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Synergistic Antimicrobial Activities of Natural Essential Oils with Chitosan Films

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ABSTRACT: The synergistic antimicrobial activities of three natural essential oils (i.e., clove bud oil, cinnamon oil, and star anise oil) with chitosan films were investigated. Cinnamon oil had the best antimicrobial activity among three oils against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus oryzae*, and *Penicillium digitatum*. The chitosan solution exhibited good inhibitory effects on the above bacteria except the fungi, whereas chitosan film had no remarkable antimicrobial activity. The cinnamon oil−chitosan film exhibited a synergetic effect by enhancing the antimicrobial activities of the oil, which might be related to the constant release of the oil. The cinnamon oil−chitosan film had also better antimicrobial activity than the clove bud oil−chitosan film. The results also showed that the compatibility of cinnamon oil with chitosan in film formation was better than that of the clove bud oil with chitosan. However, the incorporated oils modified the mechanical strengths, water vapor transmission rate, moisture content, and solubility of the chitosan film. Furthermore, chemical reaction took place between cinnamon oil and chitosan, whereas phase separation occurred between clove bud oil and chitosan.

KEYWORDS: chitosan, cinnamon, clove bud, essential oil, antimicrobial activity, physical properties

INTRODUCTION

Chitosan is a natural polysaccharide derived from the deacetylation of chitin, a major component of the shells of crustacea such as crab, shrimp, and crawfish.[1] Due to its multiple functionalities, such as biocompatibility, antimicrobial properties, and excellent film-forming properties,[2−5] chitosan has attracted considerable commercial interest from the food, medical, and chemical industries and is often used as material for coating, packaging, and wound dressing.[6−8]

To improve the antimicrobial properties of chitosan films, Zivanovic et al.[9] as well as Pelissari et al.[10] incorporated oregano in chitosan film to protect against *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. Ojagh et al.[11] and Giatrakou et al.[12] used cinnamon−chitosan and thyme−chitosan coatings to protect refrigerated rainbow trout and chicken products, respectively. Sanchez-Gonzalez et al.[13] studied the incorporation of tea tree essential oil into chitosan films to protect against *L. monocytogenes* and *Penicillium italicum*. These works attempted to explain the antimicrobial efficacy of pure chitosan and chitosan with essential oils, but the synergistic effect between chitosan and essential oils and the dynamics of antimicrobial activities of the oil−chitosan film have not been investigated.

Although many studies have confirmed the antimicrobial activities of chitosan and its oligomers, some authors doubt that chitosan in the film state could have the same effective antimicrobial action compared with chitosan in solution.[9] This indicates that the features of pure chitosan film and the mechanism of its synergistic effects with other functional compounds are still ambiguous. The antimicrobial activities of chitosan are believed to depend on its surface positive charges, which can interfere with the negatively charged residues of bacterial cell surface and lead to bacterial cell death.[14] In contrast, the major antimicrobial components of natural essential oils are related to the phenols and aldehydes, for example, eugenol in clove bud and cinnamaldehyde in cinnamon.[15−17] To understand the contingent synergies between chitosan and oil, not only should the interactions between them be investigated but also the modification of the physicochemical properties of the film containing oil.

The objective was to examine the antimicrobial activity of chitosan films incorporating several common essential oils, including clove bud, cinnamon, and star anise oil, against typical pathogenic microorganisms such as Gram-negative *E. coli*, Gram-positive *S. aureus*, and two common fungi, *A. oryzae* and *Penicillium digitatum*. The analysis of the physical properties of the film, including the microstructure feature, mechanical strength, water vapor permeability, moisture content, and solubility, was used to investigate the synergic properties of the complex films.

MATERIALS AND METHODS

Materials. The bacterial strains used in this study were *E. coli* ATCC8099, *S. aureus* ATCC6538, *A. oryzae* CGMCC 3.4259, and *P. digitatum* CGMCC 3.5752. Chitosans of three molecular weights (i.e., ≤3, 50, and 200 kDa) were purchased from Jinan Haidebei Co. Ltd. (Shandong, China). The deacetylation degree was over 85%. Clove bud oil, cinnamon oil, and star anise oil were purchased from Zhengzhou Xomolon Flavor Co., Ltd. (Zhengzhou, Henan, China). Nutrient agar medium and potato dextrose agar were obtained from Beijing Aoboxing Biotech Co., Ltd. (Beijing, China). Glycerol and acetic acid were purchased from the Beijing Chemical Factory (Beijing, China). Tween

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80 was obtained from Tianjin Jinke Fine Chemical Research Institute (Tianjin, China).

Film Preparation. Chitosan solution was prepared with 2% (w/w) chitosan in 1% (w/w) acetic acid at room temperature. After overnight agitation, the solution was filtered using a filter cloth to remove any insoluble particles. Afterward, glycerol (glycerol/chitosan = 0.5, w/w) and Tween 80 at 0.5% (w/w) were mixed into the solution, with 30 min of stirring. Essential oils (2.5, 5, 7.5, and 10%) were then added into the solution to prepare chitosan films with different oil concentrations. After 0.5 h of stirring, the film-forming solutions were treated ultrasonically for about 10 min to remove air bubbles. A solution of 15 g was cast on Plexiglas plates (8.0 × 8.0 cm) and then dried for 48 h at 25 ± 2 °C and 50 ± 2% relative humidity at constant temperature in a humidity chamber (Ningbo Southeast Instrument Co., Ltd., Zhejiang, China). The films were then peeled from the plates and placed at 50 ± 2% relative humidity at 25 °C. Pure essential oil films were prepared by adding the same amount of essential oil as in the oil−chitosan films on greaseproof paper, which had been smoothly lined into Plexiglas plates (8.0 × 8.0 cm) and then dried for 48 h under the same conditions as the film-forming solutions.

Antimicrobial Evaluation of the Chitosan Solutions and Essential Oils. The nutrient agar medium in Petri dish was inoculated with 0.1 mL 10^7-10^8 cfu/mL bacteria, whereas the potato dextrose agar was inoculated with 0.1 mL 10^7-10^8 cfu/mL mold spores. Oxford cups (inside diameter = 6.0 ± 0.1 mm, external diameter = 7.8 ± 0.1 mm, height = 10.0 ± 0.1 mm) were placed at the center of the Petri dish. Approximately 0.2 mL of 2% w/w chitosan solution or essential oil was added into the cups. Finally, bacterial strains were incubated at 37 ± 2 °C and 50 ± 2% relative humidity for 24 h. The fungal strains were incubated at 28 ± 2 °C and 50 ± 2% relative humidity for 72 h.

Antimicrobial Activities of the Chitosan Films with or without Essential Oils. The pure chitosan film and the chitosan films with clove bud oil or cinnamon oil of different contents (0, 2.5, 5, 7.5, and 10%) were prepared as the above film preparation method, respectively. The nutrient agar medium in Petri dish was inoculated with 0.1 mL 10^7-10^8 cfu/mL bacteria, whereas the potato dextrose agar was incubated with 0.1 mL 10^7-10^8 cfu/mL mold spores. The prepared films were cut into 6 mm diameter disks using a hole-puncher and then placed on microbial cultures. Bacterial strains were incubated at 37 ± 2 °C and 50 ± 2% relative humidity for 24 h, whereas fungal strains were incubated at 28 ± 2 °C and 50 ± 2% relative humidity for 72 h. The diameter of the zone of inhibition was measured using a caliper. The tests were performed in triplicate.

Dynamics of Antimicrobial Activities. The pure essential oil and chitosan films with 10% (w/w) cinnamon oil or clove bud oil were prepared as the above film preparation method, respectively. The samples were placed at 25 ± 2 °C and 50 ± 2% relative humidity before measurements. The samples were taken out to examine inhibition zone, respectively, every 3 days until the 27th day.

Film Thickness. Film thickness was determined using a digital micrometer (Chengdu Chengliang Co., Ltd., Sichuan, China). For each film, the values obtained from 10 different locations were averaged.

Mechanical Properties. ASTM D638 M,[18] a texture analyzer (TMS-Pro, Food Technology Corp., Sterling, VA) equipped with a cylinder tip, was used to determine the mechanical properties of the films. The analysis was performed using software with a texture analyzer (Texture Lab ProVersion 1.13-002, Food Technology Corp.). Each test was repeated at least five times. The film samples were placed in the middle of the two polymethacrylate plates (custom-made) with a hole 3.2 cm in diameter. The speed of the cylindrical probe (2 mm in diameter) was 1 mm/s. Puncture strength (PS, N/mm) was calculated as

$$\mathrm{PS} = F_\mathrm{p}/L \tag{1}$$

where F_p is the maximum puncture strength (N) and L is the thickness of the films (mm).

To determine the tensile strength, sample films were cut into strips 6 mm wide. The ends of the strips were mounted between cardboard grips using double-sided adhesive tape; the exposed film area was 40 × 6 mm. Initial grip separation was set to 70 mm, whereas crosshead speed was set to 1 mm/s. Tensile strength (TS, MPa) was calculated as

$$\mathrm{TS} = F_\mathrm{t}/L/W \tag{2}$$

where F_t is the maximum stretching strength (N), L is the thickness of the films (mm), and W is the width of the film samples (6 mm).

Water Vapor Transmission Rate (WVTR). The WVP of the films was measured using a Mocon Aquatran (model 1/50 G, Mocon Co., Minneapolis, MN) equipped with a coulometric phosphorus pentoxide sensor (Aquatrace). The relative humidity of the dry side was 10%, and that of the other side was 100%. The measurements were performed at 37.8 °C.

Moisture Content (MC). The MC was determined by drying small film strips in an oven at 105 °C for 24 h. The weights before and after oven-drying were recorded. Moisture content was calculated as the percentage of weight loss based on the original weight. Triplicate measurements of moisture content were conducted for each type of film; the average was taken as the result.

Water Solubility. Film solubility (S) was determined in triplicate according to the modified method proposed by Gontard et al.[19] Three pieces of each film (8 cm in diameter, about 0.6 g in total) were dried in an oven (105 ± 2 °C; 24 h) to obtain the initial dry matter weight of the films. The dried films were weighed (m_1) and then immersed into 50 mL of distilled water for 24 h at 25 ± 2 °C. After 24 h, the coagulated films were taken out of the water and dried (105 ± 2 °C; 24 h) to determine the weights of the dry matter (m_2) not dissolved in water. The weight of the dissolved dry matter was calculated as follows:

$$S\,(\%) = (m_1 - m_2) \times 100/m_1 \tag{3}$$

Size Measurement. The particle size of the film-forming solution was determined by means of dynamic light scattering (DLS) using a Delsa-Nano particle analyzer (Beckman Coulter Inc., Brea, CA). The size measurement was performed at 25 °C and at a 15° scattering angle. In DLS when the hydrodynamic size was measured, the fluctuations in time of scattered light from particles in Brownian motion are measured. The autocorrelation function $G(\tau)$ analyzing time-dependent signals was

$$G(\tau) = \mathrm{e}^{-\tau D q^2} \tag{4}$$

where D is the diffusion coefficient of the particles in the solution, τ the delay time, and q the scattering vector

$$q = \frac{4\pi n}{\lambda_0}\sin\left(\frac{\theta}{2}\right) \tag{5}$$

where n is the refractive index of media, λ_0 the wavelength of incident light in the air, and θ the scattering angle. D in eq 4 is determined by the Stokes−Einstein equation

$$D = \frac{kT}{3\pi\eta_\mathrm{s} d} \tag{6}$$

where d is the hydrodynamic size of the particles, k the Boltzmann constant (1.38×10^{-23} J/K), T the absolute temperature, and η_s the viscosity of solvent.

Morphology Measurements. The morphology of the surface and the cross section of the films were examined using scanning electron microscopy (SEM) (Hitachi S-4500, Japan). Films were mounted on aluminum stubs using glue paste and carbon paint.

Fourier Transform Infrared Spectroscopy (FT-IR). All spectra were obtained using a spectrometer GX FT-IR with a DTGS detector

Table 1. Antimicrobial Activity of Chitosan with Different Molecular Weights at Room Temperature and pH 5.6[a]

	inhibitory zone (cm^2)		
microorganism	≤3 kDa	50 kDa	200 kDa
E. coli	0.63 ± 0.02 cC	0.43 ± 0.02 bB	0.35 ± 0.01 bA
S. aureus	0.34 ± 0.01 bA	0.39 ± 0.02 bB	0.49 ± 0.01 cC
P. digitatum	0 aA	0 aA	0 aA
A. oryzae	0 aA	0 aA	0 aA

[a] Mean values in each column with different lower case letters are significantly different ($P < 0.05$). Mean values in each row with different upper case letters are significantly different ($P < 0.05$).

Table 2. Antimicrobial Activity of Different Essential Oils[a]

	inhibition zone (cm^2)		
microorganism	clove bud oil	cinnamon oil	star anise oil
E. coli	1.15 ± 0.05 aB	2.34 ± 0.08 aC	0.27 ± 0.003 bA
S. aureus	2.34 ± 0.10 bB	3.80 ± 0.10 bC	0 aA
P. digitatum	5.55 ± 0.09 dB	17.38 ± 0.14 cC	0 aA
A. oryzae	3.70 ± 0.08 cB	19.71 ± 0.16 dC	0 aA

[a] Mean values in each column with different lower case letters are significantly different ($P < 0.05$). Mean values in each row with different upper case letters are significantly different ($P < 0.05$).

(Perkin-Elmer, Fremont, CA) infrared spectrophotometer over a range of 4000−400 cm^{-1} with a resolution of 4 cm^{-1}. Deconvolution of the spectra was performed using Spectrum v5.0.1.

Statistical Analysis. Data were analyzed using Origin 8.0 and SPSS 16.0. Statistics on a completely randomized design were performed using the General Linear Models procedure with one-way ANOVA. Duncan's multiple-range test ($P < 0.05$) was used to detect the differences among the mean values.

RESULTS AND DISCUSSION

Antimicrobial Activities of Chitosan Solutions. Table 1 shows the antimicrobial activities exhibited by the inhibitory zone of the pure chitosan solutions with different molecular weights (MW) against a Gram-negative bacterium, *E. coli*, a Gram-positive bacterium, *S. aureus*, and two fungi, *P. digitatum* and *A. oryzae*. The inhibitory zone against *E. coli* increased as chitosan MW decreased, showing apparently stronger antibacterial effect on *E. coli* than on *S. aureus*. In contrast, higher MW seemed to enhance the antibacterial activity of chitosan against the Gram-positive bacterium. These observations are in accordance with the work of Zheng and Zhu,[20] in which two different antibacterial mechanisms were proposed: for the Gram-positive bacteria, chitosan of high MW could block the nutrient supply to bacteria by forming a biopolymer barrier, whereas for the Gram-negative bacteria, chitosan of low MW could easily penetrate the membrane of the microbial cell and disturb the metabolism of the cell. However, these observations are different from those in the work of No et al.,[21] in which the inhibitory effects of the chitosan with low MW had stronger bactericidal effects on *S. aureus* than on *E. coli*. In the case of fungi, none of the chitosan solutions exhibited an obvious antifungal zone, except the zone inside the Oxford cup. This observation was consistent with the descriptions in the literature;[21−23] that is, the ability of chitosan to inhibit bacteria should follow the different ways in which it inhibits fungi. Differences of antimicrobial activities obtained by other researchers were mainly due to the different experimental conditions (pH, temperature, etc.), bacteria source, chitosan characteristics, concentration, and other factors.

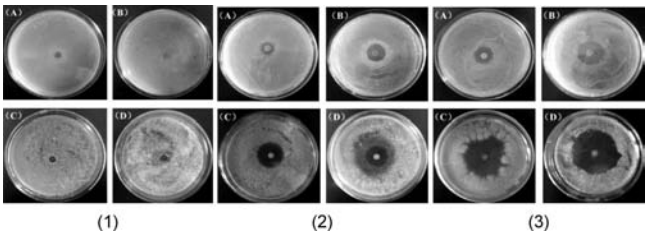

Figure 1. Inhibitory zones of the different films: (1) pure chitosan film; (2) clove bud−chitosan film; (3) cinnamon−chitosan film; (A) *E. coli*; (B) *S. aureus*; (C) *A. oryae*; (D) *P. digitatum*. The quantity of incorporated oils was 10% w/w in both clove bud− and cinnamon−chitosan films.

Antimicrobial Activities of the Essential Oils. Table 2 shows the antimicrobial activities of three essential oils (i.e., clove bud oil, cinnamon oil, and star anise oil) against the same microorganisms listed in Table 1. Under the present experimental conditions, the antifungal activity of the essential oils seemed to be better than their antibacterial activity. Cinnamon oil was also observed to exhibit stronger inhibitory effects than both the clove bud and star anise oils. In addition to the inhibition effects through direct contact with essential oil solutions, several authors noted that some fungi are also susceptible to the vapors of essential oils and could be inhibited when exposed to the atmosphere generated by the essential oils, such as oregano or cinnamon.[24,25]

Lopez et al.[25] reported that cinnamon has better antibacterial activity against *S. aureus* than against *E. coli* and better antifungal activity against *A. flavus* than against *P. islandicum*. Du[24] reported that cinnamon oil exhibits stronger antibacterial effects on *E. coli* than clove bud oil by both direct contact and vapor diffusion methods. Hosseini et al.[26] reported that clove bud oil exhibits stronger antibacterial effects on *S. aureus* than cinnamon oil. Valero and Salmeron[27] compared the antibacterial activities of 11 essential oils, including clove and cinnamon oil, against the foodborne pathogen *Bacillus cereus* grown in carrot broth. They considered cinnamon oil to be more effective than clove oil. Note that the chemical components of the essential oils, for example, clove and cinnamon, can be affected by the origin of the crop (i.e., country of origin, altitude at which it grows, and harvest season), including production process, level of purity, and preservation. These factors are very likely to lead to variability in the antimicrobial activities of the essential oils.

Antimicrobial Activities of the Chitosan Films Containing Essential Oils. Figure 1 presents the images of the inhibitory zones of the different films. Figure 2 compares the antimicrobial activities of chitosan films versus the quantity of the essential oils (A, clove bud oil; B, cinnamon oil) incorporated in a film matrix. The star anise oil was proved to have poor antimicrobial activity as shown in Table 2, and the chitosan did not form homogeneous films when star anise oil was added as well, so the star anise oil was ruled out in the experiment below. The chitosan-based films were prepared using the chitosan of 50 kDa, which could ensure that the film would have sufficient mechanical strength and less controversial antibacterial activities in the present experimental conditions. Although the chitosan of 3 kDa had better antibacterial

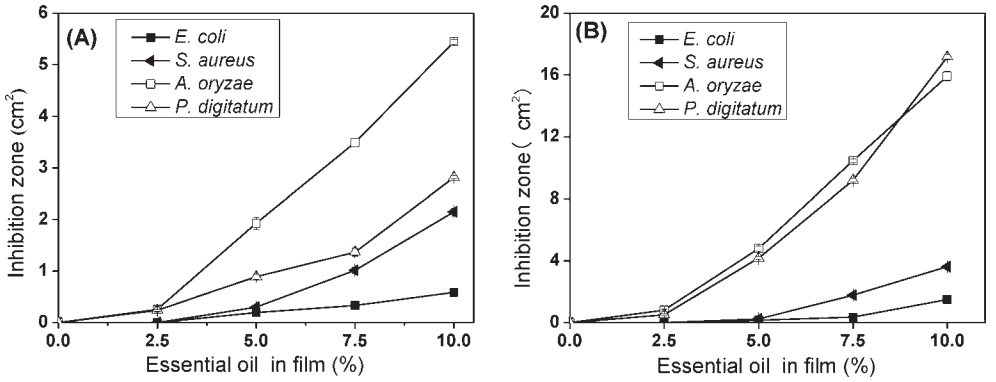

Figure 2. Antimicrobial activities of the chitosan films versus the quantity of essential oil incorporated in film-forming solutions: (A) clove bud oil; (B) cinnamon oil.

Table 3. Physical Properties of the Different Chitosan Films[a]

film	thickness (μm)	PS (N/mm)	TS (MPa)	WVP (10^{-9} g/m·s·Pa)	MC (%)	S (%)
chitosan	104 ± 1 a	28.7 ± 0.7 b	5.5 ± 0.6 c	1.58 ± 0.01 a	41.4 ± 1.7 b	20.2 ± 1.4 b
clove bud−chitosan	413 ± 1 c	4.0 ± 0.1 a	1.0 ± 0.2 a	2.57 ± 0.12 b	51.3 ± 0.1 c	32.7 ± 2.6 c
cinnamon−chitosan	310 ± 2 b	27.1 ± 1.4 b	3.0 ± 0.3 b	3.21 ± 0.09 c	37.4 ± 0.8 a	13.2 ± 0.5 a

[a] The quantity of incorporated oils was 10% w/w of the film-forming solution. Mean values in each column with different lower case letters are significantly different ($P < 0.05$)

activities, the film prepared with this polysaccharide was easily broken and thus became unusable. The oil quantity incorporated in the chitosan film-forming solution was no more than 10% because the addition of excess oil could make the film-forming solution too sticky to form a film.

No significant inhibition zone was observed for the pure chitosan film (Figures 1 and 2). The antimicrobial performance of the chitosan needs the positively charged amino groups of chitosan monomer units, which could react with the anionic groups of the microbial cell surface. Moreover, only the dissolved chitosan molecules can diffuse in agar gel and result in the formation of the inhibition zone. The chitosan molecules were fixed within the film matrix, and thus no diffusing antimicrobial agents could generate the inhibition zone. The essential oils incorporated into the film did not effectively improve the water solubility of the chitosan film (Table 3). In other words, the inhibitory zones of the films were only generated by the essential oils. Nevertheless, no bacterial growth was observed in the area directly covered by the pure chitosan film (Figure 1), indicating that the moisturized film could still be charged and exhibit local antimicrobial activity. This observation is different from that in the work of Foster and Butt,[28] who observed no antimicrobial activities of the chitosan films. This may be caused by the state of the film being too dry to be able to inhibit bacterial growth.

In Figure 2A, the variations of the inhibitory zones were not significant ($P < 0.05$) when the incorporated oil quantities were less than about 2.5% for *A. oryae* and *P. digitaum* and about 5% for *E. coli* and *S. aureus*. These values may be regarded as the minimum inhibitory concentration of the clove bud oil in the investigated film (MIC-f). When the oil quantities were higher than MIC-f, the inhibitory zone increased rapidly with the oil concentration. Moreover, the inhibitory effects of the clove bud oil on the microorganisms were observed to be in the following order: *A. oryze* > *P. digitatum* > *S. aureus* > *E. coli*. In Figure 2B, MIC-f of the cinnamon−chitosan films was also near 2.5% for *A. oryae* and *P. digitaum* and 5% for *E. coli* and *S. aureus*. The inhibitory effects of the cinnamon−chitosan film at MIC-f on the fungi were about 2−3-fold stronger than those of the clove bud−chitosan film, but both oil−chitosan films at MIC-f on bacteria were almost at the same level. When the oil quantities were higher than MIC-f, the inhibitory zone of the cinnamon increased rapidly with the increase in oil concentration. The following is the order of the inhibitory effects of cinnamon−chitosan film on the microorganisms: *A. oryze* ≈ *P. digitatum* > *S. aureus* > *E. coli*. With 10% oil incorporated in the film, the inhibitory effects of the cinnamon−chitosan film were higher than those of the clove bud−chitosan film: about 2−3-fold stronger on *E. coli*, *S. aureus*, and *A. oryae* and even 6-fold stronger on *P. digitatum*.

Dynamics of Antimicrobial Activities of the Oil−Chitosan Films. Compared with other essential oils, the cinnamon oil, having better antimicrobial activities and compatibility with chitosan in the film-forming process, was thus used to investigate the dynamic properties. Figure 3 compares the dynamics of the antimicrobial activities of the oil−chitosan films on different microorganisms (A, *E. coli*; B, *S. aureus*; C, *A. oryzae*; D, *P. digitatum*) for 27 days. The quantities of the oils incorporated in chitosan films were maintained at 10%. In all systems, the inhibitory zones of the films increased to the maximum value during the first 3 days. As described under Antimicrobial Activities of the Essential Oils, the antimicrobial activities depended on the concentration of the oils. Low quantity levels of oils led to a delay in the inhibition of bacterial growth. Only a sufficient quantity of oils showed obvious growth inhibition. The effective antimicrobial quantity of oils was also affected by the ability of oil diffusing from the film matrix, penetrating the agar gel, and evaporating into the atmosphere. These points of view have been discussed frequently in the literature.[24,25,29] In the work of López et al.,[25] the quantity of active components of the essential oils released from the polypropylene or polyethylene/

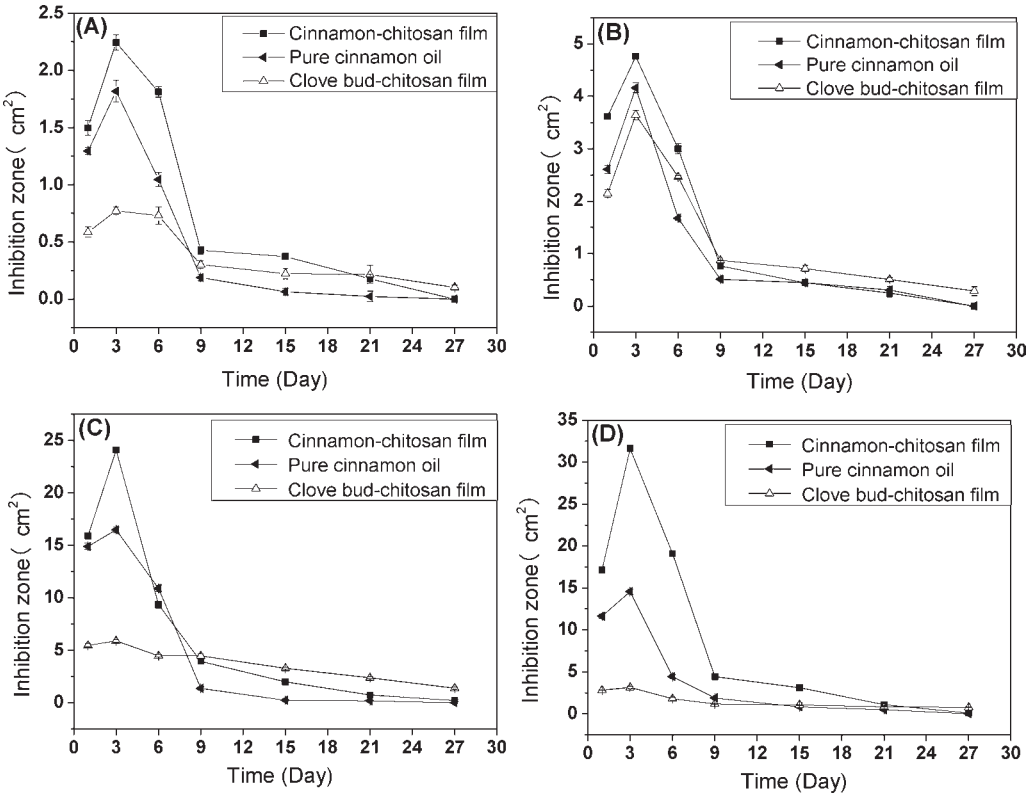

Figure 3. Antimicrobial activity changes in pure cinnamon oil, cinnamon−chitosan films, and clove bud−chitosan films against microorganisms as functions of time: (A) *E. coli*; (B) *S. aureus*; (C) *A. oryzae*; (D) *P. digitatum*.

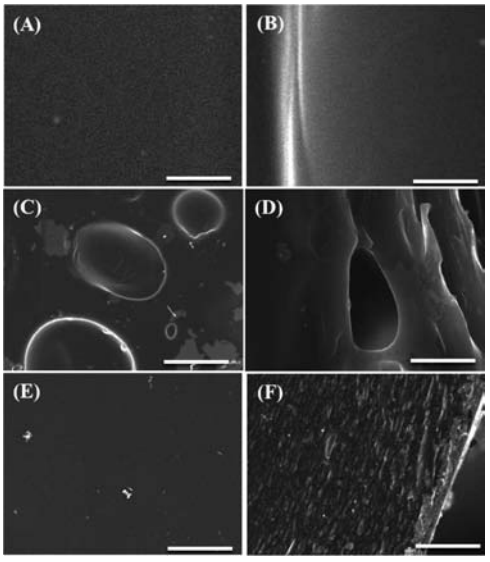

Figure 4. SEM images of the chitosan films: (A) surface of the pure chitosan film; (B) cross section of the pure chitosan film; (C) surface of the chitosan film containing 10% clove bud oil; (D) cross section of the chitosan film containing 10% clove bud oil; (E) surface of the chitosan film containing 10% cinnamon oil; (F) cross section of the chitosan film containing 10% cinnamon oil. The bar is 10 μm.

ethylene vinyl alcohol copolymer film could reach a maximum value in 6 h. Using apple-based edible films, Du[24] found that the most remarkable inhibitory effects could be observed in 24 h on the basis of two independent methods: overlay of the film on the bacteria and vapor phase diffusion. These data were faster than those of the present chitosan system. These differences may be related to the diffusion coefficient of organic species versus the molecular weight and type of polymers constituting the film matrix.[25,30,31] The inhibitory zones decreased on the fourth day and then became gradually smooth, indicating that the quantity of the residual oils in the film decreased.

In comparison with pure essential oils (Table 2), the cinnamon oil incorporated in chitosan films exhibited stronger antimicrobial activities on *E. coli* (Figure 3A), *S. aureus* (Figure 3B), *A. oryzae* (Figure 3C), and *P. digitatum* (Figure 3D) than the clove bud oil incorporated in chitosan film. Moreover, the antimicrobial activities of the cinnamon oil incorporated in chitosan films were generally stronger than those of the cinnamon oil in the pure state, although the behaviors of *A. oryzae* were somewhat abnormal. These phenomena indicate that the chitosan film matrix can reduce the released oil concentration (liquid or gaseous) through the interactions between the oils and polymeric matrix, thus enhancing the antimicrobial activities by keeping a relatively high concentration of oils in the system. However, these interactions did not change the contrast between the antibacterial activities of the two essential oils.

SEM of the Films. Figure 4 compares the SEM images of the surface and cross section of the pure chitosan film, clove bud oil−chitosan film, and cinnamon oil−chitosan film. The surface of the pure chitosan film was smooth and flat (Figure 4A). A similar surface morphology was observed in the cinnamon oil−chitosan film (Figure 4E). In contrast, many droplets with sizes between 5 and 20 μm appeared on the surface of the clove bud oil−chitosan film (Figure 4C), indicating that this essential

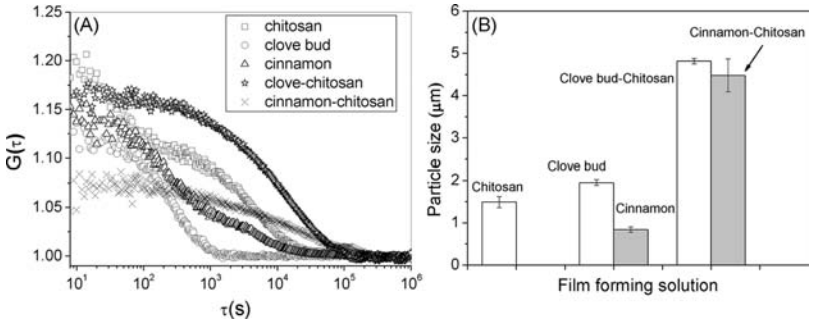

Figure 5. Size measurements of the pure chitosan solution, pure essential oils, and oil−chitosan film-forming solutions: (A) autocorrelation function curves of light scattering; (B) hydrodynamic sizes calculated according to the data from panel A. The concentration of chitosan was 2%. The concentration of oils was maintained at 10% in all cases at room temperature. The values of pH varied between 4.4 and 4.5.

oil is incompatible with chitosan molecules, creating phase separation. The cross section of the pure chitosan film was compact and uniform without pores or cracks (Figure 4B). In contrast, the chitosan film containing the clove bud oil showed a loose texture caused by the phase separation of the essential oil and polysaccharide (Figure 4D), where the cross section of the film was filled with cavities and cracks. When cinnamon oil was incorporated into the chitosan film, the cross section exhibited sheets stacked in compact layers (Figure 4F). Apparently, cinnamon essential oil is more compatible with the chitosan matrix than the clove bud oil.

Analysis of Physical Properties of the Films. Table 3 compares the thickness, puncture strength (PS), tensile strength (TS), water vapor permeability (WVP), moisture content (MC), and solubility (S) of the pure chitosan film, clove bud oil−chitosan film, and cinnamon oil−chitosan film, respectively. The thickness of the pure chitosan film was about 104 μm. When the essential oils were incorporated (10%), the microstructure of the film became loose (Figure 4D). Moreover, the thickness of the film increased about 4-fold for the clove bud oil−chitosan film and 3-fold for the cinnamon oil−chitosan film.

The values of PS and TS of the pure chitosan film were 28.7 N/mm and 5.5 MPa, respectively. These values became weaker when the oils were incorporated, particularly in the film containing clove bud oil. The loss of mechanical strength may be attributed to the breakup of the film network microstructure caused by the added oils. As noted in a previous work,[32] when the film microstructure becomes discontinuous because of incompatible substances, the distribution of the external force on each matrix bond becomes uneven, thereby leading to a decline in the mechanical strength of the system.[33] Because the compatibility of the cinnamon oil−chitosan film was better than that of the clove bud oil−chitosan film, as seen in the SEM images (Figure 4D,F), the mechanical strength of the former was higher than that of the latter.

MC is a parameter related to the total void volume occupied by water molecules in the network microstructure of the films, *S* to the hydrophilicity of the materials, and WVP to the micropaths in the network microstructure. The loose microstructure of the clove bud oil−chitosan film allowed the matrix to have a relatively high void volume and MC. The *S* values of the oil−chitosan films indicated that the clove bud oil enhanced the hydrophilicity of the film, whereas cinnamon oil reduced the hydrophilicity of the film. The water solubility of eugenol (1.44 mg/mL),[34] the major component of clove bud oil, was indeed higher than that of cinnamaldehyde (0.409 mg/mL),[34] the major component of cinnamon oil. The microstructure of the cinnamon oil−chitosan film was constituted by stacked sheets generating a number of parallel-arranged intervals and creating continuous and run-through micropaths in the film. This is perhaps why WVP was relatively higher than in the other films.

Particle Size Measurements of the Emulsion. Figure 5 compares the autocorrelation function curves of light scattering, $G(\tau)$ (τ is delay time), and the calculated hydrodynamic particle sizes of pure chitosan, essential oils, and oil−chitosan film-forming solutions. After incorporation of 10% oils in chitosan, $G(\tau)$ of the clove oil−chitosan and cinnamon oil−chitosan systems exhibited very different behaviors (Figure 5A); that is, the initial $G(\tau)$ of the former increased, whereas that of the latter decreased. Both curves shifted to the right compared with those of the pure samples. The sizes of pure chitosan, clove bud oil, and cinnamon oil solutions were 1.52 ± 0.12, 0.20 ± 0.07, and 0.85 ± 0.03 μm, respectively. The addition of oils promoted weak aggregation. The sizes increased slightly to 4.81 ± 0.11 and 4.48 ± 0.39 μm for clove oil−chitosan and cinnamon oil−chitosan solutions (Figure 5B), respectively.

Figure 6 compares $G(\tau)$ and the calculated hydrodynamic particle sizes of the oil−chitosan film-forming solutions versus real time. The initial $G(\tau)$ values of the clove bud oil−chitosan solution showed a remarkable fluctuation, and the curve progressively shifted to the right (Figure 6A) along with time. In contrast, the initial $G(\tau)$ fluctuation and curve shift of the cinnamon oil−chitosan curves were relatively small (Figure 6B). The obtained particle sizes are shown in Figure 6C, where the size of the clove bud oil−chitosan solution increased from 4.81 ± 0.07 to 7.96 ± 0.11 μm in 16 min. This is in contrast to the size of the cinnamon oil−chitosan solution, which varied only slightly between 4.48 ± 0.04 and 4.91 ± 0.04 μm (Figure 5B).

On the basis of the data of size measurements, phase separation was believed to occur in the clove bud oil−chitosan film (Figure 4C ,D), starting with the aggregation of the essential oil droplets. Considering the pK_a values of chitosan and eugenol (the major component of clove bud oil), that is, 6.5[35] and 8.55,[36] respectively, both the polysaccharide molecules and oil droplets were positively charged in an acid environment (pH 4.5). Therefore, electrostatic repulsion is probably the main reason for the occurrence of phase separation. The case of the cinnamon oil−chitosan system is more complex; thus, FT-IR analysis was used in the following section.

FT-IR of the Films. Figure 7 compares the FT-IR spectra of the pure components and oil−chitosan films in the region of 2000−650 cm^{-1}. The molecular structure of chitosan, eugenol,

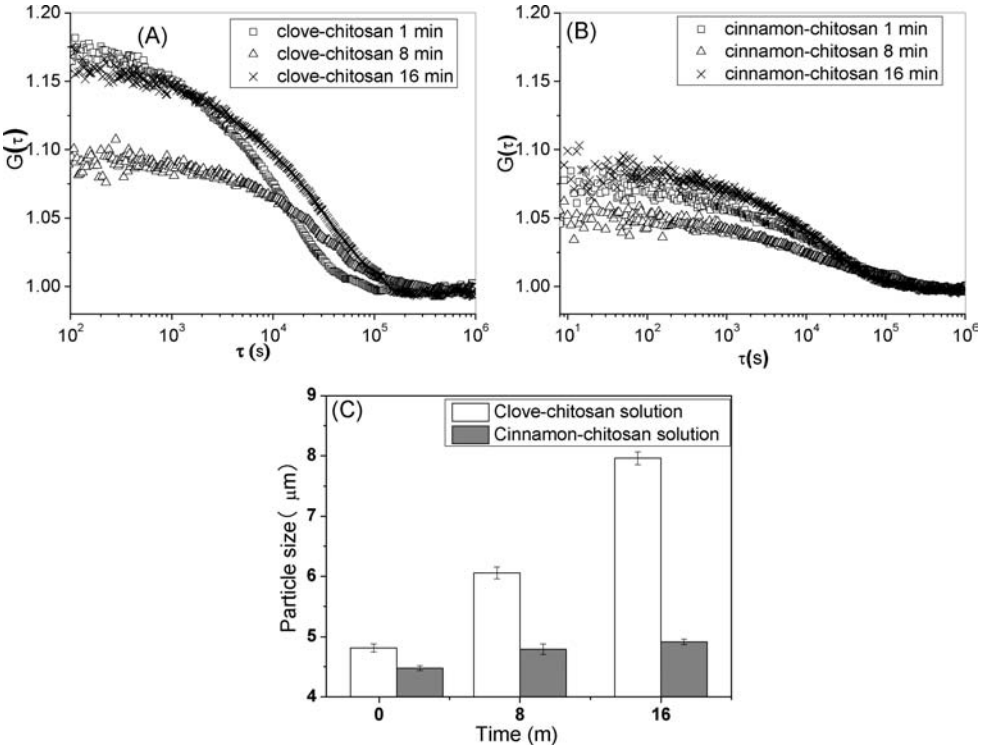

Figure 6. Size measurements of the oil−chitosan film-forming solutions versus time: (A) autocorrelation function curves of light scattering of the clove bud−chitosan solution; (B) autocorrelation function curves of light scattering of the cinnamon−chitosan solution; (C) hydrodynamic sizes calculated according to the data from panels A and B. The concentration of oils was maintained at 10% in all cases at room temperature. The values of pH varied between 4.4 and 4.5.

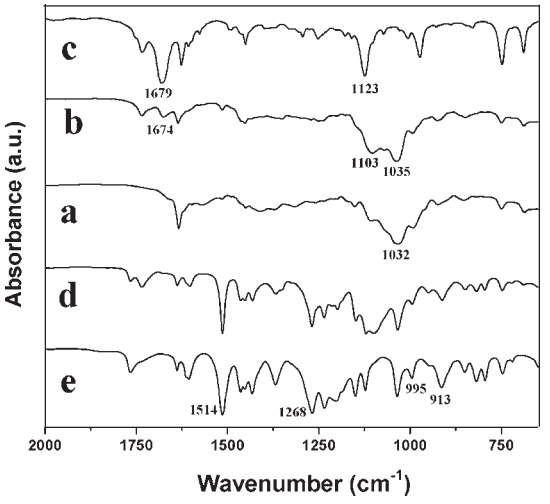

Figure 7. FT-IR spectra of the pure components and oil-chitosan films: (a) pure chitosan film; (b) cinnamon−chitosan film; (c) pure cinnamon oil; (d) clove−chitosan film; (e) pure clove bud oil. The quantity of the incorporated essential oil was maintained at 10% for the films.

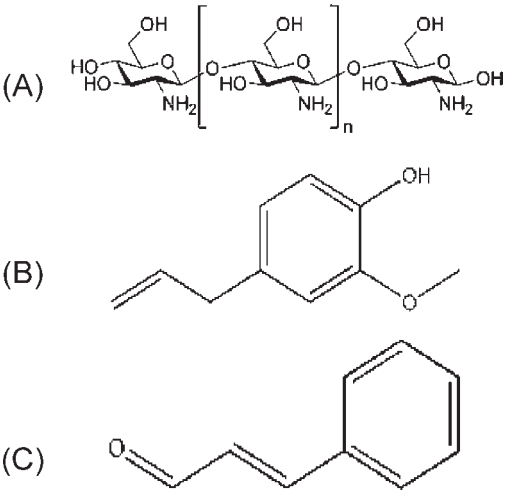

Figure 8. Molecular structure formulas: (A) chitosan; (B) eugenol; (C) cinnamaldehyde.

and cinnamaldehyde is presented in Figure 8. The large absorption at 1032 cm^{-1} in the pure chitosan film is ascribed to the stretching vibration of R−CH_2−OH, and the peak 1100 cm^{-1} is ascribed to the stretching vibration of −NH_2 stretching (Figure 7a). The characteristic peak of the cinnamaldehyde was 1679 cm^{-1}. It was caused by the stretching vibration of R−CHO conjugated with a double bond that appeared at 1623 cm^{-1} (Figure 7c).[37] After cinnamaldehyde was mixed with chitosan, the peak at 1679 cm^{-1} shifted 5 cm^{-1} to the right, and a new peak appeared at 1103 cm^{-1} (Figure 7b). This indicates that ethanol and aldehyde formed an acetal at acid condition. This interaction thickened the polysaccharide chains and led to the formation of the sheet-layer microstructure in the film. The characteristic peaks of eugenol were 1650, 1514, and 1268 cm^{-1}, with each assigned to the stretching vibration of R—C═C, aromatic ring, and phenolic hydroxyl, respectively (Figure 7e).[37] After eugenol was

mixed with chitosan, no significant shifts in these peaks were observed (Figure 7d), indicating that no new bonds were formed. These observations are in accordance with those of the SEM and size measurements.

In conclusion, among the three investigated essential oils, cinnamon oil showed the highest antimicrobial activities against *P. digitatum* and *A. oryzae* and a certain degree of inhibitory effect on Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Although the antimicrobial ability of the chitosan film was not as strong as that of the chitosan solution, the moisturized film still exhibited a certain degree of inhibitory activity. The polysaccharide film matrix indeed enhanced the antimicrobial activities of the oils by maintaining a relatively high concentration of oils in the system. Acetal was produced when cinnamaldehyde, the major constituent of cinnamon oil, and chitosan were in acidic conditions; phase separation took place between clove bud oil and chitosan.

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