Hydrogels Containing Botanical Repellents Encapsulated in Zein Nanoparticles for Crop Protection

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Abstract

Essential oils and their derivatives are eco-friendly biopesticides that can contribute to reducing the use of synthetic pesticides in agricultural pest control, offering advantages including less harm to humans and the environment. This work concerns hydrogel-based repellent systems containing botanical compounds that were emulsified or encapsulated in zein nanoparticles. The hydrogels were prepared according to a two-step process involving molding and crosslinking. They presented good rheological properties, even at elevated temperature (40 ºC), a swelling degree of about 30±1.2%, and were able to modulate the release of active compounds. The hydrogels containing botanical compounds presented high repellency (>80%) against two important agricultural pests: whitefly (*Bemisia tabaci*) and two-spotted spider mite (*Tetranychus urticae*). These repellent systems are promising for use in sustainable agriculture, since they are based on the use of natural substances for both the matrices and the active agents. It is noteworthy that the systems can be used without direct contact with plants, which minimizes any problems related to phytotoxicity.

Keywords: Hydrogel; essential oil; nanoparticles; pest control; sustainable agriculture.

1. Introduction

Overuse of synthetic pesticides in agriculture is a major source of environmental contamination affecting water, air, and soil. It has led to increased production costs, loss of productive regions, and threats to human and animal health [1]. Consequently, it is increasingly necessary to search for alternatives that have lower impacts in the environment and that can contribute to the development of sustainable agriculture [2,3].

The use of botanical pesticides has been shown to be a promising alternative to synthetic compounds. Most of these substances are produced during the secondary metabolism of plants and are important in defense against pathogens and pests. They present biological activities including the ability to control agricultural pests [4]. The literature describes several plant essential oils, as well as isolated active compounds, that present activity against insects. Geraniol, for example, is an acyclic alcohol with repellent activity, which is found in the essential oils of plants such as citronella and palmerosa [5–8]. One of the main components of clove essential oil is eugenol, a member of the phenylpropanoid chemical class, which has medicinal properties including anesthetic, bactericidal, and antifungal activities [9]. Cinnamaldehyde, another phenylpropanoid, is one of the main active compounds found in the essential oil obtained from the bark of cinnamon (*Cinnamomum* spp.). In addition to its characteristic flavor, this compound exhibits medicinal [10] and antifungal properties, and can act against agricultural pests [11,12].

The combination of compounds isolated from different plants represents an important strategy for increasing the biological activities of these essential oils. Such combinations result in unique formulations containing active agents that are not normally present together in the same plant [13]. This can assist in delaying the emergence of pest resistance, due to their different mechanisms of action [4]. However, although these compounds have great potential for use in agricultural applications, aspects such as high sensitivity to UV light, low humidity, and high temperature in the field can lead to their rapid degradation and loss of effectiveness [14].

The nanoencapsulation of compounds (mostly oily) isolated from different plants offers benefits including increased solubility, protection against premature degradation, and sustained release. Previous studies have reported the effectiveness of nanoencapsulation of these active compounds [8,15–17]. Such systems can be produced using various natural and synthetic matrices. An attractive natural matrix is zein, a protein extracted from maize, which belongs to the prolamin class of compounds. Due to its rapid

precipitation in aqueous solutions, it is widely used in the production of nanoparticles, offering the benefits of biodegradability and biocompatibility [18].

The incorporation of these systems into hydrogels constitutes an important strategy that can provide protection of the active agents and enable the development of novel systems for their application. Henson et al. (2006)[19] described a method for the preparation of hydrogels based on hydroxypropylmethylcellulose (HPMC), containing aromatic molecules as active ingredients. The hydrogels were prepared in solid form, with the desired shape and size obtained according to the final polymer concentration.

The aim of the present study was to obtain repellent formulations based on hydrogels produced from the biopolymers carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC), crosslinked in the presence of citric acid solution (a natural organic acid with multi-carboxylic structure, reason why it can be used as polymer crosslinking agent). The hydrogels were prepared with mixtures of botanical compounds (geraniol, eugenol, and cinnamaldehyde), which were either encapsulated in zein nanoparticles or emulsified in surfactant. The systems were characterized in terms of rheological stability and the release rates of the bioactive agents. The biological efficacy was evaluated against two important agricultural pests: silverleaf whitefly (*Bemisia tabaci* (Gennadius) and two-spotted spider mite (*Tetranychus urticae* Koch). The approach adopted in this study opens perspectives for the development of safer and more effective pest control systems that can contribute to the development of sustainable agriculture.

2. Materials and Methods

2.1 Materials

Geraniol (GRL – ≥ 98% purity), eugenol (EGL - ≥ 98% purity), trans-cinnamaldehyde (CND - ≥ 99% purity), Zein, Pluronic F-68, carboxymethylcellulose (CMC), and hydroxyethylcellulose (HEC) were obtained from Sigma-Aldrich (São Paulo/Brazil). Ethanol was purchased from Labsynth (São Paulo/Brazil). Acetonitrile (HPLC grade) was obtained from J. T. Baker (São Paulo/Brazil). Other analytical reagents were purchased from local suppliers (São Paulo/Brazil).

2.2 Preparation and characterization of the solutions of nanoparticles and emulsified compounds

The preparation of zein nanoparticles containing mixtures of the botanical compounds was carried out by the antisolvent precipitation method, as described by Hu and McClements (2014), with slight modifications. Zein (2% w/v) was solubilized in hydroethanolic solution (85% v/v), under agitation overnight. An aqueous solution of Pluronic F-68 surfactant (2% w/v, pH 4) was also prepared. The zein solution was purified by centrifugation (30 min at 4500 rpm), heat treatment (15 min at 75 °C), and filtration through a 0.45 μm membrane (Millipore). The particles were prepared with addition of 600 mg of each active compound to 10 mL of zein solution. Different mixtures of geraniol/eugenol and geraniol/cinnamaldehyde were used. Next, the zein solution (10 mL) was quickly added to the Pluronic F-68 solution, under magnetic stirring. The colloidal dispersion was then kept under stirring, at room temperature, until the ethanol had evaporated. The emulsions containing the active compound mixtures were prepared by adding the same amounts of the botanicals to 30 mL of the Pluronic F-68 solution, keeping it under vigorous agitation for 15 min. Losses of the active compounds during the preparation process were investigated for the nanoparticle and emulsion formulations. For the control formulations, only zein nanoparticles and surfactant were added.

The nanoparticle formulations were characterized in terms of hydrodynamic diameter, polydispersity index, zeta potential, and encapsulation efficiency. The size distribution and polydispersity index were determined using the photon correlation spectroscopy (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 temperature of 25 °C,

with the samples being diluted about 100-fold and 500-fold. The encapsulation efficiency was evaluated using the ultrafiltration/centrifugation method, as described by Oliveira et al., (2018)[8]. Quantification of the compounds was performed by high performance liquid chromatography (HPLC) described in supplementary material (S1). The total amounts of the botanical compounds (100%) present in the formulations were calculated considering the amounts added and the losses during the preparation process.

2.3 Preparation of the hydrogels

The hydrogels were prepared according to Demitri et al. (2008)[21] and Zheng et al. (2015)[22], with minor modifications. The process consisted of two steps. The first step involved the preparation of a mixture of 5% CMC and HEC, at a ratio of 5:1 (m:m), and adding nanoparticles, emulsion solutions, or water (for the control hydrogels). The different hydrogel formulations contained the following components: (i) nanoparticles with the mixture of geraniol and eugenol (NP_GRL+EGL); (ii) nanoparticles with the mixture of geraniol and cinnamaldehyde (NP_GRL+CND); (iii) nanoparticles without the botanical agents (NP_Z); (iv) emulsion with the mixture of geraniol and eugenol (EM_GRL+EGL); (v) emulsion with the mixture of geraniol and cinnamaldehyde (EM_GRL+CND); and (vi) water as the control (CTL). The solutions were mixed at 500 rpm. The CMC/HEC gel obtained was placed in a mold (2.5 x 3.5 cm), pressed to remove air bubbles, and molded into the desired shape. In the second step, the molds were immersed in citric acid solution (8 mol/L) for 6 h, for crosslinking of the hydrogel. The losses of active compounds during the crosslinking process were investigated using HPLC analysis. A schematic of hydrogel preparation is shown in Figure 1.

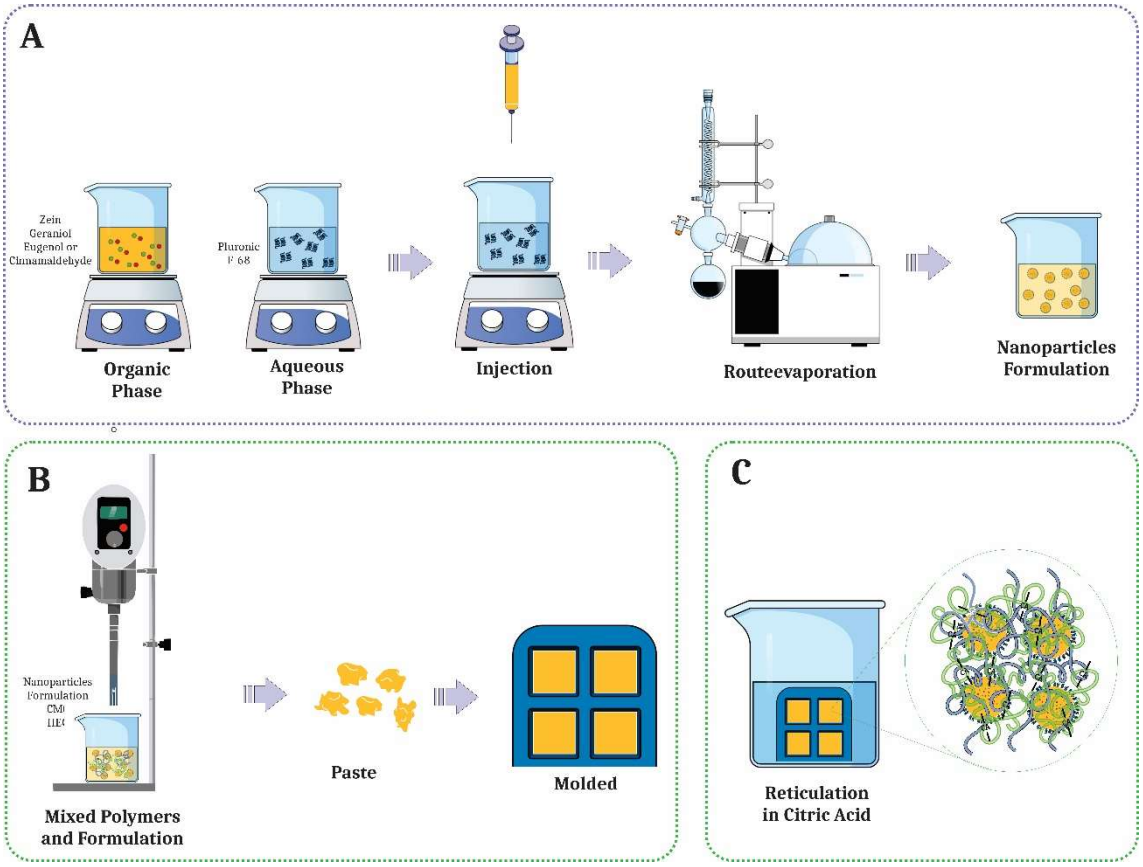


Figure 1: Hydrogel preparation scheme containing nanoparticle formulations. A) Stages of preparation of nanoparticle formulations by precipitation/solvent evaporation method. The hydrogels were prepared in two simple steps; B) the first comprised by mixing the formulations with the polymers and modeling the paste obtained; C) the second step consists in the cross-linking of the hydrogels cast in a solution of citric acid.

2.4 Characterization of the hydrogels

2.4.1 Swelling degree

Determination of the swelling degrees of the hydrogels was performed by firstly drying three replicates of each hydrogel at 30 °C, until reaching constant weight. The dried hydrogels were then immersed in deionized water (100 mL), at room temperature, for the swelling process. The hydrogels were periodically weighed and the excess water was removed using filter paper. The swelling degree (SD) was calculated as a function of time, using Equation 1:

$$SD = [(m_i - m_s) / m_s] \, x \, 100\%, \quad (1)$$

where m_i is the weight of the swollen hydrogel and m_s is the weight of the dried hydrogel.

2.4.2 Rheological properties

The stabilities of the hydrogels were evaluated by determining their rheological properties using an oscillatory rheometer (Kinexus Lab, Malvern Instruments, UK) with cone and plate geometry (diameter 20 mm, angle 0.5 rad, and 1 mm space between the plates) and Peltier temperature control. For determination of the elastic modulus (G') and the viscous modulus (G"), hydrogel samples (500 mg) were transferred to the rheometer and a frequency range of 0.1-10 Hz was applied. The shear rate was 1 Pa. The rheological properties were determined at two temperatures (25 and 40 °C), using three replicates. The rheograms were analyzed using rSpace software.

2.4.3 Fourier transform infrared spectroscopy (FTIR) and X-Ray Diffraction (XRD) Analysis

For FTIR analysis 1 mg dry sample was ground, mixed well with 100 mg KBr power, and compressed into a transparent disk. The FTIR spectra of the hydrogel and raw materials were recorded on Jasco FTIR-410 spectrometer in the range of 4000–400 cm^{-1} using an average of 128 scans with a resolution of 8 cm^{-1}. X-ray diffraction analysis of these samples was conducted on a Panalytical X´Pert Powder X-ray diffractometer equipped with Ni-filtered Cu Kα radiation (k = 1.5406) within the angle range 2θ = 5–60°. The diffractometer was functioned with 0.05° diverging, receiving slits at 40 kW and 50 mA, and a continuous scan was recorded.

2.5 *In vitro* release kinetics and release mechanisms

The release kinetics assays were performed as described by Abreu et al. (2012a)[23], with some modifications. For this, a hydrogel (averaging 4.50 g) containing the suspension of nanoparticles or emulsion was added to 100 mL of a 3% (w/v) Pluronic F68 solution and maintained under agitation at 150 rpm. Aliquots (1 mL) of the solution were collected at predetermined times, with the volume withdrawn being replaced with Pluronic F-68 solution (3%), in order to maintain a constant volume in the acceptor compartment. The amount of botanical compound released was then quantified by HPLC (the methodology is available in the Supplementary Material), with the results being expressed in %. In order to avoid any loss by evaporation, the glass chambers were covered and were only uncovered during sampling (performed in triplicate). The release data were evaluated using the zero order, first order, Higuchi, and Korsmeyer-Peppas models.

2.6 Repellent activities of the hydrogels

2.6.1 Repellent activity against whitefly (*Bemisia tabaci*)

The assays of repellent activity against whiteflywere conducted using an olfactometer (four-way arena type). The insects were reared on tomato plants (*Solanum lycopersicum*) in a breeding cages. For bioassays, insects were collected from plants using a manual sucker. It is noteworthy that for the feeding of insects were used plants grown in greenhouse, without the application of any kind of substance, ensuring that the insects did not present resistance or susceptibility. The samples evaluated were the same as described in Section 2.3, with 1 cm^3 of hydrogel being used for each bioassay. The treatments were arranged in glass connectors attached to the olfactometer pathways, with the air flow rate adjusted to 1 L/min. The air flow was obtained using a vacuum pump connected to the central outlet. In this arrangement, the air flows entered the four pathways and converged at the central point, where the *B. tabaci* were released. The distribution of the treatments was such that the likelihood of selection of a particular pathway was 50% (treatment vs. control). Therefore, two routes corresponded to the treatment and two to the control, positioned in an intercalated manner.

Each repetition corresponded to the placing of a specimen in the center, using a micropipette tip, and observing its residence time in each quadrant during a period of 10 min. The time that the insect remained in the central area of the olfactometer was considered a non-response, since this was the location where there was convergence and mixing of the four gas flows. The glass connectors and hoses were replaced every time there was a change of the treatment. Ten replicates were performed for the repellency assays. The bioassays were conducted in a room without the entrance of external light, so that the incidence of light did not affect the choice of the insect.

The results of the bioassays were expressed using the effect index (EI), in terms of the repellent effect, the attractive effect, and the "non-response", relative to the values for the control hydrogels (water only), as shown in Equation 2:

$$EI = (Et - Ec) / Ec, \quad (2)$$

where *Et* is the effect percentage (%) for the treatment and *Ec* is the effect % for the control hydrogel. A positive value indicated that the effect for the treatment was higher than that for the control, while a negative value indicated that the effect was higher for the control.

2.6.2 Repellent activity against spider mite (*Tetranychus urticae*)

The *T. urticae* mites were obtained from a rearing room (temperature 25±1 ºC, humidity 70±10%, and 12 h photoperiod), where they were kept on state cultivar of bean plants (*Phaseolus vulgaris* L.).

The first step was carried out under greenhouse conditions. Firstly, beans (*P. vulgaris*) were sown in 500 mL pots. At 30 days after germination, the plants were taken to the laboratory and placed in 6 L plastic pots, maintaining the same climatic conditions used for mite rearing. The gels were attached to a 25 cm wooden stick, using a fastener, and were placed inside the plant pots. Each formulation presented in item 2.2 (30 mL) corresponded to preparation of four hydrogel devices which were used for tests described above. The pots were assembled in pairs 17 cm apart, with one containing the gel and the other without the gel. The pots were interconnected using a 1 cm diameter plastic hose attached to orifices in the pots. A 0.5 cm² hole was made in the center of the hose, where 30 adult female *T. urticae* were transferred using a single strand brush and a stereomicroscope. The hole was then sealed with voile-like fabric. After 24 h, the leaves of the plants in each plastic container were cut and the numbers of mites and eggs were counted using a stereomicroscope. Figure S3 shows the system used for the experiments. The repellency index (RI) and the oviposition inhibition index (OI) were calculated according to Equations 3 and 4:

$$RI = \left(\frac{Nc - N}{Nc + Nt}\right) * 100 \quad (3)$$

$$OI = \left(\frac{Oc - Ot}{Oc + Ot}\right) * 100 \quad (4)$$

where *Nc* is the number of mites found on the control plant (without gel), *Nt* is the number of mites found on the plant with gel, *Ot* is the number of eggs found on the plant with gel, and *Oc* is the number of eggs found on the plant without gel. The mean repellency index and oviposition inhibition values were classified as described by Chakira et al. (2017): Class 0 (0.01-0.1%), Class I (0.1-20%), Class II (20.1-40%), Class III (40.1-60%), Class IV (60.1-80%), and Class V (80.1-100%). The numbers of mites and eggs on the treated and control plants were analyzed using t-tests for paired comparisons. The data were log-transformed prior to analysis, in order to meet parametric statistical criteria.

3. Results and Discussion

3.1 Characterization of the zein nanoparticles and emulsions containing the botanical compounds

The physico-chemical characterization of the nanoparticles (before their incorporation in the hydrogels) considered the following parameters: mean diameter (MD, nm), polydispersity index (PDI), zeta potential (ZP, mV), particle concentration (CT, particles/mL), and encapsulation efficiency (EE, %) (Table 1). The hydrodynamic diameter was smaller for the nanoemulsion droplets, compared to the nanoparticles. This was due to the nature of the preparation process, since the nanoparticles were produced by the addition of zein solution, which formed a solid matrix that encapsulated the oil[8]. The emulsion droplets also presented lower concentration and zeta potential values, compared to the nanoparticle formulations. These results indicated that the emulsions were less stable than the nanoparticles containing the mixture of active compounds.

Table 1. Characterization of the zein nanoparticles containing botanical repellents (geraniol, eugenol, and cinnamaldehyde). Being, zein nanoparticles in the absence of botanical compounds (NP); emulsion of geraniol and eugenol (EM_GRL+EGL); zein nanoparticles containing geraniol and eugenol (NP_GRL+EGL); emulsion of geraniol and cinnamaldehyde (EM_GRL+CND); zein nanoparticles containing geraniol and cinnamaldehyde (NP_GRL+CND). The parameters evaluated were the mean diameter (MD) using the dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) methods, polydispersity index (PDI), zeta potential (ZP), concentration (CT), and encapsulation efficiency (EE). The values were obtained as the mean and standard deviation for three determinations.

Formulations	MD (nm)		PDI	ZP	CT (10^{12}	EE
	DLS	NTA		(mV)	particles/mL)	(%)
EM_GRL+CND	122 ± 12	111 ± 6	0.621 ± 0.10	12 ± 2	0.12 ± 0.05	-
EM_GRL+EGL	135 ± 7	124 ± 9	0.638 ± 0.14	16 ± 3	0.15 ± 0.06	-
NP	320 ± 6	211 ± 7	0.484 ± 0.11	-11 ± 4	0.67 ± 0.21	-
NP_GRL+CND	253 ± 7	175 ± 5	0.345 ± 0.05	39 ± 3	2.91 ± 0.65	GRL 98.1 ± 0.7 CND 96.3 ± 2.1
NP_GRL+EGL	261 ± 5	158 ± 5	0.387 ± 0.09	40 ± 2	3.12 ± 0.45	GRL 99.1 ± 0.4 EGL 98.2 ± 1.1

3.2 Preparation and characterization of the hydrogels containing the emulsified and encapsulated botanical compounds

Cellulose derivatives were chosen due to their malleability, enabling hydrogels to be prepared in different shapes and sizes. The hydrogel production process involved two steps. The first step (molding process) consisted of mixing the hydrogel matrix (CMC and HEC) with the nanoparticle formulation or the emulsion, obtaining a two-phase system (particulate gel) that was transferred to rectangular molds. In this step, no crosslinking process occurred. The second step was an acidification process, where the molds were immersed in a citric acid solution (8 mol/L). During this stage, the Na^+ of the sodium carboxylate (R-COONa) linked to the CMC backbone was gradually replaced by H^+, which diffused out of the paste. The 3D network structure of the hydrogel was then constructed by physical crosslinking, together with the formation of hydrogen bonds between the polymer chains [22].

After preparation of the hydrogels, characterization tests were performed by FTIR and XRD characterization (see Supplementary Material - Figures S1 and S2, respectively). For FTIR analysis, hydrogels after citric acid cross-linking were oven dried at 50ºC for three days. The results showed that for the FTIR analyzes, the main peaks indicated are from citric acid, indicating a higher proportion of the crosslinking agent in to the hydrogels. These results were expected once the samples were dried in order to graze, and the citric acid present in the hydrogel returned to its crystalline form and due to its proportion presented higher intensity. Also, using XRD analysis, it was possible do identify that the hydrogel presented a similar pattern as CMC. Also the XRD diffractograms showed a peak at $2\theta = 14$ ° corresponding to citric acid. It is also noted that the hydrogel crystallinity is higher than observed to CMC and this may be due to the chemical cross-linking of CMC with citric acid, which causes a more organization in the hydrogels.

The control hydrogels had a swelling degree of about 27±0.4% (Figure 2). Addition of the emulsified and encapsulated compounds resulted in slight increases of the swelling degree. However, no significant differences between the formulations were observed (Two-way ANOVA test, data not shown). Tang et al (2014)[25] reported that a decrease of the CMC concentration resulted in increased swelling capacity of chitin/CMC hydrogels. According to the authors, at higher concentrations, strong hydrogen bonds and interactions occur between the hydroxyl groups of chitin and the carboxyl groups of CMC, which restrict the relaxation and expansion of the molecular chains.

The swelling degree of hydrogels is an important factor to consider in their practical applications, including the release of bioactive compounds. In this study, the hydrogels prepared with the addition of encapsulated and emulsified botanical compounds showed slight increases in the swelling degree. Zare-Akbari et al. (2016)[26] reported an increase in the swelling degree of CMC hydrogels after the incorporation of zinc oxide (ZnO) nanoparticles. According to the authors, the presence of nanoparticles with different sizes, morphologies, and surfaces resulted in greater penetration of water, in order to neutralize the osmotic pressure, which expanded the hydrogel network, hence increasing the pores and free spaces within the matrix. Zheng et al. (2015)[22] reported swelling degrees lower than 35% for hydrogels prepared using CMC at concentrations from 5 to 10%. The authors suggested that low swelling degree values contributed to increases in the mechanical properties of the hydrogels. In addition, the use of high acid concentrations resulted in high concentrations of crosslinks and a restricted polymer network dilation rate. In the present study, a high concentration of citric acid (8 mol/L) was used in the crosslinking step.

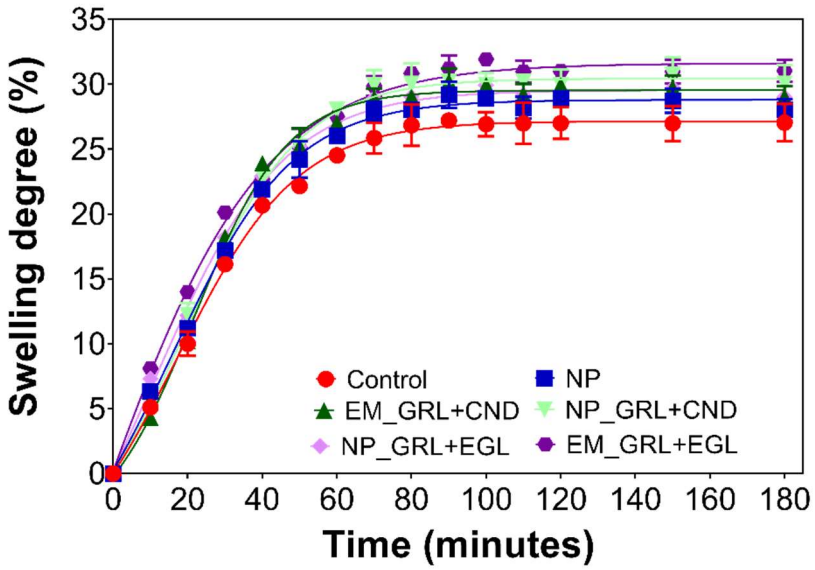


Figure 2. Swelling degrees of the hydrogels without and with encapsulated and emulsified botanical compounds: control hydrogel and hydrogel containing zein nanoparticles in the absence of botanical compounds (NP); hydrogel containing emulsified geraniol and eugenol (EM_GRL+EGL); hydrogel containing geraniol and eugenol encapsulated in zein nanoparticles (NP_GRL+EGL); hydrogel containing emulsified geraniol and cinnamaldehyde CND (EM_GRL+CND); hydrogel containing geraniol and cinnamaldehyde encapsulated in zein nanoparticles (NP_GRL+CND).

The rheological assays were performed for the different hydrogel formulations, with determination of the elastic modulus (G'), the viscous modulus (G"), and viscosity (ŋ). The analyses were performed using a frequency ramp, at two temperatures (25 and 40 °C) (Figure 3). The rheological data (G', G", G'/G" ratio, and ŋ) obtained at a frequency of 1 Hz are shown in Table 2 for all the formulations.

Table 2. Rheological parameters for the different hydrogels containing the zein nanoparticle formulations and the emulsified botanical compounds, obtained at a frequency of 1 Hz.

Hydrogel	G' (mPa) 25 ºC	G"(mPa) 25 ºC	G' (mPa) 40 ºC	G"(mPa) 40 ºC	G'/G" 25 ºC	G'/G" 40 ºC	η (mPa s) 25 ºC	η (mPa s) 40 ºC
Control	253.0	50.1	189.2	37.1	5.04	5.1	41810	31370
NP	161.9	30.5	149.7	28.1	5.30	5.3	26220	24240
EM_GRL+EGL	184.5	37.4	181.4	36.4	4.92	4.9	29970	29440
EM_GRL+CND	217.3	49.4	171.2	37.3	4.39	4.5	35460	27880
NP_GRL+EGL	170.3	32.2	170.8	32.9	5.28	5.1	27650	27690
NP_GRL+CND	204.6	40.3	160.3	30.2	5.06	5.3	33190	25960

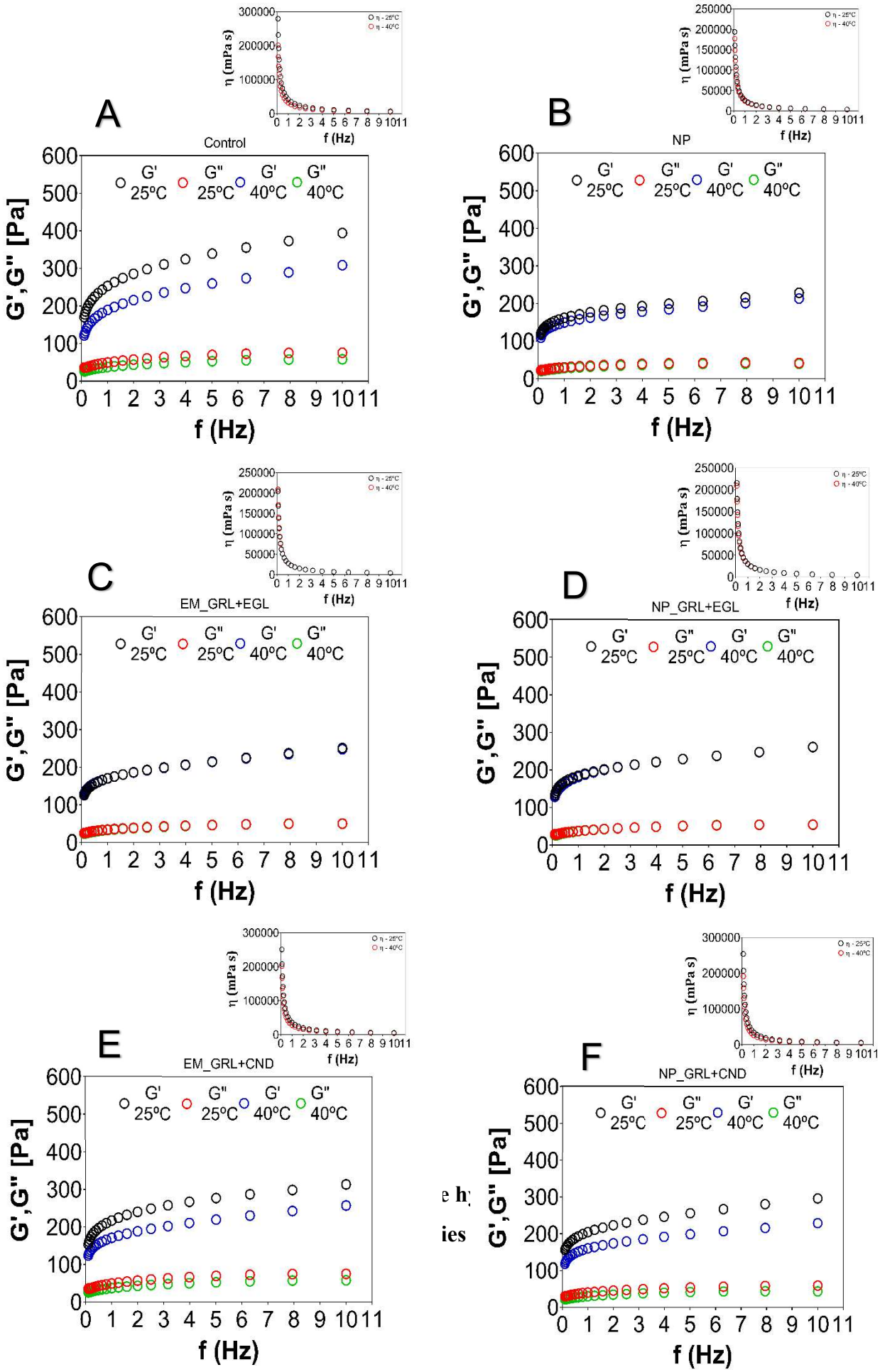
For all the hydrogels, the viscosity decreased as the frequency increased (Figure 3 inset), indicative of non-Newtonian pseudoplastic behavior. The main characteristic of a pseudoplastic material is that the apparent viscosity decreases as the shear rate increases. This is mainly due to hydrodynamic forces, which become more intense and lead to progressive rupture of the structure, with elongation of the polymeric chains in the system. This results in a new alignment with the flow of material and a reduction of viscosity [27].

As shown in Figure 3 and Table 2, the elastic modulus (G') values were higher than the viscous modulus (G') values, at both temperatures. These data indicated that the hydrogels were highly crosslinked, resulting in an organized structure, as reported by Zheng et al. (2015)[22]. The hydrogels also showed decreases of viscosity and G'/G" after addition of the formulations of botanical compounds emulsified with the surfactant, which could be explained by the presence of the surfactant and the characteristics of the emulsion. The presence of surfactant has a substantial effect on the rheological properties of solutions/hydrogels of CMC and its derivatives, as reported by Bayarri et al. (2009)[28], who found that changes in the characteristics of the dispersion medium clearly altered the

viscoelastic properties of the materials, as reflected by the G'/G" ratios. The surfactant improved the stabilization of oil-in-water emulsions, leading to the formation of layers, but hindering the cross-linking process. These features provide an explanation for the low viscosity values obtained after incorporation of the nanoparticles (Table 2).

The incorporation of the nanoparticles into the hydrogel matrix, in the presence or absence of the botanical compounds, resulted in increases of the viscoelastic parameters, especially the G'/G" ratio (Figure 3, Table 2), suggesting the formation of a stable system due to interaction between the hydrogel matrix and the nanoparticle surfaces. The incorporation of nanoparticles alone resulted in higher values of the parameters, compared to the incorporation of nanoparticles containing the botanical compounds. These findings were in agreement with the results described above, since addition of the formulations containing only the emulsified botanical compounds led to reductions of the values of the parameters evaluated, due to decreased structural organization of the hydrophilic polymeric chains in the presence of an oil phase. The increase of the G'/G" ratio indicated greater interaction between the hydrophilic polymer chains during the crosslinking process, due to the presence of zein and the surfactant used to stabilize the system.

Proteins have a strong tendency to adsorb at oil-water interfaces, resulting in better interaction between the polymers during the crosslinking process [29]. Racine et al. (2017)[30] also observed that the incorporation of solid lipid nanoparticles (SLNs) improved the rheological properties of chemically crosslinked CMC/PEG hydrogels, with increased crosslinking density of the polymer matrix, as well as modification of the release profile. In agreement with the previous studies, the present work showed that incorporation of the nanoparticles in the hydrogels led to a modest increase in swelling capacity, indicating that these hydrogels were promising candidates for the transport of encapsulated or emulsified botanical compounds.


A
Control
B
NP
C
EM_GRL+EGL
D
NP_GRL+EGL
E
EM_GRL+CND
F
NP_GRL+CND
G',G" [Pa]
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η (mPa s)
G' 25°C
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Figure 3. Rheograms for the hydrogels containing the zein nanoparticle formulations and the emulsified botanical compounds. The elastic modulus (G'), viscous modulus (G"), and viscosity (η) were determined using the frequency range 1-10 Hz and two temperatures (25 and 40 °C). A) Control hydrogel (water); B) hydrogel containing zein nanoparticles (NP); C) hydrogel containing emulsified geraniol+eugenol (EM_GRL+EGL); D) hydrogel containing geraniol+eugenol encapsulated in zein nanoparticles (NP_GRL+EGL); E) hydrogel containing emulsified geraniol+cinnamaldehyde (EM_GRL+CND); F) hydrogel containing geraniol+cinnamaldehyde encapsulated in zein nanoparticles (NP_GRL+CND).

The prepared hydrogels were used in repellent activity assays against whitefly. The EI was calculated relative to the effect of the control hydrogel (prepared only with water addition), which also underwent the crosslinking process with citric acid (Figure 4). Hence, the EI values represented the actual effect of the formulation, discounting the possible effect of citric acid on whitefly repellency. Therefore, a positive value indicated that the treatment had a greater effect, compared to the control, while a negative value indicated that the control had a greater effect.

The hydrogels containing GRL and EGL presented positive repellency, with EI values of around 1.1 and 1.5% for the encapsulated and emulsified formulations, respectively (Figure 4). The hydrogels containing the mixture of GRL and CND presented slightly higher values. These systems showed significant repellent effects, relative to the control. The repellency observed in the presence of the botanical compounds was significantly higher than for the hydrogel containing only the zein nanoparticles. Previous studies reported in the literature have demonstrated the repellent activities of different essential oils and their main active compounds against whitefly [31–33]. Deletre et al. (2016)[34] evaluated the repellent activities against whitefly of active compounds including GRL and CND, isolated from four different plant species. The compounds CND, cuminaldehyde, GRL, and citronellol presented higher repellent effects, which were concentration dependent. In the present study, the hydrogel containing CND showed the greatest effect. In other work by the same research group Deletre et al., (2015)[35], evaluation was made of mixtures of the active compounds, in addition to the essential oils. It was observed that some of the mixtures presented repellent activity, as well as the isolated compounds. For example, the repellent effect of cinnamon was mainly attributed to cinnamaldehyde, for which the effect was similar to that of the mixture. The

compounds citronellol, citronellal, and geraniol were also toxic to the whitefly, but were not as effective as citronella essential oil (containing a mixture of these compounds). Therefore, synergistic effects involving the different compounds could explain the observed behavior. Synergisitic effects of essential oil terpenoids have been well characterized with respect to toxicity [36] and feeding deterrence [37] . It should be highlighted that in the present study, only 1 cm² of the hydrogel was employed in the assays, due to the limitation of the olfactometer sample compartment, and that the concentration employed was less than 1%, so higher concentrations might provide even greater repellent effects against the whitefly.

The results indicated that employing mixtures of active compounds could be a useful option for increasing the spectrum of action of botanical compounds. There were no significant differences between the effects for the emulsions and the encapsulated compounds. This could be explained by the nature of the release of the compounds from the hydrogel. Therefore, assays of the *in vitro* release of these compounds were performed, in order to further elucidate the observed behavior.

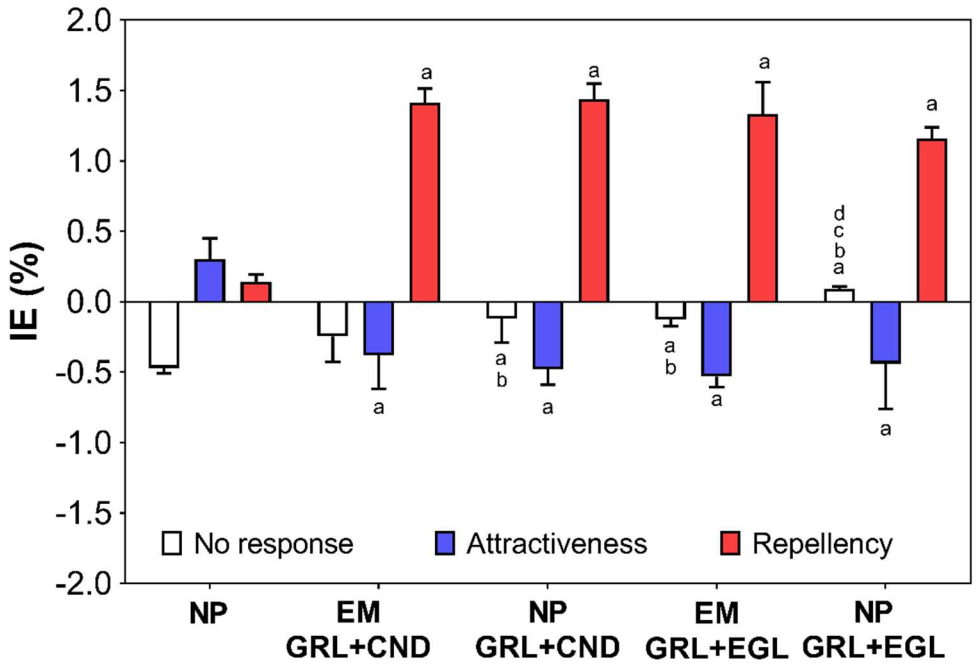


Figure 4. Effect index (EI) values for the hydrogels containing encapsulated and emulsified botanical compound mixtures, used against whitefly (*Bemisia tabaci*): hydrogel containing zein nanoparticles in the absence of botanical compounds (NP); hydrogel containing emulsified geraniol and eugenol (EM_GRL+EGL); hydrogel containing geraniol and eugenol encapsulated in zein nanoparticles (NP_GRL+EGL);

hydrogel containing emulsified geraniol and cinnamaldehyde CND (EM_GRL+CND); hydrogel containing geraniol and cinnamaldehyde encapsulated in zein nanoparticles (NP_GRL+CND). Statistically significant differences (two-way ANOVA) for treatments were investigated with significance level $p < 0.05$. For the non-response, attractiveness and repellency parameters, the indication with the letter **a** represents significant difference in relation to the treatment with NP; The indication with the letter **b** represents a significant difference in relation to treatment with EM_GRL + CND; The indication with the letter **c** represents significant difference compared to treatment with NP_GRL + CND and the indication with the letter **d** represents significant difference compared to treatment with EM_GRL + EGL.

3.3.2 Repellent activity against spider mites (*Tetranychus urticae*)

The repellent activities of the hydrogels against spider mites were determined using the system described in Section 2.5.2. The assays were performed using one hydrogel for each plant, with the hydrogel being fixed in a support, so that it did not touch the plant. Table 3 shows the results for the numbers of spider mites found on the plants with hydrogel and on the control, together with the repellency index (RI) value for each hydrogel. Table 4 presents the results for the numbers of eggs found on the plants, together with the oviposition inhibition index (OI) values.

Table 3. Numbers of mites and repellency index (RI) values for two-spotted spider mite (*Tetranychus urticae*) on plants in the presence and absence of hydrogels containing the emulsified or encapsulated botanical compounds: without hydrogel (Control); control hydrogel without botanical compounds (CTL_HYD); hydrogel containing zein nanoparticles in the absence of botanical compounds (NP); hydrogel containing emulsified geraniol and eugenol (EM_GRL+EGL); hydrogel containing geraniol and eugenol encapsulated in zein nanoparticles (NP_GRL+EGL); hydrogel containing emulsified geraniol and cinnamaldehyde CND (EM_GRL+CND); hydrogel containing geraniol and cinnamaldehyde encapsulated in zein nanoparticles (NP_GRL+CND).

Hydrogels	Number of mites (±SD) [a]			RI [c] (%)	Classification
	Treated	Untreated	*p*-values [b]		
Control	13.3 ± 6.4	13.0 ± 6.2	0.977	1.29	I
CTL_HYD	9.1 ± 5.2	12.2 ± 3.7	0.622	2.40	I
NP	11.1 ± 6.0	11.6 ± 5.9	0.955	1.35	I
EM_GRL+CND	1.1 ± 0.8	13.1 ± 3.1	<0.001	83.01	V
NP_GRL+CND	1.5 ± 1.7	19.4 ± 7.9	0.005	81.20	V
EM_GRL+EGL	0.7 ± 0.1	18.2 ± 6.2	<0.001	91.23	V
NP_GRL+EGL	1.2 ± 0.8	17.6 ± 7.4	0.001	90.12	V

[a] Average of nine repetitions; SD: standard deviation. [b] Statistical tests using the t-test for paired comparisons between the treated and control plants. [c] RI: repellency index.

622

623 **Table 4.** Numbers of eggs and oviposition inhibition index (OI) values for two-spotted
624 spider mite (*Tetranychus urticae*) on plants in the presence and absence of hydrogels
625 containing the emulsified or encapsulated botanical compounds: without hydrogel
626 (Control); control hydrogel without botanical compounds (CTL_HYD); hydrogel
627 containing zein nanoparticles without botanical compounds (NP); hydrogel containing
628 emulsified GRL and EGL (EM_GRL+EGL); hydrogel containing GRL and EGL
629 encapsulated in zein nanoparticles (NP_GRL+EGL); hydrogel containing emulsified
630 GRL and CND (EM_GRL+CND); hydrogel containing GRL and CND encapsulated in
631 zein nanoparticles (NP_GRL+CND).

632

Hydrogels	Number of eggs (±SD) [a]			OI [c] (%)	Classification
	Treated	Untreated	*p*–values [b]		
Control	5.6 ± 4.8	5.2 ± 5.1	0.918	- 5.83	0
CTL_HYD	9.4 ± 5.1	11.2 ± 8.3	0.441	1.1	I
NP	2.4 ± 1.5	2.2 ± 1.9	0.904	-8.2	0
EM_GRL+CND	0 ± 0	12.0 ± 2.7	<0.001	100	V
NP_GRL+CND	0.4 ± 0.01	11.1 ± 3.2	<0.001	97.2	V
EM_GRL+EGL	0 ± 0	13.4 ± 1.3	<0.001	100	V
NP_GRL+EGL	0.2 ± 0.04	17.6 ± 2.5	<0.001	94.4	V

[a] Average of nine repetitions; SD: standard deviation. [b] Statistical tests using the t-test for paired comparisons between the treated and control plants. [c] OI: oviposition inhibition index.

It can be seen from the results presented in Tables 3 and 4 that only the hydrogels containing the botanical compounds (either emulsified or encapsulated) led to a significant difference between the numbers of mites and eggs on the treated plants, compared to the untreated plants. For the number of mites (Table 3), the systems containing the emulsions presented the greatest difference, with EM_GRL+CND presenting RI of 83% (t: 6.26, df: 16, p<0.001), while EM_GRL+EGL showed RI of 91% (t: 4.51, df: 16, p<0.001). Both of these formulations achieved the maximum classification (V). The RI values for the systems containing encapsulated compounds were 81% for NP_GRL+CND (t: 3.24, df: 16, p=0.005) and 90% for NP_GRL+EGL (t: 3.98, df: 16, p<0.001). The hydrogels containing the encapsulated botanical compounds also presented the maximum classification (V), indicating strong repellent activity. The water control (without addition of hydrogel), the control hydrogels, and the hydrogel containing only zein nanoparticles (NP) showed no significant repellent effects, with p>0.1 and low RI values. Similar results were obtained for evaluation of the numbers of eggs (Table 4). The hydrogels containing the emulsions were more effective in preventing mite oviposition, with 100% OI for EM_GRL+CND (t: 3.42, df: 112, p<0.001) and EM_GRL+EGL (t: 3.58, df: 112, p<0.001). The NP_GRL+CND and NP_GRL+EGL hydrogels presented OI values of 97% (t: 4.57, df: 112, p<0.001) and 94% (t: 6.17, df: 112, p<0.001), respectively. These formulations received the maximum classification for prevention of mite oviposition. No effects on oviposition were observed for the control, CTL_HYD, and NP treatments, which all presented low or negative OI values.

Therefore, as observed for the whitefly (*B. tabaci*) assays, the systems containing the encapsulated or emulsified botanical compounds showed significant effects against the insects and were able to prevent mite oviposition. This was mainly due to the action of the botanical compounds, in agreement with the effects reported against *T. urticae* reported elsewhere [38–41]. Tak and Isman (2017)[42] studied the acaricidal and repellent activities of terpenes derived from different plant essential oils against *T. urticae*, as well as the effects of binary mixtures. The system used to evaluate the repellent activity was similar to that employed in the present work, involving a two-alternative test. Significant differences were found between the repellent effects of monoterpenes applied to bean and cabbage leaves. As observed in this study, CND, EGL, and GRL showed repellent activity against *T. urticae*, with RI values of 82.3±6.7%, 86.7±5.1%, and 80.2±4.1%, respectively, when the compounds were applied on bean leaves at a concentration of 10 mg/mL. It was noted that for some compounds, especially vanilla, there was an increase of repellent

activity when the compound was employed in mixtures with carvacrol and thymol. Tak and Isman (2017)[40] also pointed out that synergistic effects among botanical compounds are not rare phenomena, since these compounds are commonly found in mixtures in essential oils.

Our results confirmed that the hydrogels containing the botanical compounds showed repellent activity against two-spotted spider mite and silverleaf whitefly. Furthermore, mixing of the active compounds could improve the repellent effect, offering a promising way to increase the spectrum of action. In addition, it is worth mentioning the use of these formulations for applications in humid and high-light environments, where the stability of botanical compounds is lower. Studies in the literature [8,15,17,19] have shown that encapsulation, or association in devices, allows the protection of these compounds against external factors, increasing their effectiveness and stability. In the present study, even compounds not encapsulated in zein nanoparticles were placed in a polymeric hydrogel matrix which also already helps in their protection against external factors.

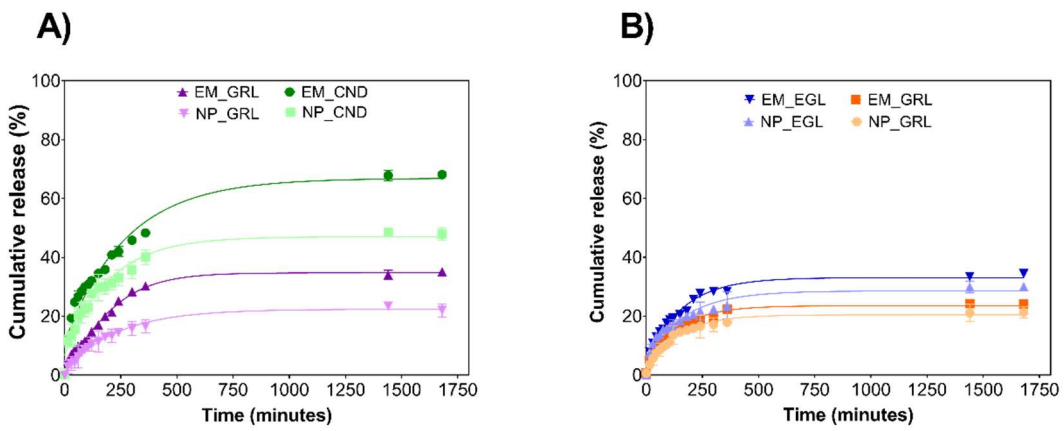
However, as also observed for the whitefly assays, there were no substantial differences between the systems containing either emulsions or nanoparticles, and in the case of the mite test, the same maximum classification (V) was obtained. In order to investigate this lack of any great difference between the emulsion and nanoparticle systems, *in vitro* release assays were performed to determine the release profiles for the active compounds in the hydrogels.

3.4 *In vitro* release assays

The *in vitro* release assays were carried out to evaluate the release profiles for the botanical compounds in the different hydrogel matrices. Figure 4 shows the cumulative release of the botanical compounds. The emulsified compounds showed faster release rates, compared to the encapsulated compounds. The most pronounced difference was observed for the systems containing the mixture of GRL and CND. For the emulsified formulation, the GRL and CND release percentages after 24 h were 35.2±2.2% and 65.8±2.7%, respectively. When the compounds were encapsulated, the release percentages were 23.4±1.4% and 48.4±1.3%, respectively. For the hydrogel containing the emulsified mixture of GRL and EGL, the release percentages were 26.5±0.1% and 35.5±0.3%, respectively, while the corresponding encapsulated formulation showed release percentages of 21.1±1.0% and 29.3±1.3%, respectively. All the hydrogels showed

biphasic release profiles, with fast initial release (up to 180 min), followed by sustained release. Similar results have been reported in other studies employing polymeric systems containing natural compounds [43–45]. For hydrogels containing nanoparticulate systems, faster release in the early stages is associated with the non-encapsulated or non-adsorbed compound present on the particle surface. For hydrogels containing emulsions, the release is faster, due to the high availability of the active compounds. Therefore, the incorporation of nanoparticles in the hydrogel restricts the mobility of the botanical compounds in the gel polymer network, resulting in lower diffusion rates, compared to hydrogels containing only emulsified compounds. Similar results were obtained by Almeida et al. (2018)[46], who prepared poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing *Cymbopogon citratus* (DC.) Stapf essential oil, incorporated in Carbopol® hydrogels. It was found that release of the essential oil from hydrogels containing the PLGA particles was slower than from hydrogels containing only the essential oil, which was attributed to the presence of a double barrier to diffusion of the essential oil, following incorporation of the nanoparticles in the hydrogels.

The diffusion of botanical compounds through hydrogels can also be investigated using the application of different mathematical models, in order to understand the release mechanisms of the system. According to the data presented in Table S5, the Korsmeyer-Peppas mathematical model provided the best fits to the data for all the compounds. The use of this model enables determination of whether the release of the active compound occurs according to Fick's diffusion law, or whether it involves other phenomena, such as swelling/relaxation of the polymer chains (case II transport). The n values for all the emulsified and nanoencapsulated botanical compounds were <0.45, indicating that diffusion was the main mechanism controlling the release of the active substances from the hydrogels. In this type of release, the release rate generally decreases progressively, because the more internalized molecules have longer distances to travel, which requires more time.



735 **Figure 5.** Cumulative release (%) of the botanical compounds from the hydrogels:
736 A) hydrogels containing a mixture of GRL and CND, either emulsified (EM) or
737 encapsulated (NP); B) hydrogels containing a mixture of GRL and EGL, either emulsified
738 (EM) or encapsulated (NP). The analyses were performed in triplicate and quantification
739 of the compounds was by HPLC.

740 The release from the hydrogel was faster for the emulsified botanical compounds,
741 compared to the encapsulated compounds. This could provide an explanation for the
742 greater repellent effect found for these systems in the biological activity tests (Section
743 3.3). It is expected that systems with faster release should provide higher repellent effects,
744 since such activity depends mainly on the concentrations of the active compounds [47].

745 However, it is possible that nanoencapsulated botanicals dispersed in hydrogels
746 could provide effects of longer duration, compared to emulsified systems. Previous work,
747 Oliveira et al (2019) [8] showed that the nanoencapsulation of botanical compounds is an
748 excellent way to reduce their degradation over time. Hence, over a longer period, under
749 weathering, hydrogels containing encapsulated compounds could offer advantages.
750 Despite lower release rates, the repellency performances of these hydrogels were similar
751 to those of the emulsified formulations, achieving the maximum repellency classification.
752
753

754 ## 4. Conclusions

755 This work describes the preparation, characterization, and evaluation of repellent
756 activity of hydrogels containing botanical compounds in emulsified or encapsulated
757 forms. The prepared nanosystems showed good physicochemical properties and were
758 successfully incorporated in hydrogels using a two-step preparation process involving
759 molding and crosslinking. For the crosslinking of the hydrogels, a citric acid solution (8
760 mol/L) was used, which ensured a high degree of crosslinking and good rheological
761 properties. The hydrogels presented high repellent activity against whiteflies and spider
762 mites, which are major agricultural pests. The findings constitute an important
763 contribution to the development of sustainable agriculture since the hydrogels are
764 composed of materials obtained from natural sources and can be used without direct
765 contact with plants.

766 ## 5. Associated Content

The Supporting Information is available free of charge on the ACS Publications website Details:

S1. Validation of the methodology for quantification of the botanical compounds by HPLC

Figure S1: FT-IR spectra of CMC (A), HEC (B), Citric Acid (C) and CMC/HEC hydrogel crosslinked with citric acid (D).

Figure S2. X-ray diffraction curves of CMC (A), HEC (b), Citric Acid (C) and CMC/HEC hydrogel crosslinked with citric acid (D).

Figure S3: Experimental model used for the mite assays (*Tetranychus urticae*).

Table S1. Correlation coefficients (r^2) and constant values for the different mathematical models applied to the release of geraniol, eugenol, and cinnamaldehyde from the hydrogels.

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Notes

The authors declare no competing financial interest.

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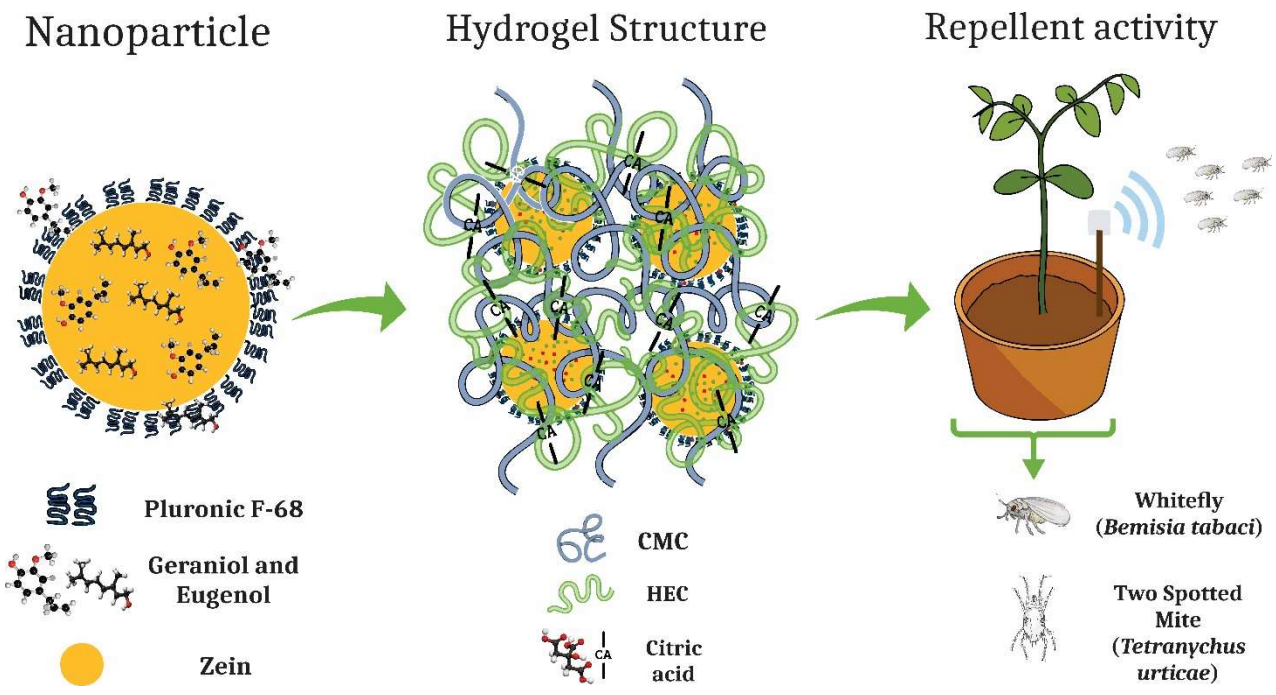

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