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A simple and green preparation of chitosan/silver nanocomposites films and studying their antibacterial activity on *Staphylococcus aureus* and *Escherichia coli*

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Abstract

A simple and eco-friendly approach has been successfully developed for the preparation of chitosan nanocomposites films loaded with silver nanoparticles using kumquat extract as a biological reducing agent. The chitosan/silver nanocomposites (CTS/Ag NCPs) films were prepared from chitosan/silver nanocomposites solution and dried for 14 h at 70^{o}C in a vacuum oven with the pressure of 0.03 Mpa. The morphology and characterization of CTS/Ag NCPs films have been also determined by FTIR, XRD, and SEM. The UV-vis spectroscopy and TEM image indicated that synthesized chitosan/silver nanocomposites have spherical shape with their uniform dispersion and their average particle size of about 20-30 nm. The prepared CTS/Ag NCPs films showed their great antibacterial activity on *Staphylococcus aureus (S. Aureus)* and *Escherichia coli (E. coli)*. Therefore, this eco-friendly method that would be used for the preparation of chitosan/Ag nanocomposites films could be competitive and alternative to the existing ones. Moreover, CTS/Ag NCPs films would have their high potential for biomedical applications (i.e, medical tape, burn treatment, etc.), opto-electronics and medical devices in the current time and in the future.

Keywords: Chitosan/Ag nanocomposites films (CTS/Ag NCPs films), Kumquat extract, *Staphylococcus aureus (S. Aureus)* bacteria, *Escherichia coli (E. coli)* bacteria, eco-friendly method.

1. Introduction

In numerous years ago, nanomaterials with their antimicrobial properties and their high surface area-to-volume ratio compared to that of other micro scale materials have drawn the scientific community's incredible attention. Actually, the nanoparticles have demonstrated their more prominent properties and efficiency due to their ability in binding to more duplicates of microbial particles and cells or their great optical, mechanical, magnetic and chemical properties, which were essentially distinctive with those of bulk materials [1]. The antibacterial activity of nanomaterials have been also examined for growth inhibitors, killing agents or antibiotic carriers [2-4].

Chitosan has been considered as an exceptionally abundant and pretty cheap natural biopolymer with great biological properties such as antitumor feature, antimicrobial activity and immune enhancing effect, which has attracted much interest of many scientists [5, 6]. Furthermore, it has been reported that the antimicrobial and antioxidant activities of chitosan were significantly enhanced due to loading chitosan with different metals [7, 8].

Silver nanoparticles (Ag NPs) have been renowned for their excellent antimicrobial properties and being nontoxic and safe molecules to human cells [9]. Therefore, they have been largely studied for their medical applications [10, 11]. A few methods to produce silver nanoparticles (Ag NPs) have been created by utilizing both physical and chemical approaches such as sonochemical and electrochemical methods, thermal decomposition, laser ablation, microwave irradiation, etc… [12-15]. However, these approaches remains restrictions of using of harmful chemicals, high operational cost and energy demand. .Therefore, the preparation of metallic nanoparticles by green synthesis to replace existent methods has been shown much attention to the scientific community in recent decades [16-20]. Green synthesis is the eco-friendly process applied in chemistry, chemical technology and engineering which was getting to be more popular and vital due to increasing global's awareness of environmental issues [21]. In fact, green synthetic methods have used new alternatives for metal nanoparticles, for instance, natural polymers (chitosan, etc.), sugars, enzymes, microorganisms, plant extracts as reductants (e.g, lemon aqueous extract, Azadirachita indica aqueous leaf extract,…) and capping agents [22-26] applied for Ag NPs green synthesis. These approaches are simple, one-step, cost-effective, energy efficient, more stable and environmental friendly [27-32].

Actually, polymer/nanoparticles blending innovation is an successful approach to get novel materials with optimized properties (i.e, electronic, magnetic, high sensitivity and antibacterial activity,...), which shows many advantages such as versatility, simplicity, inexpensiveness and non-toxicity [33, 34]. Herein, this research provided a green and simple synthesis of chitosan/silver nanocomposites films from chitosan/silver nanocomposites solution applying kumquat extract as a bio-reducing agent and examined their antibacterial properties *in vitro*. It proved that this green chitosan/silver nanoparticles' synthesis was simple, cost effective, easy to perform, stable and sustainable and created films with uniform particle size. Moreover, the synthesized chitosan films loaded with silver nanoparticles showed their antibacterial

activity against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* ***(****E. Coli)*. Therefore, the studied green chitosan/silver nanoparticles will be a new material and promising bacteriolytic agent for many applications (i.e, biomedical, food, agriculture and cosmetics, etc.) in the current time and in the future.

2. EXPERIMENTAL SECTION

2.1. Materials

Silver nitrate ($AgNO_3$) was purchased from Acros. Kumquat fruit was found from supermarkets in Can Tho City, Vietnam. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli (E. coli)* were bought from Sigma-Aldrich. Luria–Bertani broth (LB), agar powder (bacteriological grade), and sodium tripolyphosphat (STPP, 99%) were purchased from HiMedia, Mumbai, India. Chitosan was bought from Vietnam's company. All solutions were prepared with deionized water (DI H_2O) from a MilliQ system.

2.2. Methods

2.2.1. Preparation of kumquat extract

Fresh kumquats were squeezed for juice. After that, the kumquat juice was filtered, centrifuged and washed with DI water three times to obtain the kumquat extract. This extract was used for synthesis of chitosan/silver nanocomposites (CTS/Ag NCPs) in following steps.

2.2.2. Preparation of chitosan/silver nanocomposites films

Chitosan/silver nanocomposites (CTS/Ag NCPs) were synthesized by a green method using kumquat extract as a biological reducing agent. In a typical synthesis, 2 mL of STPP (1 mg in 1 mL DI H_2O) was added to 10 mL of chitosan solution (1 mg/mL in acetic acid solution of 2%) and stirred for 30 min at room temperature. After that, 1 mL of $AgNO_3$ (0.01 M) and 2 mL of kumquat extract was also quickly added into the above solution and stirred for 90 min at 70^oC. Upon the temperature and time of reaction, the reaction mixture went through a series of color changes including blue, light yellow, pink, and red, etc... The solution was then centrifuged (10000 rpm; 15 min) and washed with deionized water (DI water) to remove excess and then redisposed in DI water. The average particle size of the as-prepared chitosan/silver nanocomposite is approximately 20-30 nm.

The optimized chitosan/silver nanocomposites (CTS/Ag NCPs) films were prepared by centrifuging chitosan/silver nanocomposites solution, then drying the films for 14 h at 70^oC in a vacuum oven with the pressure of 0.03 MPa.

The photos of chitosan/silver nanocomposites (CTS/Ag NCPs) films dried for 14 h in a vacuum oven (at 70^oC, 0.03 MPa) with different volumes of $AgNO_3$ solution in the

chitosan nanoparticles' mixture have been shown in Figure 1.

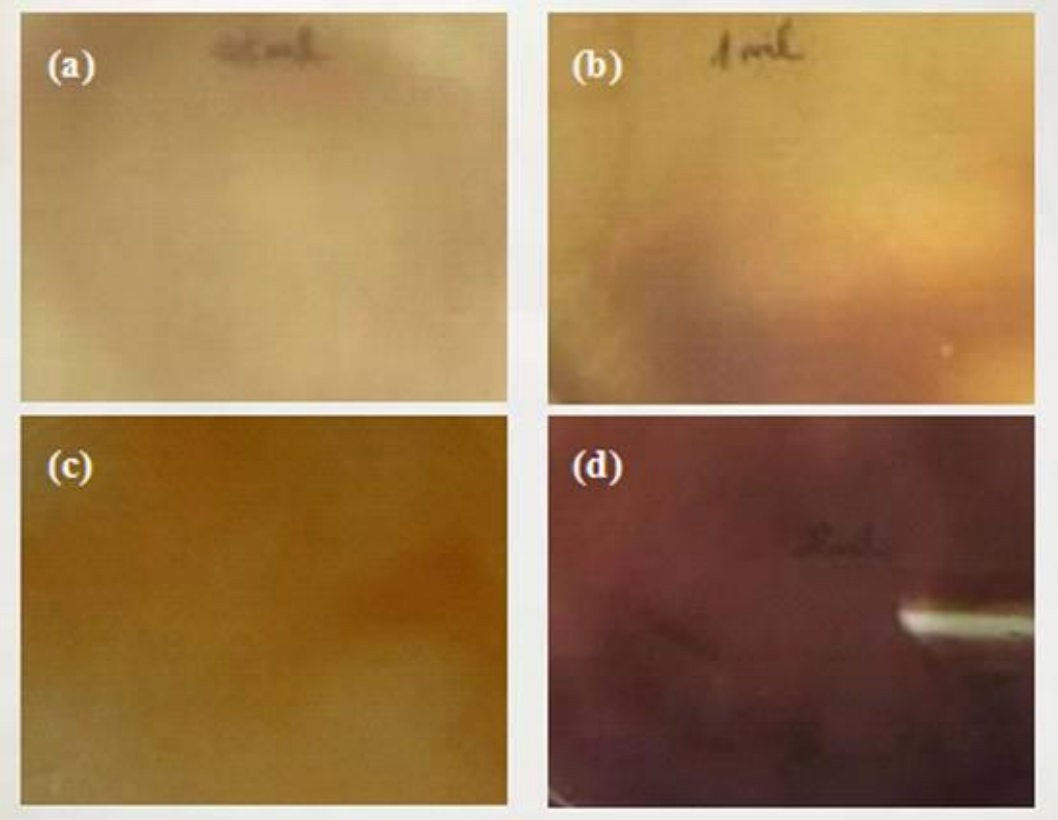

Figure 1. Photos of chitosan/silver nanocomposites (CTS/Ag NCPs) films dried in a vacuum oven for 14 h at 70°C with various volumes of $AgNO_3$ solution: (a) 0.5 mL; (b) 1 mL; (c) 1.5 mL; and (d) 2 mL, respectively.

2.2.3. Characterization

For characterization of the chitosan/Ag nanocomposites and the chitosan/Ag nanocomposites film, many examinations were conducted including the absorbance spectra of particle solutions examined by UV–vis spectrophotometry (UV-675; Shimadzu); the particle size and surface morphology of chitosan/Ag nanocomposites investigated by transmission electron microscope (TEM) with a Philips Tecnai F20 G2 FEI-TEM microscope (accelerating voltage 200 kV). In addition, fourier transform infrared spectroscopy (FTIR) spectra of chitosan/Ag nanocomposites films were found by using a Renishaw 2000 confocal Raman microscope system. Also, the phase structure of chitosan/Ag nanocomposites film was determined by a 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° and 80°. The morphology of chitosan/Ag nanocomposites film was performed by scanning electron microscope (SEM) with a JEOL JSM-6300F (SEMTech Solutions, Natick, Massachusetts, USA or Inspect S, FEI Ltd., Holland).

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

The chitosan, chitosan nanoparticles and chitosan/Ag nanocomposites solutions were vacuum dried at 70^{o}C during 14 h for making films. The antibacterial activities of the chitosan, chitosan nanoparticles and chitosan/Ag nanocomposites films were tested on two strains of *Escherichia coli (E. coli)* Gram (-) and *Staphylococcus aureus (S. aureus)* Gram (+). The bacterial suspension (30 μL of 10^6 CFU.mL^{-1}) respective of *S. aureus* and *E. coli* were applied uniformly on the surface of nutrient Luria-Bertani (LB) agar plates. The chitosan, chitosan nanoparticles and chitosan/Ag nanocomposites films have been cut into small and round films with the diameter of approximately 6 mm. These films were respectively set up on the surface of the Petri agar plates with inoculated bacteria at the equal distance points that create a top of an equilateral triangle. These plates were incubated at 37^{o}C for 24 h, after which the average diameter of the inhibition zone surrounding the disk was measured by a ruler with up to 1 mm resolution and compared to the antibacterial ability of the chitosan, chitosan nanoparticles and chitosan/Ag nanocomposites films. The mean and standard deviation (SD) reported for chitosan/Ag nanocomposites (CS/Ag NCPs) films and with microbial strain (*S. aureus* and *E. coli)* were based on three replicates.

3. RESULTS AND DISCUSSION

3.1. Characterization of the chitosan/Ag nanocomposites and the chitosan/Ag nanocomposites films

As shown in Figure 2, the UV-vis spectra of chitosan/Ag nanocomposites (CTS/Ag NCPs) exhibits with the maximum absorption peaks in the range from 401 nm to 411 nm. Herein, the plasmon resonance peaks are appropriately matched with the surface absorption of Ag nanoparticles [35]. Hence, it is demonstrated that Ag nanopaticles were created in the chitosan nanoparticles' solution. The maximum absorption peaks of chitosan/Ag nanocomposites were measured in the range of ~401-411 nm, so the average particle size of CTS/Ag NCPs can be predicted to be ~10-25 nm as compared to those of Ag nanoparticles [36]. As a result, the maximum absorption peak intensity of chitosan/Ag nanocomposites (CS/Ag NCPs) at 401 nm and 407 nm, is approximate, respectively – see Figure 2(c, e). It is clear that the size of nanoparticle at the absorption peak of 401 nm is smaller than that at the absorption peak of 402-407 nm. Thus, the optimal sample using Kumquat extract as a biological reducing agent for the chitosan/Ag nanocomposites' synthesis will be chosen for following investigations for 90 min at 70^{o}C – see in Figure 2(c).

The presence of free ions in the kumquat extract has greatly accelerated for the polyol synthesis of chitosan/Ag nanocomposites. During the synthesis, the progress of the nanoparticles production could be controlled easily through its color changes, from colorless to yellow, red-brown or blue, due to a sudden increase of the reduction rate of silver ions (Ag^+) and chitosan (high molecule mass) to become Ag and chitosan

nanoparticles (chitosan with low molecule mass). The absorption intensity of synthesized samples tends to a proportional increase of the CTS/Ag NCPs' solution color, corresponding to the increase of the reaction temperature. It demonstrated that the reaction rate of reducing agents using kumquat extract significantly affects to particle size control of synthesized CTS/Ag NCPs in the solution.

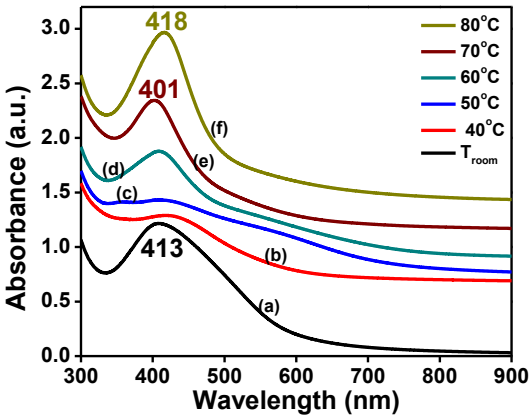

Figure 2. UV-vis spectra of chitosan/Ag nanocomposites using kumquat extract at various reaction temperatures: (a) T_{room}, (b) 40°C, (c) 50°C, (d) 60°C, (e) 70°C, and (f) 80°C, respectively.

The surface morphology of chitosan/Ag nanocomposites has been observed by Transmission electron microscopy (TEM) -see Figure 3. The TEM images of the chitosan/Ag nanoparticles demonstrate that these nanocomposites are well-dispersed, uniform and spherical with the average particle size of less than 20-30 nm. There is no agglomeration of nanoparticles perhaps due to the presence of chitosan as a capping agent. Especially, these Ag nanoparticles are uniformly diffused in the chitosan nanocomposites' matrix.

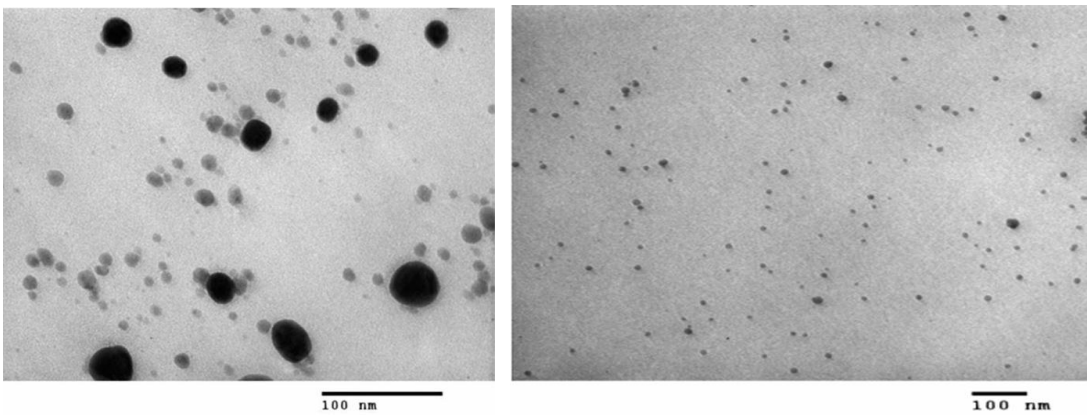

Figure 3. TEM images of chitosan/Ag nanocomposites (CTS/Ag NPs) using kumquat extract at 70 °C for 90 min.

Scanning electron microscope (SEM) was used to observe the surface morphology of fabricated chitosan/Ag nanocomposites (CTS/Ag NCPs) film – see in Figure 4. Figure 4 shows that the SEM image of silver nanoparticles shows spherical shaped particles. The size of the particles is seen within 20-30 nm. The synthesized particles are in the form of dispersions. The reduction of agglomeration is seen to occur when the chitosan is allowed to dissolve for a longer duration of time, followed by the dispersion of silver nanoparticles in the chitosan solution for about 30 min before the process of reduction.

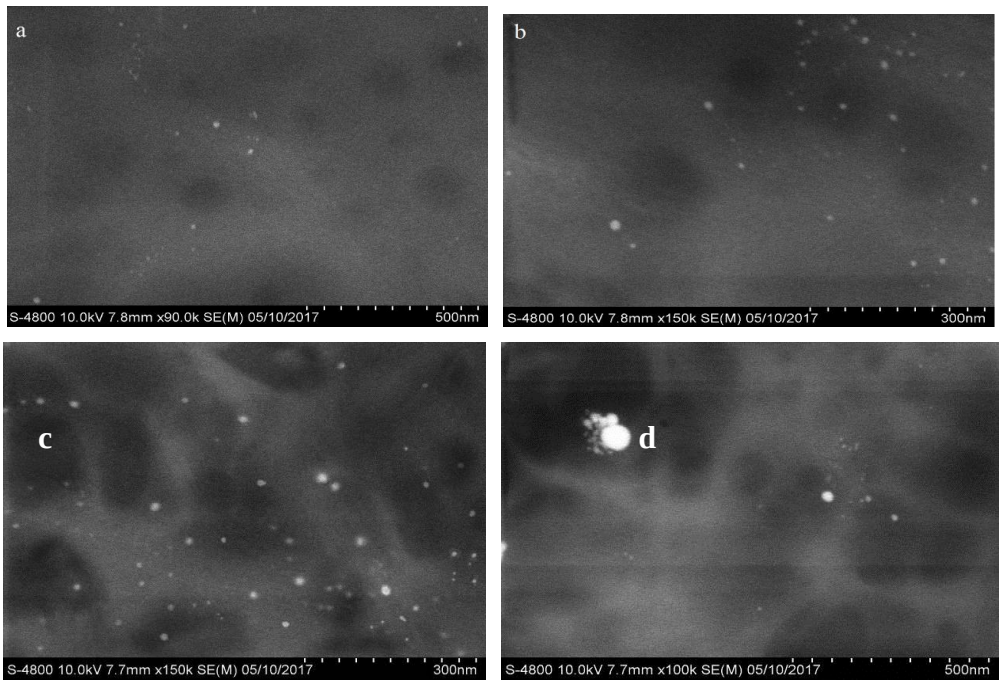

Figure 4. SEM images of chitosan/Ag nanocomposites films dried for 14 h in a vacuum oven at 70^{o}C with different volumes of $AgNO_3$ solution (0.01 M): (a) 0.5 mL; (b) 1 mL; (c) 1.5 mL; (d) 2 mL, respectively.

As shown in Figure 5, the FTIR spectrum of chitosan film displays 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 1421-1600 cm^{-1} and C-O-C band stretching at 1093 cm^{-1} [37, 38]. In the FTIR spectrum of chitosan/Ag nanocomposites (CTS/Ag NPs) film, the shifting of the chitosan peaks was observed perhaps owing to the interaction of Ag with chitosan in the nanocomposites (e.g, from 1087 cm^{-1} to ~1105 cm^{-1} and 1391 cm^{-1} (Figure 5(b) – see in Figure 5). Besides, the other significant changes that can be noticed were the reduction in the intensity of the hydroxyl (-OH) peak and the increase in the intensity of the C-O stretching, which caused by the presence of Ag nanoparticles in the chitosan film matrix.

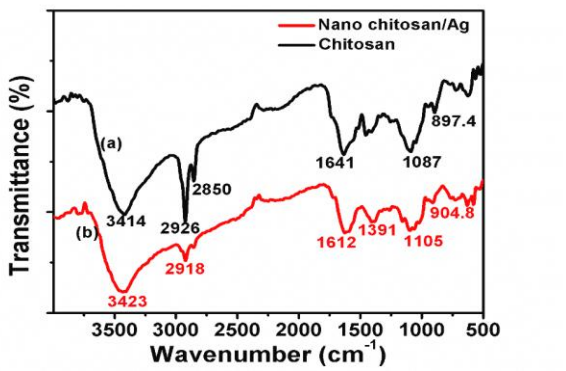

Figure 5. FTIR spectra of (a) chitosan film and (b) chitosan/Ag nanocomposites film.

The X-ray diffraction (XRD) pattern of chitosan film had a dominant peak at $2\theta = 29.3^{o}$, which demonstrated a form of amorphous structure [39]. As shown in Figure 6, the characteristic peaks for Ag nanoparticles appear at 38.14^{o}, 44.28^{o}, 65^{o}, and 78^{o}, which correspond to crystal facets of 111, 200, 220, and 311 of silver (Ag) as compared and interpreted to the standard data of JCPDS (No. 04-0783). Each crystallographic facet contains energetically distinct sites based on the atomic density. The adsorption of Ag^{+} ions changes crystalline structure and the degree of ordering of the tested sample is reduced (see in Figure 6) that matches with the previously reported result [40].

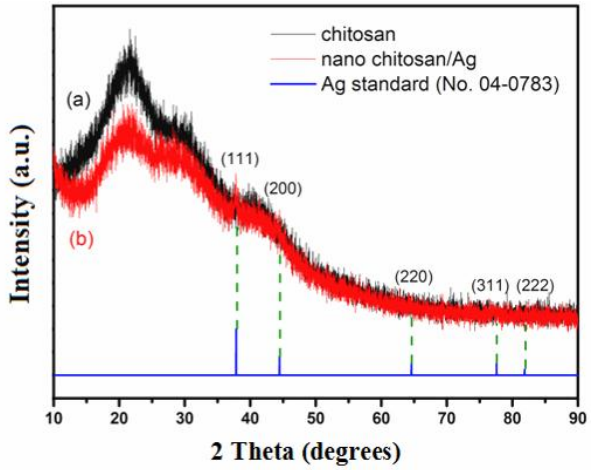

Figure 6. XRD patterns of (a) chitosan film and (b) chitosan/Ag nanocomposites film.

3.2. Antibacterial activity measurement of the chitosan/Ag nanocomposites films on Staphylococcus aureus and *Escherichia coli*

As shown in Figure 7, the result of testing the antimicrobial activity of chitosan film, chitosan nanoparticles film and chitosan/Ag nanocomposites film on two strains of

Staphylococcus aureus and *Escherichia coli* showed that the chitosan nanoparticles film formed an antibacterial halo despite of its small radii but bacteria still developed after 24 h for the chitosan nanoparticles film. With the chitosan/Ag nanocomposites film, there has been the large antibacterial halo perhaps due to the combination of Ag nanoparticles with peptidoglicans in bacterial cell walls, inhibiting the oxygen transport in the cell wall and the penetration of Ag nanoparticles into the cells, interacting and prohibiting enzymes involved in bacterial respiration [41].

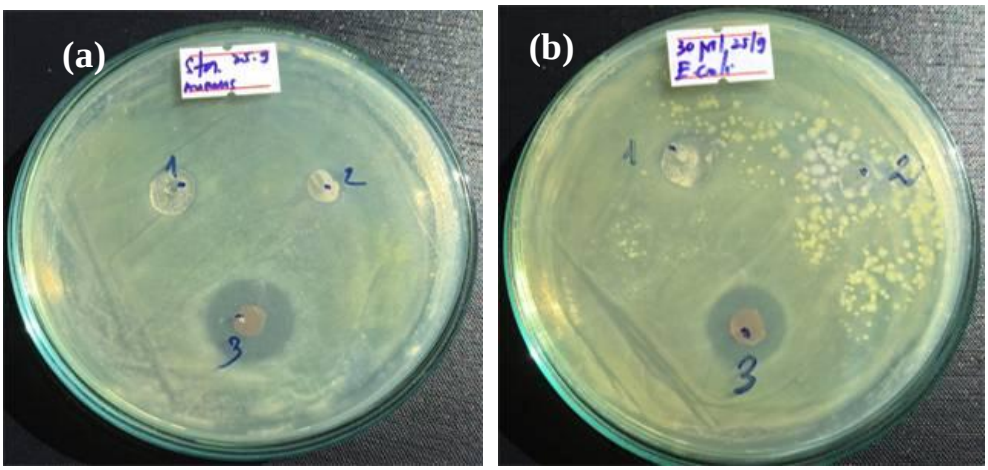

Figure 7. Colony forming units (CFUs) on agar plates treated by different substrates: (1) chitosan film, (2) chitosan nanoparticles film, (3) chitosan/Ag nanocomposites film with suspensions 30 μL of (a) *Staphylococcus aureus* (*S. aureus*) and (b) *Escherichia coli* (*E. coli*), respectively.

The chitosan/Ag nanocomposites films prepared from the chitosan/Ag nanocomposites solution with optimum conditions created clear antibacterial halos on both strains of *E. coli* and *S. aureus*. It can be seen that when the volume of bacteria spread on the plate surface changed at the levels of 30, 60 and 90 μL, the chitosan/Ag nanocomposites films still had great antibacterial activities. The chitosan/Ag nanocomposites films prepared and stored at different intervals displayed approximate antibacterial activities. The sterile halos diameters of these films were measured after 24 h of the incubation and their antibacterial activities were maintained during 48h, then their sterile halos diameters started to shorten after 48 h, and these films almost have no antibacterial activities after 72h. The observation showed that there has been a greater antibacterial efficiency (still maintained after 72 h of incubation) and larger sterile halos diameters of the chitosan/Ag nanocomposites films on *S.aureus* compared to on *E. coli* – see in Figure 8 and Tables (1, and 2).

From the above results, the fabricated chitosan/Ag nanocomposites films provided great antimicrobial activities on both strains of *E. coli* and *S. Aureus.* Actually, these films prepared and stored at different intervals displayed approximate antibacterial activities which were relatively maintained for a longtime, being suitable for medical applications in the treatment of injuries and burns.

Table 1. Results of antibacterial activity test of the chitosan/ Ag nanoparticles films on *E. coli.*

Volume of *E. Coli* suspension	**The chitosan/Ag nanoparticles films at different times**	**Antibacterial halos diameter upon time (h)**				
		24	**36**	**48**	**60**	**72**
30 µL	Film of 1 day	1.2 cm	1.2 cm	1.1 cm	0.9 cm	-
	Film of 3 day	1.3 cm	1.2 cm	1.2 cm	1.0 cm	0.8 cm
	Film of 7 day	1.3 cm	1.3 cm	1.2 cm	0.9 cm	0.8 cm
	Film of 14 day	1.1 cm	1.0 cm	1.0 cm	0.8 cm	-
60 µL	Film of 1 day	1.4 cm	1.4 cm	1.3 cm	1.1 cm	0.8 cm
	Film of 3 day	1.5 cm	1.5 cm	1.3 cm	1.1 cm	0.8 cm
	Film of 7 day	1.5 cm	1.5 cm	1.3 cm	1.0 cm	0.7 cm
	Film of 14 day	1.2 cm	1.2 cm	1.0 cm	0.8 cm	-
90 µL	Film of 1 day	1.3 cm	1.3 cm	1.1 cm	0.8 cm	-
	Film of 3 day	1.2 cm	1.2 cm	1.0 cm	0.8 cm	0.7 cm
	Film of 7 day	1.2 cm	1.2 cm	1 .0 cm	0.8 cm	-
	Film of 14 day	0.8 cm	0.8 cm	0.8 cm	0.6 cm	-

Table 2. Results of antibacterial activity test of the chitosan/ Ag nanoparticles films on *S. aureus.*

Volume of *S. Aureus* suspension	**The chitosan/Ag nanoparticles films at different times**	**Antibacterial halos diameter upon time (h)**				
		24	**36**	**48**	**60**	**72**
30 µL	Film of 1 day	1.5 cm	1.5 cm	1.3 cm	1.1 cm	0.8 cm
	Film of 3 day	1.5 cm	1.5 cm	1.4 cm	1.1 cm	0.7 cm
	Film of 7 day	1.3 cm	1.3 cm	1.3 cm	1.0 cm	0.8 cm
	Film of 14 day	1.3 cm	1.3 cm	1.3 cm	1.0 cm	0.8 cm
60 µL	Film of 1 day	1.5 cm	1.5 cm	1.2 cm	1.0 cm	0.8 cm
	Film of 3 day	1.5 cm	1.5 cm	1.3 cm	1.1 cm	1.0 cm
	Film of 7 day	1.5 cm	1.5 cm	1.3 cm	1.0 cm	-
	Film of 14 day	1.4 cm	1.4 cm	1.2 cm	1.0 cm	0.7 cm
90 µL	Film of 1 day	1.3 cm	1.3 cm	1.2 cm	1.0 cm	0.8 cm
	Film of 3 day	1.6 cm	1.6 cm	1.5 cm	1.3 cm	1.0 cm
	Film of 7 day	1.4 cm	1.4 cm	1.4 cm	1.2 cm	0.8 cm
	Film of 14 day	1.4 cm	1.4 cm	1.3 cm	1.1 cm	0.9 cm

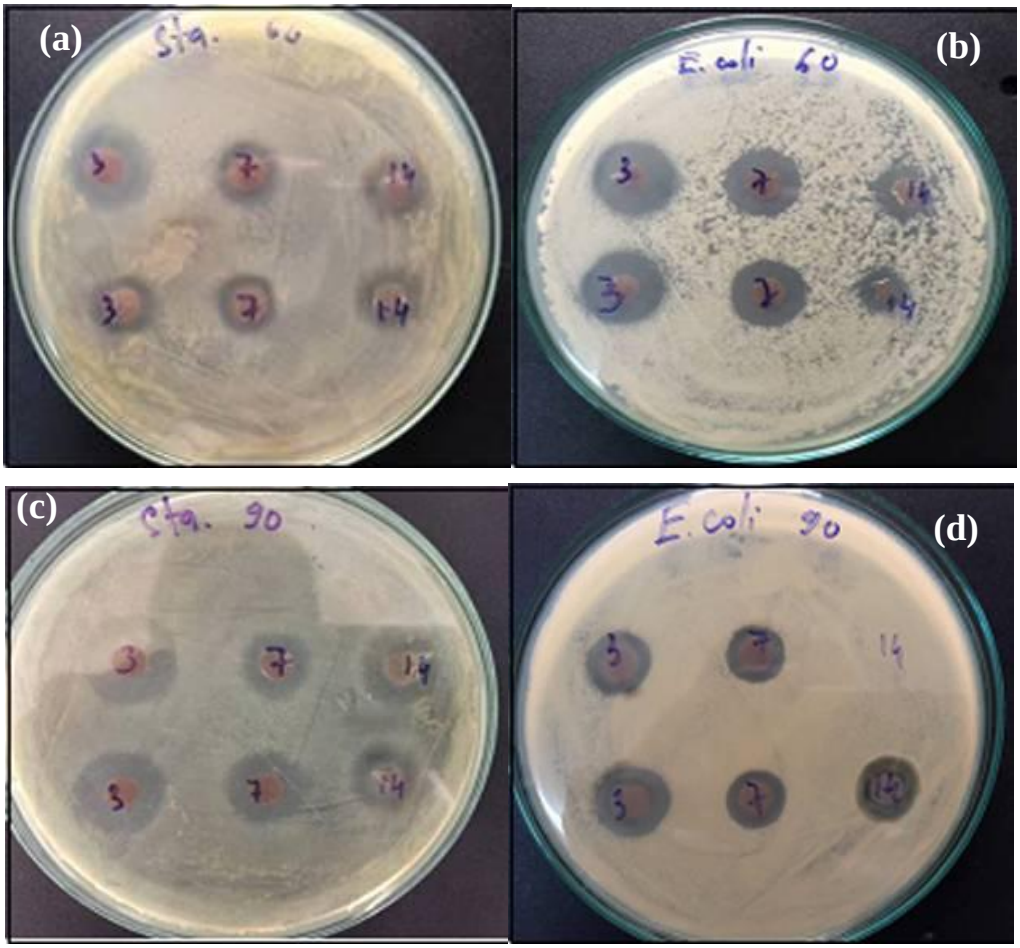

Figure 8. Colony forming units (CFUs) on agar plates treated by chitosan/Ag nanocomposites films after being stored at: (3) 3 days, (7) 7 days, and (14) 14 days with various suspension volumes of (a) 60 μL of *Staphylococcus aureus* (*S. aureus*) and (b) *Escherichia coli* (*E. coli*); and 90 μL of (c) *Staphylococcus aureus* (*S. aureus*) and (d) *Escherichia coli* (*E. coli*), respectively.

4. CONCLUSIONS

A new, simple and green preparation of chitosan/Ag nanocomposites (CTS/Ag NCPs) films using kumquat extract as a biological reducing agent have been successfully developed in this study. It proves to be an eco-friendly approach, a cost effectiveness and an efficient route for the chitosan/Ag nanocomposites'synthesis. The prepared CTS/Ag NCPs films were determined their characterization and morphology by UV-vis, FTIR, XRD and SEM. Moreover, the obtained chitosan/Ag nanocomposites (CTS/Ag NCPs) films also showed their efficient antimicrobial activities against *S. aureus* and *E. coli*. It is demonstrated that these films have sustainable antimicrobial activities and are safe in use. Therefore, they have great and promising potential to be used for biomedical applications (i.e, medical tape, ect.) and play an important role for opto-electronics and medical devices in the future.

Acknowledgements

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