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GREEN SYNTHESIS OF Cu-CHITOSAN NANOCOMPOSITE BY THE EXTRACT OF AGANONERION POLYMORPHUM LEAVES FOR ANTIBACTERIAL APPLICATION

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Abstract

Cu-chitosan nanocomposite was synthesized from $CuSO_4$ solution by the green method with the extract of Aganonerion Polymorphum leaves for antibacterial application. The results of the study sample were confirmed by modern physical methods such as Fourier Transform Infrared Spectroscopy (FTIR), powder X-Ray Diffraction (XRD), Dynamic Light Scattering (DLS), Ultraviolet-Visible spectrometry (UV-Vis), and Transmission Electron Microscope (TEM), Energy Dispersive X-Ray analysis (EDX). The optimal conditions for obtaining extracts of Aganonerion Polymorphum (AP) leaves as well as for the synthesis of Cu nanoparticles and Cu-chitosan nanocomposite were the results of this work. It is found that the optimal waterleaf ratio, optimal extraction time, optimal extraction temperature and reaction temperature, the optimal concentration of $CuSO_4$ solution are 60 g leaves/400 ml water, 15 minutes, 70 ^oC and 2 mM, respectively. The XRD patterns indicated characteristic peaks of crystal copper at two theta angles 43.1^o; 50.2^o and 74^o. Cu nanoparticles that were synthesized by the extracting solution have 50 nm in an average size and the antibacterial effect of Cu-chitosan nanocomposite for E. coli (gram-negative) and B. cereus (gram-positive) is higher than 99%.

Keywords: Aganonerion polymorphum, Antibacterial application, Copper nanoparticles, Cu-chitosan nanocomposite, Green synthesis.

1. Introduction

Today more and more nano-sized materials are used in life because it has many special properties [1]. The great concern in the world nowadays is how to make and apply nano products to life without affecting our environment. In the antibacterial field, metal nanoparticles are usually used, such as gold, silver and copper. With the same antimicrobial and antifungal properties, copper nanoparticles (Cu-NPs) are cheaper than nano-sized metal like gold and silver [2-4]. Therefore, Cu-NPs synthesis methods are nowadays attracted considerable attention.

Synthesis methods of Cu-NPs generally aim to create nanoparticles of small size and high stability to maximize the applicability. In addition, in the latest published works, important applications of Cu-NPs have been focused on testing antibacterial activity to treat and kill resistant microorganisms. The results showed that Cu-NP solution has antibacterial ability against many harmful bacteria for humans and animals such as gram-positive and gram-negative [2, 5-7].

There are many researches on Cu-NPs synthesis. However, Cu-NPs synthesis is very difficult due to copper oxidation. Recently, many Cu-NPs synthesis methods are reported, in which, stabilizing materials as chitosan, native cyclodextrins [8, 9] and ascorbic acid, N_2H_4 or $NaBH_4$ as reducing agents are used [8, 10-13]. Among those, chitosan is a biodegradable, non-toxic material, easy to produce with abundant raw materials. Thanks to chitosan's nano-metal stable capability, chitosan is often used in nano-metal synthesis. In addition, chitosan has the antibacterial ability. Antibacterial ability is due to the bond formation between a metal with chitosan forming a polymer chain of chitosan and the metal on the surface of bacteria [14, 15]. Besides, we all know that reducing agents such as $NaBH_4$, N_2H_4 in copper nanoparticles synthesis are very harmful to the environment [16-18]. In order to synthesize green and environmentally friendly compounds, it is necessary to use materials available in nature and non-toxic. Therefore, green Cu nanoparticles synthesis methods were received many attentions and widely studied.

To replace $NaBH_4$ and N_2H_4, many scientists used extracts of plant leaves as green reduced agents to synthesize Cu nanoparticles. The leaves were used to synthesize Cu nanoparticles might be Cuscuta reflexa, Plantago asiatica, Centella asiatica L., etc. The use of plant leaves extracts for the synthesis of copper nanoparticles with different purposes of dyes degradation, nitroarenes and the cyanation of aldehydes using $K_4Fe(CN)_6$ [19-21]. However, there are no studies on the use of Aganonerion Polymorphum (AP) leaves extracts for the synthesis of Cu nanoparticles. For this reason, our study attempts to synthesize Cu-NP using the extract of Aganonerion Polymorphum leaves as the reducing agent and chitosan as a stabilizer. Aganonerion Polymorphum is a plant genus of the Apocynaceae family, which is a native plant growing in Thailand, Laos, Cambodia, Vietnam, vines has 1.5-4 m long, smooth, with little white latex. AP leaves are thin, oval-shaped spear, spiked sharp, original heart or imprisonment in the root, the upper surface is brighter, 3.5-10 cm long, 2-5 cm wide, red or white flowers, and arranged 2-5 pieces in a bunch at the top. Tanaka and Nguyen [22] mentioned that in the AP trees there are many saponins, flavonoids, sterols, coumarin, tamin, fat, organic acids and twelve micronutrients. We all know saponins are antibiotic to Salmonella typhi and Klebsiella, therefore, AP is also used as a medicinal plant. In Vietnam, AP leaves are also used as an ingredient in soup and traditional medicine.

Therefore, the use of the extract of Aganonerion Polymorphum leaves and chitosan to synthesize Cu nanoparticles is very environmentally friendly.

2. Materials and methods

2.1. Materials

In this work, AP leaves were collected from Dongnai province in Southern Vietnam in April. $CuSO_4$, CH_3COOH were purchased from J.T. Baker supplier (Korea) and used directly without any further purification.

Chitosan was prepared from shrimp shell, following the procedure described in our previous work [23]. The B.cereus, E. coli were provided by the Pasteur Institute in Ho Chi Minh City and resistance testing of these two bacteria was conducted in there.

2.2. Experiments

2.2.1. Process of gaining extract solution of Aganonerion Polymorphum leaves

The Aganonerion Polymorphum leaves were washed, chopped and then extracted with distilled water under the investigated conditions. Factors affecting the extracting process such as extraction time, liquid solids ratio, extraction temperatures were examined.

Firstly, to determine the ratio of leaves to the water of the extract, extraction time (30 minutes) and extraction temperature (80 ^oC) were fixed. The extraction solution was then reacted with 5 mM $CuSO_4$ solution at pH 7, room temperature in 60 minutes. After synthesis, the density of copper nanoparticles solution was tested by Thermo Science Evolution 600 UV-Vis equipment.

Determination of the optimal extraction temperature and optimal extraction time are also carried out with the repeat procedure with changing conditions. At that time, either the extraction time (30 minutes) or optimal leaf water ratio and extraction temperature (80 ^oC) were kept unchanged, respectively.

2.2.2. Synthesis of Cu-NPs

Copper nanoparticles were obtained by pouring $CuSO_4$ solution in the extract solution, which were heating and stirring on the thermally magnetic stirrer. Factors affecting the reaction process such as reaction temperature, $CuSO_4$ concentration, pH of reaction are surveyed.

To investigate the temperature factor affecting Cu nanoparticles synthesis, the ratio between the leaf extract and 5 mM $CuSO_4$ solution of 1:10 (mL:mL) was kept constant at pH 7. The reaction temperature is changed from 50 ^oC to 80 ^oC. The procedure was repeated for investigation of $CuSO_4$ concentration and pH factors, while the other factors were kept unchanged.

2.2.3. Synthesis of Cu-Chitosan nanocomposite

The homogeneous gel solution of chitosan in 1% acetic acid at a ratio of 0.5 g/50 ml was getting mutual dissolving on a magnetic stirrer at 70 °C. The extracting solution of Aganonerion Polymorphum leaves was added to the gel and stirred for

5 minutes, the continue pouring slowly the 2 mM $CuSO_4$ solution into the above mixture and stirring for 1 hour.

The weight ratio of chitosan and $CuSO_4$ is 1:1 (g/g). The volume ratio of the extract solution and 2 mM $CuSO_4$ is 1:10 (mL). Cu-Chitosan nanocomposite was collected after stirring, filtering, washing with distilled water the Cu-Chitosan nanocomposite solution and drying at 60 °C overnight.

2.3. Characterization of Cu-Chitosan nanocomposite

The presence and the content of Cu-NPs were characterized by a Thermo Scientific Evolution 600 UV-Vis Spectrophotometer, which the testing wavelength is in 200 to 800 nm, using 1 cm quartz cuvette.

The particle size distribution of Cu-NPs and Cu-Chitosan nanocomposite was examined by DLS method using a Horiba ZS 100 with 1 cm quartz cuvette at 25 ^oC. X-ray diffraction studies of the samples were performed with an X-ray diffractometer Bruker D8 with an accelerated voltage of 40 kV, 20 mA, angle scan 2θ from 10 to 80^o, 2^o/minutes and step size of 0.03^o.

Structural features of Cu-chitosan nanocomposite was defined by the FTIR method using a Bruker Tensor 27 FTIR spectrometer. Elemental analysis of the material was analysed by the EDX method using a Horiba H-7593. The size and morphology of the Cu-Chitosan nanocomposite were examined by TEM method using a JEM-Jeol 1400.

2.4. Investigation of antibacterial activity

The ASTM 2149 method (Standard Test Methods for discovering the antimicrobial activity of restrained antimicrobial agents under dynamic contact conditions) in MOSSEL and EMB medium was applied to check the antibacterial activity of Cu-Chitosan nanocomposite for Bacillus cereus ATCC 14579 (B. cereus) and Escherichia coli ATCC 25922 (E. coli).

The test sample concentration was 1 g Cu-Chitosan nanocomposite in 50 ml distilled water. The contact time between the sample and the bacteria is one hour.

3. Results and Discussion

3.1. The extract of AP leaves

3.1.1. Effect of waterleaf ratio

In the synthesis of Cu nanoparticles, the extract of AP leaves is used as a reducing agent. UV-vis analysis shows the characterized optical density peak of the Cu-nanoparticles solution at around 450 nm.

The optimal reducing effectiveness corresponding to the ratio of extract solution (60 g leaves/400 ml water) (Fig. 1) and 5 mM $CuSO_4$ solution was 5:20. With the increase of the leaf mass, the decrease of optical density was found, suggesting that the extracted leaf mass of 60 g produced the most appropriate reducing agent for reducing Cu^{2+} ions to form copper nanoparticles.

This can be explained that with 60 g of leaves, the amount of reducing agent in the extract reaches the maximum.

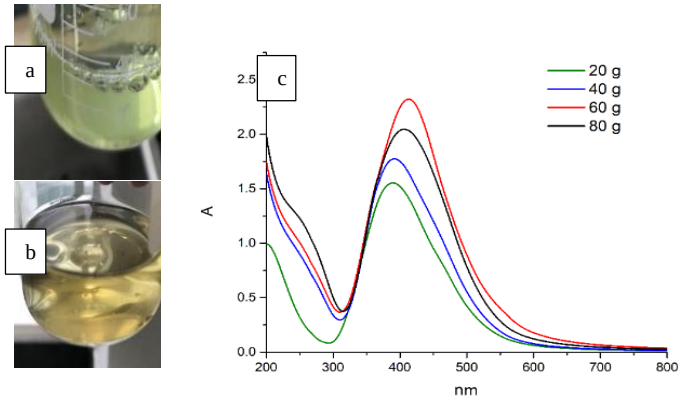

Fig. 1. (a) and (b): Aqueous extract of AP leaf and Cu-NPs solution; (c) UV-Vis spectra of Cu-NPs solutions with different extracting ratio.

3.1.2. Effect of extraction time

Fifteen minutes is the best duration for the extraction (Fig. 2). When continuing increase the extraction time, the optical density decreases, which suggests that the 15-minutes extraction time gives the most appropriate reducing agent for reducing the Cu^{2+} ions to form the copper nanoparticles. When the time is increased by more than 15 minutes, the amount of reducing agent decreases due to oxygenation by oxygen in the air.

3.1.3. Effect of extraction temperature

When fixing the waterleaf ratio and extraction time, the optimal extraction temperature is determined at 70 ºC (Fig. 3). If the temperature increases over 70 °C, the optical densities decrease, which proves that the amount of the reducing agent was decomposed at high temperatures.

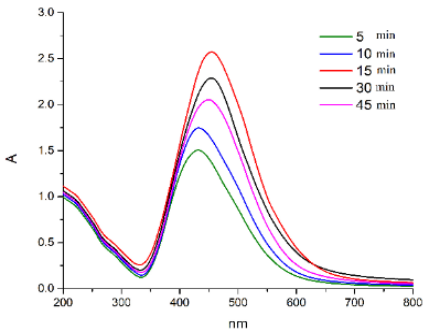

Fig. 2. UV-Vis spectra of Cu-NPs solutions with different extraction time.

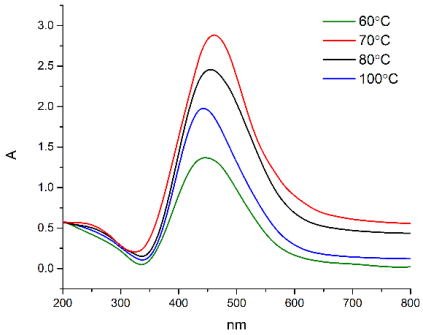

Fig. 3. UV-Vis spectra of Cu-NPs solutions with different extraction temperature.

3.2. Cu nanoparticles synthesis

3.2.1. Effect of reaction temperature

From 50 °C to 70 °C, the higher the temperature, the greater the increase in the measured optical density, the maximum value at 70 °C (Fig. 4). It can be explained

that the higher temperature the higher diffusion process making the reaction occur more intensively so copper nanoparticles could be produced more. When the temperature exceeds 70 °C reduced optical density, it indicates that the reducing agent in the extract decomposed at high temperatures above 70 °C leads to lower copper content. The smallest particle size is 220 nm and the highest uniformity of grain is found at 70 °C according to DLS results (Fig. 5).

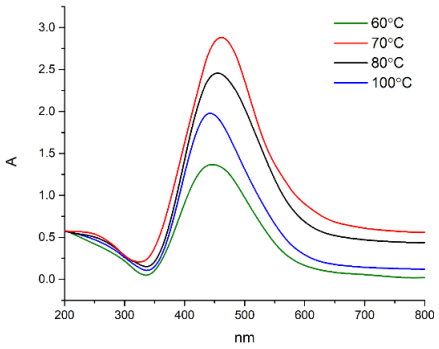

Fig. 4. UV-Vis spectra of Cu-NPs solutions with different reaction temperature.

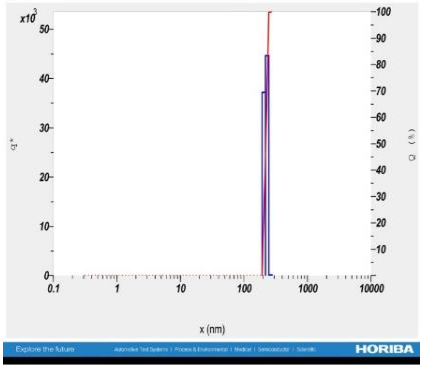

Fig. 5. Particle size distribution spectra at optimum temperature (70 ºC).

3.2.2. Effect of $CuSO_4$ concentration

Figures 6 and 7 showed that when the concentration of $CuSO_4$ solution increased from 2 mM to 5 mM, the optical density was also increased, reaching a maximum value of 5 mM. However, during three days of storage, there was the aggregation and sedimentation of copper nanoparticles in samples formed from $CuSO_4$ solutions of 3 mM, 4 mM and 5 mM. That means, except for 2 mM $CuSO_4$ solution, the nanoparticles are formed when using the $CuSO_4$ solutions of 3 mM, 4 mM and 5 mM were unstable in the surveyed condition due to easy particles collision of the higher concentration solution leading to aggregation and sedimentation of copper nanoparticles. That why 2 mM CuSO4 solution was the best selection for further experiments.

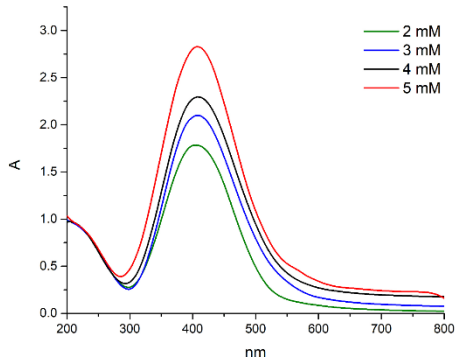

Fig. 6. UV-Vis spectra of Cu-NPs solutions with different reaction temperatures.

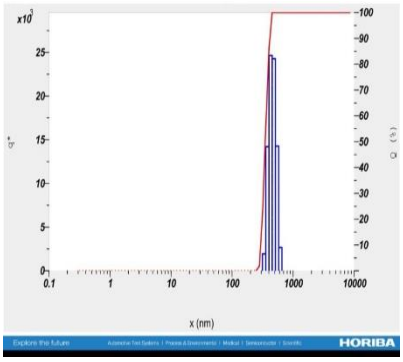

Fig. 7. Particle size distribution spectra of Cu-NPs at 2 mM $CuSO_4$ solution.

3.2.3. Effect of reaction pH

Cu-NPs size is affected significantly by pH of reaction: as the pH increases, it takes the longer reaction time. At pH 3, the Cu-NPs formation process takes only about 10 minutes. However, at pH 7, the nanoparticles formation takes up to 50 minutes. At pH 3, copper particles were formed quickly, thus, producing an unstable solution with large particles and led to the sedimentation of the solution after thirty minutes of synthesis. At pH 7, as the formation process occurred slower, the obtained copper particles were nano-sized with high homogeneity (Figs. 8 and 9). Liu et al. [24] presented the appropriate for Cu nano synthesis reaction mechanism.

At pH=3: $Cu^{2+} + C_6H_8O_6 \longrightarrow Cu^0 + C_6H_6O_6 + 2H^+$
At pH=7: $Cu^{2+} \longrightarrow Cu(OH)_2 \longrightarrow Cu_2O \longrightarrow Cu$

X-ray diffraction of copper nanoparticles includes three characteristic peaks at two thetas 43.1°; 50.2°; 74° match up the 111, 200, 220 planes of the copper crystal (Fig. 10). The results confirmed that we could synthesize copper nanoparticles from the $CuSO_4$ solution with the extract solution from AP leaves as a reduction agent.

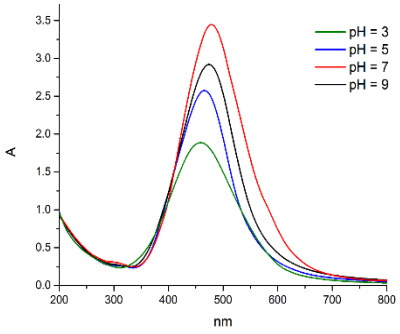

Fig. 8. UV-Vis spectra of Cu nano solutions with different reaction pH.

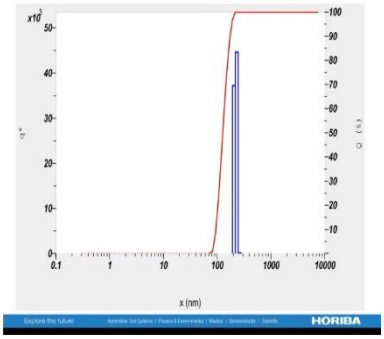

Fig. 9. Particle size distribution spectra of Cu-NPs at optimal reaction pH of 7.

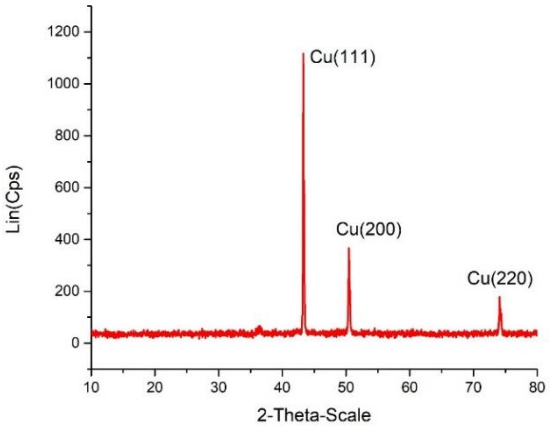

Fig. 10. XRD pattern of Cu nanoparticle.

3.3. Characterization of Cu-chitosan nanocomposite

Characteristic peaks of chitosan at 3433.14, 2876.36, 1628.76, 1338.03, 1064.47 (KBr, cm^{-1}) corresponding to OH and NH, CH, C = O, CH- OH, COC were observed in FTIR spectra of copper chitosan nanocomposite samples. The peak at 664.83 (KBr, cm^{-1}) represents the interaction between copper and chitosan, which proves that copper nanoparticles were encapsulated by chitosan (Fig. 11).

The 111, 200 planes of copper crystals are showed at diffraction angles of 43.26°, 50.62°, respectively. According to Chen et al. [15], peak at the two thetas of 20.53° indicates the presence of chitosan [15] in the composite (Fig. 12). Furthermore, CuO or Cu_2O were not observed in XRD measurements.

The elemental analysis of the sample showed that the Cu-Chitosan nanocomposite was mainly composed of copper, carbon and oxygen (Fig. 13).

The TEM image of Cu-chitosan nanocomposite (Fig. 14) showed that the Cu-NPs size is about 50 nm. Chitosan exhibits good copper-sheathing ability, as well as the ability to bond membranes. At the ratio of chitosan/$CuSO_4$, 0.5 g/0.5 g, Cu-chitosan nanocomposite achieves the smallest size and high homogeneity. When the chitosan concentration increases, the composite particle size increases, the particles are distributed unevenly, the uniformity no longer guaranteed.

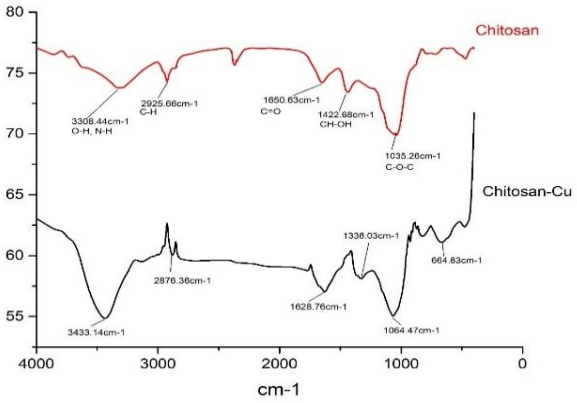

Fig. 11. FT-IR spectra of Chitosan and Cu-Chitosan composite.

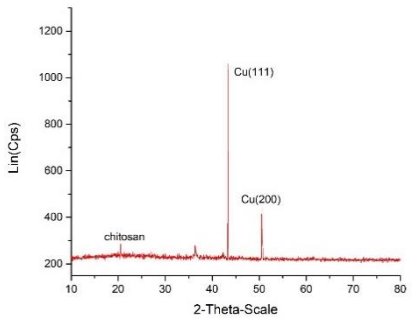

Fig. 12. XRD pattern of Cu-Chitosan composite.

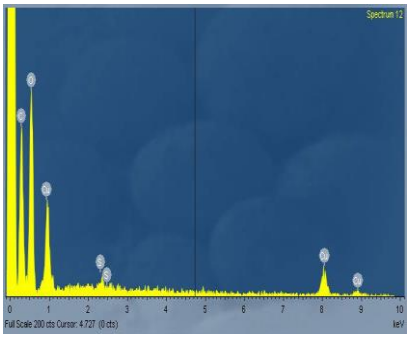

Fig. 13. EDX spectra of Cu-Chitosan nanocomposite.

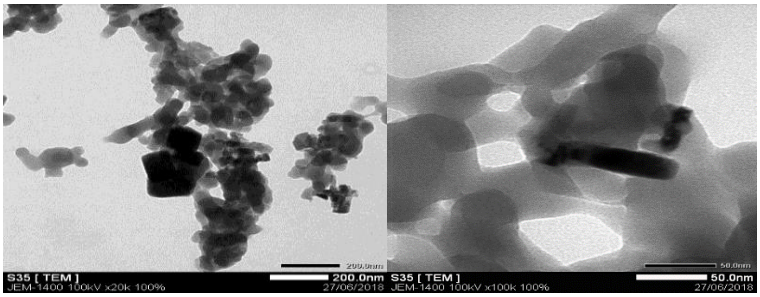

Fig. 14. TEM images of Cu-Chitosan nanoparticles.

3.3. Antimicrobial activity of Cu-chitosan nanocomposite

Cu-chitosan NPs exhibited good antimicrobial activity on two E. coli (gram-negative) and B. cereus (gram-positive). Antimicrobial activity of Cu-chitosan nanocomposite was showed in Table 1.

Table 1 indicated that 99.67% E. coli and 99.26 B. cereus were destroyed by Cu-chitosan nanocomposite. According to published works by Qi et al. [14] and Chen et al. [15], this effective antibacterial ability of Cu-chitosan nanocomposite might be due to it can alter the permeability of the cell membrane by the linkage between copper metal with chitosan forming a polymer chain of chitosan and the metal on the surface of bacteria to inhibit the growth of bacteria.

Table 1. Results of antibacterial study of Cu-chitosan nanocomposite.

Testing microorganism	Number of testing microorganism (CFU/ml)	After 1 hour exposure with Cu-chitosan nanocomposite	
		Number of living microorganism (CFU/ml)	Sterilization rate (%)
B. cereus	4.7×10^5	3500	99.26
E. coli	4.8×10^5	1600	99.67

4. Conclusions

Nanocomposite Chitosan-Cu was first synthesized by the green method using the extract solution of Aganonerion Polymorphum leaves as the reducing agent with the size about 50 nm by TEM. Nanocomposite Chitosan-Cu has antibacterial properties of greater than 99%. Nanocomposite Cu-Chitosan synthesized from this method might less affect the environment and be a promising antibacterial material.

Acknowledgements

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Abbreviations

AP	Aganonerion Polymorphum
ASTM	American Society for Testing and Material

DLS	Dynamic Light Scattering
EDX	Energy Dispersive X-Ray Analysis
FTIR	Fourier Transform Infrared Spectroscopy
IUH	Industrial University of Ho Chi Minh City
TEM	Transmission Electron Microscope
UV-Vis	Ultraviolet-Visible Spectrometry
XRD	Powder X-Ray Diffraction

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