International Journal of Engineering Science Invention
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org ||Volume 6 Issue 12|| December 2017 || PP. 76-81

A Green Approach Using River-Leaf Creeper Extract For Synthesis And Characterization Of Chitosan/Ag Nanocomposites And Study For Their Antibacterial Activity

Tran Thi Bich Quyen[1*], Tran Anh Minh[1], Nguyen Thi Phuong Thuy[1], Tran Thi Xuan Mai[2]

[1]*Department of Chemical Engineering, College of Technology, Can Tho University, 3/2 Street, Ninh Kieu District, Can Tho City, Vietnam*

[2]*Genetic Technology Laboratory, Biotechnology Research and Development Institute, Can Tho University, 3/2Street, Ninh Kieu District, Can Tho City, Vietnam*

**Corresponding Authors: Tran Thi Bich Quyen; E-mail: ttbquyen@ctu.edu.vn; Can Tho University*

Abstract*: A simple and green approach has been successfully developed to synthesize chitosan/Ag nanocomposites using River-leaf creeper 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 an average particle size of ~15-41 nm. Moreover, the synthesized chitosan/Ag nanocomposites also showed their efficient antimicrobial activity against on the E. coli bacteria. Thus, this eco-friendly method could be a competitive alternative to the conventional physical/chemical methods used for the synthesis of chitosan/Ag nanocompoites. Since, it has a potential to use in biomedical applications, opto-electronics and medical devices in future.*

Keywords: *River-leaf creeper extract, chitosan/silver nanocomposites (CTS/Ag NCPs), Escherichia coli (E. coli) bacteria, green synthesis*

Date of Submission: 25-11-2017 Date of acceptance: 08-12-2017

I. Introduction

In last years, nanomaterials have attracted considerable attention as an antimicrobial agent their high surface area to volume ratio when compared with their micro scale counterparts. Thus, nanomaterials are more efficient, since they are able to attach more copies of microbial molecules and cells [1]. Nanomaterials have been invertigated for antibacterial activity as growth inhibitors, killing agents or antibiotic carriers [2]; [3, 4].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 [5, 6].In the recent time, antimicrobial and antioxidative activities of chitosan were significant enhanced because of loading chitosan with various metals found in the previous reports [7, 8]. Among all antibacterial metals, silver nanoparticles (Ag NPs) are well known for strong antimicrobial properties, nontoxic and no harm to human cells [9]. Thus, silver nanoparticles have widely attracted attention for medical applications due to their excellent properties such as antibacterial activity [10, 11].

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… [12-15]. 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 [16-20]. 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 [21]. 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, kumquat aqueous extract,…) and capping agents [22-26]. They are simple, one step, cost-effective, energy efficient, more stable materials and environment friendly [27-32]. According to our understanding, using River-leaf creeper extract and under supporting of microwave to synthesize chitosan/silver nanocomposites have not been previously reported. River-leaf creeper is a plant with highly bioactive, which is the *Apocynaceae* familia belong to the *Aganonerion polymorphum* species. Thus, the main objective of this study was to research the synthesis of chitosan/Ag nanocomposites and investigate their antibacterial activity *in vitro*.

The chitosan/Ag nanocomposites were synthesized by green route using River-leaf creeper extract and under supporting of microwave without using any additional harmful chemical/physical methods. Herein, the synthetic method used here is simple, cost effective, easy to perform, uniform particle size, stable and sustainable. Chitosan/Ag nanocomposites (CTS/Ag NCPs) can be produced at low concentration of River-leaf creeper extract. Moreover, the synthesized chitosan/Ag nanocomposites were also evaluated their antibacterial activity on *E. Coli* bacteria. Since, it shows that the synthesized chitosan/Ag nanocomposites have a significant promise as bactericidal agent for applications (i.e, biomedical, food, agriculture and cosmetics, etc…) in the recent time and in future.

II. Materials and Methods

2.1. Materials

Silver nitrate ($AgNO_3$) was purchased from Acros. River-leaf creeper was purchased from supermarkets at Can Tho City in Vietnam. And *Escherichia coli (E. coli)* bacteria was purchased from Sigma-Aldrich. All solutions were prepared using deionized water from a MilliQ system.

2.2. Methods

Fresh River-leaf creeper was boiled with DI water at 100^{o}C 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 chitosan/Ag nanocomposites (CTS/Ag NCPs) in following steps.

2.2.2. Preparation of chitosan/Ag nanocomposites by River-leaf creeper extract

Chitosan/Ag nanocomposites (CS/Ag NCPs) were synthesized by a green method using River-leaf creeper aqueous extract as a reducing agent and under supporting of microwave. 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 River-leaf creeper aqueous 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, etc... The solution was then centrifuged (10000 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 ~15-41 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 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^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 Escherichia coli (E. coli) bacteria

The antibacterial activity of chitosan/Ag nanocomposites was tested on the *Escherichia coli (E. coli)* strain by using standard disk diffusion method. The bacterial suspension of *E. coli* (100 µL of 10^6 CFU.mL^{-1}) was applied uniformly on the surface of a nutrient agar plate before placing the disks on the plate (5 per plate). Small disks were uniform size (2 mm diameter) containing 0 µL; 10 µL; 20 µL; 30 µL; and 40 µL of chitsoan/Ag nanocomposites solution (30 µg/mL), respectively. The plates were incubated at 37^{o}C for 17 h, after which the average diameter of the inhibition zone surrounding the disk was measured by a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for chitosan/Ag nanocomposites (CTS/Ag NCPs) and with microbial strain (*E. coli)* were based on three replicates.

III. Results and Discussion

3.1. Characterization of the chitosan/Ag nanocomposites using River-leaf creeper extract

As shown in Figure 1, the UV-vis spectra of chitosan/Ag nanocomposites (CTS/Ag NCPs) exhibited with the maximum absorption peak in the range from 401-435 nm, respectively. Herein, the plasmon resonance peaks are quite match with the surface absorption of Ag nanoparticles [33, 34]. Since, it is demonstrated that Ag nanopaticles are created in the chitosan nanoparticles' solution. The maximum absorption peaks of chitosan/Ag nanocomposites measured in the range ~401-431 nm, which can be predicted the average particle size of chitosan/Ag nanocomposites being ~15-45 nm, as compared to Ag nanoparticles [33, 34].When the reaction

temperature of the chitosan/Ag mixture solution is increased, leading to the maximum absorption peaks also gradually shifted to the visible (from 401 to 411 nm) – see in Figure 1(a-d) and from 402 nm shifted to 431 nm as Figure 1(e, f). Thus, the particle size of chitosan/Ag nanocomposite's at 70^{o}C is smaller than that at 80^{o}C as shown in Figure 1(e, f). That may be due to the creation of many nuclear of silver ions (Ag^{+}) and chitosan molecules (polymers) at 70^{o}C, which is occurred bioconversion to generate chitosan/Ag nanocomposites in the mixture solution. Therefore, the optimal sample at 70^{o}C will be chosen for following investigations.

The presence of free ions in the River-leaf creeper extract solution have 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 reaction temperature. It demonstrated that reaction rate of reducing agent using River-leaf creeper extract significantly affects to particle size control of synthetic chitosan/Ag nanocomposites in the mixture solution.

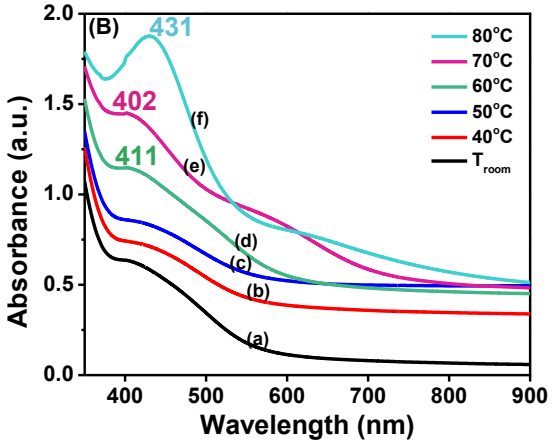

Figure 1. UV-vis spectra of chitosan/Ag nanocomposites (CTS/Ag NCPs) using River-leaf creeper aqueous extract with various reaction temperatures: (a) Troom, (b) 40^{o}C, (c) 50^{o}C, (d) 60^{o}C, (e) 70^{o}C, and (f) 80^{o}C, respectively.

Transmission electron microscopy (TEM) was used to observe the surface morphology of chitosan/Ag nanocomposites. Figure 2 shows representative TEM images of chitosan/Ag nanocomposites sample. The image of the chitosan and Ag nanoparticles reveal that the nanocomposite and that they are well disfersed and spherical in shape. Chitosan/Ag nanocomposites are uniform and spherical with average particle size less around 15-41 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.

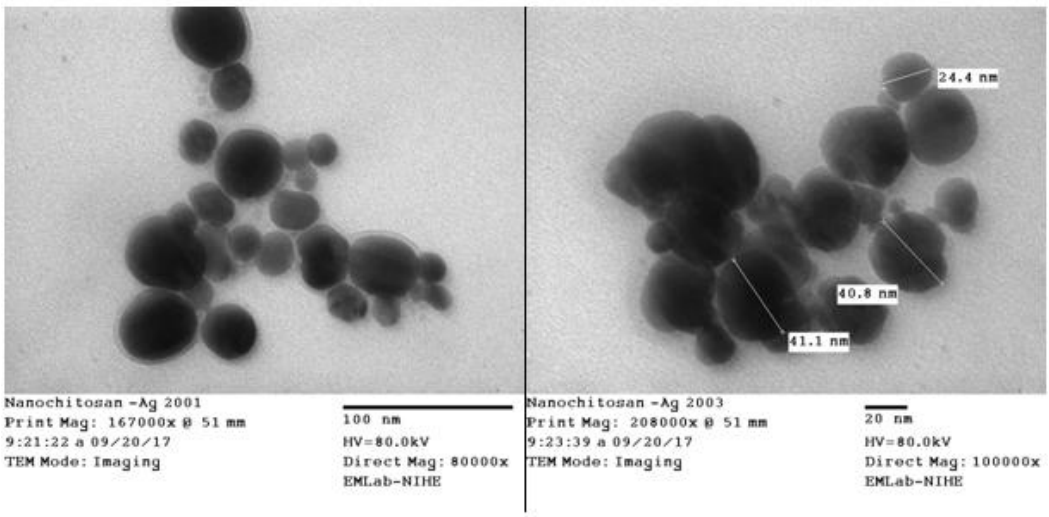

Figure 2. TEM images of chitosan/Ag nanocomposites (CTS/Ag NPs) using River-leaf creeper aqueous extract

As shown in Figure 3, the FTIR spectrum of chitosan shows the presence of bands at ~3414-3423 cm^{-1} (O-H stretching), C-H and C-N stretching at ~2926-2850 cm^{-1}, N-H bending at 1641-1612 cm^{-1}, N-H angular deformation in CO-NH plane at 1410-1600 cm^{-1} and C-O-C band stretching at 1087 cm^{-1} [35, 36]. 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 1410 cm^{-1} shifted to ~1391 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.

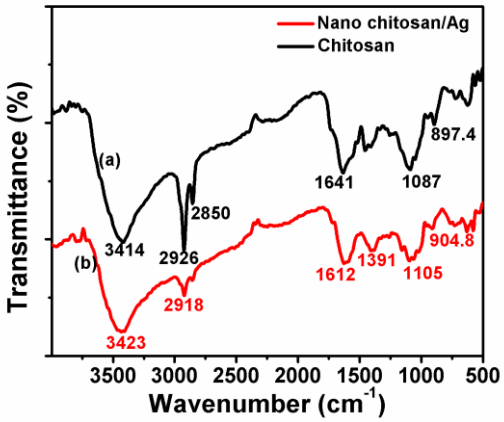

Figure 3. FTIR spectra of (a) chitosan and (b) chitosan/Ag nanocomposites using River-leaf creeper aqueous extract at 70°C for 90 min.

The X-ray diffraction (XRD) pattern of pure chitosan powder there is mainly peak at $2\theta = 29.3^{o}$, which according to literature could demonstrate crystalline structure form [37]. As shown in Figure 4, 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 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 [38].

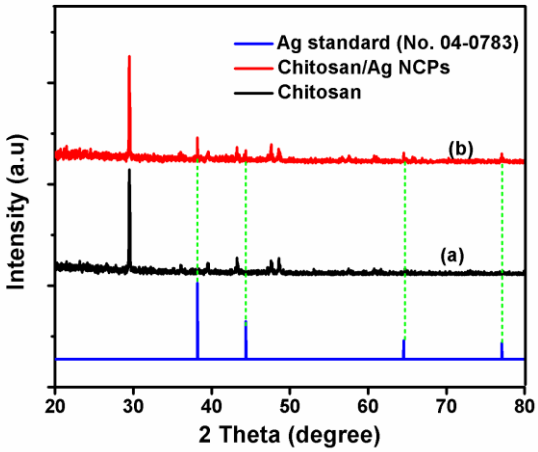

Figure 4. XRD patterns of (a) chitosan and (b) chitosan/Ag nanocomposites using River-leaf creeper aqueous extract at 70°C for 90 min.

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

The antibacterial activity of chitosan/Ag nanocomposites (30 µg/mL) was compared for *E. coli* strain in nutrient agar media using the diameter of inhibition zone in disc diffusion test with initial concentration of *E. coli* bacteria (10^{6} $CFU.mL^{-1}$). The plates were incubated for 17 h at 37°C and inhibition zones also measured. The diameter of inhibition zone (DIZ) reflects magnitude of sensitivity of the microorganism. Based on the zone of inhibition genergated, synthesized chitosan/Ag nanocomposites prove to exhibit good antibacterial activity

against *E. coli* – see in Figure 5. On the other hand, control alone did not exhibit any antibacterial activity. DIZ was measured on agar plates using a ruler with 1 mm resolution. When the volume of chitosan/Ag nanocomposites are increased, leading to increasing area of inhibition zones on the *E. coli* bacteria. On the other hand, the bacterial activity of chitosan/Ag nanocomposites is not significantly increased when the volume of chitosan/Ag nanocomposites increased from 30-40 µL. Thus, the optimal amount of chitosan/Ag nanocomposites solution for their inhibition on *E. coli* are around 20-30 µL. The results of antibacterial activities of prepared chitosan/Ag nanocomposites evaluated from the disc diffusion method are given details in Table 1.

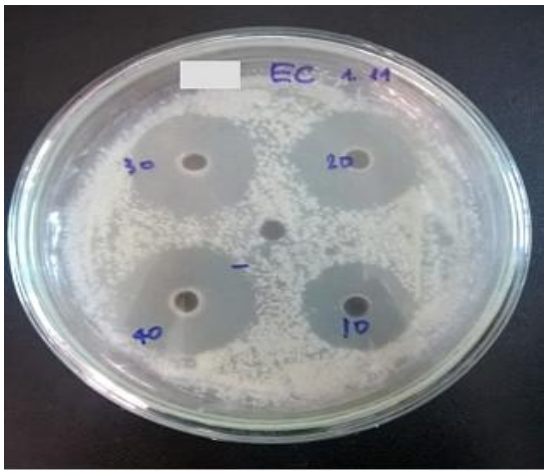

Figure 5. Representative images of agar plates containing chitosan/Ag nanocomposites with various volumes of chitosan/Ag nanocomposites solution: 0 µL; 10 µL; 20 µL; 30 µL; and 40 µL, respectively.

Table 1. Zone of inhibition (mm) obtained by disc diffusion method.

Components	**Zone of inhibition (mm)**			
Control (*E. coli*, 10^6 CFU.mL^{-1}) (-)	NZ			
Chitosan/Ag nanocomposite (L): 10; 20; 30; and 40 L, respectively	9.3	10.8	11.2	11.9

IV. Conclusions

A simple and green synthesis of chitosan/Ag nanocomposites using River-leaf creeper extract have been successfully developed in this study. It proves to be an eco-friendly, 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 about 15-41 nm in size. Moreover, the synthesized chitosan/Ag nanocomposites also showed efficient antimicrobial activity against of *E. coli* bacteria. It is demonstrated that using River-leaf creeper extract for the synthesis of chitosan/Ag nanocomposites have brought many benefits such as energy efficient, cost effective, 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.

Acknowledgements

This research is funded by Vietnam Ministry of Education and Training under grant number B2017-TCT-28ĐT.

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Tran Thi Bich Quyen "A Green Approach Using River-Leaf Creeper Extract For Synthesis And Characterization Of Chitosan/Ag Nanocomposites And Study For Their Antibacterial Activity." International Journal of Engineering Science Invention(IJESI), vol. 6, no. 12, 2017, pp. 76-81.