispersion type forces) with exposed non-polar regions on the surfaces of the zein nanoparticles, while the hydrophilic moieties that were projected towards the exterior interacted with water molecules by means of van der Waal forces and hydrogen bonds, increasing the shear plane (LI et al., 2015).

The nanopesticides presented higher nanoparticle concentrations, compared to the control formulation (nanoparticles without AIs), which could have been due to the lower stability of the latter. As pointed out by Dickson et al. (2012), who investigated the stability of hematite nanoparticles, the concentration is one of the main factors affecting the stability of nanoparticles.

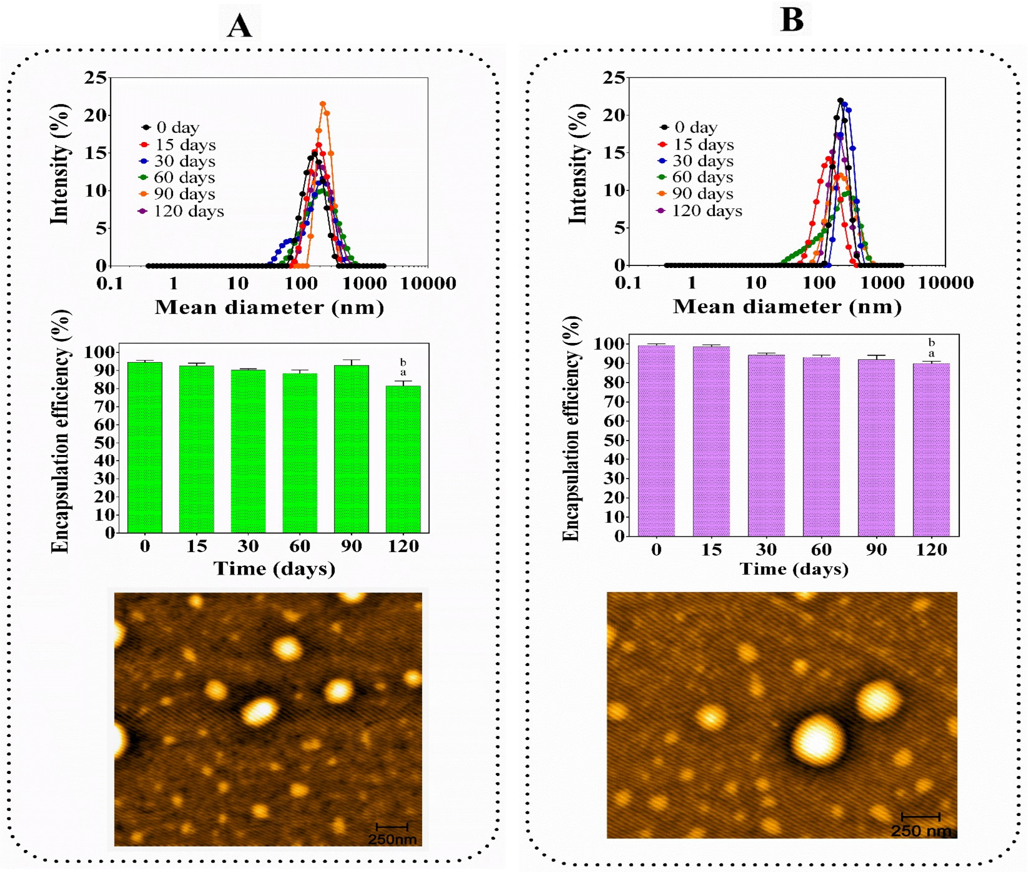
Determination of the encapsulation efficiency was also used to investigate the stability of the botanical repellents in the zein nanoparticle formulations. At first, the repellents showed high encapsulation efficiencies (> 90 %) (Figure 1). This resulted from the solubility of the AIs in the hydroethanolic solution, with the subsequent evaporation of the ethanol leading to their association with the hydrophobic zein residues. High encapsulation efficiencies of essential oils in zein nanoparticles were also found by Zhang et al. (2014), who achieved over 80% encapsulation of thymol in zein nanoparticles stabilized with sodium caseinate and chitosan hydrochloride. It can also be seen from Figure 1 that significant decreases in the encapsulation efficiencies only occurred after longer periods (90 and 120 days), probably due to losses by volatilization and particle degradation, with consequent release of the active agents to the environment. Yegin et al. (2016) prepared Pluronic F-127 polymeric nanoparticles containing geraniol, using the nanoprecipitation method, and subsequent decreases in EE values were attributed to losses by evaporation. The average EE was 52%, much lower than the values obtained in the present work. This comparison shows the effectiveness of the nanocarrier system developed here (zein nanoparticles) for the encapsulation and protection of AIs.

For comparison, emulsions of the botanical repellents were prepared with Pluronic F-68 surfactant, using the same concentrations and storage conditions, as shown in Figure S1, Supporting Information. Emulsification with the surfactant alone resulted in large decreases in the amounts of the compounds over time, due to volatilization and degradation in the solution. Chuacharoen and Sabliov (2016) prepared zein nanoparticles containing lutein, stabilized with Pluronic F-127, and lutein emulsified with this surfactant. After 30 days of storage (at 25 °C), the lutein solution emulsified with Pluronic F-127 retained only 20 % of the original amount of the AIs, while 54 % was retained in the presence of the nanoparticles. These results provide further support for the protective effect provided by encapsulation of the botanical repellents in zein nanoparticles. The results were in agreement with previous studies (CHEN et al., 2014b; CHEN; ZHANG; ZHONG, 2015; DA ROSA et al., 2015b; CHUACHAROEN; SABLIOV, 2016b; CHANG et al., 2017a, 2017b; WANG; ZHANG, 2017) and demonstrated that the zein nanoparticles possessed satisfactory characteristics for the encapsulation of hydrophobic AIs, such as the botanical repellents.

Micrographs of the nanoparticles analyzed by AFM are shown in Figure 1, from which it can be seen that the particles were spherical with smooth surfaces. The AFM images revealed high polydispersion of the formulations, with particle sizes of around 90-

250 nm, in agreement with the results obtained with the DLS technique. Similarly, Podaralla and Perumal (2012) reported spherical zein nanoparticles, using analysis by AFM.

Figure 1. Characterization of zein nanoparticles containing the botanical repellents geraniol (A) and R-citronellal (B). Size distribution curves (obtained using the DLS technique), as a function of time (upper graphs); encapsulation efficiencies of the botanical repellents, as a function of time (middle graphs); AFM micrographs of the nanopesticides (bottom images). A significance level of $p < 0.05$ was considered for the differences obtained within the same group; a*, b*, c*, d*, and e* indicate significant differences relative to days 0, 15, 30, 60, and 90, respectively.

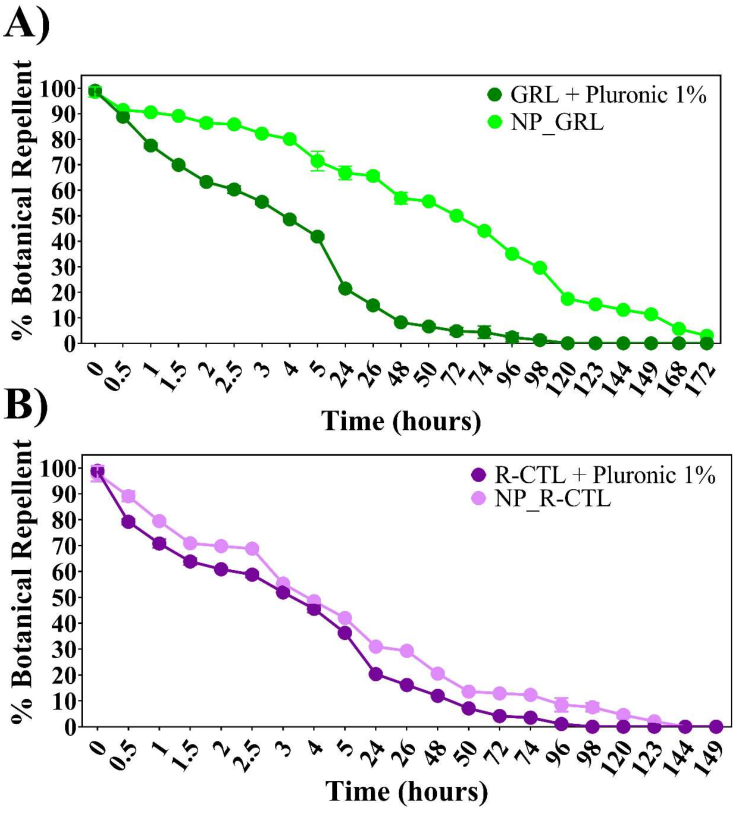


Infrared spectroscopy (FTIR) was used to investigate the interactions of the botanical repellents with the zein nanoparticles, as well as to determine whether the preparation process altered the components of the formulations. Analyses were made of the botanical repellents (GRL and R-CTL), the nanoparticle constituents (zein and Pluronic F-68), and the nanoparticles loaded with the different AIs (Figure S2). Also, a brief description of each compound can be observed in Supporting Information. In general, FTIR results showed that no new bands appeared after interaction between the botanical repellents and the nanoparticles, indicating that the interactions were essentially physical, rather than chemical. The results were also indicative of interaction between zein and the hydrophobic groups of the AIs, in agreement with the findings of Wu et al.[38], who used the same technique to characterize zein nanoparticles loaded with thymol and carvacrol.

3.2 Photostability assay

Photostability studies were performed in order to determine whether exposure to ultraviolet radiation resulted in significant changes in the concentrations of the botanical repellents after the encapsulation process, as shown in Figure 2. In all cases, encapsulation in the zein nanoparticles led to decreased degradation by UV radiation, increasing the activity of the AIs towards the target.

Figure 2. Photostability assays of the nanopesticides and repellents emulsified in surfactant (Pluronic F-68) containing A) GRL and B) R-CTL. The formulations were submitted to ultraviolet light (λ = 365 nm) using a darkroom box, with collection of samples at predetermined time intervals.



Geraniol emulsified in Pluronic F-68 showed $T_{50\%}$ (the time for 50% degradation of the compound) of 4.1 ± 0.3 h, while $T_{50\%}$ of 72.3 ± 0.5 h was obtained for the repellent encapsulated in zein nanoparticles (Figure 2), indicating that encapsulation resulted in a 17-fold decrease in degradation of the compound by UV radiation. The protective effect of the nanoparticles was lower for citronellal, with $T_{50\%}$ of 3.2 ± 0.1 for R-CTL emulsified in Pluronic F-68, while $T_{50\%}$ of 4.0 ± 0.2 was obtained for the compound encapsulated in zein nanoparticles. Therefore, in this case, a decrease of only 1.6-fold in photodegradation was obtained.

The different $T_{50\%}$ values for geraniol and R-citronellal could have been due to the nature of the interactions between the AIs and the components of the nanoparticles, as well as the locations of the interactions and the degree of exposure to the UV radiation. According to this model, a greater fraction of the citronellal molecules remained on the surface of the nanoparticles and was therefore more exposed to ultraviolet radiation (CHERAGHIAN; HENDRANINGRAT, 2016).

Similar findings have been reported elsewhere for this type of particle. Patel et al. (2010) found that zein nanoparticles decreased the rate of degradation of curcumin by around 2-fold, compared to curcumin in solution. Chuacharoen and Sabliov (2016) evaluated the degradation of lutein in zein nanoparticles and emulsified with Pluronic F-127. After 10 h of exposure to UV radiation, only 1.42 % of the lutein remained in the emulsion, while 46.53 % remained in the nanoparticle formulation. This increased stability was mainly attributed to competition for absorption of UV radiation by the zein, due to the presence of aromatic amino acids in the structure of the protein.

In order to obtain further elucidation of the photodegradation processes, zero order, first order, pseudo-first order, and pseudo-second order kinetic models were applied to the degradation curves. The use of these models can provide valuable information about the stability of AIs in solution, enabling prediction of their likely shelf-lives (KANNAN; BIERNACKI; VISCO JR., 2007). Table 2 provides the values of the photodegradation kinetic constants (k) and the correlation coefficients (r^2) obtained for the kinetic models applied to the different nanoformulations. The photodegradation of the botanical repellents prepared in the presence of Pluronic F-68 followed the pseudo-first order model. When the botanical repellents were encapsulated in zein nanoparticles, the photodegradation of geraniol followed a different kinetic model (zero order), while degradation of R-citronellal followed the pseudo-first order model. This difference could provide an explanation for the different $T_{50\%}$ values obtained for the encapsulated compounds (Table 2).

Table 2. Photodegradation constants (k) and correlation coefficients (r^2) for the kinetic models applied to the photodegradation curves.

	Photodegradation kinetic models							
Formulation	Zero order		First order		Pseudo-first order		Pseudo-second order	
Pluronic (1%)	*k* (mg mL^{-1} h^{-1})	r^2	*k* (h^{-1})	r^2	*k* (h^{-1})	r^2	*k* (mg mL^{-1} h^{-1})	r^2
GRL	0.0489	0.6679	0.0395	0.9578	0.0313	**0.9588**	0.0736	0.8534
R-CTL	0.0550	0.7087	0.0447	0.9727	0.0368	**0.9737**	0.0735	0.6858
NP_ [ ]								
GRL	0.0255	**0.9624**	0.0159	0.9236	0.0098	0.8956	0.0097	0.5434
R-CTL	0.0359	0.7130	0.0248	0.9478	0.0195	**0.9488**	0.0256	0.7053

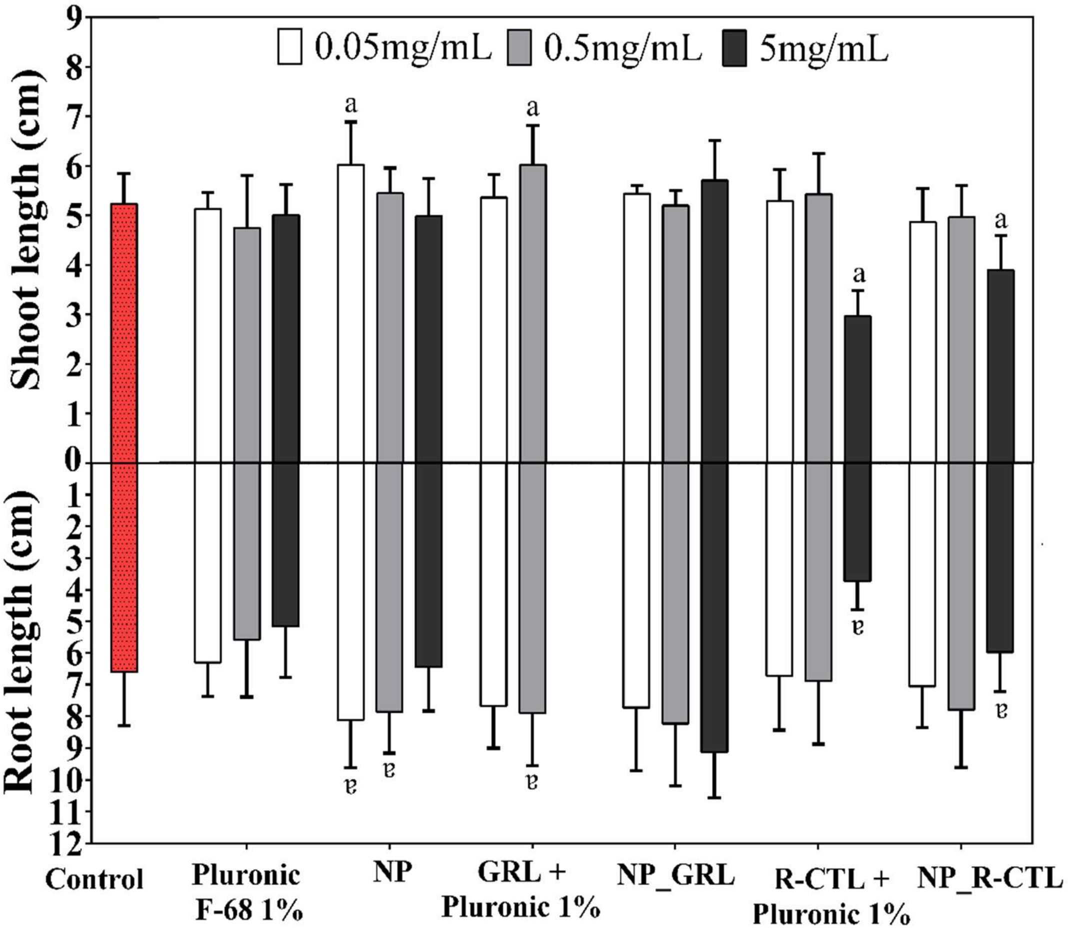
3.3 Phytotoxicity assays

Concern about possible phytotoxicity stems from the fact that many of the biologically AIs (such as monoterpenes and sesquiterpenes) present in essential oils extracted from plants can exert toxic effects in some plant species (GRANA et al., 2012). The phytotoxicity assays were also performed using bean seeds (*P. vulgaris*). Significant decreases in the germination index (GI), compared to the control, were only observed at the highest concentrations of the active agents (5 mg/mL). The greatest toxicity was found for unencapsulated GRL, with zero germination, while encapsulation of GRL in the nanoparticles resulted in GI of 80 ± 2.5%. The formulation of R-CTL emulsified with the surfactant showed GI of 64 ± 3.2%. The encapsulation of citronellal resulted in GI of 79 ± 3.5%. Xavier et al. (2012) reported that citronella oil and its constituents could influence the germination index of cowpea beans, reducing the vigor of the seeds. Encapsulation of the repellents in nanoparticles increased the germination percentage, compared to the non-encapsulated repellents, indicating a protective effect of the nanoparticles. Pérez-de-Luque and Rubiales (2009) reported that the nanoencapsulation of AIs (herbicides, fungicides, essential oils, and others) could avoid problems of phytotoxicity when applied in crops, due to decreased availability of the AIs.

Figure 3 shows the root and shoot lengths of seedlings of beans treated with zein nanoparticles containing the botanical repellents at different concentrations, as well as with the compounds in the presence of the surfactant. At concentrations of 0.5 and 0.05 mg/mL, there were no significant phytotoxic effects, compared to the control, while in some cases, there were significant increases in both root and shoot size. No phytotoxicity was observed for the zein nanoparticles without AIs or loaded with geraniol at a concentration of 5 mg/mL. However, geraniol and citronellal emulsified with Pluronic F-68 exhibited phytotoxicity towards the bean seedlings. Encapsulation in the nanoparticles reduced the phytotoxicity of R-CTL. Singh et al. (2006) also observed phytotoxic effects on the lengths of the roots and shoots of different plant species, following treatment with citronellal. The compound was reported to affect the division of meristematic cells, leading to inhibition of growth, with the greatest effect on root growth.

The effects of the formulations on the fresh and dry masses of the bean seedlings (Figures S3-A and S3-B, Supporting Information) were also investigated. As observed for the root and shoot size, the concentrations of 0.5 and 0.05 mg/mL had no adverse effects on the masses of the seedlings. At a concentration of 5 mg/mL, all the nanopesticides with surfactant reduced the fresh and dry masses of the bean seedlings, compared to the control. The nanoparticle formulations containing citronellal caused phytotoxic effects in terms of both fresh and dry mass, compared to the control, although the effects were lower than for the non-encapsulated compound. Therefore, the results indicated that the nanoencapsulation process provided protection against the phytotoxicity of the AIs, as also reported elsewhere. Perdones et al. (2016) found that pre-emergence application of the essential oil of oregano to tomato plants resulted in phytotoxic effects, which were reduced when the essential oil was encapsulated in chitosan films and polymeric nanoparticles.

Figure 3. Effects of the different formulations on the root and shoot lengths of bean seedlings (*Phaseolus vulgaris*) after treatment of the seeds: zein nanoparticles (NP); Pluronic F-68 surfactant; geraniol emulsified with surfactant (GRL + Pluronic 1%); zein nanoparticles loaded with geraniol (NP_GRL); R-citronellal emulsified with surfactant (R-CTL + Pluronic 1%); zein nanoparticles loaded with R-citronellal (NP_R-CTL); S-citronellal emulsified with surfactant (S-CTL + Pluronic 1%); and zein nanoparticles loaded with S-citronellal (NP_S-CTL). A significance level of $p < 0.05$ was considered for the differences observed between the groups with the same concentration, where * indicates a significant difference relative to the control.

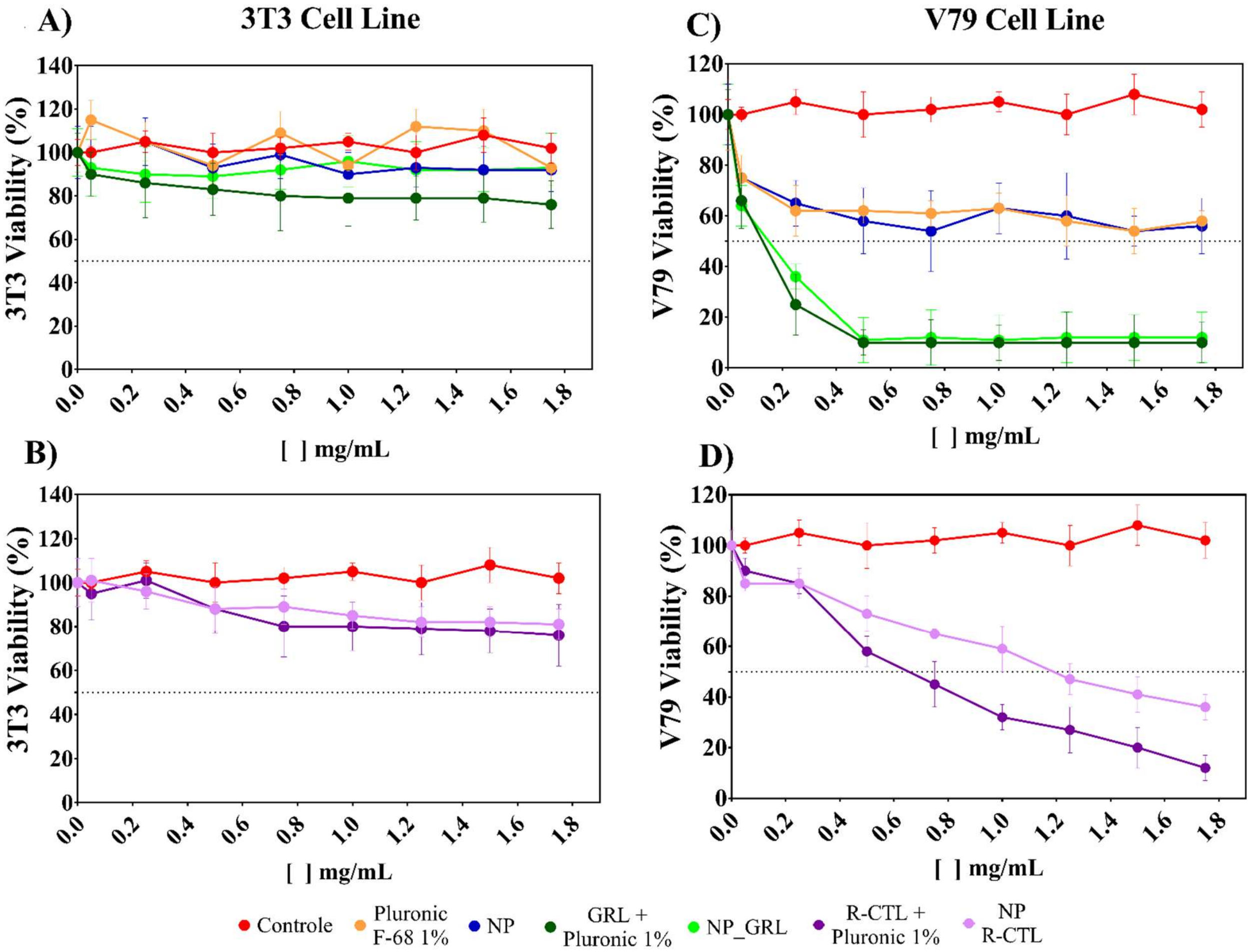


3.4 Cytotoxicity assays

Cytotoxicity studies were used to evaluate the effects of the botanical repellents and nanoformulations (with and without AIs) in nontarget organisms. Cell viability assays were performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction technique. Cytotoxic effects were investigated using two different cell lines: NIH-3T3 (mouse fibroblast cells) and V79-4 (fibroblast cells derived from mouse lung tissue). These cell types were chosen because the main routes of exposure in workers are often via the skin and by inhalation (MARUYAMA et al., 2016).

The viabilities of the two cell lines after treatment (for 24 h) with different concentrations of the formulations are presented in Figure 4. The formulations containing the botanical repellents showed greater cytotoxicity towards the V79 cell line, compared to the 3T3 cell line. In the assays performed with the 3T3 line (Figures 4-A and 4-B), it was not possible to calculate IC_{50} values, because the cell viability exceeded this value for all the concentrations tested. Only GRL emulsified with the surfactant showed a significant difference, compared to the control, with a cell viability of 76 ± 5.2 % obtained for the highest concentration tested. At the same concentration, cell viability of 93 ± 6.5 % was obtained for GRL encapsulated in the nanoparticles, demonstrating that the nanoparticles provided a protective effect against GRL toxicity. Bakkali et al.(2008) reported that different cell lines can respond differently when exposed to essential oils and their constituents, with some being more sensitive and others being more resistant. According to the authors, these compounds of natural origin do not target specific cells. Due to their lipophilic characteristics, they can enter cell membranes, disturbing the structures of the different layers, and affecting fatty acids and phospholipids.

Figure 4. Cytotoxicity of the zein nanoparticles (with and without the botanical repellents) and the botanical repellents emulsified with surfactant, using 3T3 cells (A and B) and V79 cells (C and D): zein nanoparticles (NP); Pluronic F-68 surfactant; geraniol emulsified with surfactant (GRL + Pluronic 1%); zein nanoparticles loaded with geraniol (NP_GRL); R-citronellal emulsified with surfactant (R-CTL + Pluronic 1%); zein nanoparticles loaded with R-citronellal (NP_R-CTL).



Figures 4-C and 4-D show the viability of the V79 cells after treatment with the different formulations. The Pluronic F-68 surfactant and the zein nanoparticles did not reach the IC_{50} values, at the concentrations tested. Although the cell viabilities decreased, compared to the negative control, the values remained at around 60%. The other nanoformulations containing the botanical repellents presented dose-dependent effects. The greatest cytotoxicities were observed for the nanoformulations containing GRL, with IC_{50} values of 0.16 ± 0.02 and 0.18 ± 0.03 mg/mL for emulsification with surfactant and encapsulated in the nanoparticles, respectively. Previous studies found that geraniol presented *in vitro* and *in vivo* antitumor activity towards different cell lines (CARNESECCHI et al., 2001b, 2002, 2004; SOBRAL et al., 2014). Crespo el al. (2012)

reported that geraniol affected several lipid metabolic pathways, such as that of mevalonate, as well as the biosynthesis of phosphatidylcholine, causing inhibition of cell growth and increased apoptosis.

The non-encapsulated R-citronellal presented higher IC_{50}, compared to geraniol (Figure 4-D), with a value of 0.65 ± 0.03 mg/mL. Stone et al. (2013) also observed a dose-dependent cytotoxic effect of R-citronellal in MCF-7 and Vero cell lines, with IC_{50} of 4 mM (0.625 mg/mL) for both cell types, in agreement with the IC_{50} value found in this study. When R-citronellal was encapsulated in the zein nanoparticles, the IC_{50} increased to 1.19 ± 0.03 mg/mL. This was indicative of a protective effect of the nanoencapsulation, corroborating the phytotoxicity data (Figure 3-B), where the encapsulated compound presented lower phytotoxicity to the bean seedlings, compared to the compounds emulsified with the surfactant. Zou et al. (2012) prepared and characterized zein nanoparticles used for the encapsulation of cranberry procyanidins (CPs), and evaluated the cytotoxicity of the nanoparticle formulations and CPs solution using HL-60 cells. Encapsulation of the CPs in the nanoparticles significantly decreased the cytotoxicity, compared to the CPs solution, evidencing a protective effect of the zein nanoparticles.

It is noteworthy that despite the conduct of cytotoxicity tests for formulations developed, nanomaterial safety assessment is a broad approach, which requires in-depth toxicological tests to ensure effective safety to exposed humans and animals (BROHI et al., 2017). Bahadar et al., (2016), emphasizes that the growing application of nanomaterials in many fields has increased the demand for new toxicological models, and in vivo studies are also necessary. In addition, the compounds studied (geraniol and citronellal) are known for their allergenic skin and respiratory properties (XUE et al., 2016). However, it is noted that encapsulation reduced the toxic effects of these compounds at the concentrations tested.

3.5 Insect repellent activity

The repellent activities of the nanoformulations were investigated as a function of time (0, 8, 24, and 48 hours), using three different concentrations (5, 0.5, and 0.05 mg/mL). The Pluronic F-68 solution and the control zein formulation (NP) showed significant repellent activities, compared to the control, at the highest concentration tested, with no changes as a function of time. The other concentrations (0.5 and 0.05

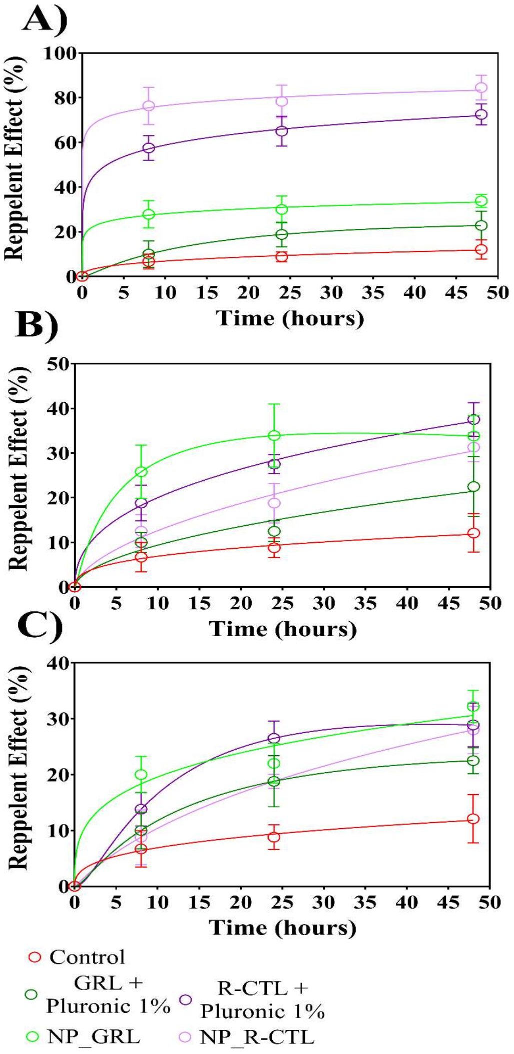
mg/mL) showed substantially lower repellent activities. At the lowest concentration, there was no significant difference, compared to the control (Figure S4 A-C).

Figure 5 shows the repellent activities of the formulations containing GRL and R-CTL. At the highest concentration tested (Figure 5A), the formulations containing R-citronellal presented the greatest repellent effects, with repellent activities of 57.5 ± 5.4% and 76.3 ± 8.3 % obtained after 8 hours for the emulsified compound and the nanopesticide, respectively. The repellent activities obtained for geraniol were lower, with values of 10.0 ± 5.9 % and 27.8 ± 6.1 % for the emulsified compound and the nanopesticide, respectively. For both botanical repellents, encapsulation resulted in higher repellent activity. A possible explanation for this could be a synergistic effect between the botanical repellent and the constituents of the nanoparticles, which also presented a repellent effect, as described previously. Werdin-González et al. (2017), found an increase in the insecticidal/fumigant activity of the essential oils of geranium (*Geranium maculatum* L.) and bergamot (*Citrus bergamia* Risso) after encapsulation in polymeric polyethylene glycol (PEG) nanoparticles. The authors reported that the greater effectiveness of the nanoformulations containing the essential oils was mainly due to alteration of the toxicokinetics and the processes affecting the AIs, such as penetration and bioavailability. In addition, the nanoparticles provided protection of the active agent against premature degradation and/or volatilization.

The repellent activity was lower at concentrations of 0.5 mg/mL (Figure 5B) and 0.05 mg/mL (Figure 5C), compared to the highest concentration tested, with values below 40 and 30%, respectively. However, the repellent activity increased as a function of time. At both concentrations, the nanopesticide formulation containing geraniol presented the greatest effect after 8 hours, with values of 25.8 ± 5.9% and 22.0 ± 3.2% for the concentrations of 0.5 and 0.05 mg/mL, respectively. At lower concentrations, the encapsulated geraniol continued to show a higher repellent effect, compared to the emulsified compound. This was not observed for the encapsulated R-citronellal, which presented repellent activity that was higher than for the control, but lower than for the emulsified compound. These findings corroborated the results presented in Section 3.4, where differences were observed in the mechanisms of photodegradation of the compounds, with the protective effect of encapsulation being lower for R-citronellal. As discussed previously, a larger fraction of the R-citronellal molecules remained on the surfaces of the nanoparticles, so after dilution of the nanopesticide suspensions, the mechanisms of release/degradation of the compounds differed, which also resulted in differences in the repellent effects.

The results showed that the botanical repellents caused irritation in the mites and exerted repellent effects, in agreement other studies demonstrating the effects of different essential oils containing geraniol and R-citronellal towards *T. urticae* (CHOI et al., 2004; YI et al., 2007; LABORDA et al., 2013). Moreover, the results show that in addition to protecting the active against photodegradation, zein nanoparticles caused a slower volatilization of botanical repellents, making their effects more durable (as observed in figure 5). The present results are promising in terms of potential applications of these nanotechnological formulations for the control of mites in a range of agricultural cultivations. Even at the lowest concentrations tested, the formulations showed repellency percentages exceeding 30%, while at these concentrations, the formulations caused no phytotoxic effects in bean, and presented low cytotoxicity.

Figure 5. Repellent activities of the botanical nanopesticides and the botanical repellents emulsified with surfactant against the two-spotted spider mite (*Tetranychus urticae*), at concentrations of A) 5, B) 0.5, and C) 0.05 mg/mL.



In conclusion, botanical repellents present in the essential oil of citronella were successfully incorporated into zein nanoparticles, with encapsulation efficiencies greater than 90%, providing effective protection of the AIs against losses by photodegradation and volatilization. The formulations showed mean nanoparticle diameters of 150-200 nm and polydispersity indices greater than 0.3, and remained stable for at least 90 days. AFM analyses revealed spherical morphology of the nanoparticles. FTIR analyses confirmed encapsulation of the botanical repellents by physical interactions with the zein protein matrix. The zein nanoparticles provided effective protection of the botanical repellents against UV radiation, especially in the case of geraniol. In phytotoxicity tests using the seeds of bean (*Phaseolus vulgaris*), concentrations of 0.5 and 0.05 mg/mL caused no phytotoxic effects on germination, while at the highest concentration (5 mg/mL), encapsulation in the nanoparticles decreased the toxic effect, hence indicating the concentration range suitable for use in agriculture. In cytotoxicity assays, the formulations showed no cytotoxic effects in 3T3 cells, with the exception of the GRL formulation emulsified with surfactant. In the case of the V79 cells, the toxic effect decreased with encapsulation of the AIs in the nanoparticles. Tests of repellent activity against the *Tetranychus urticae* Koch mite showed that the formulations had high repellent activity at the highest concentrations tested (5 mg/mL), and that the greatest effects were achieved for the formulations with R-citronellal. The greater effects of the nanopesticides were mainly due to synergism between the constituents of the particles (which also presented repellent activity) and the botanical repellents. At the lower concentrations, the formulations still presented repellent activity, and the greater effectiveness of geraniol was probably due to better protection of the compound. The zein nanoparticles provided effective encapsulation and protected these natural compounds against premature degradation, while at the same time decreasing the toxicity towards nontarget organisms. The nanopesticides developed in this work showed repellent activities against the two-spotted spider mite, even at low concentrations, while at the highest concentrations the effects of the AIs were potentiated by encapsulation. The findings open perspectives for the use of these systems in pest control in agriculture, since the formulations are environmentally friendly alternatives that can contribute to the goal of sustainability.

4. Abbreviations Used

FDA, Food and Drug Administration; GRL, geraniol; R-CTL, R-citronellal; HPLC, high performance liquid chromatography; AFM, atomic force microscopy; FTIR, fourier transform infrared spectroscopy; ATR, attenuated total reflectance; AIs, active ingredients; EE, encapsulation efficiency; NP, zein nanoparticles; NP_GRL, zein nanoparticles loading geraniol; NP_R-CTL, zein nanoparticles loading R-citronellal; GRL + Pluronic 1%, geraniol emulsified with pluronic; R-CTL + Pluronic 1%, R-citronellal emulsified with pluronic and Pluronic 1%, pluronic solution.

5. Acknowledgments

The authors are grateful for financial support provided by the São Paulo State Science Foundation (FAPESP, grant #2014/20286-9, grant #2015/15617-9) and CNPq.

6. Supporting Information

Methodology validation for botanical compounds

Quantification of the botanical repellents was performed by high performance liquid chromatography, employing an UltiMate 3000 system (Thermo Scientific). Chromeleon 7.2 software was used for acquisition and interpretation of the chromatograms. The geraniol analysis was performed using a Phenomenex Gemini C_{18} reverse phase column (150 mm × 4.6 mm, 5.0 µm) kept at 30 °C. The mobile phase was acetonitrile:water (60:40, v/v), at a flow rate of 1 mL/min. The injection volume was 100 µL and the detector wavelength was set at 210 nm. The analysis of R-citronellal was performed using a Phenomenex Kinetex C_{18} reverse phase column (150 mm × 4.6 mm, 3.0 µm) kept at 30 °C. The mobile phase was acetonitrile:water (70:30, v/v), at a flow rate of 1 mL/min. The injection volume was 100 µL and the detection wavelength was 210 nm. All the analytical curves showed correlation coefficients (r^2) higher than 0.99. The detection limits (DLs) for GRL and R-CTL were 0.39 ± 0.01 and 3.37 ± 0.12 µg/mL, respectively. The quantification limits (QLs) were 1.30 ± 0.09 and 5.61 ± 0.10 for GRL and R-CTL, respectively.

Fourier Transform Infrared Spectroscopy analysis

The spectrum of zein (Figure S2-A) showed bands between 3100 and 2800 cm^{-1}, attributed to –C-H groups of fatty acids and amino acids. Intense bands between 1800 and 1500 cm^{-1} were assigned to amides I and II, and bands from 1300 to 1500 cm^{-1} were attributed to stretching of $=CH_2$ and –C-H bonds of lipids and proteins. The spectrum of Pluronic F-68 (Figure S2-B) showed a band at 2884 cm^{-1}, assigned to stretching vibrations of –C-H groups, and bands from 948 to 1110 cm^{-1}, associated with symmetric and asymmetric stretching vibrations of C-O of ether groups. The spectrum of geraniol (Figure S2-C) showed bands associated with alcohol -OH at 3326 cm^{-1}, alkane –C-H vibration at 1669 cm^{-1}, and C=C bond vibration at 1010 cm^{-1}. The R-CTL spectrum (Figure S2-D) showed a band between 2900 and 3000 cm^{-1} corresponding to the stretching of aliphatic –C-H bonds, a band at 2700 cm^{-1} assigned to –C-H stretching, and a strong band at 1740 cm^{-1} (C=O) confirming the presence of the aldehyde group. Figure S2-E shows the infrared spectrum for the control zein nanoparticles (without AIs). A signal at 3340 cm^{-1}, indicative of the O-H group, was probably associated with the presence of water. Also present were bands associated with the characteristic groups of the zein protein, such as amides I and II. The spectrum of the zein nanoparticles loaded with geraniol (Figure S2-F) showed characteristic peaks of zein (amides I and II). An intense peak at around 3326 cm^{-1} was attributed to the alcohol groups of GRL. Shifts of the bands corresponding to the bonds -C-H (at 2967 and 2916 cm^{-1}) and –C-O- (at 1010 cm^{-1}) were also observed, probably due to interactions with the repellent. Figure S2-G shows the spectra for zein nanoparticles loaded with R-CTL, with bands corresponding to the presence of water (O-H signal at 3340 cm^{-1}), amides I and II of zein, and shifts of the aliphatic C-H bond stretching bands, indicative of interactions with the botanical repellents.

Figure S1

Stability of botanical repellents as a function of time. A significance level of $p < 0.05$ was considered for the differences obtained within the same group; a*, b*, c*, d*, and e* indicate significant differences relative to days 0, 15, 30, 60, and 90, respectively.

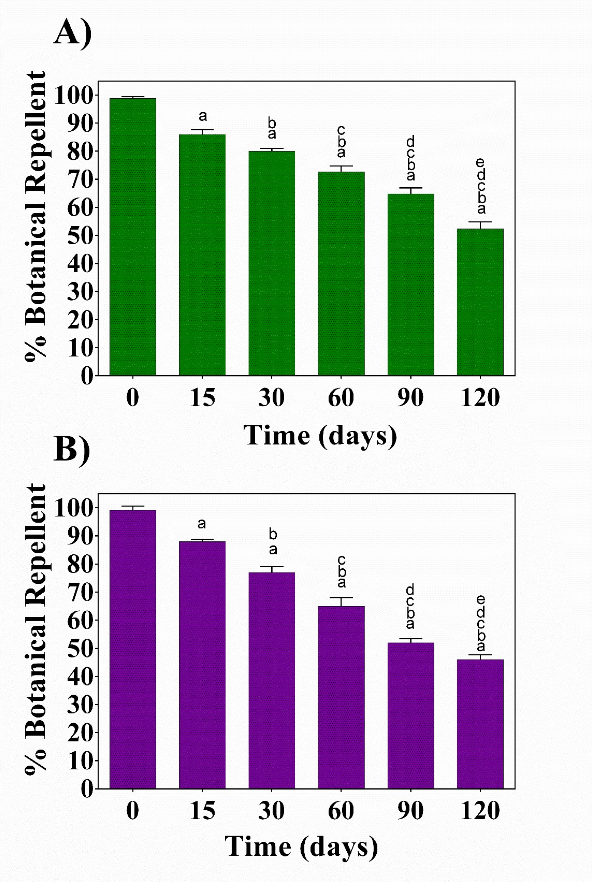


Figure S2 FTIR spectrum: A- zein powder; B- Pluronic F-68 surfactant; C- GRL; D- R-CTL; E- NP_Z; F- NP_GRL and G- NP_R-CTL

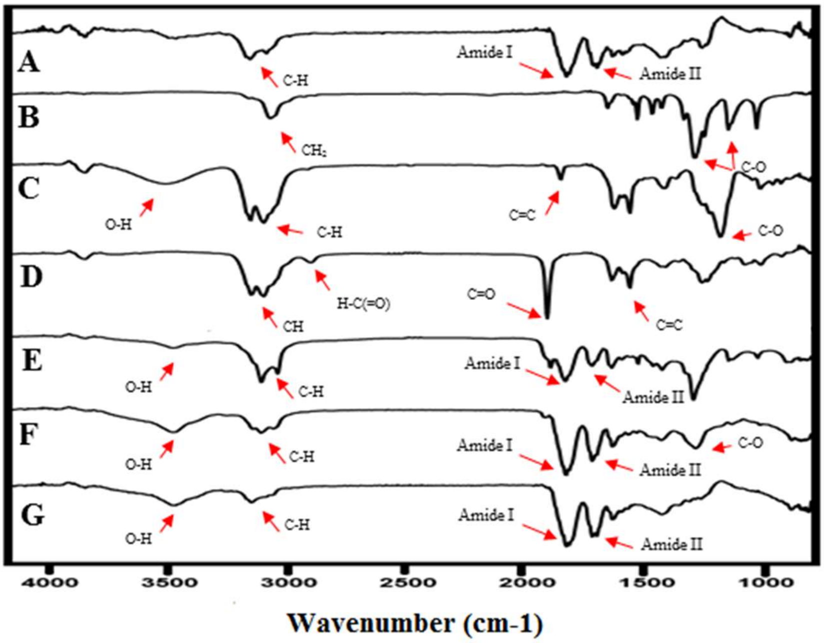


Figure S3: Effect of different formulations under fresh mass and dry mass of beans seedlings (*P. vulgaris*), after seed treatment. A) Fresh mass; B) Dry mass. Being: Zein nanoparticles (NP); Pluronic F-68 surfactant; Geraniol emulsified with surfactant (GRL + Pluronic 1%); zein nanoparticles loaded with geraniol (NP_GRL); R-Citronellal emulsified with surfactant (R-CTL + Pluronic 1%); Zein nanoparticles loaded with R-citronellal (NP_R-CTL). A significant difference of $p < 0.05$ was considered for statistical differences observed between the groups of the same concentration, where a * represents significant variations between the groups in relation to the control.

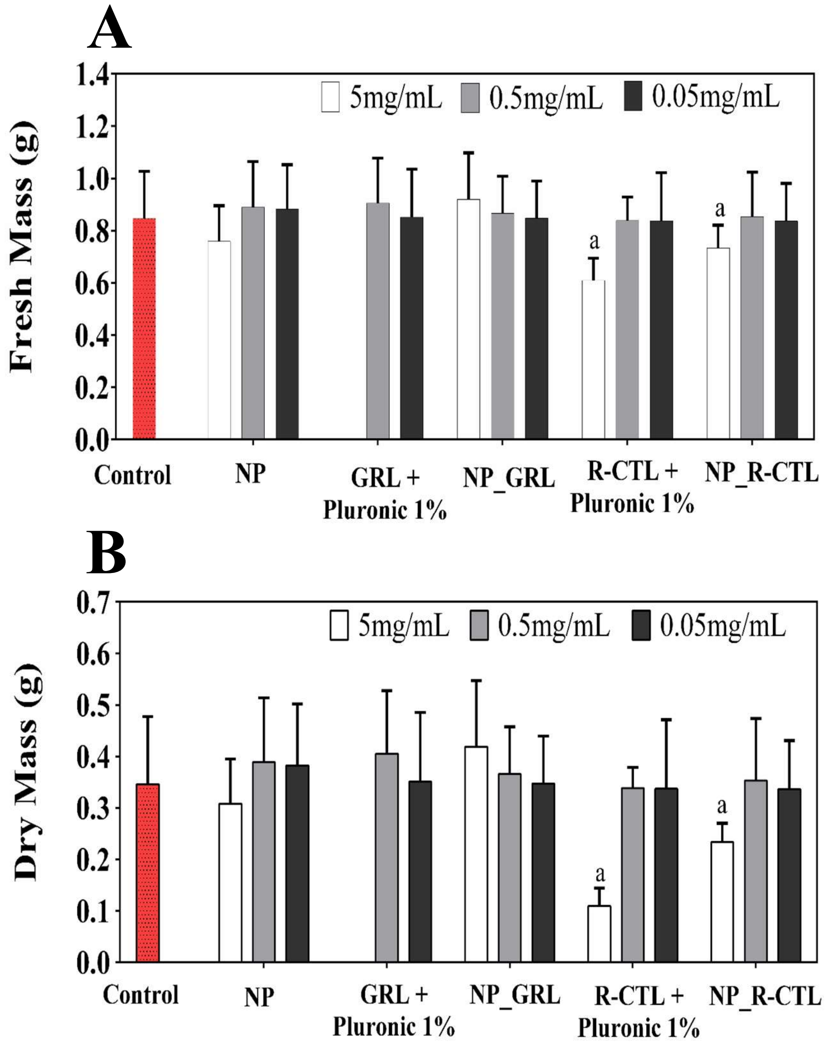
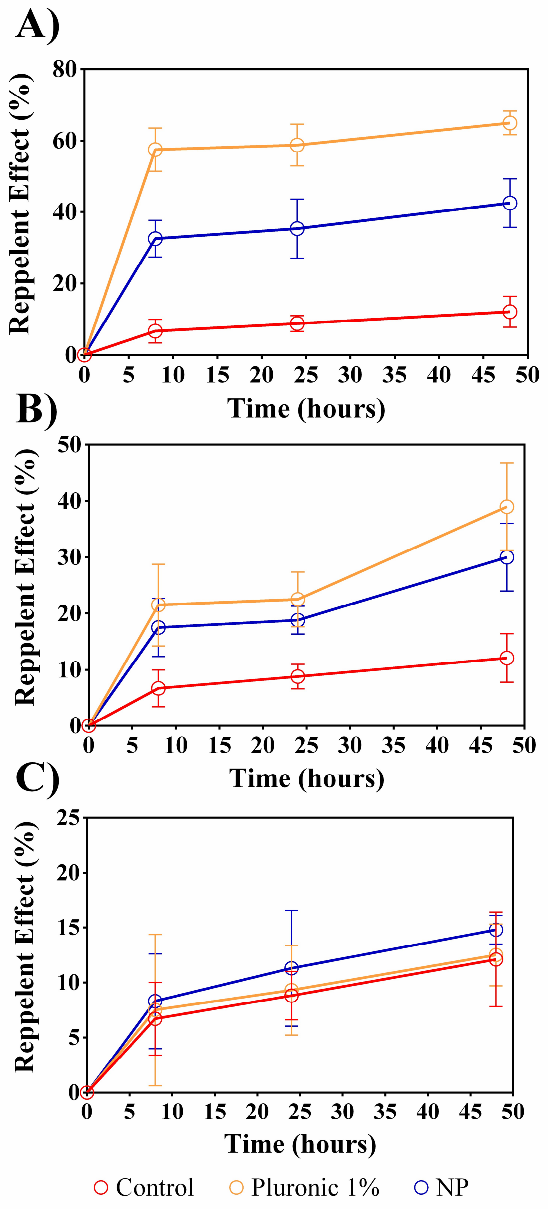
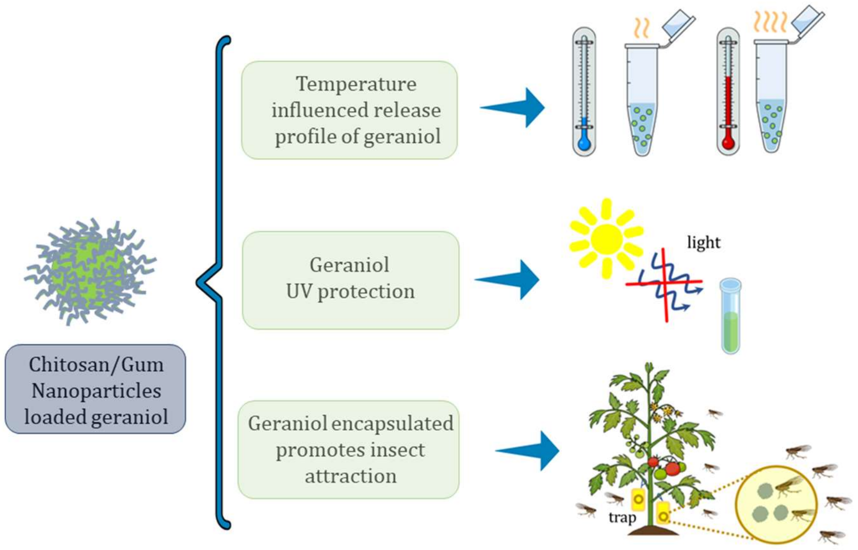


Figure S4: Repellent activity of zein nanoparticles without botanical repellents and the Pluronic F-68 surfactant against two-spotted spider mite (*Tetranychus urticae*). A) Concentration of 5 mg/mL; B) Concentration of 0.5 mg/mL and C) Concentration of 0.05 mg/mL.



CHAPTER III

Geraniol encapsulated in chitosan/gum arabic nanoparticles: a promising system for pest management in sustainable agriculture



OLIVEIRA, J. L.; CAMPOS, E. V. R.; PEREIRA, A. E. S.; NUNES, L. E. S.; DA SILVA, C. C. L.; PASQUOTO, T.; LIMA, R.; SMANIOTTO, G.; POLANCZYK, R. A.; FRACETO, L. F. Geraniol Encapsulated in Chitosan/Gum Arabic Nanoparticles: A Promising System for Pest Management in Sustainable Agriculture. **Journal of Agricultural and Food Chemistry**, v. 66, n. 21, p. 5325–5334, 2018.

Abstract

The nanoencapsulation of botanical compounds (such as geraniol) is an important strategy that can be used to increase the stability and efficiency of these substances in integrated pest management. In this study, chitosan/gum arabic nanoparticles containing geraniol were prepared and characterized. In addition, evaluation was made of the biological activity of geraniol encapsulated in chitosan/gum arabic nanoparticles towards whitefly (*Bemisia tabaci*). The optimized formulation showed a high encapsulation efficiency (>90%) and remained stable for about 120 days. The formulation protected the geraniol against degradation by UV radiation, and the in vitro release was according to a diffusion mechanism that was influenced by temperature. An attraction effect was observed for *Bemisia tabaci*, indicating the potential of this type of system for use in pest management, especially in trap devices.

Keywords: Botanical active agent, environmentally friendly, toxicity.

1. Introduction

Geraniol (2E-dimethylocta-2,6-dien-1-ol) (GRL) is an acyclic monoterpene alcohol that presents low water solubility (100 mg/L) but is soluble in most organic solvents. It is exhaled from the flowers of various plant species and is also found in the tissues of certain herbs. It is a common component of several essential oils including ninde oil (~66.0%), rose oil (~44%), palmarosa oil (~53%), and citronella oil (~25%) (MAIA; MOORE, 2011). Geraniol is widely used as a chemical component of cosmetic fragrances and for other home uses. It has various biochemical and pharmacological activities, including antimicrobial (THAKKER; PARIKH; DESAI, 2016), antioxidant (PRASAD; MURALIDHARA, 2017), anti-inflammatory (KHAN et al., 2013), and antitumor (CHO et al., 2016; DEVAKI, 2016). Geraniol also presents insect repellent and attraction properties (REIS et al., 2014; CHATTOPADHYAY et al., 2015). These effects are mainly dependent on the concentration of the compound and the insect type (BECHER et al., 2018).

Due to their biological activities towards insects, essential oils and their main active components have been identified as potential agents in pest management (DE OLIVEIRA et al., 2014b; PAVELA, 2015). The advantages of these compounds of botanical origin, rather than the more commonly used synthetic compounds, include their low persistence in the field and that fact that they generally exhibit strong selectivity and complexity (which can delay the development of resistance in target organisms) (ISMAN, 2016). However, the application of these compounds in agriculture is limited by their high sensitivity to light, humidity, temperature, and degradation by microorganisms. Hence, there is a need to develop formulations able to improve the stability and effectiveness of these natural compounds in various different applications (BENELLI et al., 2017b).

One technique that has shown considerable promise is the use of nanotechnological carrier systems capable of protecting the active agents against premature degradation, while also increasing their water solubility and promoting slower release (DE OLIVEIRA et al., 2014b). These nanostructured systems can be produced from various matrices, including biodegradable polymers such as chitosan. This is a polymer derived from chitin, found in the exoskeletons of crustaceans, and in terms of use and distribution is considered the second most widespread biomaterial (after cellulose). It is obtained by the deacetylation of chitin in an alkaline medium (HAMED; ÖZOGUL; REGENSTEIN, 2016).

A variety of methods have been described in the literature for the preparation of nanoparticles based on chitosan, the most important being ionic gelation, coacervation, co-precipitation, solvent evaporation, and microemulsion (RAMPINO et al., 2013). Some of these methods use a crosslinking process in which interlinking of chains is induced by reaction with a bifunctional substance capable of generating a three-dimensional polymer network. Various agents can be used for ionic crosslinking of chitosan in order to produce polyelectrolyte complexation nanoparticles, the main ones being sodium tripolyphosphate (WU et al., 2017), alginate (ALUANI et al., 2017), and different types of gums (TAN et al., 2016; BRAZ et al., 2017). Gum arabic is a biocompatible and biodegradable polysaccharide. The carboxyl groups present in the molecule are largely dissociated at neutral pH, with the molecule having an open, highly charged, and expanded structure. These features mean that compared to other polysaccharides, gum arabic presents greater quantities of interaction sites and negative charges capable of associating with polycationic chitosan (TAN et al., 2016). Several studies have described the use of such nanoparticles for the encapsulation of natural compounds (SOTELO-BOYÁS et al., 2017b, 2017c).

The aim of this study was to develop systems based on chitosan/gum arabic nanoparticles for loading with the botanical active agent geraniol. In addition to preparation and characterization of the nanoparticles, their photostability and release properties were evaluated in vitro at different temperatures. The biological effects of the formulation were investigated in whitefly (*Bemisia tabaci*). This study opens perspectives for the use of nanoparticles containing geraniol in pest management for sustainable agriculture.

2. Materials and Methods

2.1 Materials

Chitosan (molecular weight: 27 kDa; degree of deacetylation: 75-85%) gum arabic (molecular weight ~25×10^5 Da), Tween 80 (average micellar molecular weight 79 kDa), and the geraniol active agent were obtained from Sigma-Aldrich. Acetonitrile (HPLC grade) was obtained from J. T. Baker. The seeds were from ISLA Sementes and the substrate was from Hortaliça Mix.

2.2 Optimization of preparation of the formulation: 2^3 factorial design

The effects of the amounts of some of the components used to prepare the chitosan/gum arabic formulations were evaluated using a 2^3 factorial design performed with Statgraphics Plus statistical software. This approach is often adopted for optimization of preparation processes, since it enables evaluation of the effects of the variables using only a few experiments. For these nanoparticle formulations containing geraniol, the experimental design was expected to lead to the production of nanoparticles with average diameter of 200-300 nm, polydispersity index below 0.25, and high zeta potential, in addition to high encapsulation efficiency.

The factors studied were the concentrations of chitosan, gum arabic, and Tween 80, while the concentration of geraniol was kept constant at 5 mg/mL. The polymer concentrations were defined after initial preparation tests. The concentrations were studied at three levels: 0.15, 1, and 1.5% (g/g) of chitosan; 0.3, 1, and 3% (g/g) of gum arabic; and 0.5, 1, and 1.5% (g/g) of Tween 80. The dependent variables were the mean nanoparticle diameter (MD), polydispersity index (PI), zeta potential (ZP), and encapsulation efficiency (EE).

The nanoparticles were prepared based on the method described previously (AVADI et al., 2010), with some modifications. The procedure consisted of two main steps: emulsification, followed by a process of ionic gelation between the positive charges of the chitosan (pH 3) and the negative charges of the gum arabic (pH 7). The chitosan solution has a lower pH due to the addition of acetic acid. Firstly, solutions of chitosan and gum arabic were prepared at different concentrations, under agitation overnight at ambient temperature, with the chitosan diluted in 0.5% acetic acid solution. The solutions were filtered through a 0.45 µm syringe filter (Millipore) and the final concentrations were checked after filtration (pH 3 for chitosan and pH 7 for gum arabic). In the next step, the geraniol active agent and the Tween 80 surfactant were added to 16 mL of the chitosan solution, which was stirred at 10,000 rpm (UltraTurrax) for 5 min. The emulsion formed was then agitated slowly for around 10 min, followed by slow dropwise addition of 6 mL of the aqueous gum arabic solution to the chitosan solution. The formulation was stirred for a further 10 min and stored at 25 ºC.

2.3 Characterization of the nanoparticles

2.3.1 Mean diameter (using dynamic light scattering and nanoparticle tracking analysis) and zeta potential

Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) are complementary techniques that provide important information about the mean particle diameter of nanoparticle formulations, as well as the particle concentration. In this study, it was decided to submit the samples to analysis by the two techniques, in order to improve the accuracy of information about the nanoparticle formation process and the stability of the suspension. DLS and microelectrophoresis (zeta potential) analyses were performed using a ZetaSizer Nano ZS90 particle analyzer (Malvern Instruments) at a fixed angle of 90º and temperature of 25 ºC. For the NTA measurements, the data were collected using a NanoSight LM 10 cell (532 nm laser) and a sCMOS camera, controlled by NanoSight v. 2.3 software. The nanoparticle stability was investigated over 120 days. The stability evaluation over time is performed in order to observe eventual changes in formulations physical-chemical parameters over the storage time. Also, this is important in special if we are thinking in the potentially of these systems in the market as well as the shelf life of the product.

2.3.2 Quantification of geraniol and determination of encapsulation efficiency

Geraniol was quantified by high performance liquid chromatography, using an UltiMate 300 system (Thermo Scientific) controlled with Chromeleon v. 7.2 software. The chromatographic conditions are described in the Supplementary Material. Concentrations from 5 to 10 µg/mL were used to obtain the analytical curve. The samples were diluted in the mobile phase (acetonitrile:water, 60:40, v/v). Geraniol encapsulated in the chitosan/gum arabic nanoparticles was quantified by an ultrafiltration/centrifugation method employing regenerated cellulose ultrafiltration devices (Microcon 10 kDa, Millipore). This enabled quantification of the amount of non-encapsulated active agent, so the encapsulation efficiency (EE) could be determined by difference, considering the total amount added. The total amounts of geraniol (100%) present in the formulations were calculated taking into account the total amount added, as well as the losses during the preparation process.

2.3.3 Evaluation of interaction of geraniol with the nanoparticles using infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC)

FTIR and DSC analyses were used to investigate possible interactions between geraniol and the chitosan/gum arabic nanoparticles (DE OLIVEIRA et al., 2015). Sample pellets were obtained by centrifugation at 8000 x *g* for 15 min. The FTIR analyses employed an Agilent spectrophotometer equipped with an attenuated total reflectance (ATR) accessory and operated in the range from 400 to 4000 cm^{-1}, with 64 scans and 8 cm^{-1} resolution. The DSC analyses were performed using a DSC Q20 system (TA Instruments), under a flow of nitrogen at 50 mL/min, with heating from 0 to 300 °C at a rate of 10 °C/min.

2.3.4 Morphological analysis of the nanoparticles

Atomic force microscopy (AFM) was used to analyze the morphology of the chitosan/gum arabic nanoparticles containing the geraniol active ingredient. For this, an Easyscan 2 microscope (Nanosurf, Switzerland) was operated in contactless mode, with TapAl-G cantilevers (BudgetSensors, Bulgaria), at a scan rate of 90 Hz. Gwyddion software was used to produce images in TIFF format (256 x 256 pixels).

2.4 Release assays and release mechanisms

The release assays were performed as described previously (SOTELO-BOYÁS et al., 2017b), with some modifications. The nanoparticles containing geraniol were placed in dialysis membrane bags (1 kDa exclusion pore size, Spectra/Por) and immersed in 5% (w/v) Tween 80 solution. Aliquots were periodically collected and the compound was quantified as described above. In order to avoid any losses by evaporation, the vessels were closed and were only opened during sampling (performed in triplicate). The release assays were performed at different temperatures (20, 25, and 30 °C) in order to investigate the influence of this parameter on the profile of release of geraniol from the chitosan/gum arabic nanoparticles. The release data were evaluated using the zero order, first order, Higuchi, and Korsmeyer-Peppas models [34].

2.5 Photostability assays

The photostability assays were performed as described previously (PATEL et al., 2010), with some modifications. The formulations were subjected to ultraviolet light (365 nm) in a dark chamber and aliquots were periodically collected for determination of the amount of geraniol present in the solution. The nanoparticle formulations (100 µL) were extracted using acetonitrile (400 µL), followed by quantification of the compound using HPLC. The zero order, first order, pseudo-first order, and pseudo-second order kinetic models were applied to the photodegradation curves in order to obtain further information about the geraniol degradation process.

2.6 Biological effect on whitefly

The biological activity assays were performed with whitefly (*Bemisia tabaci*). The insects were reared on bean plants (*Phaseolus vulgaris*)in a greenhouse. For the bioassays, the insects were collected using a manual sucker. The attraction/repellency tests were conducted with a four-way olfactometer, similar to the one described previously (VET et al., 1983), which enabled recording of the insect responses to different odors, offered simultaneously. The insects were exposed to the four fields, two with only filtered air, and two with nanoparticle formulations and/or the botanical active agent. The treatments (odors) were positioned randomly in the olfactometer fields and the insects were individually introduced into the central arena of the olfactometer, where the odors derived from the four fields were mixed and the insects could move freely. The air flows passing through the odor fields were controlled at 200 mL/min with four flowmeters. For each treatment, 10 insects (repetitions) were observed during 10 min. The nanoparticles containing geraniol and geraniol emulsified with Tween 80 were tested at a geraniol concentration of 5 mg/mL. A response to the formulation was considered when the insect crossed the dotted line in the center of the arena, noting the time that it remained in such a position. This was performed every time that the insect moved to a different region. After each repetition, the olfactometer was rotated by 90° in order to avoid conditioning the insects to the laboratory environment.

3. Results and Discussion

3.1 Preparation and optimization

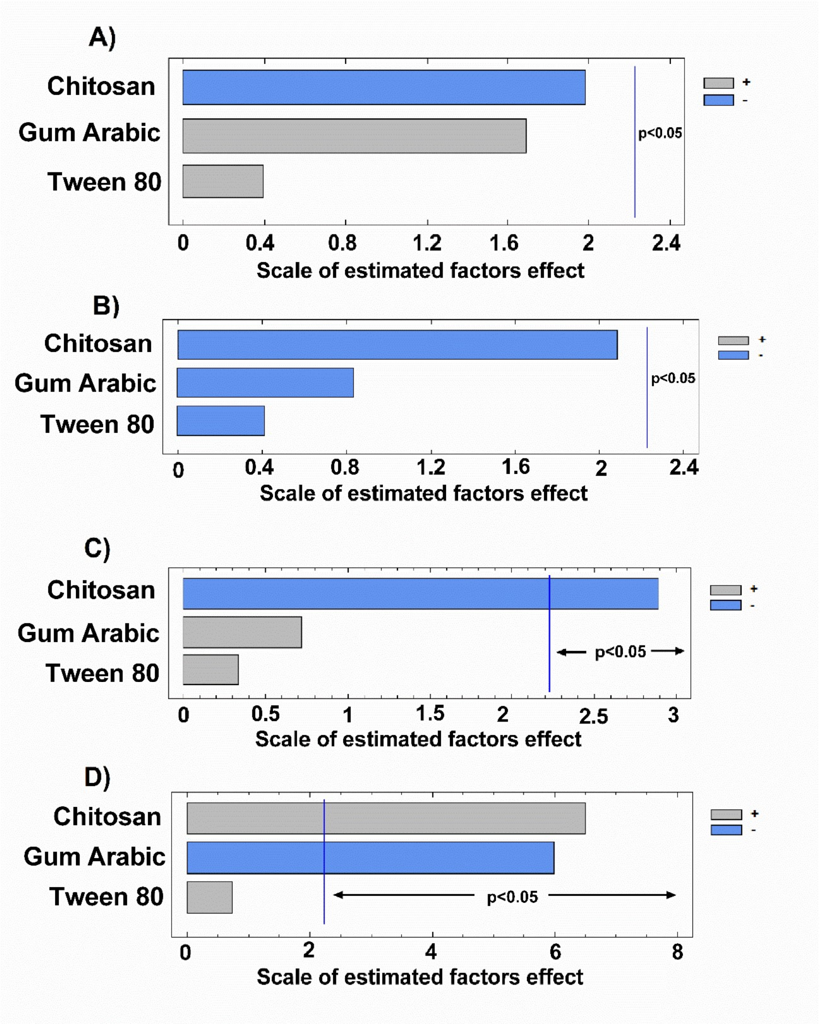
From the experimental design, it was expected to obtain a formulation with nanoparticles with size in the range 200-300 nm, polydispersity index below 0.25, high absolute zeta potential, and high efficiency of encapsulation of the botanical active ingredient. A total of 9 formulations were performed, based on the 2^3 factorial design. The effects of three factors were evaluated: chitosan concentration (X_1), gum arabic concentration (X_2), and Tween 80 concentration (X_3), each at three different levels (coded as -1, 0, and +1). The dependent variables were the mean nanoparticle size (MD), polydispersity index (PI), zeta potential (ZP), and encapsulation efficiency (EE). Table 1 provides the absolute results obtained with the factorial design applied to preparation of the chitosan/gum arabic nanoparticles containing geraniol. The ranges of values obtained were 140 - 750 nm (MD), 0.21 - 0.78 (PI), -21 - 35 mV (ZP), and 91-98% (EE).

Table 1. 2^3 factorial design applied to the formulations of chitosan/gum arabic nanoparticles containing GRL, and results obtained for mean diameter (nm), polydispersity, zeta potential (mV), and encapsulation efficiency (%). The results are shown as the means for nine formulations analyzed in duplicate. X_1, X_2, and X_3 are the factors studied in the assays (concentrations of chitosan, gum arabic, and Tween 80, respectively). The factors were varied at 3 levels (-1, 0, and 1).

Formulation	**X_1**	**X_2**	**X_3**	**Mean diameter (nm)**	**Zeta potential (mV)**	**Polydispersity index**	**Encapsulation efficiency (%)**
F1	1	1	1	235.23 ± 18.66	19.43 ± 11.03	0.21 ± 0.13	91.76 ± 2.34
F2	-1	1	1	749.46 ± 363.07	-17.75 ± 2.45	0.78 ± 0.09	95.30 ± 2.65
F3	-1	1	-1	276.04 ± 12.74	-21.93 ± 2.96	0.51 ± 0.21	98.09 ± 1.28
F4	-1	-1	-1	268.49 ± 245.13	7.00 ± 3.13	0.43 ± 0.22	97.25 ± 2.47
F5	1	1	-1	295.50 ± 81.69	18.9 ± 5.47	0.39 ± 0.13	95.15 ± 1.48
F6	1	-1	1	237.36 ± 17.86	22.26 ± 2.27	0.24 ± 0.12	95.69 ± 1.24
F7	-1	-1	1	659.72 ± 375.12	27.285 ± 0.69	0.74 ± 0.36	96.41 ± 1.12
F8	1	-1	-1	194.43 ± 21.68	35.83 ± 4.80	0.33 ± 0.12	93.18 ± 4.34
F9	0	0	0	139.85 ± 18.45	21.415 ± 2.99	0.28 ± 0.10	91.76 ± 2.34

Pareto charts (Figure 1) were used to identify the factors that significantly ($p^* <0.05$) influenced the dependent variables, as well as to study their interactions. A positive value indicates a direct relation of the factor to the variable, while a negative value indicates an inverse relationship. The values of p^* resulting from the analysis of variance (ANOVA) are summarized in Table S1 (Supplementary Material), together with the values for the effects of the dependent variables.

Figure 1. Optimization of the preparation of chitosan/gum arabic nanoparticles containing geraniol. Pareto charts showing the effects of the factors (chitosan, gum arabic, and Tween 80 concentrations) on the dependent variables: (A) mean diameter (nm), (B) encapsulation efficiency (%), (C) polydispersity index, and (D) zeta potential (mV). The blue bars indicate a negative effect of the factor on the variable; the gray bars indicate a positive effect. The data showed that the factors only influenced the polydispersity index and the zeta potential. Statistically significant differences are delimited with the vertical lines. Significance level: p <0.05.



None of the factors studied had a significant effect on the mean CS/GM_GRL particle diameter (Figure 1-A). Chitosan had a negative effect on the mean nanoparticle diameter, while gum arabic and Tween 80 showed positive effects. No significant (p <0.05) effects of the factors were observed for the efficiency of GRL encapsulation in the chitosan/gum arabic nanoparticles (Figure 1-B). On the other hand, some factors had significant effects on the CS/GM_GRL polydispersity index (Figure 1-C) and zeta potential (Figure 1-D). The statistical analysis enabled the construction of empirical models for the polydispersity index

(Equation 1) and the zeta potential (Equation 2).

$$\textbf{Polydispersity} = 0.43 - 0.32[\textbf{chitosan}]\ (r = 0.55) \quad (1)$$

$$\textbf{Zeta potential} = 12.49 + 25.45[\textbf{chitosan}] - 23.43[\textbf{gum arabic}]\ (r = 0.90) \quad (2)$$

According to Figure 1, Table S1, and the model (Equation 1), only chitosan had a significant effect on the polydispersity, indicating that changes in the concentration of this component could cause statistically significant changes in the polydispersity. Since chitosan had a negative influence on the polydispersity index, the use of higher concentrations would lead to lower polydispersity values. This could be explained by greater electrostatic interaction between the positive charges of the chitosan and the negative charges of the gum arabic, resulting in the particles having a smaller size variation. A another point is the viscosity, the increase can influence the particle size and the polydispersity values. When viscosity increases the particle size decreases and the size distribution is narrower. However, the experimental model showed a low correlation coefficient, indicating that other factors also influenced the polydispersity index values of the samples. Chitosan and gum arabic showed statistically significant affects on the zeta potential (Figure 1-D, Equation 2), while Tween 80 had no effect. The numerical value for the chitosan effect was positive, so a higher chitosan concentration resulted in a more positive zeta potential, in agreement with the fact that chitosan is a cationic polymer. The gum arabic concentration showed the opposite effect on zeta potential, with higher concentrations resulting in lower (more negative) zeta potentials, corroborating the zeta potential results obtained for the biopolymers separately. At the concentrations tested, the chitosan solutions showed zeta potentials from 30 to 60 mV, while the gum arabic solutions had zeta potentials from -40 to -18 mV.

A further consideration is that the chitosan/gum arabic nanoparticles containing geraniol were prepared using a two-step method. The first step consisted of the formation of geraniol droplets in the chitosan solution by means of the oil-in-water emulsion technique performed with Tween 80. The second step was the solidification of these droplets and the formation of nanoparticles by means of ionic bonding of the protonated amino acid groups of the chitosan (surrounding the geraniol droplets) with the carboxyl groups of the gum arabic (ABREU et al., 2012a; TAN et al., 2016). The constituents of gum arabic include salts of calcium, magnesium, and potassium, leading to negative charges of gum arabic after dissociation in water and consequently strong interaction with the positive charges of chitosan (HOSSEINI et al., 2013). In this study, no significant changes in the mean diameter or encapsulation efficiency were observed with variation of the biopolymer concentration. However, it should be noted that only modifications in the concentration were evaluated, while the effects of parameters such as pH and ionic strength, among others, were not the target of the present work. Tan et al (2016) pointed out that the formation of polyelectrolyte complexing nanoparticles is dependent on numerous factors. It was found that variation of pH and chitosan/gum arabic ratio altered the mean diameter and zeta potential of chitosan/gum arabic nanoparticles containing curcumin.

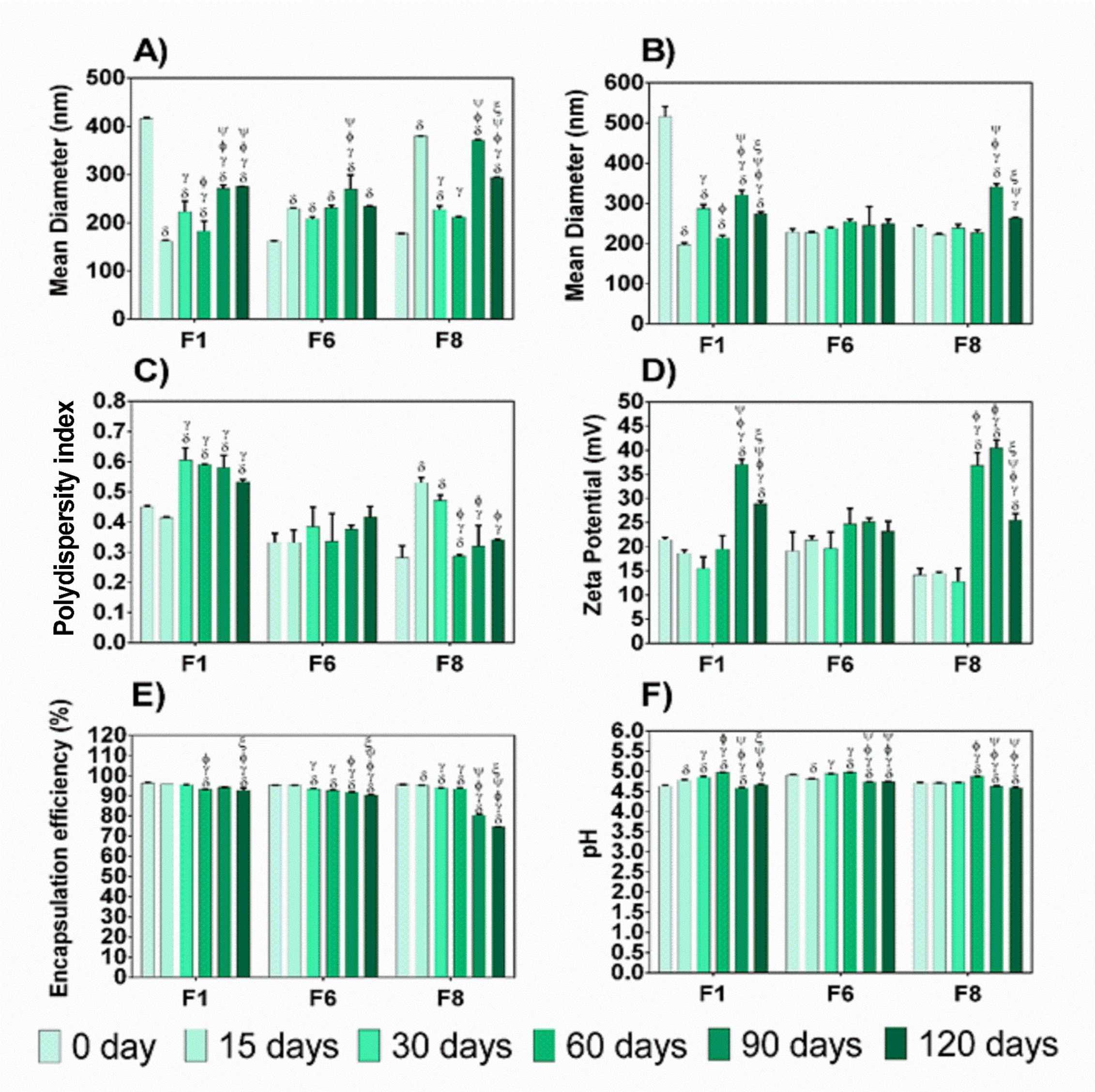
The findings were in agreement with previous studies described in the literature. Keawchaoon et al. (2011) obtained nanoparticles based on chitosan using the same method employed here, encapsulating the botanical compound carvacrol using sodium tripolyphosphate (TPP) as a crosslinking agent. The particles obtained were around 100 nm in size, with carvacrol encapsulation efficiency higher than 60%. Avadi et al., (2010) prepared and characterized nanoparticles of chitosan/gum arabic containing insulin. A 2^3 factorial design was used to investigate the effects of different factors on the preparation of the nanoparticles. It was shown that the concentrations of gum arabic and chitosan had the greatest influence on the encapsulation efficiency and mean diameter of the nanoparticles. Higher amounts of chitosan, relative to gum arabic, led to a greater capacity for ionic gel formation, decreasing the mean diameter and preventing the movement of insulin (hence increasing the encapsulation efficiency). In the present work, the factors evaluated did not have any significant effects on the encapsulation efficiency, probably due to good interaction between the polymers and the surfactant, in the proportions used, as well as the emulsification method adopted.

The results obtained using the factorial design demonstrated that the CS/GM_GRL composition mainly affected the zeta potential and polydispersity values. Therefore, tests of stability as a function of time were performed using formulations F1, F6, and F8, which presented values closer to the set of characteristics established initially.

3.2 Characterization and stability of the formulations

The results obtained for the different parameters are shown in Figure 2.

Figure 2. Physical-chemical parameters and stability evaluation of chitosan/gum arabic nanoparticles containing geraniol. The stability evaluation was performed for 120 days. (A) mean diameter (nm) by DLS, (B) mean diamter (nm) by NTA, (C) polydispersity index, (D) zeta potential (mV), (E) encapsulation efficiency (%), and (F) pH. All analyses were performed in triplicate, at 25 ºC. The stability test results showed that formulation F6 underwent the smallest changes in physico-chemical characteristics during the period of 120 days. Statistically significant differences (two-way ANOVA) for the different storage times are indicated by δ*, γ*, ϕ*, ψ*, and ξ*, corresponding to the initial time (0 days), 15 days, 30 days, 60 days, and 90 days, respectively. Significance level: $p < 0.05$.



Over the period studied, formulation F6 showed the least significant change in the mean nanoparticle diameter, with values of around 200 nm, as determined by both the DLS (Figure 2-A) and NTA (Figure 2-B) techniques. Formulations F1 and F8 showed substantial changes in the mean diameter, with a significant increases in size over the period. The polydispersity index values for formulation F6 (Figure 2-C) were in agreement with the mean diameter, with no significant changes and a mean value of 0.4. These values were consistent with those found in previous work concerning the use of natural polymers to prepare chitosan nanoparticles (ABREU et al., 2012a). The other formulations presented greater changes in polydispersity, with mean values of 0.8 and 0.6 for F1 and F8, respectively. All the formulations showed positive zeta potential values (Figure 2-D). This was mainly due to the mechanism of formation of the nanoparticles, since chitosan possesses a large quantity of protonated amino groups that can interact with the carboxylic acid groups of gum arabic, with the residual amino groups giving rise to positive zeta potential values, indicating that the chitosan molecules were located on the surfaces of the nanoparticles.

As observed for the mean diameter and polydispersity, formulation F6 showed the smallest change in zeta potential over the period. Changes in zeta potential values may be linked to the ionic changes in the medium reflecting in the zeta potential changes of the particles in solution. At the start of the trial, all the formulations showed geraniol encapsulation efficiencies exceeding 90% (Figure 2-E), followed by significant decreases over the trial period (120 days), due to the release of the active ingredient. In all cases, there were significant decreases in pH over time (Figure 2-F), which could be explained by the release of the active agent and particle degradation, resulting in alterations of the ionic species present and consequently in the pH values, corroborating the encapsulation efficiency data (Figure 2-E).

The results of the stability tests showed that formulation F6 underwent the smallest changes in physico-chemical characteristics during the period of 120 days. The parameter values were in agreement with those reported previously for this type of particle. Abreu et al. (2012b) prepared and characterized nanoparticles composed of chitosan/cashew gum for encapsulation of the essential oil of rosemary (*Lippia sidoides*). The particles presented polydispersity above 0.4 ± 0.01 and the best formulation had an average size of 405 ± 52 nm, zeta potential of 28 ± 10.2 mV, and encapsulation efficiency of 78 ± 2.33%.

Formulation F6 was selected for use in the remaining assays. The same composition (1,5% of chitosan, 0,3% of grum Arabic and 1,5% of Tween 80) was also used to prepare control formulations without addition of the geraniol active agent.

The morphology of CS/GM_GRL was investigated using atomic force microscopy (AFM), which showed that the nanoparticles were spherical (Figure 3-A) with mean diameter of 160.4 ± 4.9 nm (for analysis of 100 nanoparticles using the Gwyddion software), in agreement with the results of the DLS and NTA analyses (Figure 3-B). The DLS and NTA results indicated the existence of a wide particle size range, as also observed in the AFM micrographs. In other work, Harika et al., (2016) used AFM to evaluate the size and morphology of chitosan/gum kondagogu (*Cochlospermum gossypium*) nanoparticles loaded with cefixime, which were found to be spherical and dense, with average size of 83.5 ± 16.2 nm.

Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were used to investigate the interaction of geraniol with the CS/GM nanoparticles and the effect of the preparation process on the components of the formulation.

Figure 4-A shows the infrared spectra of the components of the nanoparticles, geraniol, and the chitosan/gum arabic nanoparticles. The chitosan spectrum (Figure 4-AI) showed characteristic vibrational bands of OH and NH groups (at 3330 cm^{-1}), amide III and NH_3 groups (1535 cm^{-1}), and stretching vibrations of CO bonds (1070 and 1020 cm^{-1}). The gum arabic spectrum (Figure 4-AII) showed typical bands at 3330 cm^{-1}, characteristic of O-H stretching of the glucosidic ring. Bands at 1600 and 1420 cm^{-1} corresponded to symmetric and asymmetric stretching vibrations of COO-, respectively, while bands in the region 1280-400 cm^{-1} could be attributed to carbohydrates. The geraniol spectrum (Figure 4-AIII) presented specific bands of alcohol OH groups (3326 cm^{-1}), CH stretching vibrations of alkanes (2967 and 2916 cm^{-1}), and vibrations of C=C bonds (1669 cm^{-1}). The spectrum of the nanoparticles prepared without addition of the active ingredient (Figure 4-AIV) showed characteristic bands of the chitosan and gum arabic polymers, with a band at 2925 cm^{-1} corresponding to stretching vibration of CH bonds and bands at 1600 and 1420 cm^{-1} attributed to stretching vibration of carboxylic acid salts. The spectrum of the geraniol-loaded nanoparticles (Figure 4-AV) showed the characteristic bands of chitosan and gum arabic, together with shifts at around 2967 and 2916 cm^{-1}, corresponding to CH stretching vibrations, and at 1669 cm^{-1} (C=C bonds), confirming the interaction with geraniol and encapsulation of the active ingredient [25].

The results of the DSC analyses are shown in Figure 4-B, where the heat flux (W/g) is plotted as a function of temperature (°C). The thermograms for chitosan (Figure 4-BI) and gum arabic (Figure 4-BII) showed characteristic endothermic peaks at approximately 106 °C, associated with the evaporation of volatile substances such as water, which in the case of chitosan could be attributed to the presence of hydrogen bonds with hydroxyl groups. Figures 4-BIII and 4-BIV show thermograms of the chitosan/gum arabic nanoparticles without and with geraniol, respectively. The thermogram of the chitosan/gum arabic nanoparticles without the active compound showed a reduction of the endothermic peak temperature, due to the interaction of the charges and nanoparticles formation (TAN et al., 2016). In addition, the presence of geraniol decreased the temperature of the endothermic peak from 77 °C (without geraniol) to 70 °C (with geraniol). Decreases in the crystallinity temperature are indicative of structural changes in the chitosan (DUDHANI; KOSARAJU, 2010). Therefore, the process of formation of the nanoparticles (crosslinking) appeared to have influenced the observed peaks. A lower temperature was observed for the nanoparticles containing GRL (Figure 4-BIV), probably due to the interaction with the encapsulated hydrophobic component.

Figure 3. Morphology and size distribution of the chitosan/gum arabic nanoparticles containing geraniol. (A) Atomic force microscopy (AFM) micrograph and (B) size distributions obtained using the DLS and NTA techniques. The results show the spherical morphology of nanoparticles that presented an average diameter of 220.4 ± 10.2 nm for the DLS technique and 178.3 ± 8.9 nm for NTA. The analyzes correspond to the F6 formulation, which presented greater stability as a function of time. The AFM image was processed using Gwyddion® software. The NTA and DLS data were treated using GraphPad Prism 6® software.

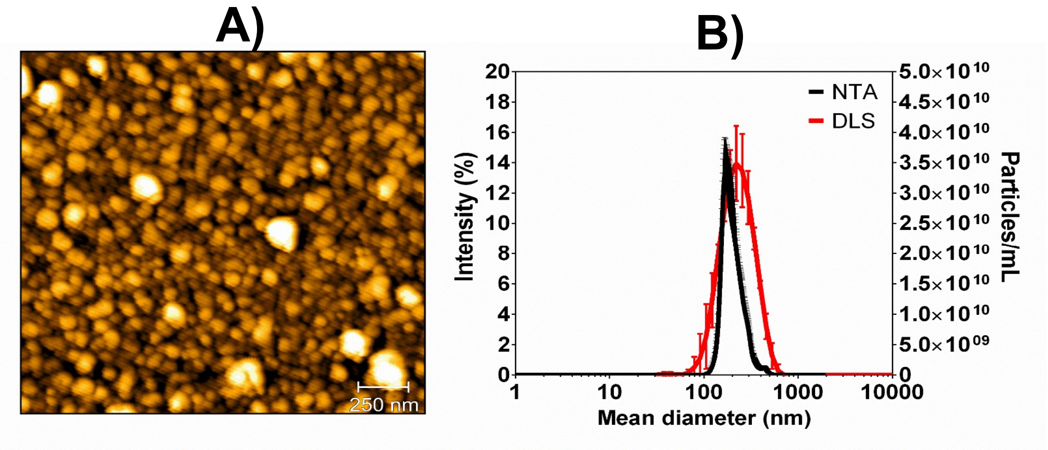
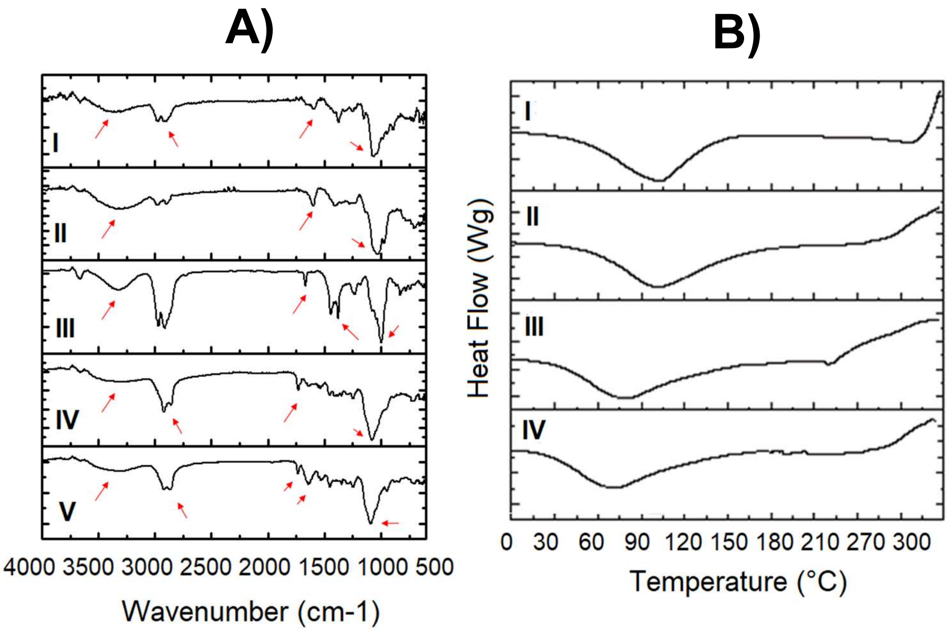


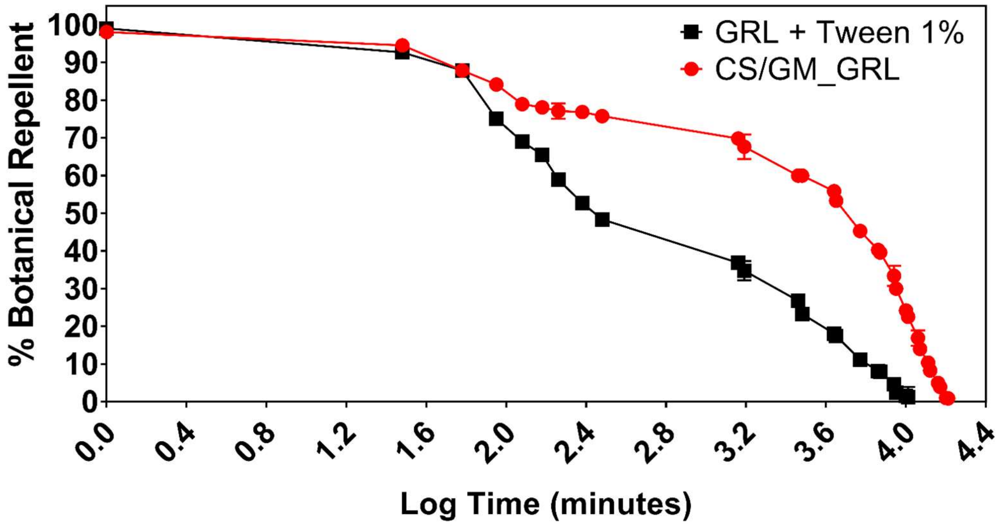
Figure 4. Characterization of the nanoparticles using infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). (A) FTIR graphs: I - chitosan powder, II - gum arabic powder, III - geraniol, IV - control nanoparticles (without the presence of geraniol), and V - nanoparticles containing geraniol. (B) DSC graphs: I - chitosan powder, II - gum arabic powder, III - control nanoparticles (without the presence of geraniol), and IV - nanoparticles containing geraniol. The results evidenced the interaction of geraniol with components of the nanoparticles, such as the shift of the characteristic chitosan bands (at 2967 and 2916 cm^{-1}) and a decrease in the crystallinity temperature (from 77 to 70 °C).



3.3 Photostability assays

Degradation due to exposure to ultraviolet radiation is one of the main problems that can decrease the effectiveness of botanical active agents in the environment. Therefore, photostability tests were performed in order to determine whether the encapsulation process altered the degradation of geraniol (Figure 5).

Figure 5. Increased geraniol photoprotection after encapsulation in chitosan/gum arabic nanoparticles. The graph shows the photodegradation curves for chitosan/gum arabic nanoparticles containing GRL (-●- CS/GM_GRL), compared to GRL emulsified with surfactant (-■- GRL + Tween 1%). The assays were performed in triplicate, at 25 °C, with the samples being subjected to ultraviolet light (365 nm), in a dark chamber, at a distance of 10 cm. The quantification was performed by HPLC after extraction into acetonitrile. The results showed that the chitosan/gum arabic nanoparticles were able to protect geraniol from degradation by UV radiation.



The chitosan/gum arabic nanoparticles provided effective protection of the botanical active ingredient against UV radiation. Degradation of 50% ($t_{50\%}$) of the emulsified GRL was observed after exposure for 4.2 ± 0.1 h, while for the encapsulated geraniol the same percentage was reached after 74.1 ± 0.4 h. Hence, the encapsulation process increased the durability of the geraniol by about 18.5-fold. In previous work, Zhao et al. (2016) encapsulated octyl methoxycinnamate in chitosan/gum arabic microcapsules using the coacervation method. It was found that encapsulation protected the active agent against degradation by UV radiation, with degradation of 25% after 9 h, compared to approximately 70% degradation of the unencapsulated compound.

Different kinetic models were applied to the photodegradation curves in order to investigate the GRL photodegradation mechanism. The values of the photodegradation kinetic constants (*k*) and correlation coefficients (r^2) for the models are listed in Table S2 (Supplementary Material). For GRL emulsified with Tween 80, the best fit to the photodegradation curves was obtained using the pseudo-first order kinetic model, while for

CS/GM_GRL, the best fit was provided by the zero order model. These results were in accordance with the different $t_{50\%}$ values and reflected the advantage of the formulation for use in environmental applications, since it provided protection against premature degradation of the active agent and maintenance of the concentrations required for effective action.

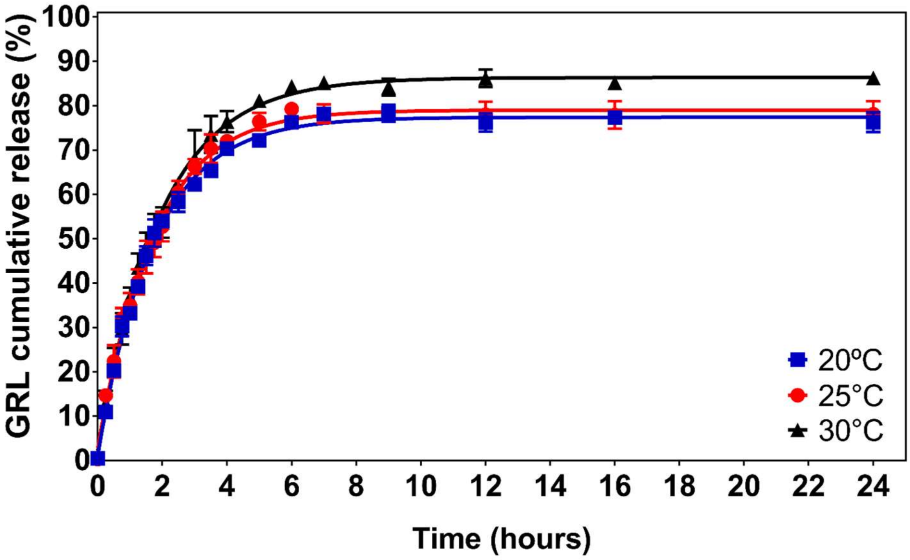
3.4 Release assays and release mechanism

The release assays were used to investigate the profile of GRL release from the CS/GM nanoparticles. It should be noted that the assays were carried out at different temperatures, since this is an important factor to consider in the development of formulations for agricultural applications, especially in the case of botanical compounds (most of which are volatile). Changes in temperature can directly influence diffusion of the active compound, as well as interactions between the nanoparticle components, consequently affecting the release of the active agent. Different mathematical models were applied in order to understand the release mechanism.

The 1% concentration of surfactant (Tween 80) used to prepare the nanoparticles was much higher than the minimum micellar concentration (0.012 mM). Therefore, tests were not performed using the emulsified geraniol, because solubilization of the active agent in the micelles would represent a release system, making it unsuitable for comparison with release of the active agent encapsulated in the carrier system. Hence, it was decided to study the influence of temperature variation on the release of the compound.

The GRL release profile (Figure 6) differed according to the temperature used (20, 25, and 30 °C). Initially, no significant differences were observed in the geraniol release profiles at the different temperatures, with 50% release of the compound after around 1.7 h. However, significant differences were observed after 5 h, with 72.1 ± 1%, 76.4 ± 1.9%, and 81.1 ± 1.4% release at 20, 25, and 30 °C, respectively. Hence, there was greater release of geraniol as the temperature increased. After this period, the release showed no significant difference for temperatures of 20 and 25 ºC, with 78 ± 2% release after 7 h. At 30 ºC, there was 85 ± 1.9% release over the same period, evidencing that an increase in temperature could lead to greater geraniol release. Similar changes in the release profile as a function of temperature were observed previously for chitosan nanoparticles loaded with the plant hormone gibberellic acid (GA) (PEREIRA et al., 2017). Higher release values were obtained at 30 °C, compared to 25 °C, which was attributed to greater relaxation of the polymer chains at higher temperatures and consequent loss of interaction between GA and the amino groups of chitosan.

Figure 6. Release of geraniol from the chitosan/gum arabic nanoparticles. The graph shows the geraniol cumulative release curves (%) obtained at different temperatures: (■ 20 ºC), (● 25 ºC), (▲ ºC). The analyses were performed in triplicate and quantification was by HPLC. The results showed that the GRL release profile differed according to the temperature used, increasing with increasing temperature.



Different release models were applied to the curves shown in Figure 6, in order to investigate the release mechanism. According to the data shown in Table S3, the Higuchi model provided the best fit to the profile of GRL release from the CS/GM nanoparticles, for all the temperatures studied. This indicated that the mechanism of release of GRL from this matrix was mainly due to diffusion following Fick's law, and was temperature dependent. The results could be explained by the characteristics of the gelation/crosslinking of the chitosan and the gum arabic, with temperature being an external factor that affected these properties. According to Azevedo et al, (2014), who prepared chitosan/tripolyphosphate nanoparticles containing vitamin B2, solutions of nanoparticles acquire greater or lesser viscosity at temperatures below or above 30 °C, respectively, with this temperature being defined as the lower critical solution temperature (LCST). An increase in the release constant occurs due to the interaction with the encapsulated compounds (hydrophobic or hydrophilic). The results obtained here using the release models supported the data illustrated in Figure 4-

B, where the fusion temperatures of the compounds changed after encapsulation, indicative of interaction with the encapsulated hydrophobic component.

3.5 Biological effect on whitefly (*Bemisia tabaci*)

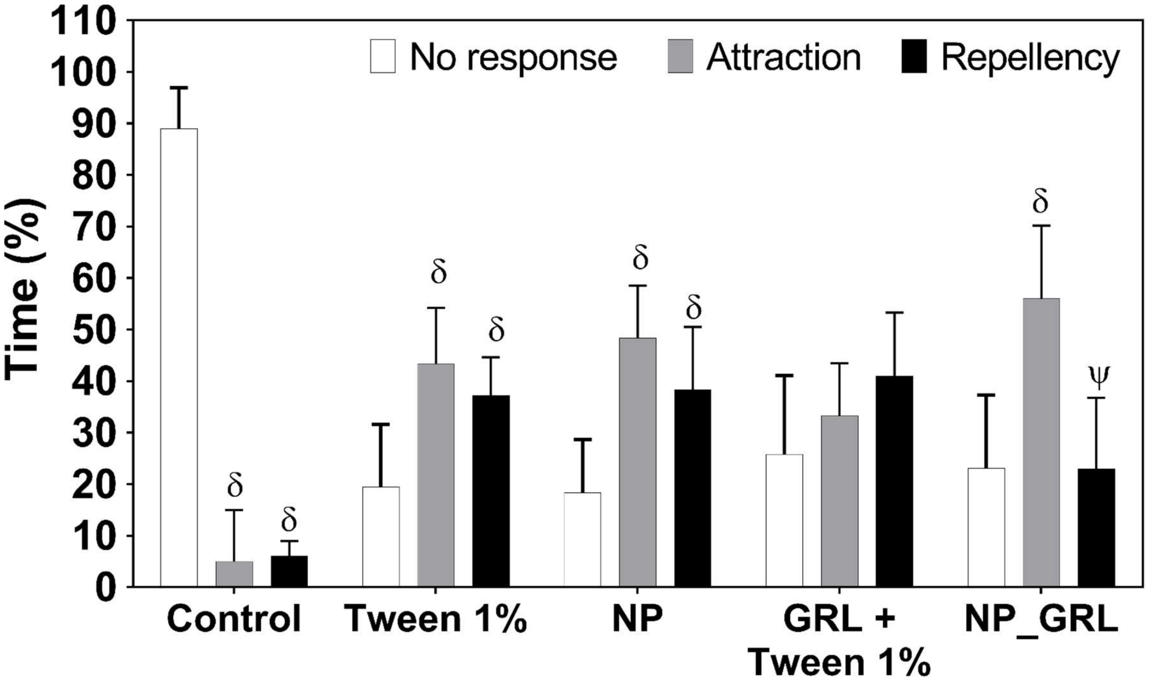
Significant differences were observed in the times that the insects remained without exhibiting a response in the attraction and repellency field, depending on the presence or absence of the different test substances (Figure 7). In the absence of the test substances, there was no observable effect on the insects. Treatment using the formulation of nanoparticles containing geraniol resulted in significant differences in the times spent by the insects in the different odor fields, reflecting an important attraction effect caused in the whitefly. It is likely that the encapsulation and consequent modified release of geraniol probably resulted in a more homogeneous concentration of the compound within the olfactometer during the period of the experiment, which enhanced the attraction effect. Homogeneity of the geraniol concentration was also assisted by the nature of the equipment used (olfactometer), in which flowmeters maintained constant flows of air through each odor field.

Sadeh et al., (2017b) recently reported that the attraction of whitefly to the essential oil of rosemary (*Rosmarinus officinalis* L.) was associated with the composition and concentration of the volatile compounds present in the plant. Hussein et al. (2017) observed an attraction effect of the essential oil of lemon eucalyptus (*Corymbia citriodora*) in whitefly. In these studies, it was found that the concentrations of the compounds played fundamental roles, while the attraction effects were also influenced by environmental and geographical factors, as well as stress induced by abiotic factors. Kim et al., (2009, 2017) reported the attraction effect of geraniol and its metabolites in the ambrosia beetle (*Platypus koryoensis*), with the concentrations of the compounds being determining factors that affected the behavior of the insects.

The responses of insects to odors are directly related to sensory inputs from multiple chemical receptors, which send different signals to the brain. A particular chemical compound may induce a behavioral response by activating or inhibiting certain receptors, while causing no effects in others (BECHER et al., 2018). Chemical ecological studies have indicated that higher concentrations are often required for insects to exhibit repellency behavior, since the receptors concerned have higher response thresholds (PRICE et al., 2011), while at lower concentrations, compounds may exert attraction activity. These effects have sometimes been assumed to provide a general framework for chemical insect communication (DAVIS, 1985). However, in many cases, such a general principle may not be applicable. For example, Hao et al. (2013) found that although the concentration of a given compound affected the behavioral response of *Aedes albopictus*, only the compound citronellal caused behavior in line with the general principle described above, while the compounds geraniol and linalool induced different behavioral responses at the different concentrations tested. The authors also pointed out that the evaporation rate is an important factor influencing the behavioral response. Further studies using biochemical markers and molecular tools are needed in order to obtain a better understanding of the mechanisms underlying these complex processes.

The chitosan/gum arabic nanoparticles modulated the release of GRL, causing an attraction effect in the whitefly, indicating the potential of this system for use in pest management, especially using trap devices (SAHA; CHANDRAN, 2017). Traps are widely employed in pest management, so the use of natural compounds as attractants represents a more environmentally friendly control method (THOMAS, 2016). Traps are valuable tools for the control and monitoring of insect populations, as well as for determining the need for control and the timing of intervention practices (CORRÊA-FERREIRA, 2012).

Figure 7. Response of the whitefly (*Bemisia tabaci*) in the 4-way olfactometer assay. The formulations tested were chitosan/gum arabic nanoparticles (CS/GM), Tween 80 (1%), geraniol emulsified with surfactant (GRL + Tween 1%), and chitosan/gum arabic nanoparticles loaded with geraniol (CS/GM_GRL). The results showed that the chitosan/gum arabic nanoparticles containing geraniol caused a significant attraction effect in the whitefly. The data were submitted to statistical analysis (two-way ANOVA), considering a significance level of $p < 0.05$. For the same group, δ* indicates a significant difference relative to a zero response, and ψ* indicates a significant difference in terms of the degree of attraction.



In summary, the chitosan/gum arabic nanoparticles containing the active agent geraniol developed in this study showed good colloidal properties and were also able to protect the active compound against degradation by UV radiation. The biological results with whitefly showed that the nanoparticle formulation containing geraniol presented significant attraction activity and could be used in trap systems. The results open perspectives for the use of botanical compounds in pest control and monitoring, indicating that nanoencapsulation can enhance the efficacy of botanical compounds, improving their stability and decreasing degradation rates.

4. Abbreviations used

GRL, geraniol; HPLC, high performance liquid chromatography; AFM, atomic force microscopy; FTIR, Fourier transform infrared spectroscopy; DSC, differential scattering calorimetry; DLS, dynamic light scattering; NTA, nanoparticle tracking analysis; ATR, attenuated total reflectance; EE, encapsulation efficiency; MD, mean nanoparticle diameter; PI, polydispersity index; ZP, zeta potential; CS/GM, chitosan/gum arabic nanoparticles; CS/GM_GRL, geraniol-loaded chitosan/gum arabic nanoparticles; Tween 80 1%, Tween 80 surfactant solution; GRL + Tween 80 1%, geraniol emulsified with the surfactant; LCST, lower critical solution temperature.

5. Acknowledgments

The authors are grateful for the financial support provided by the São Paulo State Science Foundation (FAPESP, grants #2014/20286-9 and #2015/15617-9) and CNPq. We also thank Lais Cristina Marquardt for sample preparation and collection of particle size data.

6. Supporting Information

Validation of methodology for quantification of geraniol by HPLC

Quantification of the botanical repellents was performed by high performance liquid chromatography, using an UltiMate 3000 system (Thermo Scientific). Chromeleon 7.2 software was used for acquisition and interpretation of the chromatograms. Analysis of geraniol employed a reverse phase Phenomenex Gemini C18 column (150 mm × 4.6 mm; 5.0 µm) maintained at 30 °C. The mobile phase was acetonitrile:water (60:40, v/v), at a flow rate of 1 mL/min. The injection volume was 100 µL and the detector wavelength was 210 nm. Concentrations of 5-10 µg / mL were used for analytical curve construction. The analytical curve showed a correlation coefficient (r^2) >0.99. The limits of detection (LD) and quantification (LQ) were 0.39 ± 0.01 and 1.30 ± 0.09 µg/mL, respectively.

Table S1. Estimated effects and p-values for the dependent variables studied (mean diameter, polydispersity index, zeta potential, and encapsulation efficiency).

Factors	**Dependent variables**							
	MD		PDI		ZP		EE	
	Estimated effect	p-value	Estimated effect	p-value	Estimated Effect	p-value	Estimated effect	p-value
Chitosan	-247.79	0.0758	-0.32	**0.0161***	25.45	**0.0001***	-2.81	0.0634
Gum arabic	49.05	0.7032	0.03	0.7438	-23.43	**0.0001***	-0.55	0.6881
Tween 80	211.82	0.1213	0.08	0.4898	2.85	0.4828	-1.12	0.4227

Table S2. Values of the photodegradation constants (*k*) and correlation coefficients (r^2) for the kinetic models applied to the photodegradation curves shown in Figure 4.

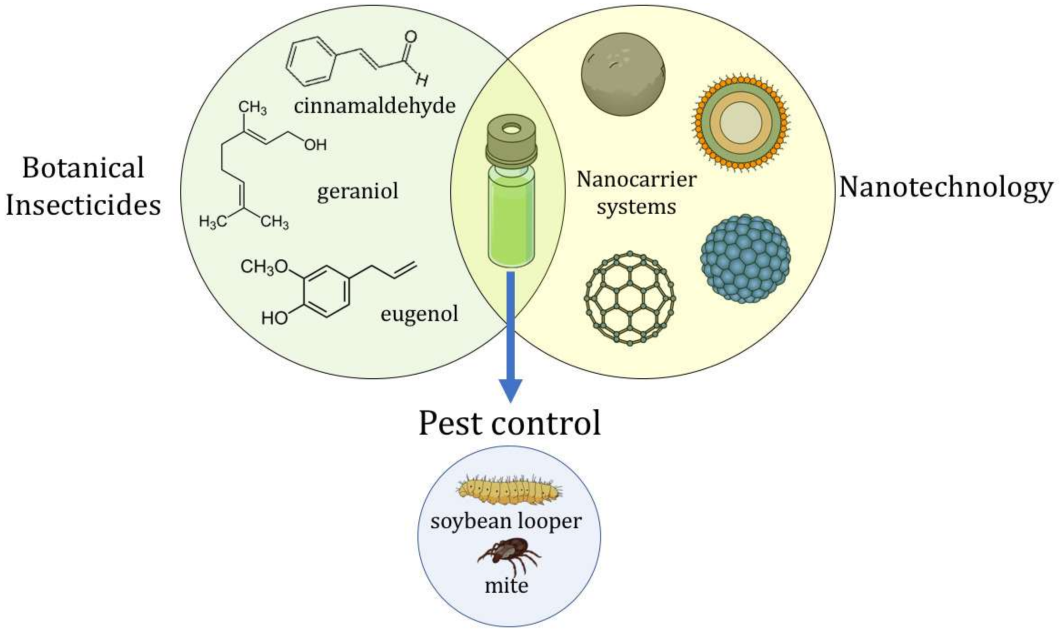
	Photodegradation kinetic models							
Formulation	Zero order		First order		Pseudo-first order		Pseudo-second order	
Tween 1%								
GRL	0.0285	0.7646	0.0230	0.9681	0.0194	**0.9791**	0.0364	0.6578
NP_CS/GM								
GRL	0.0169	**0.9722**	0.0159	0.8495	0.0053	0.9648	0.0278	0.4658

Table S3. Correlation coefficients (R^2) for the different mathematical models applied to the release of geraniol from the chitosan/gum arabic nanoparticles, at different temperatures.

	Mathematical models								
	Zero order		**First order**		**Higuchi**		**Korsmeyer-Peppas**		
Temperature (°C)	*k* (h^{-1})	r^2	*k* (h^{-1})	r^2	*k* ($h^{-1/2}$)	r^2	*k* (h^{-1})	*n*	r^2
20	18.47	0.9273	0.2376	0.7790	42.16	**0.9840**	3.486	0.70	0.9780
25	19.78	0.9719	0.2491	0.8702	43.20	**0.9964**	3.559	0.61	0.9944
30	21.34	0.9545	0.2738	0.8364	45.82	**0.9940**	3.621	0.68	0.9908

CHAPTER IV

Convergence of botanical compounds with nanotechnology for effective pest control systems



OLIVEIRA, J.L; CAMPOS, E. V.R.; COSTA, T. G.; LIMA, R.; DELLA-VECHIA, J. F.; SOARES, S. T.; ANDRADE, D. J.; GONÇALVES, K. C.; NASCIMENTO, J.; POLANCZYK, R. A.; FRACETO, L. F. Convergence of botanical compounds with nanotechnology for effective pest control systems.

Submetido para **Pest Management Science**

Abstract

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*). 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, 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. 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, transgenic) in agriculture since World War II. As a result, food production has increased significantly (SHIVA, 2016b). Despite the great advancements, such practices have also brought several health and environmental implications (soil and water contamination, toxicity to non-target organisms) (PELLEGRINI; FERNÁNDEZ, 2018). 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 (SHELEF et al., 2018).

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 (ISMAN MURRAY B, 2015). 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 (MIRESMAILLI; ISMAN, 2014).

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 (ZHU et al., 2015; REIS et al., 2016; LUCIA et al., 2017; OLIVEIRA et al., 2018). 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. In addition to bactericidal and antifungal properties (ABBASZADEH et al., 2014), it is also known for pest control properties (XU et al., 2015; SAAD; ABOU-TALEB; ABDELGALEIL, 2018). 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 (LIU et al., 2014; JEON et al., 2017; ZAIO et al., 2018).

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 (CAMPOS et al., 2018b). 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 increase effectiveness (because of a combined efficacy underpinned by different modes of action) and retarding the development of resistance by pests (ISMAN, 2017). 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 (PANT; DUBEY; PATANJALI, 2016).

In this context, innovative formulations based on nanoencapsulation have been shown to improve stability and efficacy of natural compounds (DE OLIVEIRA et al., 2014a). Protection against premature degradation coupled with sustained release and increased solubility of active compounds have been reported in numerous studies for nanoencapsuled botanical pesticides (BILENLER TUGCA et al., 2015; DA ROSA et al., 2015a; DAI et al., 2018; OLIVEIRA et al., 2018). Such nanostructured systems can be produced from different matrices (natural and synthetic). A particular example is zein, which belongs to 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 (PALIWAL; PALAKURTHI, 2014a).

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. Ethanol was purchased from Labsynth.

Acetonitrile (Grade HPLC) was obtained from J.T. Baker. 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; MCCLEMENTS, 2014)), 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) 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 analyzes 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 analyzes 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 (OLIVEIRA et al., 2018). 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) 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.

2.6. Cytotoxicity assays

Cytotoxicity assays were conducted according to the cell viability method, measured in terms of reduction of tetrazolium dye (MTT test) (GRILLO et al., 2014). 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 germholic and eugenol emulsified with Pluronic (EM_GRL+EGL) and Pluronic solution (NP_GRL+CND), geraniol and cynnamite emulsified with Pluronic (EM_GRL+CND), geraniol and eugenol 1%). 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 cotton 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.

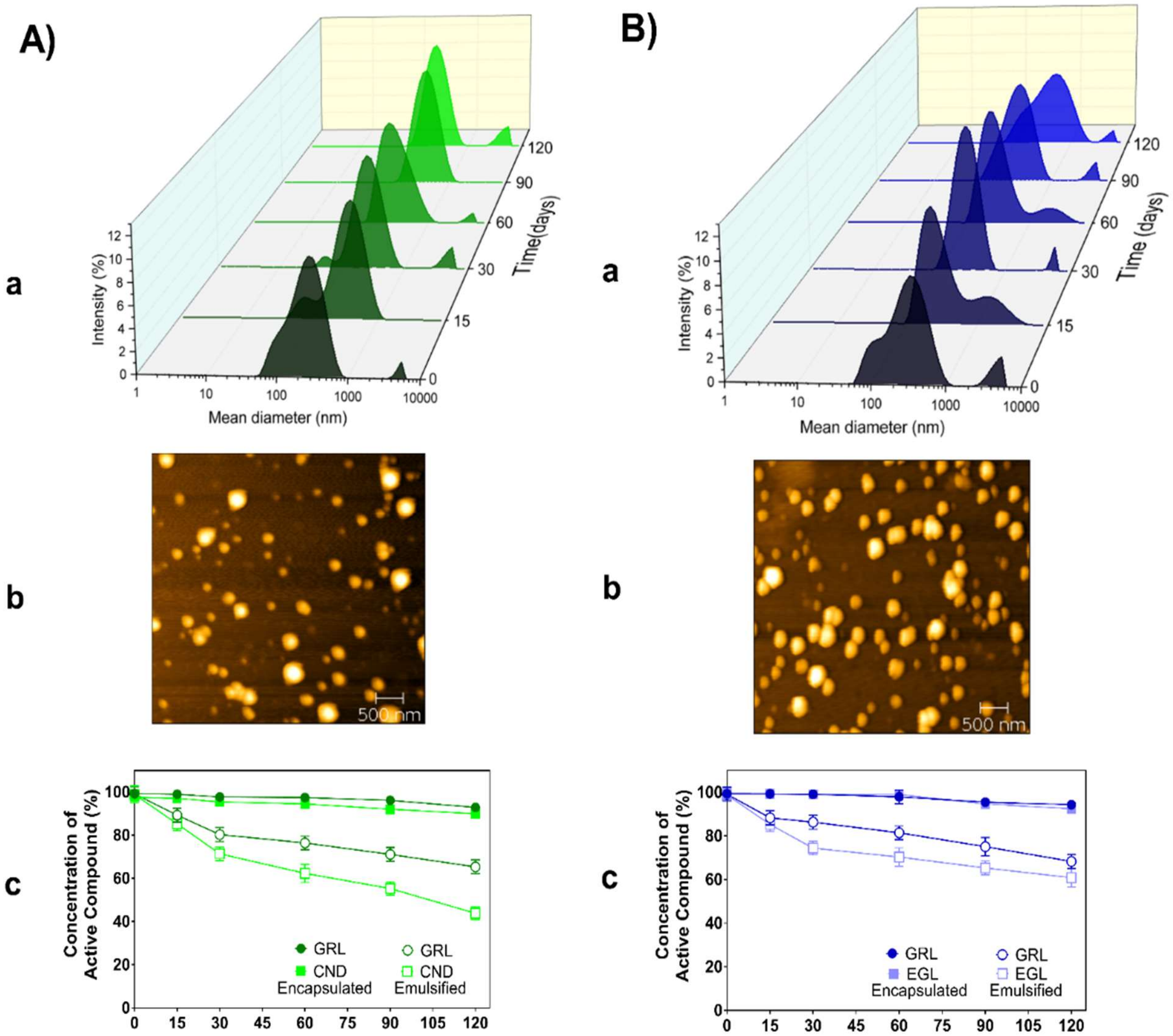
Formulations	MD (nm)		PDI	ZP (mV)	CT ($x10^{12}$ particles/mL)	EE (%)
	DLS	NTA				
NP	302 ± 8	232 ± 9	0.52 ± 0.09	-15 ± 1	0.8 ± 0.1	-
NP_GRL+CND	234 ± 5	156 ± 6	0.38 ± 0.02	43 ± 2	3.3 ± 0.8	GRL 99 ± 1 CND 97 ± 2
NP_GRL+EGL	282 ± 3	160 ± 8	0.34 ± 0.05	41 ± 2	3.2 ± 0.7	GRL 99 ± 1 EGL 98 ± 1

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 indicate a low stability of these formulations, which prevented the continuation of the analyzes after 15 days of storage due to precipitation and phase separation, and were therefore not included in the extended stability analyzes (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.

Both 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 remaining stable until the final analysis. Furthermore, the prepared nanoparticle formulations showed few changes in size distribution as a function of time (Figure 1 A and B). The AFM micrographs (Figure 1 - Ab and Bb), show spherical morphology and smooth surface of the nanoparticles. They also show high polydispersity of the formulations, with particle size distribution between 90 and 550 nm. This corroborates with the polydispersity index (Figure S1-C) that shows values higher than 0.3. This indicate good physicochemical stability of the prepared nanoparticle formulations, which is extreme importance for commercial applications that require storage over long periods. These results also corroborate a previous study by our research group (OLIVEIRA et al., 2018) that prepared zein nanoparticles for encapsulation of geraniol and R-citronellal separately. The results of that study had also shown that the prepared nanoparticle formulations were stable over time, with mean size of 200 nm, polydispersion index of 0.3, and zeta potential of -20 mV.

The encapsulation efficiency was also investigated in the current study (Figure 1- Ac and Bc). It was observed that, as shown in the previous work of Oliveira et al., (2018), the encapsulation index of the botanical compounds was high (>98%) in zein nanoparticles. This is likely to be due to the strong interaction between the studied compounds and the hydrophobic part of the zein. For the encapsulated compounds, it was observed that there was any significant decrease in encapsulation efficiency only at the extended storage times (90 and 120 days). This is most likely because of the loss of compounds due to volatilization, and/or degradation of the particles and release of the compounds. This is still a major improvement in stability as there was a much greater degradation when preparations were made only by emulsifying the substances with a surfactant. For encapsulated GRL and CND, 92± 2 and 90 ± 2 % of the active substance were available after 120 days, while emulsions has 65 ± 3 and 44 ± 2 % of the active substances, respectively. Similar results were obtained for encapsulated formulations containing GRL and EGL, that had 94 ± 1 and 92 ± 2 % of the substances, while emulsified forms had 68 ± 3 and 61 ± 4 % of the compounds respectively. It appears that when the compounds are not encapsulated, they are more prone to loss due to volatilization and degradation than when they are encapsulated in nanoparticles. The results of this study therefore provide further evidence that encapsulation can protect actives ingredients against rapid volatilization and degradation (CHEN; ZHANG; ZHONG, 2015; CHUACHAROEN; SABLIOV, 2016b; OLIVEIRA et al., 2018). Scremin et al., (2018) 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: Characterization of zein nanoparticles containing geraniol (GRL), cinnamaldehyde (CND) and eugenol (EGL). A) nanoparticles containing the GRL and CND mixture (NP_GRL + CND); B) nanoparticles containing the GRL and EGL (NP_GRL + EGL) mixture. Size distribution measured by DLS as a function of time (I); Micrographs for the nanoparticles containing the AFM (II) technique and the degradation of the active as a function of time encapsulated and emulsified (III).

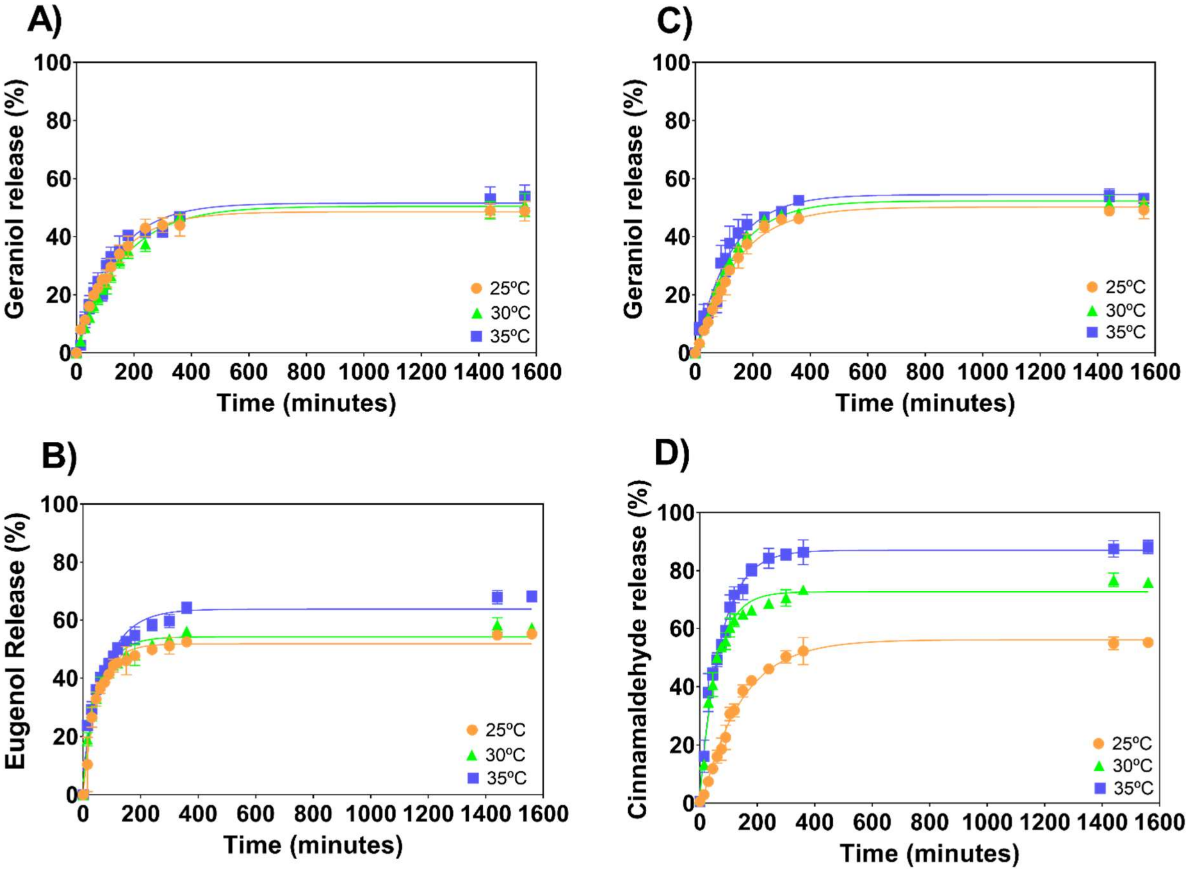


The formulations containing mixture of active compounds were more stable as a function of time (Table 1, Figure 1 and Figure S1). In addition, the formulations were able to protect the active substance against degradation in solution. Thus, the nanoparticle formulations containing mixtures of geraniol 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 substances 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 (YAWS, 2015). 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) 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 lipophilic than trans-cinnamaldehyde probably makes it more difficult for eugenol to diffuse from inside the nanoparticles to the external medium.

Figure 2: Cumulative release curves for active compounds (%) obtained at different temperatures: (orange ● 25 ° C), (green ▲30 ° C) e (blue ■ 30 ° C). For the nanoparticles containing geraniol and eugenol in A) geraniol and in B) eugenol; for nanoparticles containing geraniol and cinnamaldehyde in C) geraniol and in D) cinnamaldehyde. Analyzes were performed in triplicate and quantification was done by HPLC.



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 (PEPPAS; NARASIMHAN, 2014). 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	2.82×10^{-4}	0.3414	1.166	0.7123	1.372	0.3897	**0.8441**
EGL	0.0011	0.2751	1.61×10^{-4}	0.1872	1.031	0.5185	1.695	0.2612	**0.6799**
NP_GRL+CND									
GRL	0.0084	0.4439	3.71×10^{-4}	0.2783	1.285	0.6888	0.4261	0.5493	**0.7899**
CND	0.0096	0.4482	3.87×10^{-4}	0.2672	1.446	0.6904	2.1551	0.5805	**0.7762**
	30 ºC								
NP_GRL+EGL									
GRL	0.0105	0.5072	3.58×10^{-4}	0.3228	1.287	0.7493	0.6727	0.4045	**0.8288**
EGL	0.0025	0.3081	1.46×10^{-4}	0.2957	1.059	0.5613	1.709	0.2107	**0.7937**
NP_GRL+CND									
GRL	0.0082	0.4329	3.46×10^{-4}	0.2688	1.321	0.6812	0.6825	0.5184	**0.7781**
CND	0.3631	0.8971	5.95×10^{-4}	0.7196	6.206	0.9117	2.8282	0.7321	**0.9633**
	35 ºC								
NP_GRL+EGL									
GRL	0.0103	0.4952	3.45×10^{-4}	0.2502	1.308	0.7375	0.7175	0.4138	**0.8187**
EGL	0.0842	0.6865	1.19×10^{-4}	0.7252	3.311	0.9192	1.3512	0.3183	**0.9791**
NP_GRL+CND									
GRL	0.0069	0.3979	2.99×10^{-4}	0.2967	1.322	0.6433	1.262	0.5273	**0.7825**
CND	0.3821	0.8971	6.33×10^{-4}	0.7441	6.474	0.9152	3.132	0.6849	**0.9654**

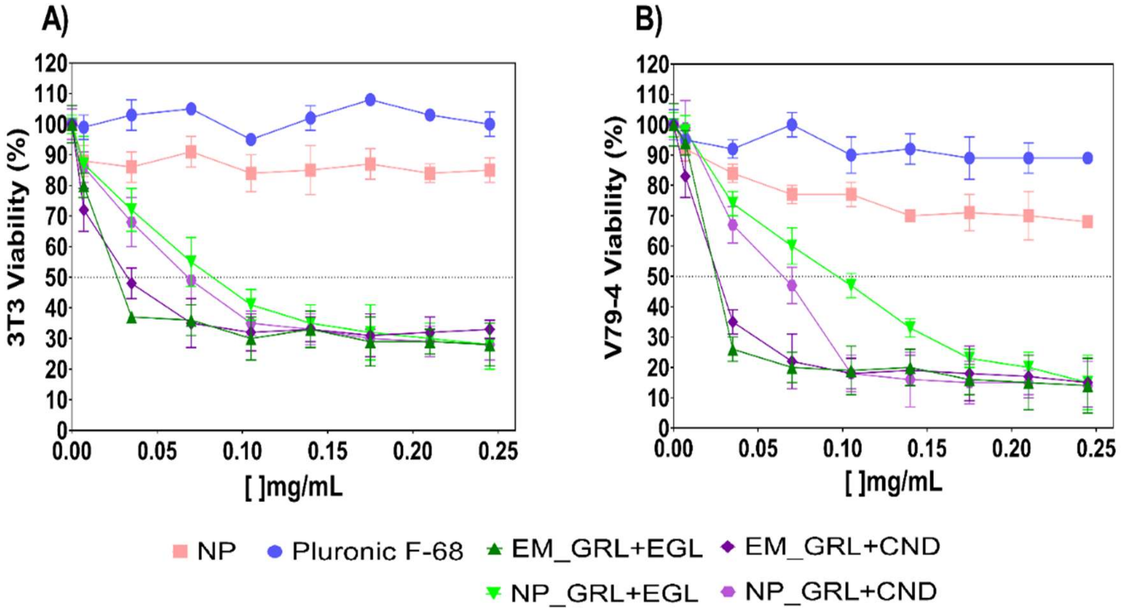
According to the data presented in Table 2, it is possible to observe that the mathematical model that best fits for all active compounds was the Korsmeyer-Peppas model. Through use of this model, it is possible to determine if the release of the active substances followed Fick's law of diffusion, or 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). 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 (DE OLIVEIRA et al., 2018). Campos et al., (2018c) 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 Al-tamimi et al., (2016) essential oils and their active components can have cytotoxic effects amongst other biological activities. Indeed, cytotoxicity of has been reported in literature for geraniol (CARNESECCHI et al., 2001a; QUEIROZ et al., 2017), eugenol (FUJISAWA et al., 2002; HO; HUANG; CHANG, 2006) 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 (OLIVEIRA et al., 2018). Campos and co-workers (2018b) 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) 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: Cytotoxicity of zein nanoparticles (with and without botanicals) and botanicals emulsified with surfactant using 3T3 (A) and V79 (B) cells. Being: ■ nanoparticles of zein (NP); ● Surfactant Pluronic F-68; ▲ 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).



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), 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.

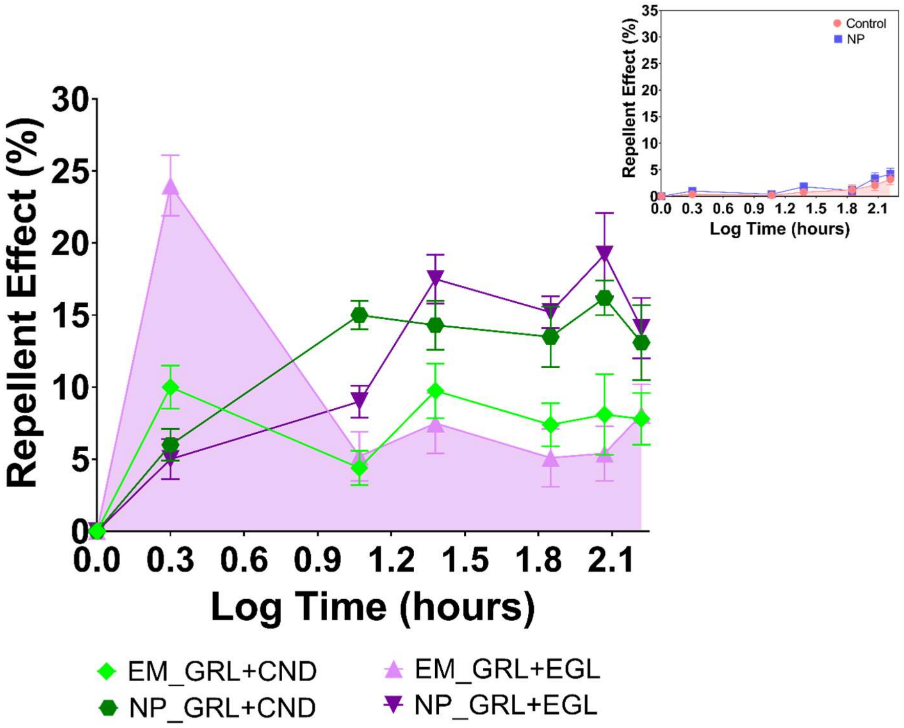
In a previous study, Tak; Isman., (2017) evaluated acaricidal and repellent activity of different terpenes derived from plant essential oils, in addition to the effect of binary mixtures against *T. urticae.* The authors tested twice the concentration used in the present study (10 mg mL^{-1}) and obtained 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 (DELETRE et al., 2015; REIS et al., 2016; PASCUAL-VILLALOBOS et al., 2017). In a previous study, our research group (Oliveira et al., 2018) 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 (EL-ZEMITY; REZK; ZAITOON, 2009; DA CAMARA et al., 2015; REDDY; DOLMA, 2018). 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: Repellent activity of nanoparticle formulations containing the mixture of botanical compounds and emulsified botanical compounds in surfactant against *Tetranychus urticae.* Inset - ● Control repellent activity (water) and nanoparticle formulation in the absence of botanical compounds. Control (water); ■ zein nanoparticles (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).



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 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, it is also noteworthy that, 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. (2018c) 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. Paulraj et al., (2017) 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) 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.

Table 4: Biological effects on mortality and mass of larvae and pupae of *Chrysodeixis includes* fed with artificial diets treated with emulsified and nanoencapsulated botanicals. Laboratory evaluation at 25 ± 2 °C, 70 ± 10% relative humidity and 12-hour photoperiod. 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.

Formulations	Mortality (%)	Larvae mass (mg)	Pupae mass (mg)	Oviposition
Control	1.5 ± 0.9	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. It is also worth highlighting that 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.

5. Acknowledgments

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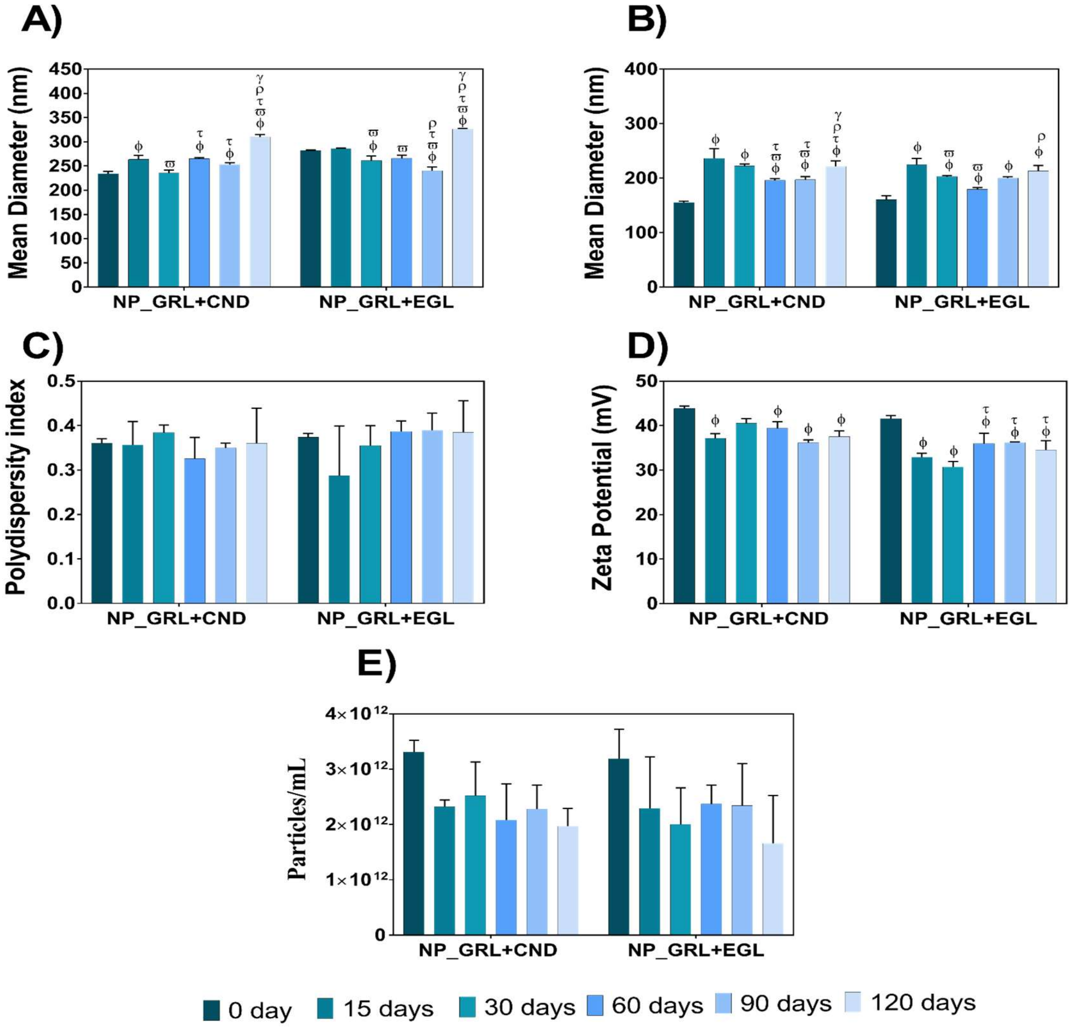
6. Author contribution

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 final version.

SUPPORTING INFORMATION

Figure S1: Physical-chemical parameters and stability evaluation of zein nanoparticles containing botanical compounds. The stability evaluation was performed for 120 days. (A) mean diameter (nm) by DLS, (B) mean diamter (nm) by NTA, (C) polydispersity index, (D) zeta potential (mV), (E) nanoparticles concentration (particles/mL). All analyses were performed in triplicate, at 25 ºC. Statistically significant differences (two-way ANOVA) for the different storage times are indicated by δ*, γ*, ϕ*, ψ*, and ξ*, corresponding to the initial time (0 days), 15 days, 30 days, 60 days, and 90 days, respectively. Significance level: $p < 0.05$.



CONCLUSÕES GERAIS E PERSPECTIVAS

A associação de compostos naturais com nanotecnologia tem ganhado destaque como formulações alternativas visando aplicações em agricultura sustentável. A crescente preocupação com os impactos ambientais causado pelo uso excessivo dos pesticidas sintéticos aliado ao surgimento de pragas resistentes tem contribuído para o avanço das pesquisas na área. Como demonstrado no primeiro capítulo dessa tese, o número de trabalhos científicos na área tem aumentado, no entanto, apesar de alguns produtos já estarem disponíveis no mercado, a quantidade ainda é limitada em relação as formulações convencionais. Isso deve-se principalmente a alguns fatores que limitam a expansão na utilização deste tipo de formulação. A falta de marcos regulatórios e também a falta de padronização na composição dos óleos essenciais bem como de formulações naturais, tem sido um dos principais entraves para o setor. Outro importante fator a se destacar, são os estudos de toxicidade e também destino desses materiais no ambiente. Inúmeros trabalhos descrito na literatura apresentam apenas etapas de preparo e caracterização e/ou algum efeito biológico, no entanto não se preocupam em demonstrar efeitos tóxicos por exemplo a organismos não-alvo.

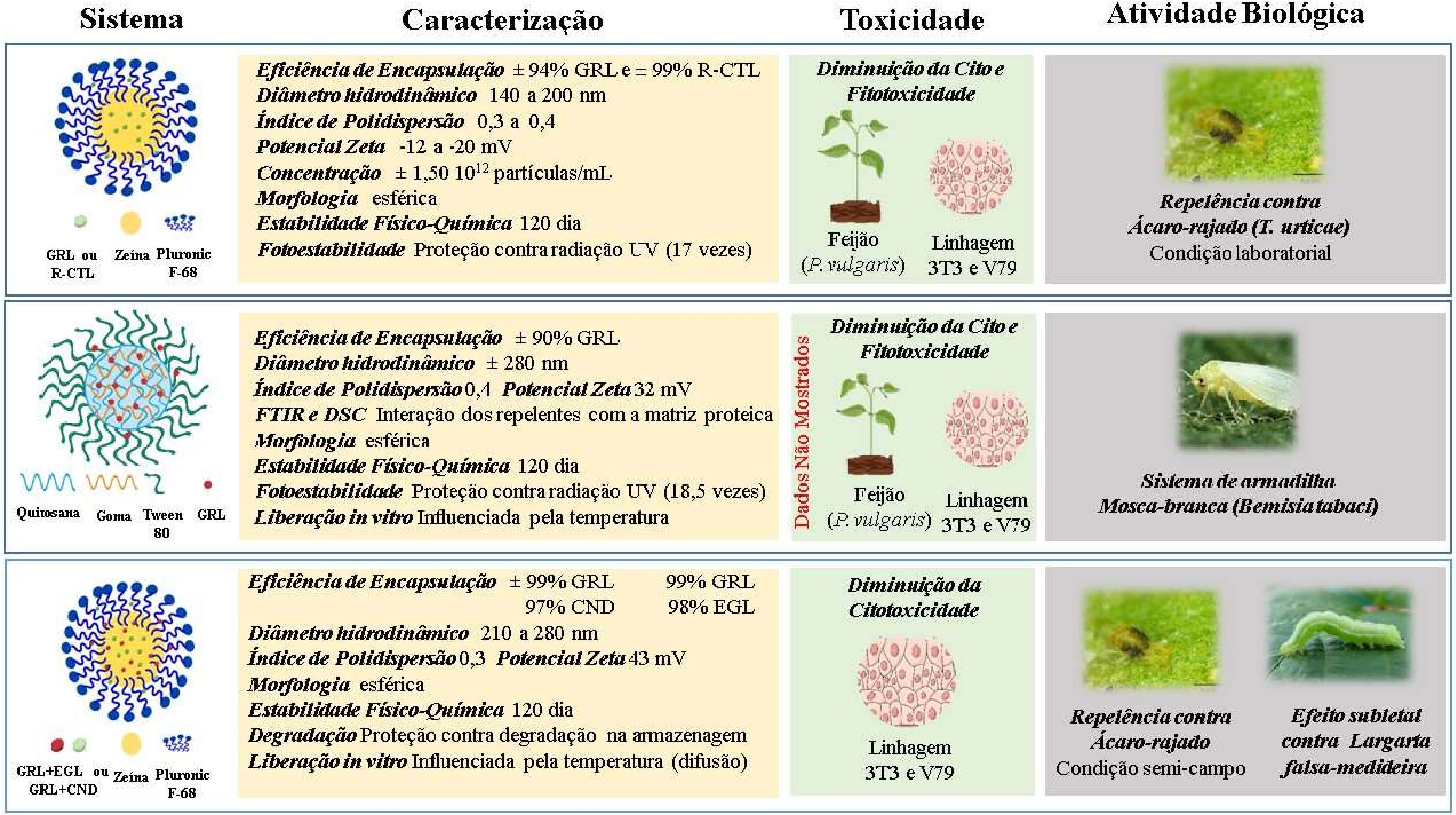
Neste contexto, a presente tese apresentou o desenvolvimento de diferentes sistemas carreadores para compostos botânicos visando o controle de pragas agrícolas e aplicações na agricultura sustentável. Destaca-se ainda que foram conduzidos ensaios para avaliação da toxicidade desses sistemas para organismos não-alvo (animal e vegetal). No segundo capítulo são apresentando os resultados para as nanopartículas de zeína contendo os compostos botânicos geraniol e R-citronelal. Ambos compostos apresentaram eficiências de encapsulação superiores a 90%, sendo que a nanoencapsulação proporcionou efetiva proteção contra perdas por fotodegradação e volatilização. As formulações apresentaram-se esféricas, com diâmetro médio de 150-250 nm, sendo que o índice de polidispersão foi superior a 0,3 e mantiveram-se estáveis por cerca de 90 dias. Os testes de fitotoxicidade em espécies de feijão (*Phaseolus vulgaris*) e tomate (*Solanum lycopersicum*), bem como os de citotoxicidade em células 3T3 e V79 mostraram que a nanoencapsulação diminuiu o efeito tóxico dos compostos botânicos. Ademais, os testes de atividade repelente contra o ácaro *Tetranychus urticae* Koch mostraram que as formulações de nanopartículas apresentaram atividade repelente superior ao composto emulsionado mesmo em baixas concentrações.

Já o terceiro capítulo apresenta os resultados para as nanopartículas de quitosana/goma arábica contendo o composto geraniol. Após os testes de otimização, foram obtidas partículas com diâmetro médio de 250 nm, índice de polidispersão de 0,2 e eficiência de encapsulação de 90%. Ademais, as nanopartículas apresentaram estabilidade físico-química por um período de 120 dias. A encapsulação aumentou em cerca de 18 vezes a durabilidade do geraniol frente a degradação por radiação UV em comparação com o composto apenas emulsionado. Os ensaios de liberação *in vitro* mostraram que o aumento de temperatura também aumentou a liberação do geraniol, sendo o mecanismo de liberação predominantemente por difusão. Os resultados biológicos com mosca-branca realizados em olfatômetro mostraram que as nanopartículas contend o geraniol apresentaram significante atividade atrativa para o inseto. Isto devido principalmente a liberação sustentada do composto, permanecendo em faixa de concentração que ocasionou atração. Sendo assim, o sistema desenvolvido aprensentou potencialidade para ser utilizado no controle e monitoramento desses insetos, especialmente em sistemas de armadilhas.

Por fim, no quarto capítulo foram preparadas nanopartículas de zeína contendo mistura de compostos ativos botânicos (geraniol, eugenol e cinamaldeído). Os sistemas apresentaram morfologia esféricas, com diâmetro médio de 270 nm e índice de polidispersão de 0.3. Os compostos botânicos apresentaram eficiência de encapsulação superior a 80% sendo que as formulações mantiveram-se estáveis por um período de 120 dias. A encapsulação dos compostos permitiu uma proteção contra a degradação durante o processo de armazenagem. A liberação *in vitro* , mostrou um perfil de liberação sustentada dependente da temperatura e através de difusão. A encapsulação dos compostos nas nanopartículas de zeína também permitiu uma diminuição nos valores de IC_{50} nos ensaios de viabilidade cellular, indicando um efeito protetor das nanopartículas. Ademais, nos ensaios biológicos contra duas imporantes pragas agrícolas ácaro-rajado e lagarta falsa-medideira, os sistemas nanoestruturados contendo os compostos botânicos apresentaram efetividade superior em relação aos compostos apenas emulsionados. Em especial, os ensaios de atividade repelente contra ácaro-rajado as formulações foram testadas em condições de semi-campo, e em média aumentaram em cerca de 1,8 vezes a efetividade. Já para os ensaios com lagarta falsa-medideira as emulsões apresentaram elevada mortalidade, no entanto, no efeito subletal as nanopartículas apresentaram maior efeito, isto principalmente devido a liberação sustentada dos compostos ativos.

A Figura 1 apresenta um resumo dos principais resultados obtidos ao longo desta tese, para os diferentes sistemas desenvolvidos. São apresentandos resultados para o preparo e caracterização, estabilidade físico-química, toxicidade, cinética de liberação i*n vitro* e também atividade biológica. Ademais são apresentandas uma representação esquemática da organização estrutural das nanopartículas desenvolvidas.

Figura 1: Resumo dos resultados e representação esquemáticas das nanopartículas desenvolvidas. No topo, representação das nanopartículas de zeína contendo os compostos botânicos geraniol e citronelal; No meio, representação das nanopartículas de quitosana/goma arábica contendo o composto botânico geraniol; Na base, representação das nanopartículas de zeína contendo a mistura dos compostos botânicos geraniol e eugenol e cinamaldeído.



Sistema	Caracterização	Toxicidade	Atividade Biológica
GRL ou R-CTL Zeína Pluronic F-68	***Eficiência de Encapsulação*** ± 94% GRL e ± 99% R-CTL ***Diâmetro hidrodinâmico*** 140 a 200 nm ***Índice de Polidispersão*** 0,3 a 0,4 ***Potencial Zeta*** -12 a -20 mV ***Concentração*** ± 1,50 10^{12} partículas/mL ***Morfologia*** esférica ***Estabilidade Físico-Química*** 120 dia ***Fotoestabilidade*** Proteção contra radiação UV (17 vezes)	***Diminuição da Cito e Fitotoxicidade*** Feijão (*P. vulgaris*) Linhagem 3T3 e V79	***Repelência contra Ácaro-rajado (T. urticae)*** Condição laboratorial
Quitosana Goma Tween 80 GRL	***Eficiência de Encapsulação*** ± 90% GRL ***Diâmetro hidrodinâmico*** ± 280 nm ***Índice de Polidispersão*** 0,4 ***Potencial Zeta*** 32 mV ***FTIR e DSC*** Interação dos repelentes com a matriz proteica ***Morfologia*** esférica ***Estabilidade Físico-Química*** 120 dia ***Fotoestabilidade*** Proteção contra radiação UV (18,5 vezes) ***Liberação in vitro*** Influenciada pela temperatura	Dados Não Mostrados ***Diminuição da Cito e Fitotoxicidade*** Feijão (*P. vulgaris*) Linhagem 3T3 e V79	***Sistema de armadilha Mosca-branca (Bemisia tabaci)***
GRL+EGL ou GRL+CND Zeína Pluronic F-68	***Eficiência de Encapsulação*** ± 99% GRL 97% CND 99% GRL 98% EGL ***Diâmetro hidrodinâmico*** 210 a 280 nm ***Índice de Polidispersão*** 0,3 ***Potencial Zeta*** 43 mV ***Morfologia*** esférica ***Estabilidade Físico-Química*** 120 dia ***Degradação*** Proteção contra degradação na armazenagem ***Liberação in vitro*** Influenciada pela temperatura (difusão)	***Diminuição da Citotoxicidade*** Linhagem 3T3 e V79	***Repelência contra Ácaro-rajado*** Condição semi-campo ***Efeito subletal contra Largarta falsa-medideira***

Em resumo, os resultados apresentando ao longo desta tese demonstram que ambos sistemas nanocarreadores desenvolvidos (zeína e quitosana/goma arábica) se mostram eficazes para o carreamento de pesticidas botânicos. Ademais, as formulações apresentaram baixa toxicidade para organismos não-alvo, além de apresentarem efetivo controle de importantes pragas agrícolas testadas. Destaca-se ainda a importância dos trabalhos desenvolvidos, os quais levaram em consideração não apenas o preparo e caracterização dos sistemas nanoestruturados mais também estudo de toxicidade e atividade biológica. Ademais, os resultados mostraram que a nanotecnologia juntamente com os pesticidas botânicos podem ser utilizadas amplamente em programas de manejo integrado de pragas, inclusive em sistemas de armadilhas (utilizados para o monitoramento e controle).

Por fim, os resultados apresentado são promissores para a utilização desses sistemas especialmente em agricultura sustentável. No entanto, vale destacar que o caminho para a comercialização desses sistemas ainda é longo, sendo necessário importantes estudos de aprimoramento dessas formulações. Cabe ressaltar que o próprio processo de escalonamento desses sistemas é um importante fator a ser estudado. Ainda dentro do processo de produção a estimativa de custos dessas formulações também é importante, uma vez que muitas formulações convencionais acabam apresentando um custo menor em relação a formulações nanotecnológicas. Porém destaca-se que neste sentido se optou pela utilização principalmente de matrizes com baixo custo e com maior disponibilidade (zeína e quitosana). Outro importante aspecto está na utilização de compostos ativos isolados, os quais apresentam custo elevado, devido principalmente ao processo de isolamento. Sendo assim a utilização e teste da eficácia também do óleo essenciais das plantas pode ser uma alternativa.

Sendo assim, a presente tese apresenta as potencialidade desses sistemas para a melhora da estabilidade e eficácia dos pesticidas botânicos visando controle de pragas em agricultura e potencial diminuição dos impactos ambientais em associação com o aumento da segurança alimentar.

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