

Communication

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Communication

Enhanced Inhibitory Effect of Chitosan-Ag Nanoparticles Hydrogel on *Alternaria alternata* in Jujube

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Abstract: Silver ion (Ag⁺) are recognized for their potent antibacterial properties; however, their utilization in food preservation is restricted by factors such as inadequate stability, a transient antibacterial impact, and the potential for human absorption. Hydrogel-based antibacterial agents are capable of exerting their antibacterial effects by establishing appropriate microenvironments, which facilitate the efficient delivery of antimicrobial molecules to the contaminated regions of food. In previous studies, we observed that chitosan (CS) and silver nitrate (AgNO₃) were able to reduce Ag⁺ to silver nanoparticles (AgNPs) with enhanced antibacterial properties. This not only significantly extended the stability of the antibacterial agent but also demonstrated exceptional inhibitory capabilities against pathogenic bacteria, including common grape contaminants such as *P. expansum*, *A. niger*, and *B. cinerea*. Utilizing ultrasound-induced complexation technology, this study has successfully synthesized a chitosan-silver (CS-AgNPs) hydrogel. The efficacy of this hydrogel against the pathogenic fungus responsible for jujube black spot disease has been thoroughly investigated. The morphology structures of CS-AgNPs hydrogels were characterized by TEM, FTIR and UV-vis. The results indicated that AgNPs ranging from 80 to 110 nm were uniformly dispersed within the gel matrix and remained stable for extended periods at room temperature. In vitro antibacterial assays and fractional inhibitory concentration index (FICI) studies revealed that the pronounced antibacterial efficacy of the CS-AgNPs hydrogel was primarily attributed to the additive antibacterial effect of CS and AgNPs. These insights underscore the significant potential of CS-AgNPs hydrogels in combating fungal diseases of jujube, offering innovative strategies and materials for the management of agricultural pathogens.

Keywords: chitosan-Ag nanoparticles; jujube; black spot disease; FICI; additive effect

1. Introduction

The jujube black spot disease, caused by the pathogen *Alternaria alternata* (*A. alternata*), poses a significant threat to human health due to the production of harmful secondary metabolites. A recent survey indicated that the disease prevalence was below 10% in early August. Nonetheless, the situation escalated sharply, with the infection rate surging to 20-30% just before the harvest. During

the post-harvest period, the disease incidence surged to over 50% within a mere 30 days, leading to devastating losses. Therefore, it is imperative to implement strategies during the post-harvest storage phase of jujubes that effectively suppress the growth and reproduction of *Alternaria* fungi [1]. These interventions are pivotal for extending the shelf life and maintaining the quality of jujubes.

Chitosan (CS) and its derivatives are natural biopolymers renowned for their exceptional biocompatibility and degradability, playing crucial roles in postharvest fruit preservation and antimicrobial fields [2–4]. Nonetheless, the efficacy of chitosan in suppressing pathogenic fungi in fruits is subject to certain constraints. Its adsorption capacity is insufficient, and it faces challenges in forming a stable film, which hinders its broader application in various fields. For instance, the decay rate of jujubes coated with chitosan reached 26% after 42 days storage [5]. Zhang et al. [6] reported that chitosan exhibits an inhibitory effect on *Aspergillus* but is ineffective against *Penicillium*. Therefore, integrating chitosan with other fungal inhibitors is anticipated to more effectively reduce the incidence of black spot disease in jujubes.

The amalgamation of metal oxides or metal nanoparticles with chitosan serves as an effective strategy to enhance their antibacterial capabilities. For instance, the integration of nanomaterials such as titanium dioxide (TiO₂) [7], zinc oxide (ZnO) [8,9], silver (Ag) [10–12], and copper oxide (CuO) [13] into chitosan has been a integration, providing innovative opportunities for the innovation of antibacterial preservation materials [14]. Silver nanoparticles (AgNPs) boast superior antibacterial efficacy, exceptional thermal stability, and reduced toxicity compared to other metal nanoparticles [15–17]. Nevertheless, the direct application of AgNPs may pose potential health risks, particularly to the human liver. The researchers have explored the combination of AgNPs with various biopolymers, including starch, chitosan, and cellulose, which substantially diminishes the release of AgNPs to mitigate this [18–20]. Our research group's previous studies have established that ultrasonic treatment enables chitosan to efficiently reduce AgNO₃, thereby synthesizing silver nanoparticles (AgNPs) [21,22]. These AgNPs are subsequently encapsulated within the chitosan matrix, forming chitosan-silver nanoparticles (CS-AgNPs). This approach effectively circumvents the issue of AgNPs agglomeration and oxidation in air. Furthermore, the CS-AgNP gel has been shown to effectively inhibit the growth and reproduction of fungal pathogens such as *P. expansum*, *A. Niger* and *B. cinerea* in grapes [23].

In this study, we have utilized the in-situ generated CS-AgNPs gel for the prevention and treatment of black spot disease in jujubes during storage. The experimental results indicate that the CS-AgNPs gel exhibits a significant inhibitory effect on the genus *Aspergillus*. Additionally, a comprehensive investigation into the additive inhibitory mechanism of CS and AgNPs on fungi was conducted, employing the fractional inhibitory concentration index (FICI). This research presents a novel approach for the preparation of natural antimicrobial and preservation materials.

2. Results and Discussion

2.1. Fungal Identification

The colonies display a flat, dense, velvety structure that proliferates rapidly, initially appearing as a dark white and then transitioning to light or dark green hues (Figure 1). The morphological examination and phylogenetic tree analysis have conclusively confirmed the identification of the fungus as *A. alternata* (Figure 2).

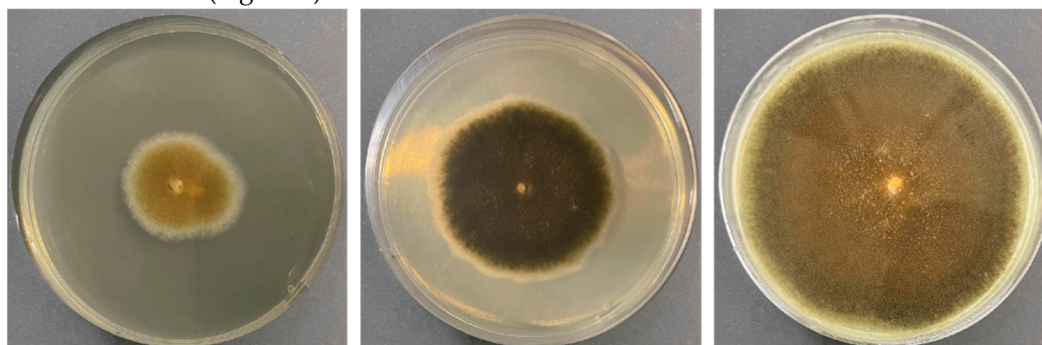


Figure 1. Identification of dominant pathogenic fungus causing black spot disease in jujube.

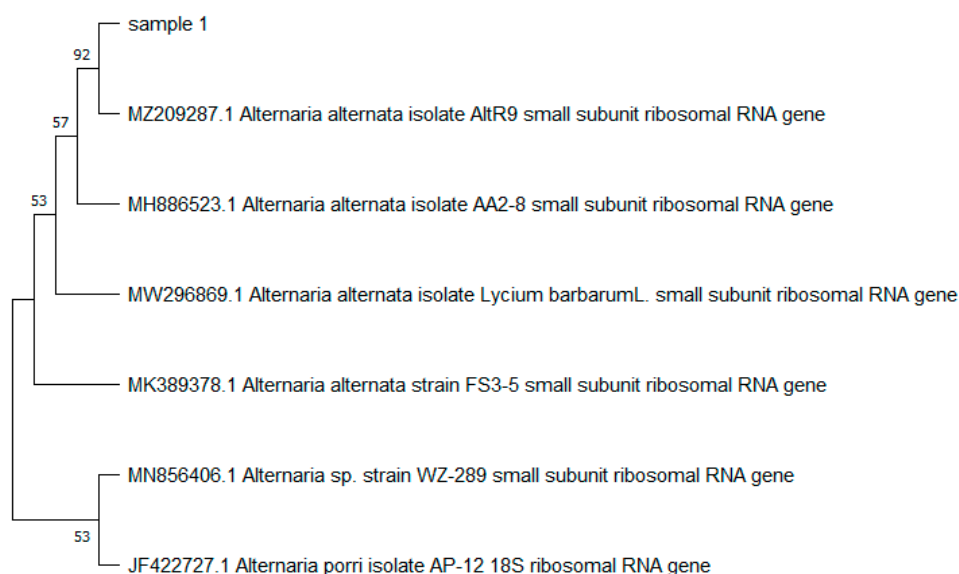


Figure 2. Fungal species identification by phylogenetic tree analysis.

2.2. CS-AgNPs Hydrogel Characterization

The CS-AgNPs hydrogel exhibited outstanding gelation properties (Figure 3a). The morphology of the AgNPs was assessed through transmission electron microscopy (TEM) images, which revealed the formation of AgNPs ranging in size from 80 to 110 nm (Figure 3b).

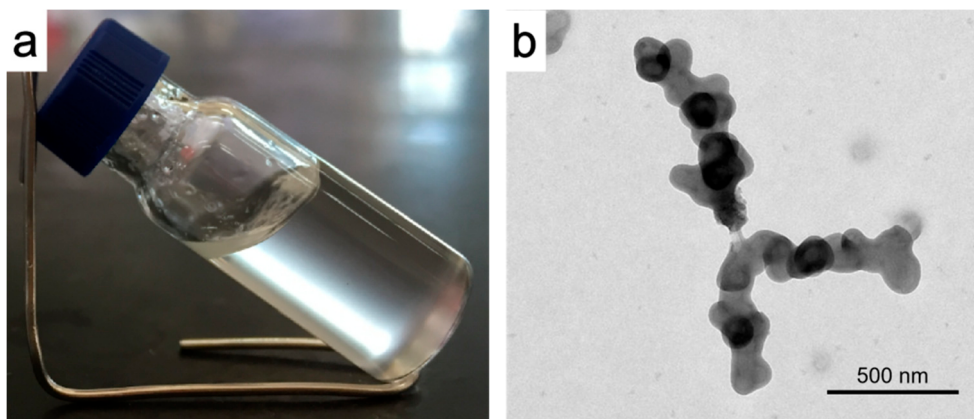


Figure 3. Image of CS-AgNPs Hydrogel (a); TEM image of AgNPs (b).

The FTIR spectra of the CS powders and the CS-AgNPs xerogels were confirmed the complexation between CS and Ag^+ . The amide band at 1600 cm^{-1} underwent a blue shift, and the C-N band at 1420 cm^{-1} a red shift from CS to CS-AgNPs xerogel (Figure 4a). These shifts revealed that both -OH and - NH_2 groups in the CS chains took part in the coordination complexation. This is consistent with previous research reports [23]. The time-dependent UV-vis absorption spectrum indicated that the process is not time dependent, which suggested a high degree of stability in the complexation between Ag^+ and CS polymer chains. (Figure 4b).

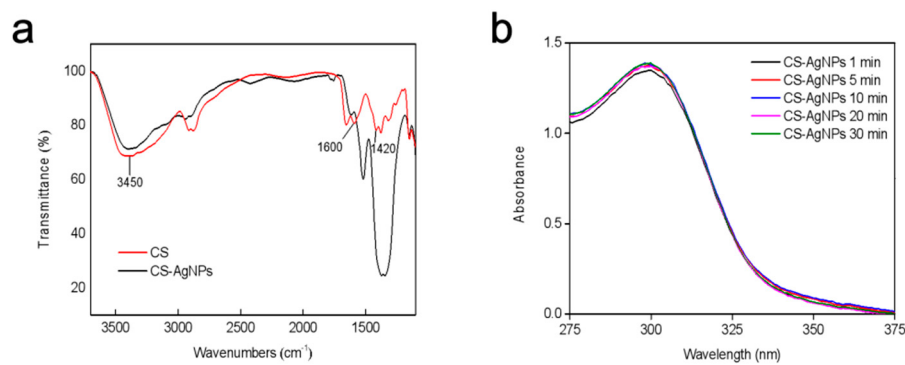


Figure 4. FTIR spectra of CS powders and CS-AgNPs xerogels (a); The time dependent UV-Vis absorption spectra of CS-AgNPs at concentration of 80 mM (b).

2.3. Inhibition of CS-AgNPs Hydrogel on *A. alternata* In Vitro

The inhibitory effects of CS, AgNO₃, and CS-AgNPs gels against the fungus *A. alternata* are depicted in Figure 5. Compared to the control group (CK), the presence of *A. alternata* was not observed on the filter paper treated with CS. Although the inhibitory effect was minimal, with the inhibition zone being barely detectable. This implies that while Alternarin cannot proliferate on CS, it does not exert a significant inhibitory influence on the fungus. The filter paper treated with Ag⁺ displayed a distinct antibacterial circle, indicating that Ag⁺ effectively inhibits *A. alternata* fungi. The CS-AgNPs gel demonstrated a substantial inhibitory zone against *A. alternata* fungi, with the zone of inhibition expanding over time, signifying a pronounced inhibitory action.

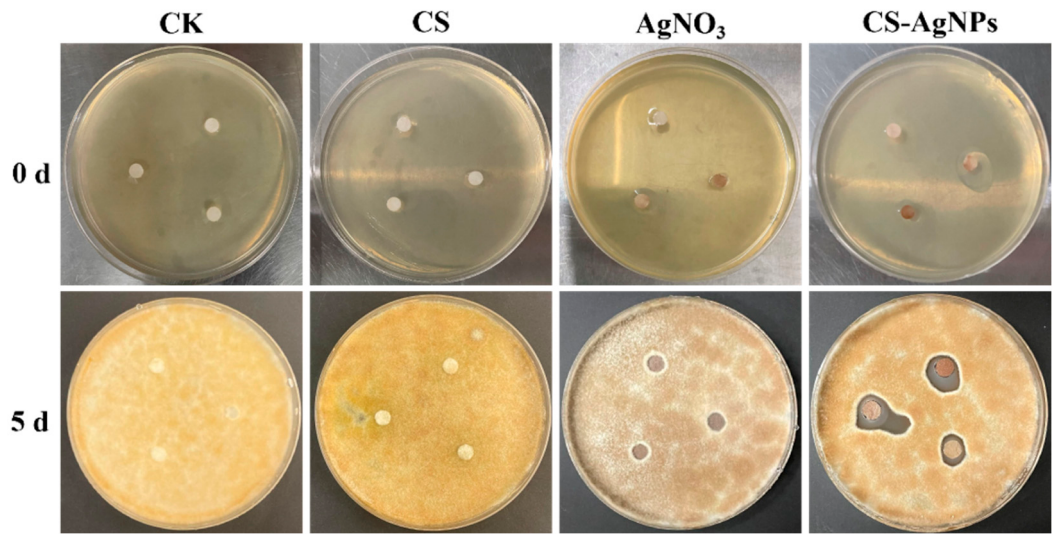


Figure 5. The antibacterial effect of different concentrations of CS-Ag xerogels bacteriostatic agent on colony growth of *A. alternata*. (0.62 wt% CS-AgNPs hydrogel, CS 1.7 mg·mL⁻¹, Ag⁺ 4.5 mg·mL⁻¹).

2.4. Quantitative Assay of the Antimicrobial Activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CS and CS-AgNPs for *A. alternata* were determined by microdilution, and the results are shown in Table 1.

Table 1. The MIC and MBC of CS and AgNPs on *A. alternata*.

Sample	MIC (ppm)	MBC (ppm)
CS	125	271
AgNO ₃	7.8	31.2

CS-AgNPs 1:3	3.8	/
CS-AgNPs 1:2	4.2	/
CS-AgNPs 1:1	5.4	/
CS-AgNPs 2:1	6.8	/
CS-AgNPs 3:1	7.1	/

The research findings clearly demonstrate that the antimicrobial effect of AgNO₃ on *A. alternata* is significantly superior to that of chitosan (CS), exhibiting a more pronounced antimicrobial efficacy. Furthermore, the inhibitory effect of CS-AgNPs was not significantly different from that of AgNO₃. According to the equation (Details in Section 3.6.2), the fractional inhibitory concentration index (FICI) was calculated based on the MIC value of the CS-AgNps mixture, and the interaction between CS and AgNps was analyzed. The change of the inhibitory concentration effect of CS-AgNps on the growth of *A. alternata* were determined. Antimicrobial testing on different proportions of AgNPs and CS solution mixture revealed FICI values ranging from 0.52 to 0.97, which indicated CS and AgNPs showed obvious additive effect instead of synergistic effect against *A. alternata*.

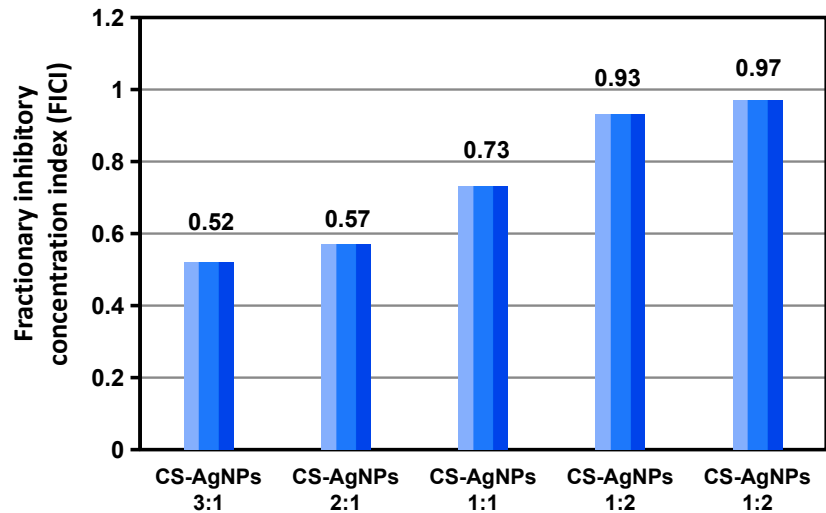


Figure 6. The variation of the fractional inhibitory concentration index of CS-AgNPs solution mixtures measured for the inhibition of *A. alternata*.

2.5. Residue of Silver in Jujube

The residual Ag⁺ content in jujubes treated with CS-AgNPs gel was measured, revealing an increasing trend in the concentration of Ag⁺ over time. The Ag⁺ concentration peaked at a saturation level of approximately 0.34 mg/kg on the 10th day, as depicted in Figure 7. It is inferred that when the Ag⁺ residue concentration is at this level, it poses no health risks to humans according to the literature reference [23].

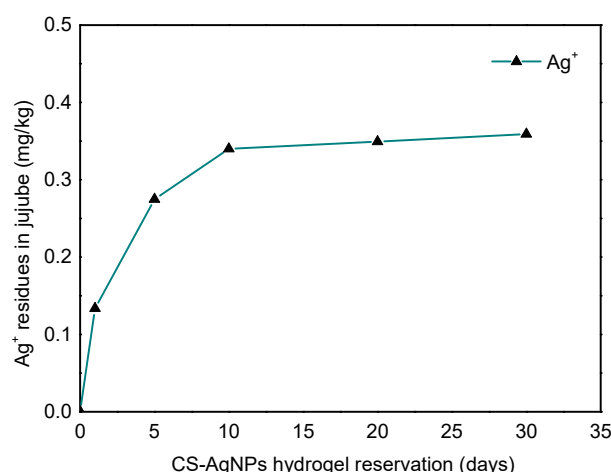


Figure 7. The change of Ag residues in jujube after CS-AgNPs Hydrogel preservation.

3. Materials and Methods

3.1. Fruit Material and Chemical Material

Jujubes were purchased from Aksu, Xinjiang, China. All fruits were free from mechanical damage or infection. The surface of the selected fruits was disinfected with 75% ethanol for 5 min, washed twice with sterile water and air-dried.

Low molecular weight chitosan (85% deacetylated) and agar powder (bacteriological grade) were procured from Sigma-Aldrich, USA. Silver nitrate (AgNO_3 , 99.8%; Energy), acetic acid (glacial, 99.100%; Sinopharm), sodium citrate (95%; Sinopharm) and sodium hydroxide (NaOH, Sinopharm) 4-nitrophenol (95%; Merck) were used as received without further purification. All other chemicals used in this study were of analytical grade.

3.2. Fungal Isolation, Identification, Culture and Spore Suspension

The procedure refers to previous literature [23]. The procedure involved washing the jujubes affected by black spot disease with sterile water, collecting the filtrate, and diluting it. Subsequently, 100 μL of the diluted solution was evenly spread onto a petri dish containing potato dextrose agar (PDA) medium and incubated at 28°C for 7 days. The fungal isolates were then purified through three rounds of subculturing on PDA.

The major isolates were obtained and characterized using macroscopic and microscopic observation methods. The isolated fungi were separately cultured on PDA at 28°C for 7 days. Spore suspensions were obtained by rinsing the cultures with sterile water containing 0.05% (v/v) Tween-80 and then adjusted to 1×10^9 CFU/L.

3.3. Characterization

UV-vis spectrum was recorded on Shimadzu UV-2550 spectrometer (Japan). Fourier transform infrared spectroscopy (FTIR) was carried out in Bruker EQUINOX-55 (Germany). Transmission electron microscopy (TEM) was taken using Hitachi H-600 (Japan) operated at 80 kV. Inductively coupled plasma-mass spectrometry (ICP-MS) was recorded on ThermoFisher iCAP Q (U.S.).

3.4. Preparation of CS-AgNPs Hydrogel

The 1 mL CS-AgNPs hydrogel was prepared by adding 100 μL 0.2 M NaOH to 800 μL 0.5 wt % CS solution (in 1 % acetic acid), followed by adding 100 μL freshly prepared 0.3 M AgNO_3 solution with vigorous shaking for 2 seconds. The Critical Gelation Concentration (CGC) of the hydrogel was

visually recognized by the reversed vial test method. Confirmation that in this case the CS-Ag complex systems have been obtained will be described below based on TEM, UV-vis and FTIR measurements.

3.5. Zone of Inhibition Test

All experimental operations are carried out in a clean workbench.

3.5.1. Preparation of Fungal Suspension

Pick up 3 needles of pathogenic fungi strains into the test tube containing 10 mL sterile water, and then shake the test tube well to make 10^{-1} fungi suspension.

3.5.2. Antibacterial Experimental Method

The research methodology adheres to the established protocols as described in previous literature [23].

Preparation of bacteriostatic tablets: Prepare the qualitative filter paper into a circular paper with a diameter of 6 mm with a punch, then put these filter paper into an autoclave at 121°C for 20 min, then dry for later use. Immerse the sterilized and dried 6 mm filter paper disc into different bacteriostatic solutions.

Fungal inoculation: inoculate 500 μ L fungal suspension on the culture medium, mix evenly, then cover the petri dish and dry it at room temperature for 5 min.

Put the filter paper that treated with antibacterial agent gently on the medium coated with bacteria by using sterilized tweezers, and put 3 pieces on each medium. The distance between the centers of each filter paper is more than 30 mm, and the distance from the edge of the petri dish is more than 20 mm. The filter paper was tightly attached to the medium, covered the petri dish was and placed at 28°C for 5 d observation

After pasting the filter paper, gently press the filter paper with tweezers to make it tightly adhere to the culture medium and cover the culture dish, place it in a 28°C incubator and culture for 5 days. Measure the diameter of the bacteriostatic circle with a vernier caliper and record the results.

3.6. Quantitative Assay of the Antimicrobial Activity

3.6.1. The Minimal Inhibitory Concentrations (MIC) and the Minima Bactericidal Concentrations (MBC)

The MIC and the MBC were determined by binary serial microdilution assay performed in 96-well microtiter plates. The sterile water was used for media, buffer preparation, and dilutions, physiological. Incubation for antibacterial assays was done in a shaker-incubator at 28°C.

In a 96-well plate setup, the first well was prepared with 100 μ L of Potato Dextrose Agar (PDA) and 100 μ L of LCS (or AgNO_3) as a sterile control. Wells 2 through 11 received 95 μ L of PDA, 50 μ L of LCS (or AgNO_3) antimicrobial agent, and 50 μ L of bacterial suspension. Well 12 contained 195 μ L of PDA and 5 μ L of bacterial suspension. The concentration of chitosan (CS) ranged from 1000 mg/L down to the MIC and MBC values of 1.95 mg/L, while the concentration of AgNO_3 ranged from 1000 mg/L to 1.95 mg/L. The plates were incubated at 28°C for 48 hours to monitor the growth and proliferation of *Alternaria* fungi, thereby establishing the lowest inhibitory concentration (MIC) and the lowest bactericidal concentration (MBC).

3.6.2. Fractional Inhibitory Concentration Index (FICI)

To assess the effects of chitosan (CS) and silver nanoparticles (AgNPs), the antimicrobial activity of a CS-AgNPs mixture with varying volume ratios was quantitatively evaluated using the MIC method. The MIC values of the individual components served as a baseline, from which the FICI was established during the MIC experiment. Subsequently, the FICI results were used to refine the effective concentration range for the synergistic action.

$$FICI = \frac{MIC (CS-AgNPs)}{MIC (CS)} + \frac{MIC (CS-AgNPs)}{MIC (AgNPs)}$$

The values of FICI are interpreted as follows [24] : synergistic ($FICI \leq 0.5$), additive ($1 \geq FICI > 0.5$), indifferent ($4 \geq FICI > 1$), antagonistic ($FICI \geq 4$).

3.7. Residue of Silver in Jujube

The research methodology adheres to the established protocols as described in previous literature [23]. Rinse and dry the fresh dates, extract the edible portions after pitting, and blend them into a smooth puree. Weigh 3.0 g of the homogenized sample into a polytetrafluoroethylene digestion tank, then added 6 mL nitric acid and 1 mL hydrogen peroxide. After digestion completed, dilute the digestion solution to 50 mL with ultrapure water before testing. The blank experiments were conducted simultaneously. All of experiments were repeated three times. ICP-MS was used to detect Ag^+ residue in jujube after CS-AgNPs hydrogel preservation.

$$X = \frac{(c-c_0) \times V \times f}{m \times 1000}$$

X — the silver content in sample (mg/kg);

c — determination value of silver in sample solution (ng/mL);

c_0 — determination value of silver in sample blank solution (ng/mL);

V — constant volume of sample (mL);

f — dilution ratio of sample;

m — the weight of sample (g).

4. Conclusions

In this paper, the CS-Ag complex hydrogels were rapidly synthesized through physical crosslinking, exhibiting exceptional stability and long-term preservation at ambient temperature. The synthesized nanoparticles exhibited a quasi-spherical morphology, with an average diameter of 80 nm and displayed commendable monodispersity, as evidenced by TEM images. FTIR spectra confirmed the presence of polymer chains attached to the metallic surface. The CS-AgNPs gel demonstrated effective disease control in jujubes, exhibiting a strong inhibitory effect on the growth of *A. alternata* in vitro studies. The antibacterial performance of CS-Ag complex can be explained as a additive effect of chitosan and silver. CS serves a dual role by not only stabilizing Ag^+ to avert oxidation and aggregation but also by converting Ag^+ into AgNPs, which possess superior antimicrobial efficacy. This transformation significantly enhanced the antimicrobial capabilities of the CS-AgNPs gel. Antimicrobial assays revealed FICI values ranging from 0.52 to 0.97, indicating that CS and AgNPs exhibited a clear additive effect rather than a synergistic effect against *A. alternata*. Moreover, the application of CS-AgNPs Hydrogel not only enhances food safety by preventing fungal contamination but also ensures that no detectable residues remain after a simple water wash. This hydrogel thus improves the safety of jujubes and serves as a viable alternative to fungicides for controlling fungal diseases in fruit.

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