Zein Nanoparticles Impregnated with Eugenol and Garlic Essential Oils for Treating Fish Pathogens

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Abstract

The supply of food derived from aquaculture has increased significantly in recent years. The aim of this industrial sector is to produce sustainable products to meet the needs of consumers, providing food security and nutritional benefits. The development of aquaculture has faced challenges including disease outbreaks that can cause substantial economic losses. These diseases can be controlled using chemicals such as antibiotics. However, the indiscriminate use of these substances can have major negative impacts on human health and the environment, with the additional risk of the emergence of resistant organisms. The present manuscript describes the use of phytotherapy in association with nanotechnology, in order to obtain a more effective and less harmful system for the control of bacterial diseases in fish. Zein nanoparticles associated with eugenol and garlic essential oil were prepared through antisolvent precipitation and characterized. Zein nanoparticles are promising carriers system as zein protein are biodegradable and biocompatible and in this way good candidate for the encapsulation of active ingredients. The system presented good physicochemical properties, with average particle diameter of around 150 nm, polydispersity index lower than 0.2, and zeta potential around 30 mV. High encapsulation efficiency was obtained for the active compounds, with values higher than 90%, and the compounds were protected against degradation during storage (90 days). The nanoparticle formulations containing the botanical compounds also showed less toxicity in the tests performed with biomarker (*Artemia salina*). In addition, the systems showed bactericidal activity against the important fish pathogenic bacteria *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Streptococcus iniae in vitro*. The present study open new perspectives for the use of botanical compounds, in combination with nanotechnology, to treat fish diseases caused by bacteria, contributing to a more sustainable fish chain production.

Keywords: Polymeric nanoparticles, botanical compounds, nanotechnology, antimicrobials.

1 Introduction

Aquaculture, which has a long history as a subsistence or commercial activity, has become an important sector involved in food production worldwide. The increase of fish farming, low water quality, and inappropriate feeding, has resulted in the occurrence of fish diseases, hence compromising the sustainability of aquaculture. Diseases lead to reduced commercial value of the fish, increased mortality, and decreased growth rates [1]. There are pathogens which can affect aquaculture such as, *Aeromonas hydrophila* [2], *Edwardsiella tarda* [3,4] and *Streptococcus iniae* [5,6], causing mainly skin ulceration, hemorrhagic septicemia, internal hemorrhage, pale gills, lateral line necrosis, lethargy, exophthalmia and anorexia [7–9].

These fish diseases are controlled using antibiotics such as oxytetracycline, florfenicol, amoxicillin, and erythromycin, with the use and recommended dosage varying according to the legislation of each country [5,10,11]. However, these antibiotics can cause bacterial resistance, resistant bacteria may be present in the wastewater stream from farms or agro-industrial plants, and can be transferred to food crops or surface waters [12]. The problems associated with the conventional control methods have stimulated the search for alternative methods for the control of fish diseases that are both effective and environmentally safe.

Essential oils derived from plants are promising candidates to substitute antibiotics and other chemotherapeutic agents in aquaculture, offering advantages including low environmental impacts, high biodegradability, low toxicity, and reduced costs for fish farmers. Their low persistence results in a reduction of residues in fish, while their different modes of action result in slower rates of development of resistance in disease organisms [13,14]. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene) is the main phenolic compound in clove essential oil. In aquaculture, this compound has been used as an anesthetic and bactericidal for different fish species [15–20]. In terms, garlic (*Allium sativum*), a member of the Liliaceae family, is one of the oldest vegetables widely used due to its strong antibacterial activity [21]. Allicin is the main antibacterial compound present in garlic essential oil. In aquaculture, garlic and/or its constituents have presented antimicrobial activity against both Gram-positive and Gram-negative bacteria [21,22].

Although essential oils and their derivatives present valuable properties, there are certain limitations concerning their application on a large scale in aquaculture, especially due to their low aqueous solubility, low stability, and high sensitivity to ultraviolet irradiation and elevated temperatures [23]. Therefore, new technologies are required in order to overcome the limitations of essential oils. In particular, nanotechnological techniques have shown potential for the encapsulation and release of essential oils [23,24]. Natural polymers have attracted great interest for the

development of release systems for active agents. Zein is one of the most widely used natural polymers, which accounts for 45-50% of the protein in maize [25]. Scientific interest in zein has mainly been due to its ability to form low cost biodegradable flexible films and resistant hydrophobic coatings which provide protection against microbial attack, indicating its suitability for producing micro/nanoparticles used as delivery systems for nutrients and drugs [25,26].

Given the above background, the aim of the present work was to prepare and characterize an antimicrobial system based on zein nanoparticles containing isolated and a combination of eugenol and garlic oil, as well as to evaluate its effectiveness in controlling fish pathogenic bacteria (*Aeromonas hydrophila*, *Edwardsiella tarda*, and *Streptococcus iniae*). To know the possible toxicity of nanoparticles loaded with eugenol and garlic oil, the toxicity was determined in tests using *Artemia Salina*. It should be noted that there have been no previous studies concerning the development of zein nanoparticles for release of botanical antimicrobials to control bacteria that cause infections in fish. This study provides new perspectives for the use of nanoparticles to control diseases in fish, hence contributing to the sustainable development of aquaculture.

2 Results and Discussion

2.1. Nanoparticle preparation and physicochemical characterization

In this study, zein nanoparticles was develop as a new carrier system for two different botanical compounds, as an alternative formulation for treat fish pathogens. The antisolvent precipitation method was used to prepare the system that consisted essentially of hydroethanolic zein solution injection into an aqueous solution. Due to the strongly hydrophobic characteristics of zein (associated with a high proportion of cationic amino acids), it is soluble in binary solvents containing alcohol, which can be exploited for the preparation of nanoparticles [27]. The zein has a high isoelectric point (pI = 6), which results in a tendency to aggregate protein chains in formulations with neutral / basic pH [28]. For this reason, the surfactant Pluronic F-68 was used as a stabilizing agent during the preparation of the particles. The process of formation of the zein nanoparticles containing the botanical compounds is shown schematically in Figure 1.

[Figure 1]

Table 1 presents the initial characterization of the nanoparticles formulations, with measurements of mean diameter (MD), polydispersity index (PDI), zeta potential (ZP), nanoparticle concentration (CT), and encapsulation efficiency (EE).

[Table 1]

The results (Table 1) showed that the addition of the active compounds resulted in an increase in the average diameter of the nanoparticles, in comparison with the control nanoparticles (ZNP), being more significant for the nanoparticles with isolated actives. Both the control nanoparticles and the nanoparticles containing the botanical compounds showed polydispersity rates below 0.2. It is also observed that all formulations showed values of zeta potential around 30 mV. In addition, botanical compounds showed high interaction with the zein matrix, with encapsulation efficiency values greater than 96%.

In addition to the initial physical-chemical characterization, the stability of the formulations was evaluated according to the storage time (0, 7, 15, 30, 60 and 90 days). The results (Figure 2-A and B) showed that the average diameters of the control nanoparticles and the nanoparticles containing eugenol and garlic, as well as the mixture of the two compounds, increased significantly as a function of the storage time, as evidenced by the measurements using the DLS and NTA techniques. However, it should be noted that a greater increase in the average particle diameter was observed for the control nanoparticles, compared to the nanoparticles containing active botanical compounds. The control nanoparticle (ZNP) showed a mean diameter value of 141 ± 1 nm and 132 ± 2 nm in the initial time, for the DLS and NTA techniques, respectively. At 90 days these values were 303 ± 19 nm and 323 ± 4 nm. For ZNE, the mean initial diameter values for DLS and NTA were 183 ± 3 nm and 190 ± 4 nm and within 90 days they reached values of 280 ± 2 nm and 296 ± 3 nm. The nanoparticles containing isolated garlic oil (ZNG) initially presented values of mean diameter of 195 ± 7 nm and 151 ± 2 nm, for DLS and NTA. At 90 days these values were 284 ± 3 nm and 272 ± 5 nm, respectively. Finally, the nanoparticles containing the mixture of active compounds showed mean diameter values of 146 ± 7 nm and 161 ± 4 nm in the initial time for DLS and NTA, reaching values of 247 ± 9 nm and 232 ± 4 nm in 90 days for same techniques.

The results for polydispersity index presented in Figure 2-C show a significant increase for all particles over the analyzed period. It is also noteworthy that these results corroborate those presented by the mean diameter (DLS and NTA), since the control nanoparticles (ZNP) showed more change in the values compared to those containing the botanical compounds. This profile was also observed for the zeta potential results (Figure 2-D), where all formulations showed a

significant decrease in the zeta potential values, as a function of time. However, as with the other data, the greatest reduction was observed for the control nanoparticles (ZNP). These had an initial zeta potential value of 31 ± 1 mV decreasing to a final value of 19 ± 1 mV. The data set shows us that all systems showed a tendency to agglomerate, since they increased values of size and polydispersity index and decreased values of zeta potential. However, nanoparticles containing botanical compounds are more stable (90 days) than control nanoparticles (without active).

The physicochemical characterization and stability evaluation of nanotechnology-based formulations is an important phase in the development of these systems. In this work, both the control nanoparticles and the nanoparticles containing the botanical compounds presented polydispersity index lower than 0.2, indicating good homogeneity of the size distribution of the nanoparticles. The four formulations presented high zeta potential values, while high encapsulation efficiencies (>99%) showed that both eugenol and garlic oil had strong affinity for the carrier system. The results also showed that the mean diameters of nanoparticles formulations increased significantly as a function of storage time, as evidenced by the measurements using the DLS and NTA techniques.

It can be concluded from the results of physical-chemical stability as a function of time, that the nanoparticles started to lose stability, with the beginning of the formation of aggregates. An increase in the mean diameter (DLS and NTA), an increase in the polydispersity index and a decrease in the zeta potential were observed. However, an important observation was that the addition of botanical compounds led to greater stability when compared to the control formulation. The results obtained here are in agreement with previously published studies. Oliveira et al. (2018)[24] characterized zein nanoparticles prepared by the same method, which were used for the encapsulation of geraniol and citronellal. The formulations showed an average diameter of 80 to 200 nm, polydispersion index > 0.3 and zeta potential of 10 to 33 mV. It was also found that formulations containing botanical compounds showed greater stability compared to nanoparticles without the active agents.

[Figure 2]

The morphology of the nanoparticles containing the botanical compounds (eugenol, garlic oil and the mixture) were investigated by atomic force microscopy (AFM) and are shown in Figure 3-C, D and E. The size distribution graphs for the nanoparticles analyzed by the DLS and NTA techniques are also shown (Figure 3- A and B). It is possible to observe that all nanoparticles presented spherical morphology and corroborating the polydispersity index data (Figure 2-C), we can observe different particle sizes. Through the Gywdion software, nanoparticle size

measurements were performed. The zein nanoparticles containing eugenol (Figure 3-C) showed a mean diameter value for AFM techniques of 160 ± 5 nm. The nanoparticles loaded with garlic oil (Figure 3-D) showed values of 180 ± 7 nm. The zein nanoparticles containing the mixture of eugenol and garlic oil (Figure 3-E) showed values of 148 ± 8 nm, respectively.

The AFM technique is widely used to characterize nanoparticle systems containing botanical compounds. In this study, both formulations presented spherical particle morphology. The mean diameters found by the NTA and DLS techniques were larger, in comparison to this technique that shows smaller mean diameters. This could be mainly attributed to the drying process used to prepare the samples for AFM analysis. Chen and Zhong (2015)[29] prepared zein nanoparticles stabilized with gum arabic, which were used for the encapsulation of peppermint essential oil. AFM analyses revealed that the nanoparticles were spherical, with a mean diameter of 160.7 ± 37.4 nm and a size range from 120 to 196 nm.

[Figure 3]

2.1.1. Active compound stability during storage

In order to evaluate the capacity of zein nanoparticles in protecting botanical compounds against degradation during storage time, a comparison was made between these formulations and emulsions of botanical compounds prepared with only the surfactant Pluronic F-68. Figure 4 shows the results for quantification of botanical compounds during storage for 90 days, with A) presenting the results for nanoparticles containing isolated compounds and in B) containing their mixture. It is observed (Figure 4-A) that in the case of the emulsion containing only eugenol, it presented 91.2 ± 1.9% of active compound in the initial time, and in the final time (90 days) there was a significant reduction, reaching a value of 25.9 ± 0.01 %. For emulsion containing isolated garlic oil, the decrease was lower. In the initial time, it showed 96.5 ± 0.01% of the active compound, with a reduction to 88.5 ± 0.03% after 90 days. A similar profile for eugenol was observed for the emulsion prepared with the mixture of active compounds (Figure 4-B). However, for garlic oil, the reduction in the percentage of active compound was greater when prepared together. Because, this formulation presented values of 90.3 ± 0.27% of the active compound at initial time, with a reduction to 54.2 ± 0.06% after 90 days

When we analyze nanoparticle formulations, it is observed that the reduction in the percentage of active compound was significantly smaller compared to emulsions, showing the protective effect. For nanoparticle formulations containing only eugenol (ZNE) the percentage of initial active compound was 99.1 ± 0.9% and after 90 days it was 82.8 ± 0.8%. For nanoparticle formulations containing only garlic oil (ZNG), there was a reduction of approximately 4.4%.

Presenting an initial value of 99.8 ± 0.2% and reaching a value of 95.4 ± 0.1% within 90 days. A similar profile was observed for the nanoparticles containing the mixture of botanical compounds (ZNEG). They initially presented percentages of active of 96.1 ± 0.1 and 97.2 ± 0.02%, for eugenol and garlic oil, respectively. After 90 days of storage, these values were 85.4 ± 0.12% and 90.6 ± 0.82%.

Here, active compound stability during storage was evaluated. For both systems (nanoparticles and emulsion), eugenol showed greater loss during storage, which could be attributed to its higher volatility, compared to garlic oil [30]. Nonetheless, the losses of the active compounds were lower when they were encapsulated in the zein nanoparticles. These results were in agreement with previous studies showing the potential of encapsulation to reduce the degradation of active compounds [24,31–33]. Piletti et al. (2017)[34] evaluated the increase in thermal stability of eugenol following its encapsulation in β-cyclodextrin. Antimicrobial activity of the inclusion complex was observed, even after heat treatment at 80 °C, which was two times higher than the volatilization temperature of the eugenol molecule, hence confirming the thermal protection. Scremin et al. (2018)[35] microencapsulated eugenol in matrices composed of carrageenan, rice bran protein, and serum albumin, using a spray drying method. The encapsulation provided protection of about 30%, compared to the unencapsulated compound. Therefore, the zein nanoparticles produced in the present work were effective in reducing losses of the botanical compounds by hydrolysis, volatilization, and photodegradation, hence increasing the efficacy of the compounds.

[Figure 4]

2.2. Evaluation of the bactericidal activity of the nanoparticles

Figure 3 shows the results for bactericidal activity of the formulations using the disk diffusion assay. Results are presented for three different bacteria pathogenic to fish, *Aeromonas hydrophila* (Figure 5-A), *Edwardsiella tard* (Figure 5-B) and *Streptococcus iniae* (Figure 5-C). It is observed that for the negative control (C), as well as for the control of nanoparticles (ZNP) and only the Pluronic F-68 surfactant (PLU), no significant differences were observed in the mean diameter of halo. The addition of botanical compounds, whether in the form of emulsions or nanoparticles, resulted in a significant increase in halo values, indicating activity against bacteria. Except for the isolated garlic oil emulsion, which showed no significant difference compared to the controls for *Edwardsiella tard* (Figure 5-B) and *Streptococcus iniae* (Figure 5-C). However, it is worth noting that when encapsulated in the nanoparticles, it showed significant activity in relation to the controls. We also observed that mostly the nanoparticles containing the botanical compounds showed a greater effect (higher halo values), in relation to the emulsified compounds. In addition, the

formulations (emulsions and nanoparticles) containing the mixture of botanical compounds showed the greatest effects, compared to the isolated compounds, indicating a synergistic effect

[Figure 5]

For *in vitro* bactericidal activity assays, the results are interesting, since the nanoparticles containing botanical compounds synthetized in this study presented bactericidal activity against three different bacteria species. It should be noted that the nanoparticles containing the active compounds showed a superior effect in relation to the emulsified compounds. The greatest bactericidal effect was observed for the formulation containing the mixture of botanical compounds, which is mainly due to synergistic effects among them.

Previous studies [36–39] have demonstrated that compounds derived from plants can be successfully used in aquaculture for purposes including control of diseases, stimulation of immunity in fish [40], reducing stress (due to the sedative and anesthetic properties of the compounds), and increasing the shelf life of refrigerated and frozen fish [41]. Sutili et al. (2014)[20] evaluated the effectiveness of eugenol against the pathogenic bacterium *Aeromonas hydrophila*, as well as the effect of this compound on hematological and immune parameters in *Rhamdia quelen* (silver catfish). It was found that fish infected with *A. hydrophila* and treated with sub-inhibitory concentrations of eugenol (5 and 10 $mg.L^{-1}$) showed improved survival rates, as well as significantly reduced erythrocyte hemolysis caused by the bacteria. In addition, at these concentrations, no alterations were observed in the hematological and immune parameters of the catfish. Thomas et al. (2014)[42] showed that a nanoemulsion containing lime oil was effective in treating bacterial infections (*P. aeruginosa*) in tilapia (*O. mossambicus*), both *in vivo* and *in vitro*. In both cases, use of the oil in the form of a nanoemulsion led to improved results. In earlier work, Thomas et al. (2013)[42] demonstrated the effectiveness of nanoemulsions of neem oil against the bacterium *Aeromonas salmonicida*, in tests using catfish (*Clarias batrachus*). Gholipourkanani; Buller; Lymbery, (2019)[38] prepared nanoemultions to compare the bactericidal and bacteriostatic characteristics of *Eucalyptus globulus*, *Origanum vulgare*, *Lavendula angustifolia* and *Melaleuca alternifoli*, against three pathogens, *A. hydrophila, S.inae, P. damselaedamselae.* The nanoemulsion of *Origanum vulgare* turned out to have a stronger bactericidal activity, compared to the other treatments.

Another observed feature was the similarity between the results obtained for eugenol and garlic oil in the encapsulated and emulsified formulations. This showed that the surfactant micelles could also act as a release system and promote biological activity similar to that found for the encapsulated systems. However, an important finding was that the stability data (Figure 2) showed that the lost of the active agents was faster for the emulsified compounds, compared to the

encapsulated forms. The results indicated that the biological activity of the zein nanoparticle formulations could be higher over time, compared to the emulsions with Pluronic F-68, since the encapsulation acted to reduce volatilization, especially of eugenol.

2.3. Acute toxicity test with *Artemia salina*

In order to evaluate the toxicity of formulations containing botanical compounds, a biomarker (*Artemia salina*) belonging to the aquatic saline system was used. Table 2 shows the results of the LC_{50} for the tested formulations. Through the results it is possible to observe that for all formulations, when the active ingredients were encapsulated into the nanoparticles, there was a significant increase in the LC_{50} values. This therefore indicates that the nanoparticles had a protective effect against the toxicity of botanical compounds. It is also worth noting that formulations in nanoparticles increased by about 2-fold the LC50 values, and isolated for garlic oil when encapsulated values increased nearly 20-fold.

[Table 2]

Many studies have proposed the use of plant essential oils and/or their derivatives, with promising results obtained for these compounds in aquaculture. However, it is essential that as for synthetic compounds, the use of botanical substances must be performed in a way that guarantees environmental sustainability. Most of the reported studies used *in vitro* or *in vivo* tests to evaluate the effectiveness of these compounds, but on a small scale. Hence, different effects might be observed on an industrial scale. Botanicals can become toxic, depending on the concentration used and the route of administration [31].

It is in this context of toxicity assessment that the prepared formulations were subjected to an acute biomarker toxicity test (*Artemia salina*). The results showed that when encapsulated in the nanoparticles, the botanical compounds showed higher LC_{50} values compared to the only emulsified compounds. This indicates a significant decrease in the toxic effect of the active compounds after encapsulation. There are studies in the literature that used *Artemia salina* to assess the toxicity of botanical compounds [43,44] and also of nanoparticles [45,46]. Mota et al., (2020)[47] prepared and characterized poly (lactide-co-glycolide) (PLGA) nanoparticles containing hydroethanolic extract of *Sambucus nigra* L. After preparing and characterizing the system, the authors also evaluated toxicity using *Artemia salina* as a model. As found in this study, the authors observed that after the encapsulation the toxicity of the extract was lower, showing the protective effect of the nanoparticles. According to the authors, this may occur because in nanoparticle systems the compound is not fully available, being encapsulated in the nanoparticle matrix.

Although, *A. salina* is a salt water organism, it has been used as a general bioindicator in ecotoxicology by several world organizations such as OECD and USEPA for aquatic environments. *Artemia* sp. is one of the most valuable test organisms available for several applications, including toxicology and ecotoxicology researches. There is a tendency to use an *Artemia salina* assay in toxicological tests that screen chemical compounds with possible biological activity due to several advantages such as: well-known biology and ecology, low cost of the organisms, as well, speed and convenience of the tests. For example, as *in vitro* and *in vivo* toxicity assessment of selenium nanoparticles showed low cytotoxicity against macrophages and *Artemia nauplii,* they can be proposed as a biocompatible nano-biomedicine against bacterial infections [48].

Also in a previous study[49], it was showed that zein nanoparticles were stable when tested in saline solution. The results showed that there were no changes in mean diameter values of nanoparticles determined by DLS and NTA. Despite the decrease in zeta potential values (explained due to the greater ionic strength of the saline medium, changing the ionic balance), the formulation remained stable in solution due importance of steric hindrance effect of Pluronic F-68 into the zein nanoparticles stabilization.

3. Conclusions

The present work describes the preparation and characterization of zein nanoparticles containing eugenol and garlic essential oil. The antisolvent precipitation method was used to prepare the systems, resulting in high encapsulation efficiencies (>90%) for active agents, indicative of good interaction with the protein matrix. The formulations presented spherical morphology, mean diameters around 150 nm, polydispersity indexes of 0.2 and zeta potentials around 30 mV.

The nanoparticle formulations showed variable stability over time, but formulations containing botanical compounds showed greater stability in relation to control particles in the analyzed time (90 days). The nanoparticles protected the active compounds from degradation during storage. The results of disk diffusion assays showed that the zein nanoparticles containing eugenol and garlic essential oil provided greater inhibition of the bacteria *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Streptococcus iniae*, compared to other formulation. Significant differences in the bactericidal effects were observed between the encapsulated and emulsified botanical compounds. However, the emulsified compounds degraded faster in solution. In addition, toxicity tests on *Artemia salina* showed that the encapsulation of botanical compounds reduced their toxic effect, but maintained their bactericidal effect. Such results show the potential of the system.

To date, there have been few studies concerning the use of botanical compounds associated with nanotechnology in aquaculture. The present work demonstrated the effectiveness of

nanoparticles containing eugenol and garlic essential oil for inhibiting the growth of Gram-positive and Gram-negative bacteria that are major causative agents of fish diseases. These formulations are viable, effective, and an option that produces less damage for the treatment of diseases in fish, enabling reduction of the amounts of the active agents, as well as improving the stability of the natural compounds. However, it is clear that further toxicological studies should be performed in order to obtain a better understanding of the effects of such nanotechnological systems. Studies using different concentrations could also provide important information about the bactericidal activity potential of nanoparticle systems containing botanical compounds. Nonetheless, the present findings open new perspectives for the use of botanical compounds, in combination with nanotechnology, to treat fish diseases caused by bacteria. Encapsulation can not only protect the active compounds against degradation, but also contribute to their increased effectiveness. Although, present results shed light on a new perspective for the development of an innovative product, further investigation is required to identify other damage effects in aquatic organisms.

4. Materials and Methods

4.1 Materials

Garlic oil (98%), eugenol (99%), zein, and Pluronic F-68 were purchased from Sigma-Aldrich. Acetone, ethanol were isopropanol purchased from Labsynth. The solvent employed for the chromatographic analyses was HPLC-grade acetonitrile (JT Baker, Phillipsburg, New Jersey). The bacterias *Aeromonas hydrophila* (ATCC-7966), *Edwardsiella tarda* (ATCC-15947), and *Streptococcus iniae* (ATCC-29177) were purchased from PASTLABOR-Brazil).

4.2 Preparation of zein nanoparticles loaded with eugenol and/or garlic oil

The nanoparticles loaded with eugenol (ZNE), garlic oil (ZNG), the mixture of both (ZNEG) and nanoparticles without actives compounds (ZNP) were prepared by the anti-solvent precipitation method described by [50], with slight modifications. Zein (1% w/v) was dissolved overnight in hydro-ethanolic solution (85% v / v). Subsequently, the zein solution was adjusted to pH 4.5 with 1.0 $mol.L^{-1}$ HCl, followed by filtration through a 0.45 μm membrane (Millipore) to remove insoluble particles. Separately, an aqueous Pluronic F-68 solution (1% w/v) was prepared and the pH was adjusted to 4.0. For the preparation of the nanoparticles, 100 mg of eugenol and/or garlic oil were dissolved in the pre-treated zein solution (4 mL), the zein containing the active compound was injected (syringe with a needle of 25 mm) into the surfactant solution (Pluronic F-68, 16 mL), under magnetic stirring (200 rpm). The resulting colloidal dispersion was kept under stirring (200 rpm) at room temperature for solvent evaporation. The lost volume was compensated for by the addition of

water at pH 4.0, which resulted in final concentrations of 5 mg/mL for both formulations. In the case of garlic oil nanoparticles, isopropanol was used as the solvent. Control emulsions were also produced, containing only botanical compounds and Pluronic F-68 surfactant for comparison (PLU's). For these formulations, initially the botanical compounds were added in the same concentration as used for nanoparticles preparation. Subsequently, the surfactant solution was added in the final volume of 30 mL. The mixture was then strongly stirred (600 rpm) using a magnetic stirrer until an emulsion was formed.

4.3 Physicochemical characterization of the nanoparticles

4.3.1 Mean diameter, polydispersity index, and zeta potential

Photon correlation spectroscopy and microelectrophoresis were used to determine the hydrodynamic diameter and polydispersity index, and zeta potential of the nanoparticles, respectively. The analyses were performed using a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) at a fixed angle of 90° and 25 °C and nanoparticle suspensions was diluted 1000x in deionized water. The results were expressed as means of three determinations. Also, particle concentration and size distributions were performed 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 stabilities of the formulations were evaluated using measurements at predetermined time intervals (after 0, 15, 30, 60 and 90 days).

4.3.2 Active compound stability during storage

The amount of oil encapsulated in the nanoparticles was determined by the ultrafiltration/centrifugation method, in which the nanoparticle suspension was filtered using regenerated cellulose filters with 10 kDa exclusion pore size (Microcon, Millipore), only allowing passage of the unencapsulated oil. The eugenol and garlic oil in the ultrafiltrate were quantified by HPLC. The amounts of unencapsulated compounds were obtained as the difference between the total amount (100%) of compound added in the system and the amount that was not associated with the nanoparticles, enabling determination of the encapsulation efficiencies of the colloidal systems. The equations of the analytical curves used for quantification were as follows: garlic oil = 0.06629x + 0.1336 (r^2 = 0.990); eugenol = 4.939x + 28.89 (r^2 = 0.9943). For the purpose of comparison, emulsions were prepared containing the same concentrations of the botanical compounds (not encapsulated), and quantification by HPLC was also performed as a function of time.

4.3.3 Morphological analysis by atomic force microscopy (AFM)

The morphology of the nanoparticles was investigated using the AFM technique, employing an EasyScan 2 Basic AFM (Nanosurf, Switzerland) operating in tapping mode. Dilutions

of the nanoparticles were performed and the suspensions were placed on silicon plates previously prepared with removal of the silicon dioxide to facilitate interaction/adhesion of the samples on the plates. The images (256 x 256 pixels, in TIFF format) were captured in time mode, in a range of 10 seconds and were analyzed using Gwyddion software.

4.4 Evaluation of the bactericidal activity of the nanoparticles using disk diffusion assay

The bactericidal activity of the nanoparticles was evaluated against three different pathogenic bacteria (*Aeromonas hydrophila*, *Edwardsiella tarda*, and *Streptococcus iniae*) which cause fish infections. The bacteria were cultured for 24 h in Tryptic Soy Broth (TSB) medium and were then counted in a Neubauer chamber, using Trypan Blue staining, in order to obtain a concentration of 1 x 10^8 CFU.mL^{-1}. After counting, the bacteria were inoculated in Petri dishes containing blood agar. Filter paper disks (0.6 cm diameter) were added on the surface of the agar containing the bacteria and a 6 µL aliquot of each dispersion of nanoparticles was pipetted on each disk. The treatments were performed using 5 mg.mL^{-1} suspensions of zein nanoparticles alone (ZNP) and nanoparticles containing botanical compounds: zein nanoparticles with eugenol (ZNE), zein nanoparticles with garlic oil (ZNG) and zein nanoparticles co-loaded with eugenol and garlic oil (ZNEG). Emulsions of pluronic with eugenol (PLUE), pluronic with garlic oil (PLUG), pluronic with mixture (PLUEG) and only pluronic (PLU) were used as controls. The Petri dishes were prepared in triplicate and were divided into those containing the emulsified compounds and those containing the nanoencapsulated compounds. The plates were incubated for 24 h, followed by measurement of the halo diameters and calculation of the mean and standard deviation of the three measurements. Statistical analysis was performed by ANOVA followed by Tukey's test ($p < 0.05$ significance level), using GraphPad Prism 7 software.

4.5 Acute toxicity test with *Artemia salina* species

For this test, small nauplii of *Artemia salina* were used, 24 h before the test, synthetic seawater was prepared in a 1L Erlenmeyer, mixing 30 g of "Sera Premium®" salt (Sera GmbH, Heinsberg), in 1 L of water (pH = 7.2). 30 g of Artemia salina cysts (INVE Aquaculture) were added to this mixture. This suspension was kept under intense aeration through a porous stone. 12-well polystyrene plates were used for the test, containing the treatments (volume 5 mL/well). Two nauplii were transferred for each well with the aid of a micropipette and were exposed to different concentrations of nanoparticles and emulsions containing eugenol and garlic oil (1, 1.8, 5.82 and 10.47 mg.L^{-1}) and exposed to the test conditions (20 ± 2 ºC, approximately 1000 lx (photoperiod 16-

h light/8-h dark). After 48 h, the organisms were analyzed and determined the concentration that affected the mobility of 50% of the population (LC_{50}), with a confidence interval of 95%. Exceptionally for the formulation of nanoparticles containing only garlic oil, due to the lack of mortality in the tested concentrations, higher concentrations (1, 10, 100 and 1000 $mg.L^{-1}$) were tested to identify LC_{50} values. The results were analyzed using Statgraphics software.

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6. Authors contributions

A. I. S. L., E. V. R. C., J. L. O. and L. F. F., performed nanoparticles preparation and characterization; A. I. S. L, M. G. and R. L., performed *in vitro* biological assays. A. I. S. L, R. F. C. and V. L. S. S. C. performed *in vitro* acute toxicity test with *Artemia salina.* All authors corrected the manuscript and L. F. F performed the final adaptations.

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Notes

The authors declare no competing financial interest.

Figure Captions

Figure 1. Scheme for zein nanoparticles preparation containing botanical compounds.

Figure 2. Physicochemical parameters and stability evaluation of zein nanoparticles containing botanical compounds (eugenol and garlic oil). The stability evaluation was performed for 90 days. (A) Mean diameter (nm) by DLS; (B) mean diameter (nm) by NTA; (C) polydispersity index; (D) zeta potential (mV). All analyses were performed in triplicate, at 25 ºC.

Figure 3. Size distribution graphs for the nanoparticles analyzed by DLS (A) and NTA (B) techniques. Morphology observed by AFM for (C) zein nanoparticles containing eugenol (ZNE), (D) zein nanoparticles loaded with garlic oil (ZNG) and (E) zein nanoparticles with mixture botinical compounds (ZNEG).

Figure 4. Stability of the botanical compounds during storage. The analyses were performed by HPLC, in triplicate. The percentages of the botanical compounds are shown for the emulsions and the nanoparticles, being: A) Emulsions and nanoparticles containing the active compounds isolated B) Emulsions and nanoparticles containing the mixture of active compounds, eugenol (EGL) and garlic oil (GO).

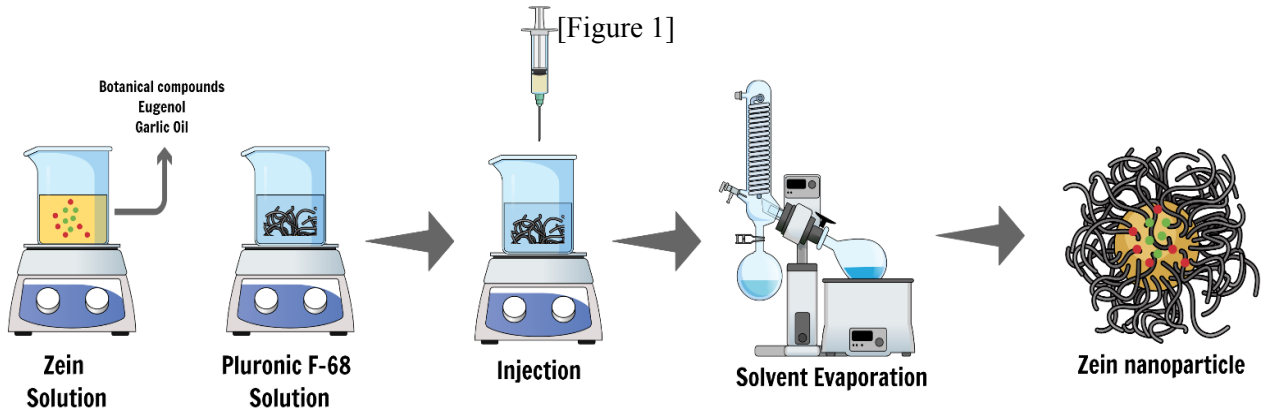
Figure 5. Mean halo diameters (cm) obtained for treatment of the bacteria *Aeromonas hydrophila* (A), *Edwardsiella tarda* (B), and *Streptococcus iniae* (C) after exposure to the different formulations: Positive control (C), Pluronic F-68 Surfactant (PLU), surfactant and eugenol (PLUE), surfactant and garlic oil (PLUG), surfactant plus eugenol and garlic essential oil (PLUEG), nanoparticles control (ZNP), control zein nanoparticles (ZNP), zein and eugenol nanoparticles (ZNE), zein nanoparticles and garlic essential oil (2B), zeina nanoparticles containing eugenol and garlic essential oil (0.5) (ZNEG). The values are the means of three determinations. Statistically significant differences (one-way ANOVA) for the different treatments are indicated by α*, ψ*,β* and φ* corresponding to negative control (C), PLUE, PLUG and PLUEG. Significance level: $p < 0.05$.

Table Captions

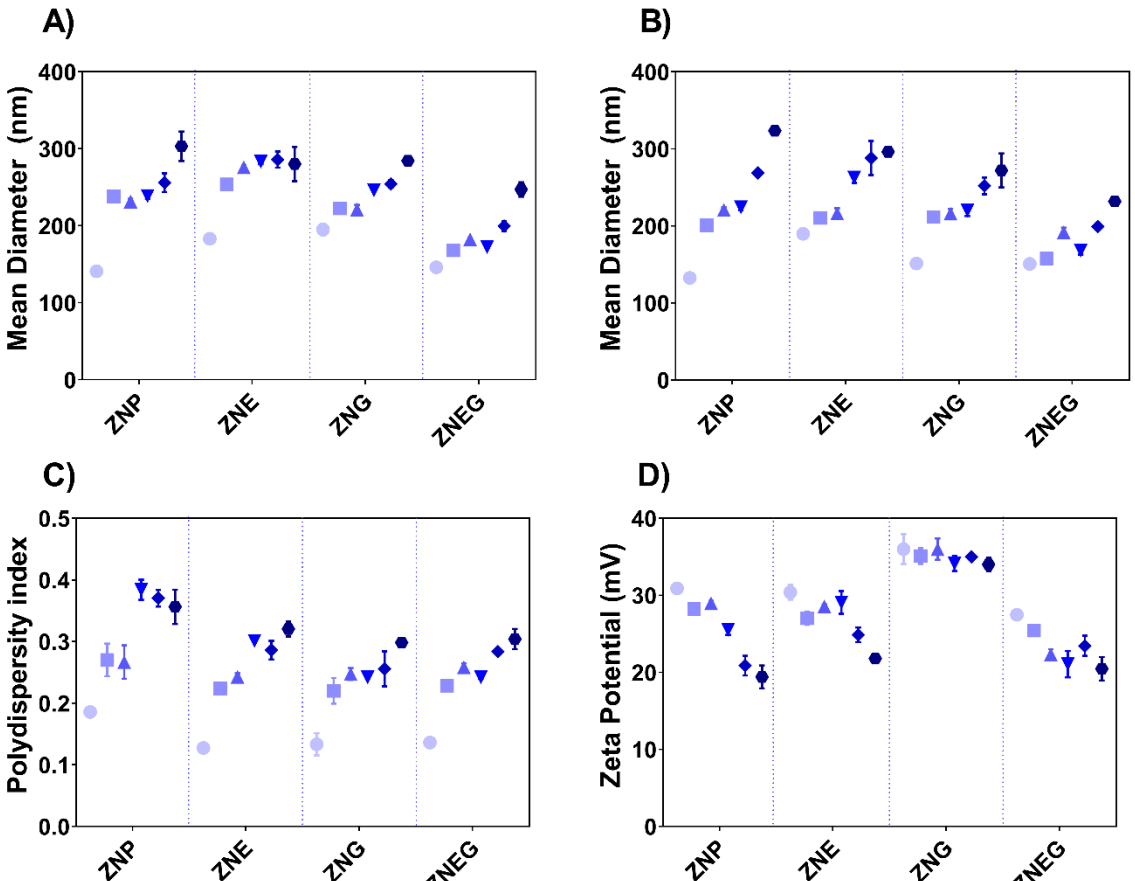
Table 1. Physicochemical characterization of the control nanoparticles (ZNP), nanoparticles loaded with eugenol (ZNE), nanoparticles loaded with garlic oil (ZNG) and the nanoparticles loaded with eugenol and garlic oil (ZNEG). The parameters analyzed were: mean diameter (MD) measured by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA), polydispersity index (PDI), zeta potential (ZP), nanoparticle concentration (CT), and encapsulation efficiency (EE).

Table 2. LC_{50} concentration for emulsified and nanoencapsulated botanical compounds evaluated in *Artemia salina* species

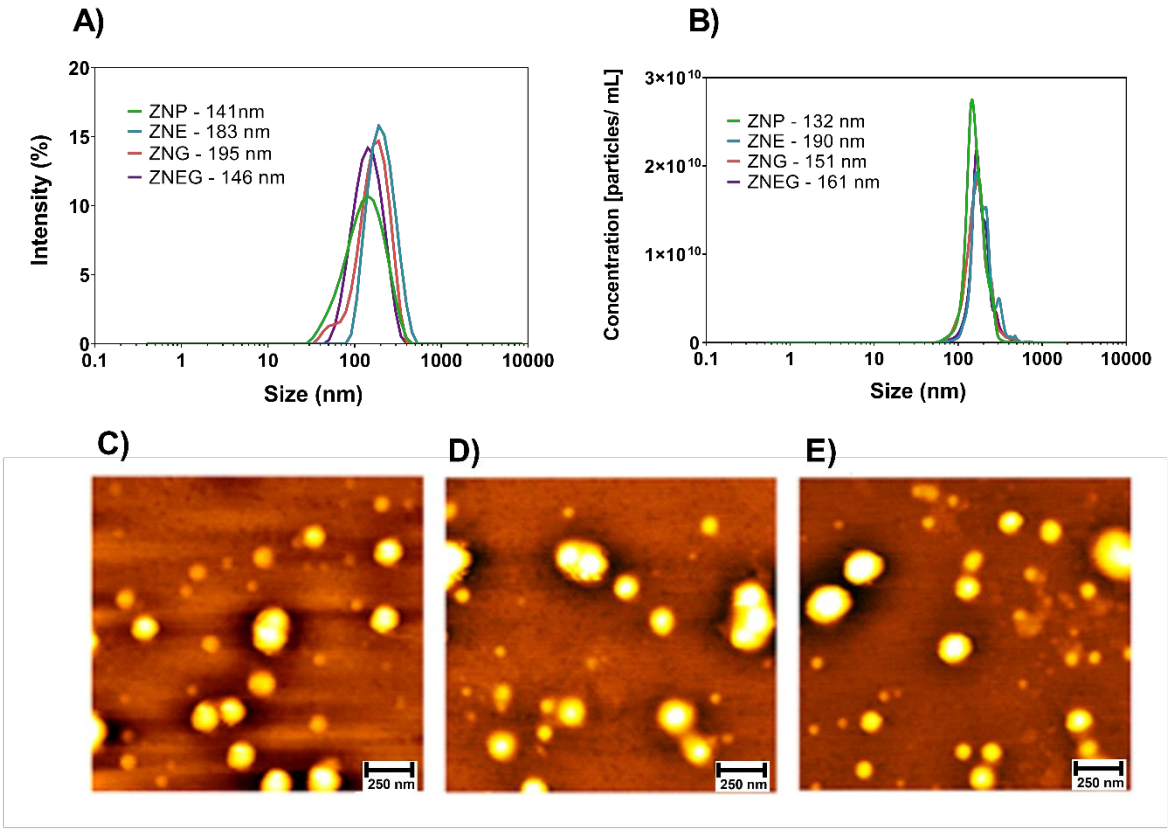
[Figure 1]



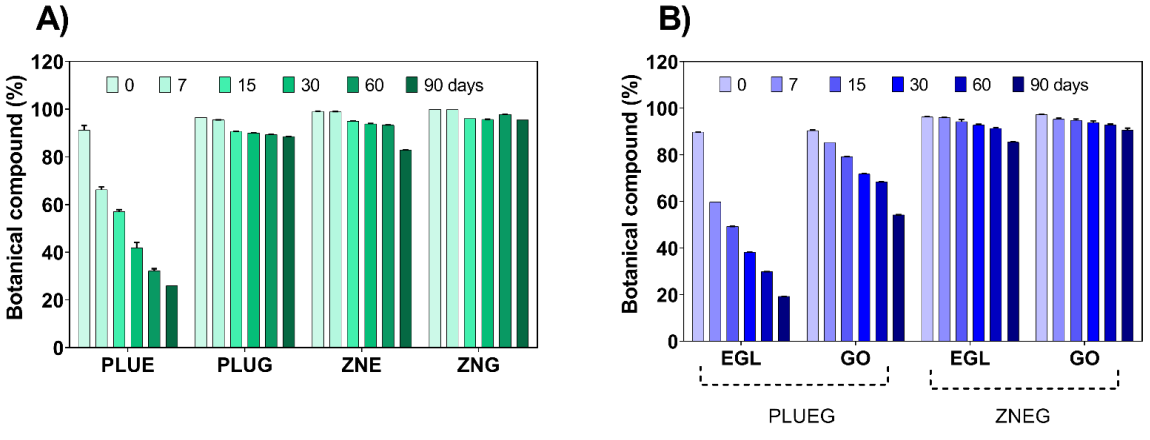
[Figure 2]



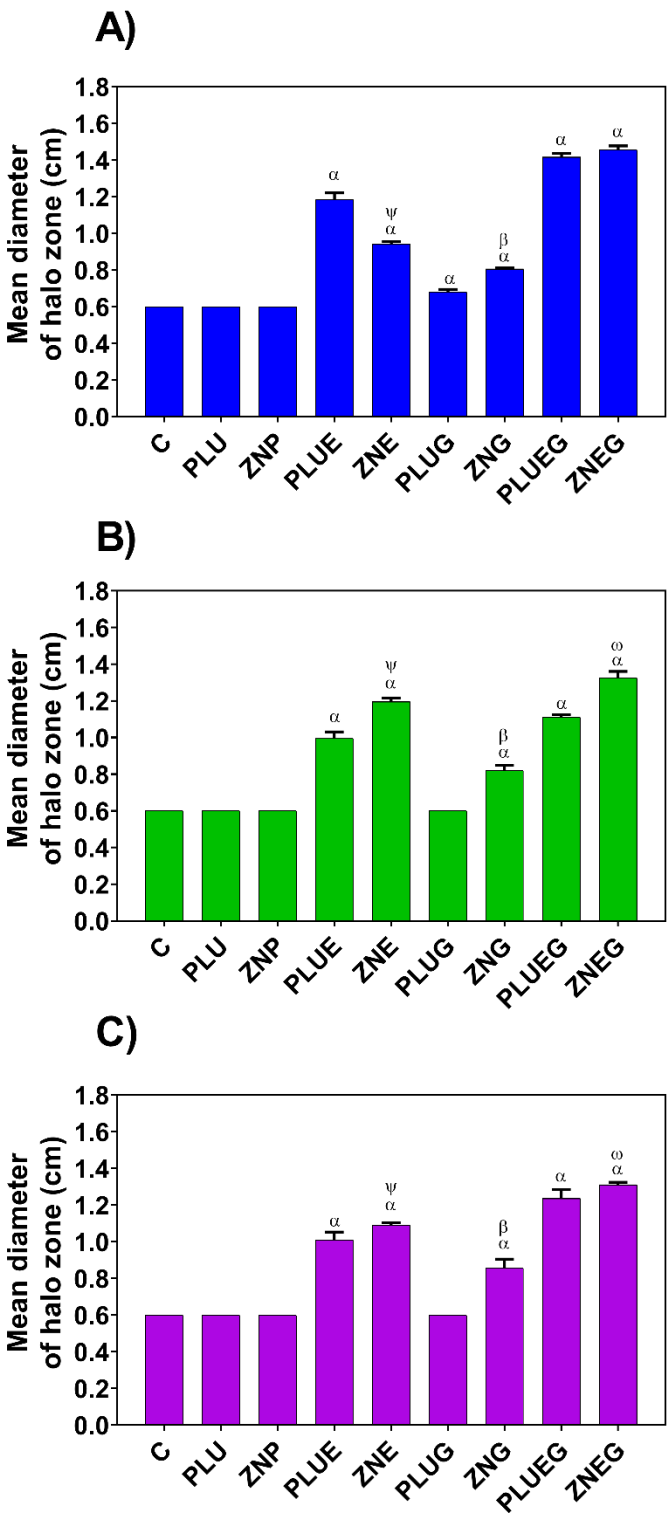
[Figure 3]



[Figure 4]



[Figure 5]


A)
B)
C)
Mean diameter of halo zone (cm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
PLU
ZNP
PLUE
ZNE
PLUG
ZNG
PLUEG
ZNEG
α
ψ α
β α
ω α

[Table 1]

Formulations	MD (nm)		PDI	ZP (mV)	CT (x10^{12} particles/mL)	EE (%)
	DLS	NTA				
ZNP	141 ± 1	132 ± 2	0.185 ± 0.04	31 ± 2	4.9 ± 0.9	-
ZNE	183 ± 3	190 ± 4	0.127 ± 0.005	31 ± 1	3.55 ± 1.9	99.1 ± 0.9
ZNG	195 ± 7	151 ± 2	0.133 ± 0.010	36 ± 2	3.82 ± 1.3	99.8 ± 0.2
ZNEG	146 ± 7	161 ± 4	0.136 ± 0.070	27 ± 1	3.9 ± 0.5	E 96.3 ± 0.1 G 97.2 ± 0.02

*E: eugenol; G: garlic oil

[Table 2]

Treatment	LC_{50} (mg L^{-1})
PLUE	2.27 (1.32-2.65)
PLUG	2.01 (1.71-2.37)
PLUEG	1.72 (1.38-2.43)
ZNE	6.23 (5.52-6.93)
ZNG	61.22 (52.79-70.07)
ZNEG	5.82 (4.10-5.33)

*Emulsions of Pluronic with eugenol (PLUE), Emulsions of pluronic with garlic oil (PLUG), Emulsions of Pluronic with mixture (PLUEG), zein nanoparticles with eugenol (ZNE), zein nanoparticles with garlic oil (ZNG) and zein nanoparticles co-loaded with eugenol and garlic oil (ZNEG).

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