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Comparative study of chitosan/Ag nanocomposites synthesis and test their antibacterial activity on *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

A green and simple method has been successfully developed to synthesize chitosan/Ag nanocomposites using Kumquat extract and River-leaf creeper extract as biological reducing agents. It is indicated to be an eco-friendly and green method, so it is suitable for a feasible synthesis of chitosan/Ag nanocomposites with cost effectiveness. The prepared chitosan/Ag nanocomposites have been characterized by UV-vis, Transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD). Result showed those chitosan/Ag nanocomposites have been obtained with average particle size of ~15-25 nm (using kumquat extract) and ~15-41 nm (using river-leaf creeper extract). Moreover, the synthesized chitosan/Ag nanocomposites also showed their efficient antimicrobial activity against Staphylococcus aureus and Escherichia coli. This new combined material has been observed to have significantly higher antimicrobial activity than its components do at their corresponding concentrations. The presence of a small percentage (2.75%, 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. Therefore, this eco-friendly method could be competitive and alternative to the existing ones that would be used for synthesis of chitosan/Ag nanocomposites. Thus, it would be highly potential to be used in biomedical applications, opto-electronics and medical devices in the future.

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1 INTRODUCTION

Nanomaterials are more efficient since they are able to attach more copies of microbial molecules and cells in last years (Luo *et al.*, 2008). Nanomaterials have been investigated for antibacterial activity as growth inhibitors, killing agents or antibiotic carriers (Qi *et al.*, 2004; Cioffi *et al.*, 2005; Kumar *et al.*, 2005).

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 (Gu *et al.*, 2003; Wan *et al.*, 2011). In the recent time, antimicrobial and antioxidative activities of chitosan have been significantly enhanced because of loading chitosan with various metals found in the previous reports (Liau *et al.*, 1997; Du *et al.*, 2009).

Among all antibacterial metals, silver nanoparticles (Ag NPs) are well known for their strong antimicrobial properties and in addition they are nontoxic and harmless to human cells (Reneker *et al.*, 2008). Thus, silver nanoparticles have soon become subjects taking much attention to medical applications due to their excellent properties such as antibacterial activity (Chen *et al.*, 2006; Roe *et al.*, 2008).

A number of methods for producing Ag NPs have been developed using both physical and chemical approaches such as sonochemical and electrochemical methods, thermal decomposition, laser ablation, microwave irradiation, etc. (Tang, 2001; Bae *et al.*, 2002; Zhang *et al.*, 2004; Kim *et al.*, 2005). However, they still have limitations such as use 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 (Panigrahi *et al.*, 2005; Qian *et al.*, 2005; Huang *et al.*, 2006; Pal *et al.*, 2007; Pal *et al.*, 2008).

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 globe's concern is about environmental problems in recent years (Thuesombat *et al.*, 2014). 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, *Azadirachta indica* aqueous leaf extract, kumquat aqueous extract, etc.), and capping agents (Bar *et al.*, 2009; Prabhu *et al.*, 2012; Gopinath, 2013; Mittal *et al.*, 2013; Rafique *et al.*, 2017). They are simple, one step, cost-effective, energy efficient, more stable, and environmentally friendly (Kong *et al.*, 2010; Badawy, 2011; Kharissova *et al.*, 2013; Ahmed *et al.*, 2016; Benelli, 2016).

It is known that using of Kumquat extract and River-leaf creeper extract as biological reducing agents to synthesize chitosan/silver nanocomposites has not been previously reported. Herein, Kumquat, which is a Fortunella japonica species of the Rutaceae familia, was used as a reducing agent for bioconversion of silver ions (Ag+) to nanoparticles (Ag0). River-leaf creeper is also a plant with high bioactivity, which is the Aganonerion polymorphum species of the Apocynaceae familia. Accordingly, the main objective of this paper is to research a feasible synthesis of chitosan/Ag nanocomposites and to investigate their antibacterial activity in vitro.

Herein, the synthesis of chitosan/Ag nanocomposites proposed a green route choosing River-leaf creeper extract and Kumquat extract as biological reducing agents without additionally using any harmful chemical/physical methods. Consequently, this synthetic method is simple, cost effective, easy to perform, stable, and sustainable with uniform particle size. Now, chitosan/Ag nanocomposites (CTS/Ag NCPs) can be produced at low concentration of Kumquat extract and River-leaf creeper extract. Moreover, the synthesized CTS/Ag NCPs were also evaluated by their antibacterial activity on *Staphylococcus aureus* and *Escherichia coli*. *S. aureus* (also known as *golden staph*) is a Gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a member of the normal flora of the body, frequently found in the nose, respiratory tract, and on the skin. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery. *E. coli* is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Hence, it shows that this new material has a significant promise to become as a bacteriolytic agent for applications (i.e., biomedical, food, agriculture and cosmetics, etc.) in the recent time and in future.

2 MATERIALS AND METHODS

2.1 Materials

Silver nitrate ($AgNO_3$) was purchased from Acros. Kumquat and River-leaf creeper were purchased from supermarkets at Can Tho City in Vietnam. *S. aureus* and *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 Methods

2.2.1 Preparation of extract

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

Fresh River-leaf creeper was boiled with DI water at 100ºC for 10 min and obtained the River-leaf creeper extract mixture. After that, the River-leaf creeper extract was filtered to obtain a juice extract from River-leaf creeper. This River-leaf creeper aqueous extract was used for synthesis of CTS/Ag NCPs in following steps.

2.2.2 Preparation of chitosan/Ag nanocomposites

CTS/Ag NCPs were synthesized by a green method using various reducing agents of Kumquat extract and River-leaf creeper extract. In a typical synthesis, 1 mL of $AgNO_3$ (0.01 M) was added to 40 mL of chitosan solution (1.5 mg/mL in acetic acid solution 2%). After that, 1 mL of Kumquat extract or River-leaf creeper extract was quickly added and stirred at 70ºC for 90 min. Upon temperature and time of reaction, the reaction mixture went through a series of color changes that included blue, light yellow, pink, and red. The solution was then centrifuged (10000 rpm; 15 min) and washed with deionized (DI) water to remove excess. And then redispersed in DI water. The average particle size of the as-prepared CTS/Ag NCPs is of the range ~15-25 (using Kumquat extract) and ~15-41 nm (using River-leaf creeper extract.

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 CTS/Ag NCPs were obtained by using a Renishaw 2000 confocal Raman microscope system. The phase structure of CTS/Ag NCPs was determined by an X-ray diffractometer (Bruker D8 Advance, Germany) with Cu K_α source operated at 40 kV and 30 mA. A scan rate of 0.05 deg^{-1} was used for 2θ between 10º and 80º. The particle size and surface morphology of CTS/Ag NCPs 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 CTS/Ag NCPs on S. aureus and E. coli bacteria strains

To determine the minimum inhibitory concentration (MIC) of the CTS/Ag NCPs, 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 CTS/Ag NCPs solution and grown overnight at 37°C. The minimum concentration of the CTS/Ag NCPs 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 examine the bactericidal activity of the CTS/Ag NCPs, 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 CTS/Ag NCPs or with the CTS/Ag NCPs 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 hours.

3 RESULTS AND DISCUSSION

3.1 Characterization of the CTS/Ag NCPs

As shown in Figure 1, the UV-vis spectra of CTS/Ag NCPs exhibited with the maximum absorption peak in the range from 401-411 nm (Figure 1(A)) and 401-435 nm (Figure 1(B)), respectively. Herein, the plasmon resonance peaks are appropriately matched with the surface absorption of Ag nanoparticles (Tsuji *et al.*, 2003). Hence, it is demonstrated that Ag NPs are created in the chitosan nanoparticles' solution. The maximum absorption peaks of CTS/Ag NCPs measured in the range ~401-411 nm, so the average particle size of

CTS/Ag NCPs can be predicted to be in the range of ~15-25 nm, and respectively, the former in the range of ~401-435 nm, so the latter in the range of ~15-45 nm, as compared with Ag NPs (Bar *et al.*, 2009; Rafique *et al.*, 2017). Result that the maximum absorption peak intensity of CTS/Ag NCPs respective at 401 nm and 407 nm is approximate (Figure 1A (c, e)), and the maximum absorption peaks are also gradually shifted to the visible (from 401 to 411 nm) (Figure 1B(a-d)), and from 402 nm shifted to 431 nm (Figure 1B(e, f)). Thus, the particle size of CTS/Ag NCPs at 70°C is smaller than that of CTS/Ag NCPs at 80°C as shown in Figure 1B (e, f). That may be due to the creation of many nuclei of silver ions (Ag^+) and chitosan molecules (polymers) at 70°C, which occurred bioconversion to generate CTS/Ag NCPs in the mixture solution. As known, the absorption peak in the range at 401 nm has nanoparticle size smaller than that of the absorption peak at 402-407 nm. Thus, the optimal sample using Kumquat extract as a reducing agent for the CTS/Ag NCPs' synthesis will be chosen for following investigations respective for 90 min at 70°C (Figure 1A(c)).

The presence of free ions in the Kumquat extract solution and the River-leaf creeper extract solution has greatly accelerated for the polyol synthesis of CTS/Ag NCPs. During the synthesis, the obtained CTS/Ag NCPs could easily monitor the progress of the nanoparticles production through its changes of color, 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 reaction rate of reducing agents using Kumquat extract and River-leaf creeper extract significantly affect to particle size control of synthesized CTS/Ag NCPs in the mixture solution.

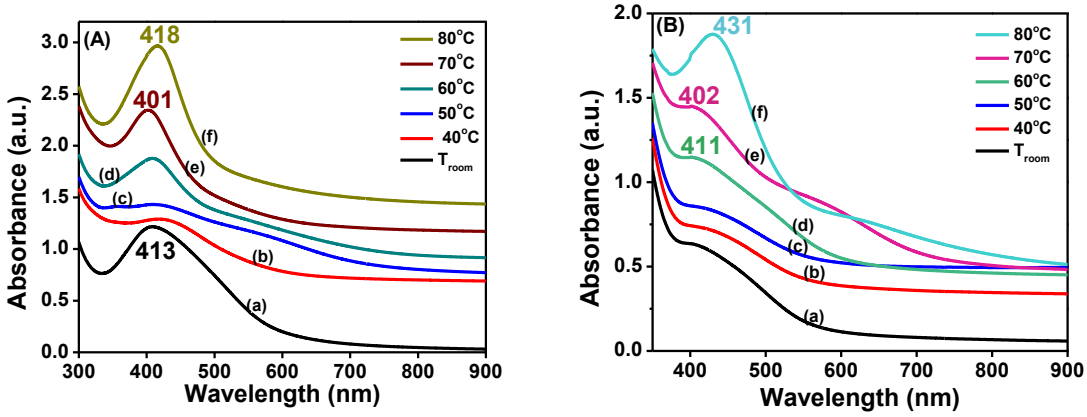

Fig. 1: UV-vis spectra of chitosan/Ag nanocomposites (CTS/Ag NCPs) using: (A) Kumquat extract, and (B) River-leaf creeper extract with various reaction temperatures: (a) T_{room}, (b) 40°C, (c) 50°C, (d) 60°C, (e) 70°C, and (f) 80°C, respectively

TEM was used to observe the surface morphology of chitosan/Ag nanocomposites. Figure 2 shows representative TEM images of CTS/Ag NCPs sample. The image of the CTS/Ag NCPs reveals the shape of nanocomposite: uniform and spherical. CTS/Ag NCPs have these properties with the average particle size in the range of ~15-25 nm (Figure 2(a, b)) and of ~15-41 nm (Figure 2(c, d)). 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 (Figure 2). As shown in Figure 2, the particle size of CTS/Ag NCPs using kumquat extract as a reducing agent are round 15-25 nm smaller than that of CTS/Ag NCPs using river-leaf creeper extract being ~15-41 nm. Therefore, the optimal sample using kumquat extract as a biological reducing agent will be chosen to synthesize CTS/Ag NCPs for the following investigations.

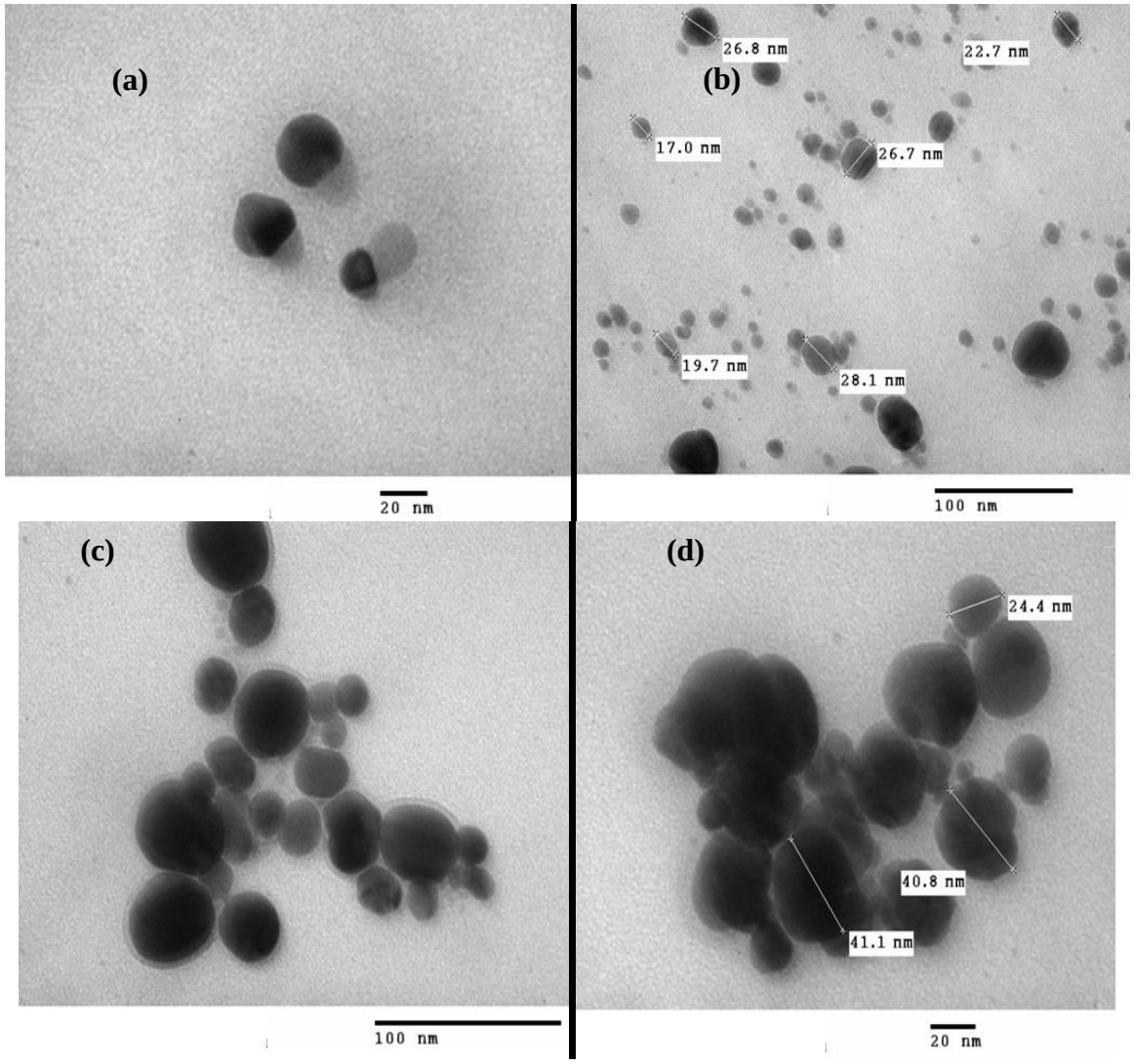

Fig. 2: TEM images of CTS/Ag NPs using Kumquat extract (a, b) and River-leaf creeper extract (c, d), at 70ºC for 90 min

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 1421-1600 cm^{-1} and C-O-C band stretching at 1093 cm^{-1} (Saraswathy *et al.*, 2001; Ali *et al.*, 2011). In the FTIR spectrum of CTS/Ag NPs, the shifting of the chitosan peaks is observed perhaps due to the interaction of Ag with chitosan in the nanocomposite (e.g. from 1421 cm^{-1} shifted to ~1411 cm^{-1} (Figure 3(b)). 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 occurred by the presence of Ag NPs the chitosan matrix and the formation of the mixture solution of CTS/Ag NPs.

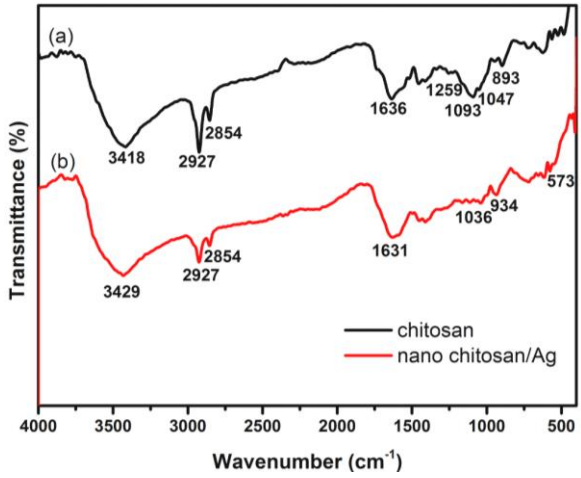

Fig. 3: FTIR spectra of (a) chitosan and (b) chitosan/Ag nanocomposites using kumquat extract at 70°C for 90 min

The X-ray diffraction (XRD) pattern of pure chitosan powder has a dominant peak at 2θ = 21°, which according to literature could demonstrate a form of amorphous structure (Webster, 2007). As shown in Figure 4, the characteristic peaks for Ag NPs appear at 38.14°, 44.28°, 65°, 78°, and 81.7° which correspond to crystal facets of {111}, {200}, {220}, {311}, and {222} of 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 (Figure 4) do agree with the previously reported result (Modrzejewska, 2009).

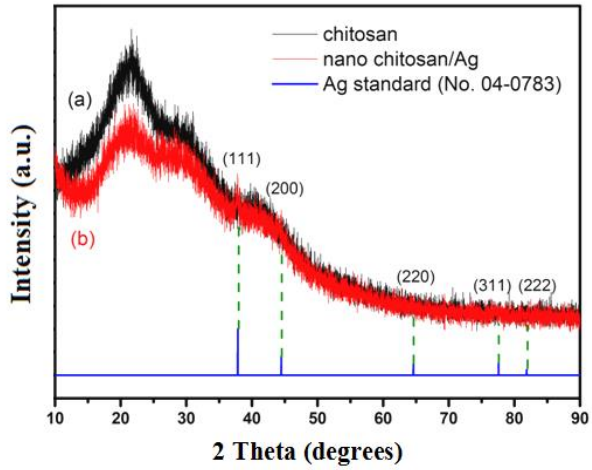

Fig. 4: XRD patterns of (a) chitosan and (b) chitosan/Ag nanocomposites using kumquat extract at 70°C for 90 min

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

The effect of the CTS/Ag NCPs on the growth of GFP-expressing *E. coli* and *S. aureus* was investigated by monitoring culture turbidity (Table 1). This growth was completely inhibited at CTS/Ag NCPs volumes ≥10 µL. This volume (10 µL) of the CTS/Ag NCPs was considered to be the MIC of *E. coli*, whereas a volume of 90 µL was found to be the MIC of *S. aureus*. Besides, inhibition with 100 µL chitosan nanoparticle was lower growth as compared to bacterial growth using CTS/Ag NCPs (Table 1).

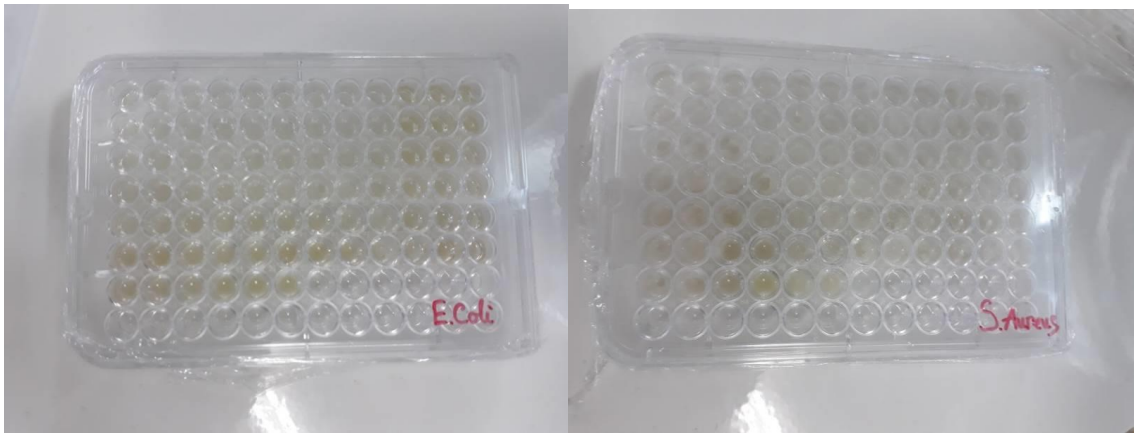

Fig. 5: Representative images of 96 wells per agar disk (*E. coli* and *S. aureus* bacteria) containing chitosan/Ag nanocomposites with various volumes of CTS/Ag NCPs solution: 0 µL; 10 µL; 20 µL; 30 µL; 40 µL; 50 µL; 60 µL; 70 µL; 80 µL; 90 µL; and 100 µL, respectively

Table 1: MIC values of the CTS/Ag NCPs samples against *E. coli* and *S. aureus*

Inhibitory percentage (%)	*E. coli* inhibited (%)			*S. aureus* inhibited (%)		
	Chitosan	Chitosan nanoparticles	Chitosan/Ag nanocomposites	Chitosan	Chitosan nanoparticles	Chitosan/Ag nanocomposites
100	82	85	96	83	85	91
90	79	82	95	72	79	89
80	79	81	87	75	74	87
70	76	81	82	80	80	82
60	78	78	83	81	85	87
50	78	82	87	81	84	89
40	75	81	84	79	80	84
30	80	80	85	81	83	84
20	66	68	82	70	73	84
10	67	69	81	73	74	89

4 CONCLUSIONS

A green and simple approach for the synthesis of CTS/Ag NCPs using Kumquat extract and River-leaf creeper extract have been successfully developed in this study. It is proved to be an eco-friendly, green approach for a synthesis of CTS/Ag NPs, providing a cost effectiveness and an efficient route for the CTS/Ag NCPs' synthesis. It indicated that synthesized chitosan/Ag nanocomposites have uniform, very well capped particle structures, respective about 15-25 nm (using kumquat extract) and around 15-41 nm (using river-leaf creeper extract) in size. Moreover, the synthesized CTS/Ag NCPs also showed efficient antimicrobial activity against of *S. aureus* and *E. coli* bacterial strains. The CTS/Ag NCP was found to have significantly higher antimicrobial activity than its components at their respective concentrations. The presence of a small percentage (2.75%, 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. Fluorescence spectroscopy indicated that the bacterial growth stopped immediately after exposure of *S. aureus* and *E. coli* to the CTS/Ag NCPs, with the release of cellular green fluorescent protein into the medium at a faster rate than with chitosan. It is demonstrated that using Kumquat extract and River-leaf creeper extract for the synthesis of CTS/Ag NPs may have many benefits such as energy efficiency, cost effectiveness, protection of human health (non-toxic to humans in minute concentrations) and environment, hence bringing out safer and less waste products. Therefore, it has great 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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