Synthesis and Characterization of Chitosan Nanoparticles Loaded Botanical Extracts with Antifungal Activity on *Colletotrichum gloeosporioides* and *Alternaria* species

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Abstract

In this study, chitosan nanoparticles (CSNPs) and chitosan nanoparticles-botanical extracts: EEA-CSNPs (ethanolic blueberry extract added chitosan nanoparticles) and EMN-CSNPs (extract methanol of nanche added chitosan nanoparticles) were characterized and evaluated *in vitro* ongrowth of *Alternaria alternata* isolated from Fig and Rosemary and *Colletotrichum gloeosporioides* isolated from Papaya and Soursop. From particle size distribution characterization, the size of nanoparticles increased after EEA incorporation. On the other hand, the smallest value of Z-average particle size was for the EMN-CSNPs. Zeta potential value decreased for CSNPs and EEA-CSNPs. However, when EMN is incorporated to CSNPs, the value is increased. From the results, it can be seen that the most stable suspension was EMN-CSNPs. After incorporation of *Byrsonima crassifolia* to CSNPs, no changes were observed in characteristic absorption bands for the FTIR spectra. However, after *Vaccinium corymbosum* incorporation to the CSNPs, changes were seen. For *in vitro* evaluation, CSNPs without EEA caused the total germination and sporulation inhibition of *A. alternata* from Rosemary. Incorporation of EMN to CSNPs improved the control of *C. gloeosporioides* with amycelial growth inhibition of 79% isolated from papaya and 82% isolated from soursop. In both isolated there were total germination inhibition. Overall, a synergistic effect between the chitosan and EMN was observed.

Keywords

Zeta Potential, *In Vitro*, Fungi, Blueberry, Nanche

1. Introduction

The main agents associated with deterioration and post-harvest losses in a wide variety of horticultural products, affecting their commercialization are *Colletotrichum gloeosporioides* and *Alternaria alternata*. An alternative for the control of phytopathogenic fungi is the synthesis of nanoparticles from chitosan. Nanoparticles are known to be more reactive and therefore more efficient in their antimicrobial activity [1] [2] due to the large area of contact with the microbial membrane and consequently the agglomeration on the surface of the cell wall of the fungus [3]. Among the existing methods for the synthesis of nanoparticles, the method of nanoprecipitation (solvent displacement), has many advantages. It is fast, easy to perform, and the experimental can be carried out in one step. In addition, the formation of nanoparticles is instantaneous [4] [5]. Antimicrobial compounds can be present in different plant extracts obtained from leaves, flowers, seeds, roots and stems. About this, numerous reports have demonstrated their positive effect against a great diversity of phytopathogens such as bacteria and fungi [6]. Nanche leaves (*Byrsonima crassifolia)* have fatty acids, diterpenes, phenolic compound and monoterpenes and remarkable antifungal effect in the growth inhibition [7] [8]. Blueberries (*Vaccinium corymbosum)* are known for their rich bioactive compounds, including flavonoids, phenolic acids, tannins, and anthocyanins, which individually or synergistically have biological properties [9] [10]. Chitosan polysaccharide has taken on enormous importance in the control of postharvest pathogenic microorganisms through the development of biodegradable edible coatings and films containing natural antimicrobials; it also has elicitor properties that enhance the natural defenses of fruit, vegetables and grains. Chitosan is an excellent carrier of other functional substances. It has been use for encapsulation of different compounds such as essential oils [11], RNA [12], antibiotics [13], drugs for cancer therapy [14], nutraceuticals [15] and vitamins [16], among others, potentializing the combined properties of the encapsulating agent and chitosan. Therefore, the incorporation of blueberry fruit extracts and nanche leaves extracts into chitosan nanoparticles may enhance the antimicrobial function. The aim of this work was to characterize and study the antifungal activity of chitosan nanoparticles incorporated with botanic extract for use in coatings or packaging in commodities postharvest. This is the first time that incorporation of botanic extracts into chitosan nanoparticles and their combined antifungal activity has been reported in the literature.

2. Materials and Methods

2.1. Materials

Medium molecular weight chitosan (deacetylation degree 75% - 85%; Sigma-Aldrich) was used for the preparation of chitosan nanoparticles. Glacial acetic acid and methanol were purchased from Fermont Chemicals Inc. Ethanol was bought from J.T. Baker.

2.2. Methods

2.2.1. Nanoparticles Synthesis

Nanoparticles were synthesized following the procedure of [11]. Briefly, chitosan (0.05% w/v) was dissolved in acetic acid (1% and 2% v/v) to obtain the solvent phase. Therefore, 5 mL of this phase was dropped to the non-solvent phase (80 mL of methanol) by using a peristaltic pump (Cole Parmer, Model Masterflex C/L) and kept under stirring. Once the nanoparticles solution was formed, it was placed in a rotavapor at 40°C witha speed of 50 rpm (BüchiRotavapor R-114, Heating Bath B-491) until methanol was removed. Finally, it was stored under refrigeration at 4°C. For the nanoparticles with the botanicalextract, it was added to the non-solvent phase (methanol and ethanol) at a concentration of 5%.

2.2.2. Preparation of Extracts

This study was carried out in the Biotic Products Development Center in Yautepec, state of Morelos, México. Two different plant species reported with medicinal properties were evaluated for their antifungal activity: *Byrsonima crassifolia* (L.) Kunth, and *Vaccinium corymbosum.* They were collected in Yautepec within the state of Morelos, in this sampling site the climate is wet tropical with annual precipitancy of 754.6 to 1187 mm. Once harvested, leaves and fruits were sorted, discarding damaged or diseased material. Plant material was dipped in 1% sodium hypochlorite, rinsed with distilled water, air-dried, macerated with the aid of a blender and a grinder and stored in amber bottles until further use.

Leaves powders (50 g) were extracted with methanol (*B.crassifolia*) and fruits of *V. corymbosum* in ethanol with 500 ml for 24 h in each solvent at room temperature according to [17]. After extraction step, the leaves and fruits extracts were filtered and concentrated in a rotary evaporator (Buchi R-114, LabortechnikFlawil, Switzerland) and then stored at 4°C in amber bottles until use.

2.3. Nanoparticles Characterization

2.3.1. Zeta Potential

The zeta potential and size distribution of chitosan nanoparticles (CSNPs) and chitosan nanoparticles-botanical extracts: EEA-CSNPs (ethanolic blueberry extract added chitosan nanoparticles) and EMN-CSNPs (extract methanol of nanche added chitosan nanoparticles) was analyzed DLS (Dynamic Light Scattering) using a Zetasizer Nano-ZS90 (Malvern Instruments). 3 mL of sample were placed in a quartz cuvette and analyzed.

2.3.2. Confocal Raman and Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectrum of the plant extracts, chitosan nanoparticles and chitosan nanoparticles-botanical extract were obtained by using a Confocal Micro Raman equipped with an ATR module of Selenium Zinc from 650 - 4000 cm^{-1}.

2.4. Test Microorganism and Antifungal Activity

Colletotrichum gloeosporioides were isolated from *Carica papaya* L. and *Anno-*

na muricata L., *Alternaria alternata* from *Ficuscarica* and *Rosmarinus officinalis*at Morelos, México and the isolates were maintained on Potato Dextrose-Agar (PDA) in petri plates at temperature 28˚C. To maintain pathogenicity of the fungus, periodic inoculations and reisolations from infected fruitsand leaves were carried out.

Nanoparticles synthesized were added to PDA after sterilization media and poured into Petri plates (60 × 15 mm). A five-mm agar disc of a 9 days old colony of the pathogen was placed at the center of each plate and incubated at 28˚C for 8 to 14 days in the dark. The colony diameter was recorded for each treatment until fungal colonies in the control treatment reached the edge of the plate. The percentage of inhibition was% I = [(C - T)/C] × 100 where C represents the growth in the non-amended control and T in the treatment. For *in vitro* studies sporulation and germination were measured as previously described [18]. There were six replicates for each treatment. Control Petri plates contained only PDA and Sportak as commercial fungicide was used for comparison.

2.5. Statistical Analysis

A completely randomized design was used for statistical analysis. One-way analysis of variance (ANOVA) with a significance level of $P < 0.05$ was applied. Similarly, when significant differences were found, a comparison of means was performed using Tukey's multiple comparison test. A confidence interval of 95% was employed. The analysis was performed using a SigmaStat 3.5 program.

3. Results

3.1. Zeta Potential

Figure 1 shows the size distribution profiles for chitosan nanoparticles and the nanoparticles added with the botanical extracts. In Figure 1(a), the size distribution for CSNPs is observed. Z-average particle size obtained was 406.6 ± 25.5 nm and Zeta potential value was −12.3 to −12.9. Figure 1(b) and Figure 1(c) show the profiles for chitosan nanoparticles added with the botanical extracts having a Z-average particle size of 521.8 ± 85.9 nm and 304.2 ± 31.7 nm and Zeta potential values of −4.85 to −7.86 mV and −40.0 to −43.8 mV for EEA-CSNPs and EMN-CSNPs, respectively.

Comparing the size distribution of CSNPs, EEA-CSNPs and EMN-CSNPs it can be seen, that the narrower size distribution was for EEA-CSNPs, followed by EMN-CSNPs and finally the CSNPs. On the other hand, the smallest value of Z-average particle size was for the EMN-CSNPs (304.2 ± 31.7 nm), followed by CSNPs (406.6 ± 25.5 nm) and finally EEA-CSNPs (521.8 ± 85.9 nm).

Zeta potential value for CSNPs was −12.3 to −12.9 mV. When EEA-CSNPs is added to CSNPs Zeta potential value decreased (−4.85 to −7.86 mV). However, when EMN-CSNPs are incorporated to CSNPs, the value is increased (−40.0 to −43.8 mV). Addition of bioactive components to chitosan solutions, affects the
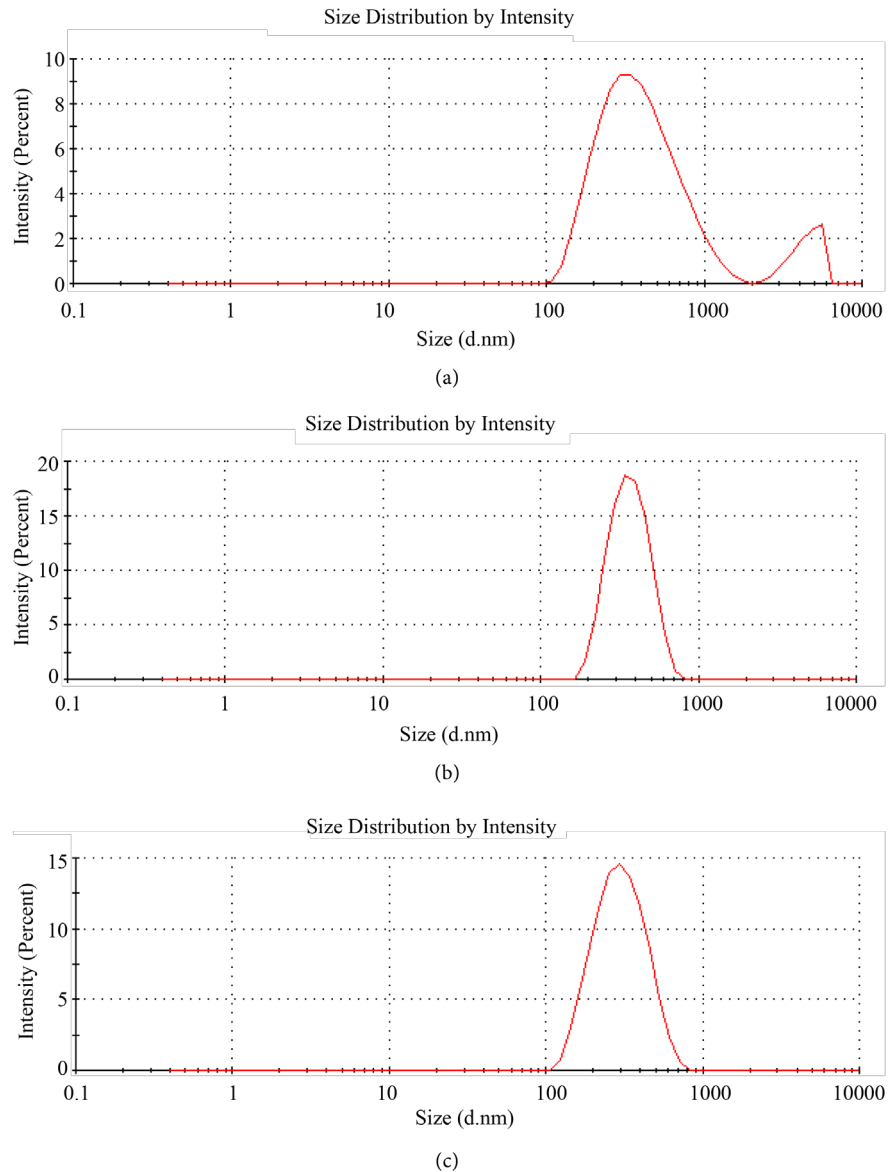
Figure 1. Zeta potential distribution for: (a) CSNPs; (b) EEA-CSNPs and (c) EMN-CSNPs.

surface positive charges of chitosan changing the stability of the nanoparticles. It has been reported in the literature, that a stable suspension must have a Zeta potential minimum value of ± 30 mV [19]. From the results, it can be seen that the most stable suspension was EMN-CSNPs. Also, the lowest particle size (304.2 ± 31.7 nm), was obtained for EMN-CSNPs.

3.2. Confocal Raman and Fourier Transform Infrared Spectroscopy (FTIR)

In Figure 2, the FTIR spectra of the CSNPs, botanical extracts and CSNPs loaded with the different botanical extracts are observed. In Figure 2(a) results for ethanolic extract from *Vaccinium corymbosum* are shown, and in Figure

2(b) results for methanolic extract from *Byrsonima crassifolia* are seen.

The characteristic absorption bands for CSNPs are: between 3750 and 2750 cm^{-1} related to -CH, -OH and -NH stretching, at 2921 cm^{-1} corresponding to -CH, at 1635 cm^{-1} related to amide I (-NH$_2$), at 1375 cm^{-1} for -CH deformation and at 1027 cm^{-1} corresponding to stretching vibrations of C-O-C for the aromatic ring. The band between 878 and 676 cm^{-1} belongs to pyranoside ring (**Figure 2(a)** and **Figure 2(b)**).

On the other hand, in **Figure 2(a)** for the EEA, the characteristic absorption
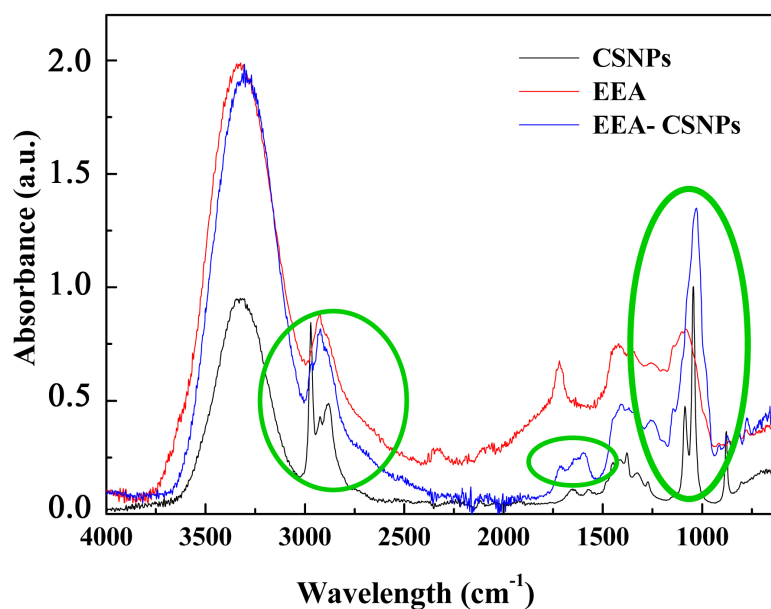
(a)
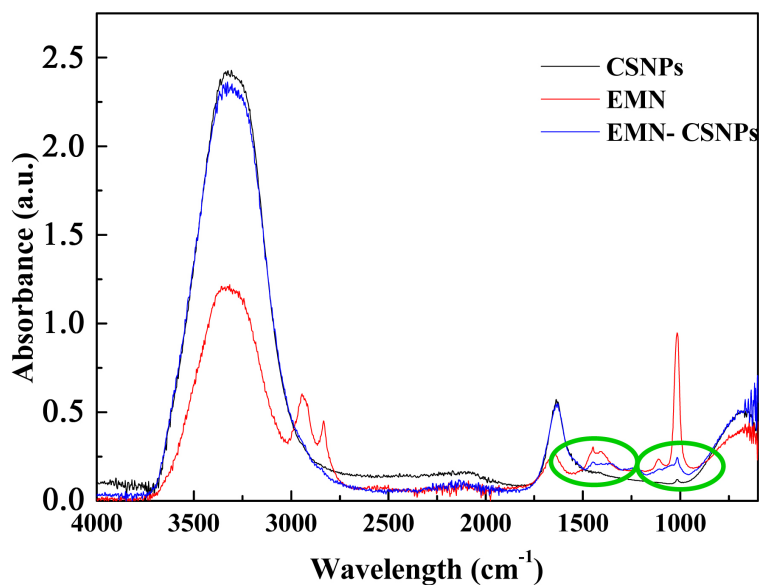
(b)

Figure 2. FTIR spectra of (a) CSNPs, EEA and the EEA-CSNPs; and (b) CSNPs, EMN and EMN-CSNPs.

bands are almost similar, only varying in intensity. They main peaks are: between 3750 - 3100 cm^{-1} for-CH and -OH, at 2900 cm^{-1} corresponding to -CH, at 1717 and 1594 cm^{-1} for -C=O and at 1079 cm^{-1} for C-O-C.

After *Vaccinium corymbosum* incorporation to the CSNPs (**Figure 2(a)**). The bands at around 2900 cm^{-1} corresponding to -CH, the bands at 1717 and at 1594 cm^{-1} for -C=O and the band at 1079 cm^{-1} for C-O-C were broadened (green circle), indicating EEA incorporation into the CSNPs. However, after incorporation of the EMN to CSNPs, for *Byrsonima crassifolia* small changes (green circle)were observed in characteristic absorption bands in the FTIR spectra (**Figure 2(b)**).

3.3. Antifungal Activity

Table 1 shows the results *in vitro* evaluations of chitosan C (2%) and the CSNPs and EEA-CSNPs (0.05%) on *A. alternata* development after 8 - 14-day incubation at 28˚C. The results obtained were significantly ($P < 0.05$) different among treatments, the CSNPs and EEA-CSNPs (250 and 500 µl) shown high micelial inhibition and germination inhibition on *A. alternata* from Fig and Rosemary. The highest sporulation was obtained in CS treatment (9.3×10^7 spores/ml) followed by the EEA-CSNPs (250 to 500 µl) treatments with few spore formation (0.33 a 1.57×10^7 spores/ml) and CSNPs (500 µl) treatments shown 0.00 spores/ml in *A. alternata* from Rosemary. Similar results were published by [20] which tested the antimicrobial activity of chitosannanoparticles with and without thyme essential oil against *C. gloeosporioides*. The results showed that oil addition caused the total inhibition of this fungus. In this work, the results showed that CSNPs (500 µl) without EEA caused the total germination inhibition of *A. alternata* from Rosemary.

Table 1. Effect of chitosan, chitosan nanoparticles and chitosan nanoparticles added with ethanolic extract of cranberry fruits, in two isolates of *A. alternata* (Fig) and(Rosemary) after an incubation period of 7 - 8 days at 28˚C.

Treatments	*A. alternata*			
	Fig	Rosemary		
	Mycelial inhibition (%)	Micelial inhibition (%)	Germination inhibition (%)	Sporulation (spores ml^{-1})
PDA	0.0**a**	0.0**a**	26.0**a**	1.63×10^8**a**
CS 2%	6.0**a**	1.2**a**	37.5**a**	9.3×10^7**b**
CSNPs 0.05% (250 µl)	50.2**b**	47.7**b**	41.1**a**	1.7×10^7**c**
CSNPs 0.05% (500 µl)	83.1**c**	83.0**c**	100**c**	0.00**d**
EEA-CSNPs 0.05% (250 µl)	50.3**b**	43.8**b**	45.2**ab**	1.57×10^7**c**
EEA-CSNPs 0.05% (500 µl)	83.3**c**	76.5**c**	75.1**b**	1.33×10^6**c**

C = chitosan; CSNPs = chitosan nanoparticles; EEA = ethanolic blueberry extract. Mean in the same column followed by different letters are statistically different according to the Tukey test ($P < 0.05$).

Table 2 shows the results *in vitro* evaluations of chitosan CS (2%) and the CSNPs and EMN-CSNPs (0.05%) on *C. gloeosporioides* from papaya and soursop development after 8 - 14-day incubation at 28˚C. The results obtained were significantly ($P < 0.05$) different among treatments. The PDA and CS control showed the lowest mycelial inhibition, EMN-NPQ with 500 and 750 μl and the commercial fungicide showed the highest mycelial inhibition. A dose response effect was observed in the treatments of CSNPs and EMN-CSNPs as the volume increase, increased the mycelial inhibition in both isolates of *C. gloeosporioides*. Inhibition of germination was 0% to 11% in the treatments with CS and CSNPs, with the treatment of EMN-CSNPs and the fungicide of 47% to 100% inhibitionon *C. gloeosporioides* from papaya, similar values were obtained over *C. gloeosporioides* from soursop. Chookhongkga, Sopondilok, and Photchanachai [21] evaluated the effect of chitosan nanoparticles on mycelial growth of *Rhizopus* sp., *C. capsici*, *C. gloeosporioides* and *A. niger*, finding lower mycelial growth of 2.8, 2.2, 2.4 and 5.5 mm, respectively, at 0.6% concentration. Other studies showed that applying chitosan nanoparticles at 0.1% inhibited mycelial growth of *A. alternata*, *Macrophomina phaseolina* and *Rhizoctonia solani* [22]. Zahid, Alderson, Ali, Maqbool, y Manickam [23] reported that low molecular weight chitosan nanoparticles at 1% concentration had the best inhibitory effect on conidial germination.

4. Discussion

From the above results, it can be seen that CSNPs were more effective than

Table 2. Inhibition of growth of two isolates of *C. gloeosporioides* (papaya and soursop), after an incubation period of 7 - 8 days at 28˚C, in nutrient medium with chitosan, chitosan nanoparticles and chitosan nanoparticles added with extract Methanol from nanche leaves.

Treatments	C. gloeosporioides			
	Papaya		Soursop	
	Mycelial inhibition (%)	Germination inhibition (%)	Mycelial inhibition (%)	Germination inhibition (%)
PDA	0.0a	0.0a	0.0a	0.0a
CS 2.5%	9.4b	0.0a	9.0a	2.0a
CSNPs 0.05% (250 μl)	19.4b	0.0a	33.0b	7.0b
CSNPs 0.05% (500 μl)	53.4c	0.0a	49.0c	8.0b
CSNPs 0.05% (750 μl)	57.0c	11.0b	59.4d	7.0b
EMN-CSNPs (250 μl)	44.0d	47.0c	52.8dc	10.0b
EMN-CSNPs (500 μl)	71.2e	100d	82.0e	100c
EMN-CSNPs (750 μl)	79.6f	100d	82.0e	100c
Sportak 0.01 ml	80.0f	100d	82.0e	100c

CS = chitosan; CSNPs = nanoparticles of chitosan; EMN = extract methanol of nanche.

EEA-CSNPs in the inhibition of *A. alternata* in Fig and Rosemary. On the other hand, EMN-CSNPs were more effective than CSNPs for the mycelial and germination inhibition of *C. gloeosporioides* from Papaya and Soursop.

Particle size and Zeta potential are important parameters that affects the antimicrobial properties of nanoparticles. It has been reported in the literature that the plasma membrane of fungi is the most susceptible place of attack of chitosan nanoparticles causing leakage of cellular components, or acting as a chelating agent causing the unavailability of nutrients necessary for growth of fungi or binding of nanoparticles to fungi DNA [24]. Then, small nanoparticles with Zeta potential near ± 30 mV are more effective in fungi attack [15] [19] (Zhang *et al.*, 2016; Müller *et al.*, 2001).

From the experimental results, CSNPs were smaller in size (406.6 ± 25.5 nm) and had a Zeta potential value (−12.3 to −12.9 mV) is not nearest to ± 30 mV compared to EEA-CSNPs whose average particle size was 521.8 ± 85.9 nm and Zeta potential was −4.85 to −7.86 mV. On the other hand, average particle size of EMN-CSNPs (304.2 ± 31.7 nm) was smaller than for CSNPs (406.6 ± 25.5 nm) and Zeta potential value was (−40.0 to −43.8 mV) which is nearest to ± 30 mV, compared to the value obtained for CSNPs (−12.3 to −12.9 mV). Therefore, due to particle size and Zeta potential values obtained, more antifungal activity was found for CSNPs in comparison to EEA-CSNPs, against *A. alternata*, and for EMN-CSNPs in comparison to CSNPs, agaisnt *C. gloeosporioides.*

5. Conclusions

The smallest value of Z-average particle size was for the EMN-CSNPs, followed by CSNPs and finally EEA-CSNPs. Addition of bioactive components to chitosan solutions, affects the surface positive charges of chitosan changing the stability of the nanoparticles.

EEA incorporation was evidenced by FTIR. For *in vitro* evaluation CSNPs without EEA caused the total germination inhibition of *A. Alternata* from Rosemary. The incorporation of EMN to CSNPs improved the control of *C. gloeosporioides* isolated from papaya and soursop showing a synergistic effect between the chitosan and the EMN.

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