A GREEN AND SIMPLE SYNTHESIS OF CHITOSAN/Ag NANOCOMPOSITES AND STUDY FOR THEIR ANTIBACTERIAL ACTIVITY ON *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*

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ABSTRACT

A green and simple approach has been successfully developed to synthesize chitosan/Ag nanocomposites using kumquat extract as a biological reducing agent. It indicates to be an eco-friendly and green method for the synthesis providing a cost effective and an efficient route for the chitosan/Ag nanocomposites' synthesis. The prepared chitosan/Ag nanocomposites have been characterized by UV-vis, TEM, FTIR, and XRD. Result showed those chitosan/Ag nanocomposites have been obtained with average particle size ~15-25 nm. Moreover, the synthesized chitosan/Ag nanocomposites also showed their efficient antimicrobial activity against *S. aureus* and *E. coli*. The chitosan/Ag nanocomposite was found to have significantly higher antimicrobial activity than its components at their respective concentrations. The presence of a small percentage (2.5 %, w/w) of metal nanoparticles in the nanocomposite was enough to significantly enhance inactivation of *S. aureus* and *E. coli* as compared with unaltered chitosan. Thus, this eco-friendly method could be a competitive alternative to the conventional physical/chemical methods used for the synthesis of chitosan/Ag nanocomposites. Since, it has a potential to use in biomedical and cosmetic applications.

Keywords: Chitosan/silver nanocomposites (CS/Ag NCPs), *Escherichia coli (E. coli)* bacteria, environmental friendly, green synthesis, Kumquat extract.

1. INTRODUCTION

In the recent time, antimicrobial and antioxidative activities of chitosan were significantly enhanced because of loading chitosan with various metals found in the previous reports [1, 2].

Chitosan is a natural biopolymer extremely abundant and relatively cheap. It has attracted significant interest by a lot of scientists due to its biological properties such as antitumor activity, antimicrobial activity and immune enhancing effect [3, 4].

Among all antibacterial metals, silver nanoparticles (Ag NPs) are well known for strong antimicrobial properties, nontoxic and no harm to human cells [5]. Thus, silver nanoparticles have widely attracted attention for medical applications due to their excellent properties such as antibacterial activity [6, 7].

A number of methods for producing silver nanoparticles (Ag NPs) have been developed using both physical and chemical approaches such as sonochemical and electrochemical methods, thermal decomposition, laser ablation, microwave irradiation, etc. [8-12]. However, they are also related to the limitations as using of toxic chemicals, high operational cost and energy needs. Therefore, considerable interest has been paid to the preparation of metallic nanoparticles by green synthesis in recent years [13-17].

Therefore, green synthesis is the green environment friendly processes in chemistry, in chemical technology and engineering; which are becoming more popular and much needed since the global's concern is about environmental problems in recent years [18]. Green synthetic methods have been used new alternative for metal nanoparticles as well as Ag NPs synthesis using natural polymers (chitosan, etc.), sugars, enzymes, microorganisms, plant extracts as reductants (e.g, lemon aqueous extract, Azadirachita indica aqueous leaf extract, etc.) and capping agents [19-21]. They are simple, one step, cost-effective, energy efficient, more stable materials and environment friendly [22-24].

According to our understanding, using kumquat aqueous extract to synthesize chitosan/silver nanocomposites have not been previously reported. Thus, the main objective of this study was to research the synthesis and characterization of chitosan/Ag nanocomposites. The chitosan/Ag nanocomposites were synthesized by green route using kumquat aqueous extract without using any additional harmful chemical/physical methods. Herein, the synthetic method used here is simple, rapid reaction time, cost effective, easy to perform, uniform particle size, stable and sustainable. Chitosan/Ag nanocomposites (CS/Ag NCPs) can be produced at low concentration of kumquat aqueous extract. Moreover, the synthesized chitosan/Ag nanocomposites have a significant promise as bactericidal agent for applications (i.e, biomedical, food, agriculture and cosmetics, etc.) in the current time and in future.

2. MATERIALS AND METHOD

2.1. Materials

Silver nitrate ($AgNO_3$), sodium tripolyphosphate (STPP, > 98 %) were purchased from Acros. Kumquat fruit (~3 months old, green shell) was purchased from a garden at Phong Dien, Can Tho City in Vietnam. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli (E. coli)* were purchased from Sigma-Aldrich. Luria–Bertani broth (LB) and agar powder (bacteriological grade) were purchased from HiMedia, Mumbai, India. Chitosan was bought from Vietnam's company. All solutions were prepared using deionized water from a MilliQ system.

2.2. Method

2.2.1. Preparation of kumquat extract

Fresh kumquat (200 g) was squeezed and obtained the kumquat juice mixture. After that, the kumquat juice was filtered, centrifuged and washed with DI water for three times to obtain a juice extract (~100 mL) from kumquat. This kumquat aqueous extract was used for synthesis of chitosan/Ag nanocomposites (CS/Ag NCPs) in following steps.

2.2.2. Preparation of chitosan/Ag nanocomposites by kumquat extract

Chitosan/Ag nanocomposites (CS/Ag NCPs) were synthesized by a green method using kumquat aqueous extract as a reducing agent for the bioconversion of chitosan polymer and silver ions into chitosan and Ag nanoparticles. In a typical synthesis, 2 mL of sodium tripolyphosphate (STPP) solution (1 % in H_2O) was added to 40 mL of chitosan solution (2 mg/mL in acetic acid solution 2 %) and stirred for 30 min at 50 ^oC to obtain chitosan nanoparticles. And then, 1 mL of $AgNO_3$ (0.01 M) was added to the above solution mixture and after 1 min, 2 mL of kumquat aqueous extract was also quickly added and stirred for 90 min at 70 ^oC. The solution was then centrifuged (12000 rpm; 15 min) and washed with deionized water (DI water) to remove excess. And then redispersed in DI water. The average particle size of the as-prepared chitosan/Ag nanocomposite is ~15-25 nm.

2.2.3. Characterization

The absorbance spectra of particle solutions were examined by UV–vis spectrophotometry (UV-675; Shimadzu). Fourier transform infrared spectroscopy (FTIR) spectra of chitosan/Ag nanocomposites were obtained by using a Renishaw 2000 confocal Raman microscope system. The phase structure of chitosan/Ag nanocomposite was determined by an X-ray diffractometer (Rigaku Dmax-B, Japan) with Cu K_α source operated at 40 kV and 100 mA. A scan rate of 0.05 deg^{-1} was used for 2θ between 10^o and 80^o. The particle size and surface morphology of chitosan/Ag nanocomposites were examined by transmission electron microscope (TEM) with a Philips Tecnai F20 G2 FEI-TEM microscope (accelerating voltage 200 kV).

2.2.4. Preparation for studying antibacterial activity of chitosan/Ag nanocomposites on Staphylococcus aureus (*S. aureus*) *and Escherichia coli (E. coli) bacteria strains*

To determine the minimum inhibitory concentration (MIC) of the chitosan/Ag nanocomposites, the green fluorescent protein (GFP)-expressing *S. aureus* and *E. coli* at numbers of 10^6 cfu/mL was inoculated into LB medium supplemented with various concentrations (volumes) of chitosan/Ag nanocomposites solution (with amount of Ag in chitosan/Ag nanocomposites being 23.7 mg/kg from analysis result of ICP) and grown overnight at 37 °C. The minimum concentration of the chitosan/Ag nanocomposites which gave cultures that did not become turbid was taken to be the MIC. The cultures that were not turbid were re-inoculated into fresh LB containing ampicillin at 100 μg/mL.

To study the bactericidal activity of the chitosan/Ag nanocomposites, GFP-expressing *E. coli* and *S. aureus* were grown overnight for each well (96 well/disk) in 150 μL LB ampicillin medium at pH 6.3. The cells were harvested by centrifugation and resuspended in 300 μL LB. Three 100 μL portions of the cell suspension were inoculated into 50 mL volumes of fresh LB ampicillin media, without the chitosan/Ag nanocomposites or with chitosan/Ag nanocomposites using various concentrations (100 μL, 90 μL into 10 μL DI H_2O, 80 μL into 20 μL DI H_2O. During the cells incubation at 37 °C, the optical densities at 595 nm (OD600) of the cultures were determined using a UV–visible spectrophotometer (SPEKOL 1200, Analytikjena, Jena,

Germany); and GFP-expressed fluorescence was determined using a fluorescence spectrophotometer (Varian Cary Eclipse, Palo Alto, CA, USA) with the excitation wavelength set at 400 nm. Numbers of viable E. coli and S. aureus were determined by plating serially ten-fold dilutions of bacterial culture on ampicillin supplemented LB-agar wells/plate which were incubated at 37 °C for 24 h.

3. RESULTS AND DISCUSSION

3.1. Characterization of the chitosan/Ag nanocomposites

As shown in Figure 1, the UV-vis spectra of chitosan/Ag nanocomposites (CS/Ag NCPs) exhibited with the maximum absorption peak in the range from 401-411 nm, respectively. Herein, the plasmon resonance peaks are quite matchable with the surface absorption of Ag nanoparticles [25, 26]. Since, it is demonstrated that Ag nanoparticles are created in the chitosan nanoparticles' solution. The maximum absorption peaks of chitosan/Ag nanocomposites measured in the range ~401-411 nm, which can be predicted the average particle size of chitosan/Ag nanocomposites being ~15-25 nm, as compared to Ag nanoparticles [25, 26]. Result that the maximum absorption peak intensity of chitosan/Ag nanocomposites (CS/Ag NCPs) at 401 nm and 407 nm are approximate – see Figure 1 (c, e), respectively. As known, the absorption peak in the range at 401 nm has nanoparticle size smaller than that of the absorption peak at 407 nm. Thus, the optimal sample will be chosen for following investigations respective for 90 min at 70 ^{o}C – see in Figure 1(c).

The presence of free ions in the kumquat extract solution has greatly accelerated for the polyol synthesis of chitosan/Ag nanocomposites. During the synthesis, we could easily monitor the progress of the nanoparticles production through its color changes from colorless to yellow, red-brown or blue, etc due to a dramatic increase in the reduction rate of silver ions (Ag^{+}) and chitosan (high molecule mass) become Ag and chitosan nanoparticles (chitosan with low molecule mass). The absorption intensity of synthesized samples tend to proportional increase to the chitosan/Ag nanocomposites' solution color, corresponding to increase the concentration of $AgNO_3$ solution. It demonstrated that reaction rate of reducing agent using kumquat extract significantly affects to particle size control of synthetic chitosan/Ag nanocomposites in the mixture solution.

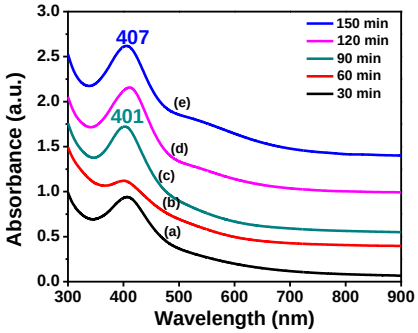

Figure 1. UV-vis spectra of chitosan/Ag nanocomposites using kumquat extract at 70 ^{o}C with various reaction times: (a) 30 min; (b) 60 min; (c) 90 min; (d) 120 min; and (e) 150 min, respectively.

Transmission electron microscopy (TEM) was used to observe the surface morphology of chitosan/Ag nanocomposites. Figures 2(a, b) shows representative TEM images of chitosan/Ag nanocomposites sample. The image of the chitosan and Ag nanoparticles reveal that the nanocomposite (non-core/shell structure) and that they are well dispersed and spherical in shape. Chitosan/Ag nanocomposites are uniform and spherical with average particle size ~15-25 nm. There is no agglomeration of nanoparticles may be due to the presence of chitosan as a capping agent. Especially, these particles are uniformly mixed in a chitosan matrix – see in Figure 2. Moreover, the average particle size of chitosan/Ag nanocomposites are diffused in the aqueous solution with large amount of particles in the range from 15 nm to 30 nm as shown in Figure 2(c).

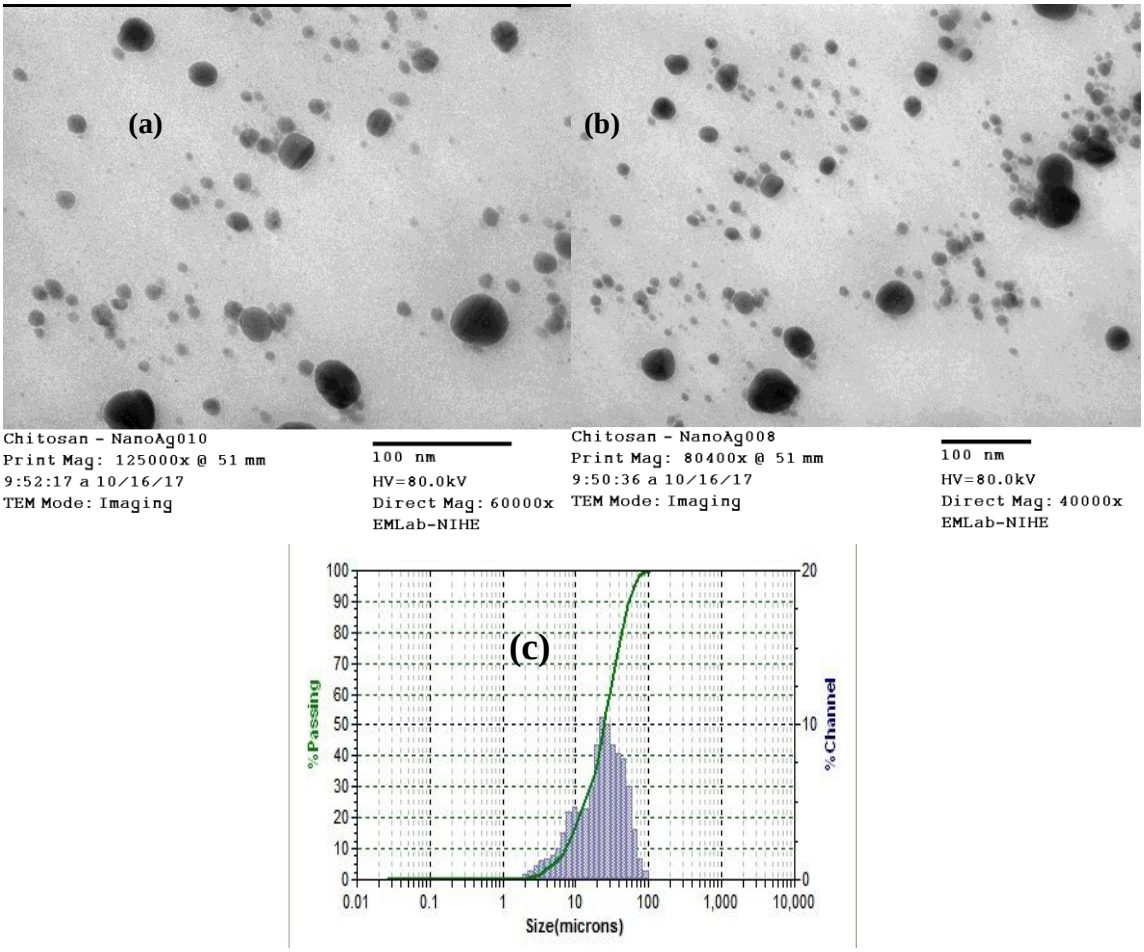

Figure 2. TEM images (a, b) of chitosan/Ag nanocomposites (CS/Ag NPs); and (c) DLS image of chitosan/Ag nanocomposites (CS/Ag NPs) in the solution mixture using kumquat aqueous extract at 70 ^{o}C for 90 min, respectively.

As shown in Figure 3, the FTIR spectrum of chitosan shows the presence of bands at ~3418-3429 cm^{-1} (O-H stretching), C-H and C-N stretching at ~2927-2854 cm^{-1}, N-H bending at 1636-1631 cm^{-1}, N-H angular deformation in CO-NH plane at 1421-1636 cm^{-1} and C-O-C band stretching at 1093 cm^{-1} [27, 28]. In the FTIR spectrum of chitosan/Ag nanocomposites, the shifting of the chitosan peaks is observed which may be due to the interaction of Ag with

chitosan in the nanocomposite (e.g, from 1470 cm^{-1} shifted to ~1451 cm^{-1} (Figure 3(b) – see in Figure 3). Besides, the other changes that are significantly noticeable the reduction in the intensity of the hydroxyl (-OH) peak and the increase in the intensity of the C-O stretching, which is occurred when the presence of Ag nanoparticles in the chitosan matrix and formed the mixture solution of chitosan/Ag nanocomposites.

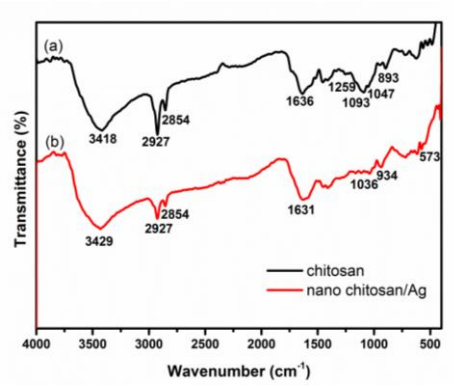

Figure 3. FTIR spectra of (a) chitosan and (b) chitosan/Ag nanocomposites using kumquat extract at 70 ^{o}C for 90 min.

The X-ray diffraction (XRD) pattern of pure chitosan powder there is mainly peak at $2\theta = 21^{o}$, which according to literature could demonstrate amorphous structure form [29]. As shown in Figure 4, the characteristic peaks for Ag nanoparticles appear at 38.14^{o}, 44.28^{o}, 65^{o}, 78^{o}, and 81.7^{o} which correspond to crystal facets of {111}, {200}, {220}, {311}, and {222} of silver (Ag) as compared and interpreted to standard data of JCPDS (No. 04-0783). Each crystallographic facet contains energetically distinct sites based on atom density. The adsorption of Ag^{+} ions changes crystalline structure and the degree of ordering of the tested sample be reduced – see in Figure 4, which agrees to the previously reported result [30].

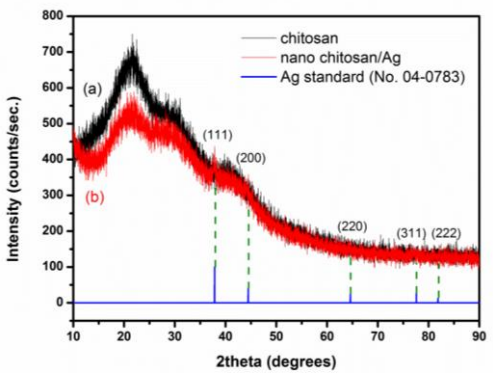

Figure 4. XRD patterns of (a) chitosan and (b) chitosan/Ag nanocomposites using kumquat extract at 70 ^{o}C for 90 min.

3.2. Antibacterial activity measurement of the chitosan/Ag nanocomposites on *S. aureus* and *E. coli* bacteria strains

The effect of the chitosan/Ag nanocomposites (with amount of Ag in chitosan/Ag nanocomposites being 23.7 mg/kg from analysis result of ICP) on the growth of GFP-expressing

E. coli and *S. aureus* was investigated by monitoring culture turbidity (Table 1). Growth was completely inhibited at chitosan/Ag nanocomposites volumes ≥ 10 μL. This volume (10 μL) of the chitosan/Ag nanocomposite was considered to be the MIC of *E. coli*, while a volume of 90 μL was found to be the MIC of *S. aureus* (Figure 5). Besides, inhibition with 100 μL chitosan nanoparticle was lower growth as compared to bacterial growth using chitosan/Ag nanocomposites (Table 1).

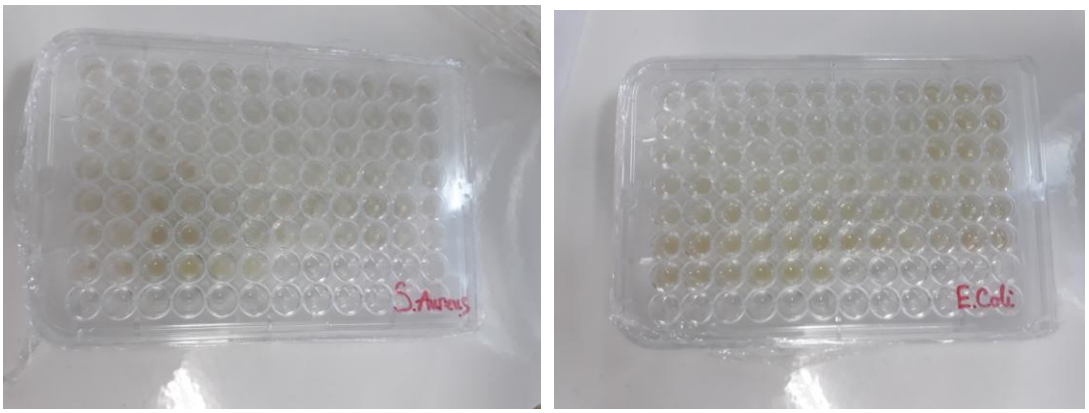

Figure 5. Representative images of 96 wells per agar disk (*S. aureus* and *E. coli* bacteria) containing chitosan/Ag nanocomposites with various volumes of chitosan/Ag nanocomposites solution: 0 μL; 10 μL; 20 μL; 30 μL; 40 μL; 50 μL; 60 μL; 70 μL; 80 μL; 90 μL; and 100 μL, respectively.

Table 1. MIC values of the chitosan/Ag nanocomposite samples against *E. coli* and *S. aureus.*

Inhibital percentage (%)	*E. coli* inhibited (%)			*S. aureus* inhibited (%)		
	Chitosan	Chitosan nanoparticles	Chitosan/Ag nanocomposites	Chitosan	Chitosan nanoparticles	Chitosan/Ag nanocomposites
100	86	88	96	80	82	93
90	85	86	90	72	79	89
80	81	84	86	71	78	81
70	81	88	88	70	73	78
60	80	82	85	71	75	77
50	78	81	81	71	74	79
40	74	79	84	70	76	75
30	64	68	82	69	71	74
20	60	64	80	67	70	74
10	57	59	79	73	70	78

4. CONCLUSION

A simple and rapid green synthesis of chitosan/Ag nanocomposites using kumquat extract have been successfully developed in this study. It proves to be an eco-friendly, rapid green

approach for the synthesis providing a cost effective and an efficient route for the chitosan/Ag nanocomposites' synthesis. It indicated that synthesized chitosan/Ag nanocomposites have uniform, very well capped particle structures ~15-25 nm in size. It is demonstrated that using kumquat extract for the synthesis of chitosan/Ag nanocomposites have brought many benefits such as energy efficient, cost effective, rapid reaction time, protecting human health (non-toxic to humans in minute concentrations) and environment leading to safer products and lesser waste. Therefore, it has greatly potential and promising to use in biomedical applications and plays an important role in opto-electronics and medical devices in future.

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