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Chitosan-CuO Nanoparticles as Antibacterial *Shigella dysenteriae*: Synthesis, Characterization, and *In Vitro* Study

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Abstract

The synthesis of chitosan- CuO nanoparticles was studied. This research's aims were biosynthesis CuO nanoparticles, synthesis of chitosan-CuO nanoparticles, and used as an antibacterial agent of *Shigella dysenteriae*. CuO nanoparticles and chitosan-CuO nanoparticles were characterized by FTIR spectroscopy and X-ray diffraction, respectively. CuO nanoparticle was synthesized by the reaction between leaf extract of sweet star fruit (*Averrhoa carambola* L.) and copper sulfate pentahydrate. Chitosan-CuO nanoparticles were synthesized by a heating method. The suspension of chitosan-CuO nanoparticles was used as an antibacterial agent with a paper disk method. The result showed that the Cu-O group at CuO nanoparticles was detected at a wavenumber of 503, 619, 767, and 821 cm^{-1}. The crystallite size of the CuO nanoparticles was 4.25 nm. Cu-O group bonded at N-H and O-H groups and detected at 3406 cm^{-1} from the FTIR spectra of chitosan-CuO nanoparticles. The average inhibition zone of chitosan-CuO nanoparticles at concentration 2.500, 5.000, 7.500, and 10.000 ppm to *Shigella dysenteriae* were 13.57±1.55; 14.90±1.20; 15.97±0.76 and 17.03±1.80 mm, respectively.

1. Introduction

Scientists develop metal nanoparticles (MNPs) such as CuO nanoparticles (material scientists, pharmacists, biologists, and chemists). The scientists are developing these MNPs with high-efficiency and low-cost material sources [1]. The CuO nanoparticles have electronic, optical, catalytic, and magnetic activity [2].

Several routes can synthesize CuO nanoparticles. Chemical route (called chemical methods) such as sol-gel [3] and precipitation [4, 5] and non-chemical route [2]. A non-chemical route is called a green synthesis method. This method is effective, eco-friendly. No need for high temperatures, pressures, and toxic chemicals can be minimized [2]. Toxic chemicals can be substituted by some plant components, such as roots, leaves, stems, seeds, and fruits [6]. Some plant components can act as a bioreduction to produce CuO nanoparticles [1, 2, 7]. The product of CuO nanoparticles can be used as an antibacterial agent [2, 7, 8], super-strong materials, sensors, and catalysts [2].

Chitosan, an organic polymer, is produced by deacetylation of chitin, and it has the name α (1–4)-linked 2-amino 2-deoxy β- D glucopyranose [9]. The properties of chitosan will increase with the modification process at chitosan's chemical structure (-NH_2 group). The modification process of chitosan can be done by two methods, i.e., physical and chemical methods [10]. Chitosan can be used as supporting materials with metal oxide nanoparticles and applied as antibacterial agents [11, 12, 13]. We studied that chitosan combined with zinc nanoparticles can act as an antibacterial agent. With the increase of concentration of chitosan-metal nanoparticles, its antibacterial agent properties are increasing [10, 11]. This fact showed, there is an effect of metal nanoparticles on chitosan. This effect is inhibiting the growth of bacteria.

Laha *et al.* [14] reported that the synthesis procedure of CuO nanoparticles is easy compared to other metal-based nanoparticles. CuO nanoparticles' properties are easy to release out of the human body, easily mix with polymers such as chitosan, and relatively stable and unique of chemical and physical properties [15, 16]. CuO nanoparticles combined with chitosan have many advantages, such as the properties of antibacterial [13, 15, 17, 18, 19]. However, the chitosan modified CuO nanoparticle as antibacterial gram-negative such as *Shigella dysenteriae* is not widely studied. *Shigella dysenteriae* is a gram-negative bacterium. The cell wall comprises a thin membrane of peptide polyglycogen, and an outer membrane is lipopolysaccharide phospholipids and lipoprotein [20].

We biosynthesized CuO nanoparticles through a green synthesis. The CuO nanoparticles product can be interacted by chitosan structure through primary amine or OH group of chitosan [13, 21]. FTIR spectroscopy and X-ray diffraction (XRD) are used to characterize chitosan, CuO nanoparticles, and chitosan supported CuO nanoparticles (chitosan-CuO nanoparticles). Chitosan-CuO nanoparticles were applied as antibacterial of *Shigella dysenteriae*. Based on the fact that the properties of chitosan as an antibacterial [22] and copper nanoparticles can interact with the bacterial surface [2].

2. Methodology

2.1. Equipment/Material

Chitosan (DD 87%, CV. Ocean Fresh Bandung, West Java, Indonesia). Acetic acid glacial (CH_3COOH 100%, Merck), potassium hydroxide (KOH ≥ 85%, Merck), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$, 99-100.5%, Merck), and Salmonella Shigella Agar (Merck). Distilled water and *Shigella dysenteriae* (our laboratory, Microbiology laboratory). Sweet star fruit leaf (*Averrhoa carambola* L.) (Palembang, South Sumatera, Indonesia). FTIR Spectrophotometer (Shimadzu Prestige-21) and X-Ray diffraction (Shimadzu 6000).

2.2. Preparation of aqueous leaf extract of sweet star fruit

About 50 g of sweet star fruit leaves (clean) was used in this study. Cleaned leaves of sweet star fruit were sliced into small shapes and mixed with 100 mL distilled water in a 250 mL Erlenmeyer flask. The mixture was boiled at 90°C (15 minutes), and then the mixture could cool at room temperature. The mixture was separated, and the filtrate was stored in the refrigerator for further study [23].

2.3. Biosynthesis of CuO nanoparticles

Biosynthesis of CuO nanoparticles was synthesized according to Nasrollahzadeh *et al.* [24] procedure with slight modification. About 100 mL of leaf extract of sweet star fruit was added to 250 mL Erlenmeyer flask contained 50 mL of 0.1 M $CuSO_4$ $5H_2O$ solution. The mixture was boiled at 80°C until the mixture's color changed from slightly green to deep black. The mixture could cool at room temperature for one night to form precipitation. The residue was separated from filtrate, and residue (CuO nanoparticles) was washed with distilled water (several times). CuO nanoparticles were dried in an electric oven at 50°C until dry.

2.4. Synthesis of chitosan supported CuO nanoparticles (chitosan-CuO nanoparticles)

CuO nanoparticles (0.1 g) was suspended in 50 mL of acetic acid 10% (v/v). Chitosan (0.1 g) was added to this solution. The mixture was stirred continuously for 30 min, after which 1 M of KOH solution was added dropwise to the solution until the pH was 10. The solution was continued by heating on a hot plate at 60°C (3 h). Finally, this solution could cool at room temperature for one night to form precipitation. The filtrate was separated from the residue. The residue was washed several times with distilled water until the filtrate of residue has neutral pH. The residue was dried at 50°C in an electric oven until dry (3 h) [24].

2.5. Characterization

The functional group of chitosan-CuO nanoparticles, CuO nanoparticles, and $CuSO_4$ $5H_2O$ were analyzed by FTIR Spectrophotometer (Shimadzu Prestige-21) help of KBr pellets and spectra were recorded at a range of 4500–500 cm^{-1}. X-ray diffraction (Shimadzu 6000) was used to calculate the crystalline size of CuO nanoparticles and evaluate the crystalline level of chitosan-CuO nanoparticles, CuO nanoparticles, and copper (II) sulfate pentahydrate. The operational condition of X-ray diffraction is Cu $K\alpha$ X-ray tube at 1.5406 Å, 30 kV, and 10 mA with scan speed/duration time 10.000 deg. min^{-1} and the 2θ range of 0°–60°.

2.6. *In* vitro study of chitosan-CuO nanoparticles against *Shigella dysenteriae*

2.6.1. The preparation of the suspension chitosan-CuO nanoparticles, acetic acid solution 1% v/v, and chitosan solution

In this study, the concentration of chitosan-CuO nanoparticles was prepared in 10.000 (C1), 7.500 (C2), 5.000 (C3), and 2.500 ppm (C4), respectively. Acetic acid solution 1% v/v (B) and chitosan solution (A, 2.500 ppm, without modified CuO nanoparticles) were prepared as control solutions.

2.6.2. The preparation of Salmonella Shigella agar as media of the agar diffusion method

The Salmonella Shigella Agar solution was sterilized in an autoclave, 15 mL of Salmonella Shigella Agar was poured into Petri dishes, and then they were solidified as the first layer. 10 mL of fresh inoculum suspension of *Shigella dysenteriae* (approximately 1.0×10^8 CFU/mL) was spread on each agar plate's surface as the second layer. Finally, the sterile paper disks (6 mm diameter) were dropped by 10 µL the suspension of chitosan-CuO nanoparticles, acetic acid (1% v/v), and chitosan solution, respectively. The paper disk contained the sample was placed aseptically in the second layer. The Petri dishes were incubated at 37°C for 24 h, and the inhibition zones of bacterial growth were measured after 24 h. *In vitro* study prepared with triplicate.

3. Result and Discussion

3.1. Biosynthesis of CuO nanoparticles

The documentation of the biosynthesis of CuO nanoparticles can be seen in Figure 1. The leaf extract of sweet star fruit (Figure 1a) and copper (II) sulfate pentahydrate solution (Figure 1b) is used for the biosynthesis of CuO nanoparticles. This biosynthesis's heating method with continuous stirring until the mixture of leaf extract of sweet star fruit and copper (II) sulfate pentahydrate solution had a deep dark (Figure 1c). The CuO nanoparticles were obtained from this biosynthesis, as shown in Figure 1d and e.

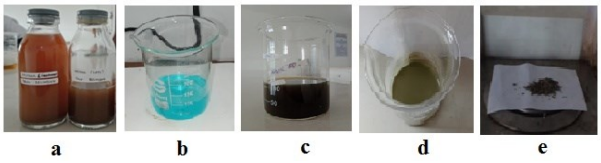

Figure 1. Photograph of water extract of sweet star fruit leaf (a), copper (II) sulfate pentahydrate (b), a mixture of water extract of sweet star fruit leaves and a solution of copper sulfate pentahydrate after boiling at 80°C (c), the resulting product from CuO nanoparticles - wet (d) and dry (e).

The biosynthesis mechanism of CuO nanoparticles is a reduction or an oxidation mechanism [24]. Sweet star fruit leaf extract contains natural ingredients or metabolites (primary or secondary) such as alkaloids, carbohydrates, glycosides, phytosterols, resins, phenols, tannins, diterpenes, flavonoids, proteins and amino acids, quinones, and phlobatnins [25]. It had a crucial position in converting metal ions to specific metal nanoparticles. These metabolites are responsible for reducing or oxidizing agents to nano-sized metal oxide [26]. Metal salt, reducing, and stabilizing or capping agents are three components for controlling the size of metal oxide nanoparticles [27] and illustrated, as seen in Figure 2.

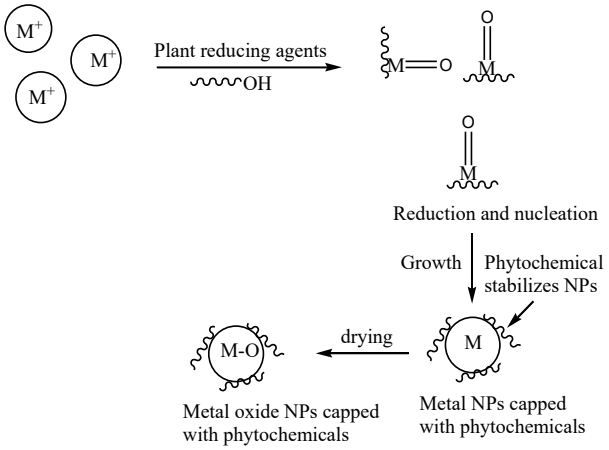

Figure 2. Illustration of the biosynthesis mechanism of metal oxide nanoparticles with a green synthesis method [28].

3.2. Synthesis of chitosan-CuO nanoparticles

CuO nanoparticles can be bonded by –OH and $-NH_2$ groups of chitosan structure through two steps: effect acid and alkaline solution [29]. In the first step, when the acetic acid solution was added to the chitosan compound and CuO nanoparticles, CuO was changed into Cu^{2+} ion, and Cu^{2+} ion can interact with –OH and $-NH_2$ groups of chitosan structure through the coordination bonds [30]. In the second step, the addition of alkali compound (OH^-) until pH of 10 and the factor of heating, the Cu^{2+} ion can be converted again to form CuO [17]. Schematic reaction and the product chitosan- CuO nanoparticles can be seen in Figures 3 and 4.

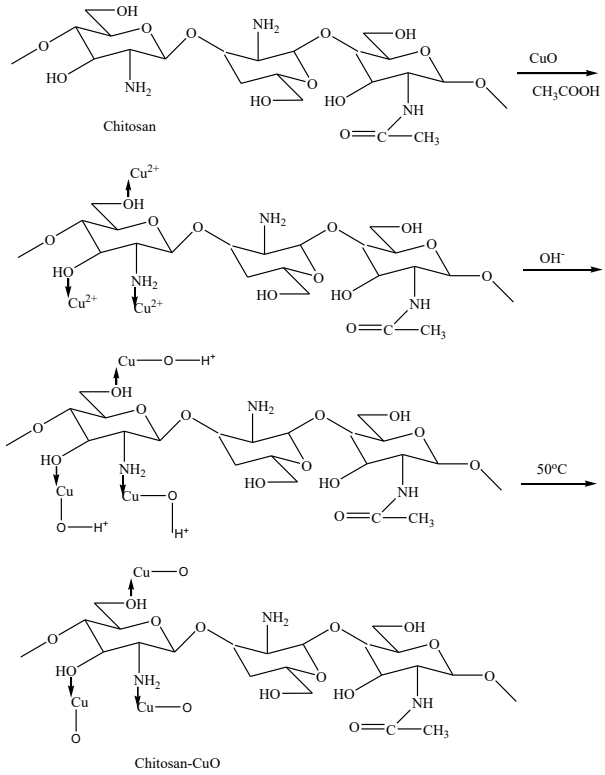

Figure 3. The schematic reaction of synthesis chitosan-CuO [17, 30].

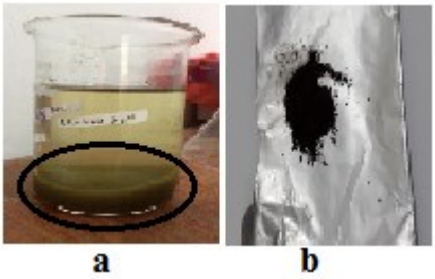

Figure. 4. The photograph of chitosan-CuO nanoparticles (wet, a) and dry (b)

3.3. Analysis of functional group

FTIR spectra of chitosan, copper (II) sulfate pentahydrate, CuO nanoparticles, and chitosan-CuO nanoparticles are shown in Figure 5. The main bands in copper (II) sulfate pentahydrate (Figure 5a) are: stretching vibration of O-H groups detected at 3134-3385 cm^{-1}. The stretching vibration asymmetric of H_2O appeared at 1625 cm^{-1}. Bending vibration of O-H groups at 1091 cm^{-1}. SO_4^{2-} non-degenerate and degenerate mode detected at 997 cm^{-1} and 661 cm^{-1} respectively [31].

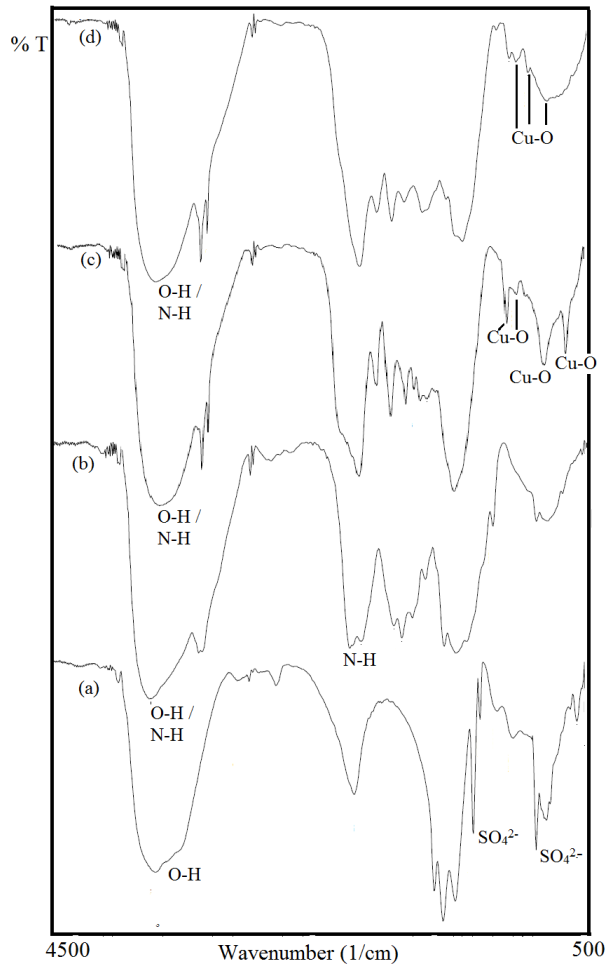

Figure 5. FTIR spectra of copper (II) sulfate pentahydrate (a), chitosan (b), CuO nanoparticles (c), and chitosan-CuO nanoparticles (d)

The prominent bands in the FTIR spectra of chitosan (Figure 5b) show: the peak at 3427 cm^{-1} is stretching vibrations of the O-H group and overlaps with the N-H group. 2920 and 1381cm^{-1} are stretching and bending vibration of the C-H group, respectively, and 1656 cm^{-1} is a bending vibration of the N-H group [32, 33]. The deformation vibration of primary amine was detected at 1421 cm^{-1} [34]. The peak at1091 cm^{-1} is stretching vibration of the C–O group [35].

FTIR spectra of CuO nanoparticles result from molecular interactions between the media of the aqueous leaf extract of sweet star fruit and CuO nanoparticles (Figure 5c). The broad peak at 3369 cm^{-1} is stretching vibration of O-H groups from alcohols and phenols, but Taran *et al.* [36] reported that the band at 3369 cm^{-1} is N-H stretching. C-H groups' stretching vibration appeared at 2848-2918 cm^{-1} [7]. The peak at 1317 cm^{-1} is the C-O group's vibration [24]. The vibration band at 1099 cm^{-1} is the stretching vibration of the C=O group. C=C stretching detected at 1612 cm^{-1}. 1444 cm^{-1} is a band of O-H bending, and 1249 cm^{-1} is C-O stretching [37]. The Cu-O group's stretching vibration was detected in wave number 503, 619, 767, and 821 cm^{-1}. All these wavenumbers are confirming the formation of CuO nanoparticles [7, 38, 39].

The spectra FTIR chitosan-CuO nanoparticles (Figure 5d) showed that stretching vibration of –OH and $-NH_2$, bending vibration of N-H, and stretching vibration of the C–O group are shifted from 3427 to 3406; 1595 to 1523 and 1091 to 1068 cm^{-1}, respectively. This fact showed that the chitosan framework is supported by CuO nanoparticles [21], and there is an interaction between the chitosan structure and CuO nanoparticles [17]. In these spectra, the Cu-O group's stretching vibration appeared at 619, 721, and 783 cm^{-1} [7, 38, 39]. Diffractogram XRD of chitosan-CuO nanoparticles confirmed the evidence of CuO nanoparticles at chitosan structure.

3.4. Analysis of the physical structure

Diffractograms of chitosan, copper (II) sulfate pentahydrate, CuO nanoparticles, and chitosan-CuO nanoparticles were presented in Figure 6. The narrow peaks of copper (II) sulfate pentahydrate in Figure 6a showed an excellent quality crystalline nature and showed the highest peak at 2θ ~ 18° [39]. The X-ray diffractogram of chitosan is a semi-crystalline form (Figure 6b) with two strong diffractions at 2θ = 10° and 20°. Two strong diffractions showed the semi-crystalline and high degree of crystalline morphology of chitosan, respectively. Plenty of O-H and NH_2 groups at the chitosan framework play an important role in intra and intermolecular hydrogen bonds [32].

The Diffractogram of CuO nanoparticles (Figure 6c) was showed in crystalline. The form of these peaks was sharp and narrow. Diffraction angles of CuO nanoparticles are 15.99°, 18.33°, 21.37°, 22.61°, 23.87°, 27.13°, 29.19°, 31.26°, 32.65°, 36.97°, 51.51° and 56.59°. These diffraction angles have sharp and narrow diffraction peaks, indicating that the synthesized CuO nanoparticles are crystalline [36].

The biosynthesized CuO nanoparticles' crystal size was 4.25 nm if calculated by Debye Scherrer's formula:

$$D = (0.9.\lambda / \beta.\cos\theta)$$

D, λ, β and θ were the average crystallite size, the wavelength of X-ray used (1.5406 Å), the full width at half maximum (FWHM) and the Bragg's angle respectively [26]. Figure 6d showed that the diffraction angles of chitosan-CuO nanoparticles are 18.34°, 21.35°, 24.27°, 27.96°, and 56.31°. Chitosan peak at 21.35° and another peak of CuO nanoparticles were observed at 56.31° [40], which indicates CuO peaks at diffractogram chitosan-CuO nanoparticles although with lesser intensity. The crystallinity of chitosan changed after substituted by CuO nanoparticles. The diffractogram is shown in figure 6d. The effect of decreasing the crystallinity of chitosan, a hydrogen bond in the chitosan structure becomes weak because of the insertion of CuO nanoparticles into the chitosan functional group [41, 42].

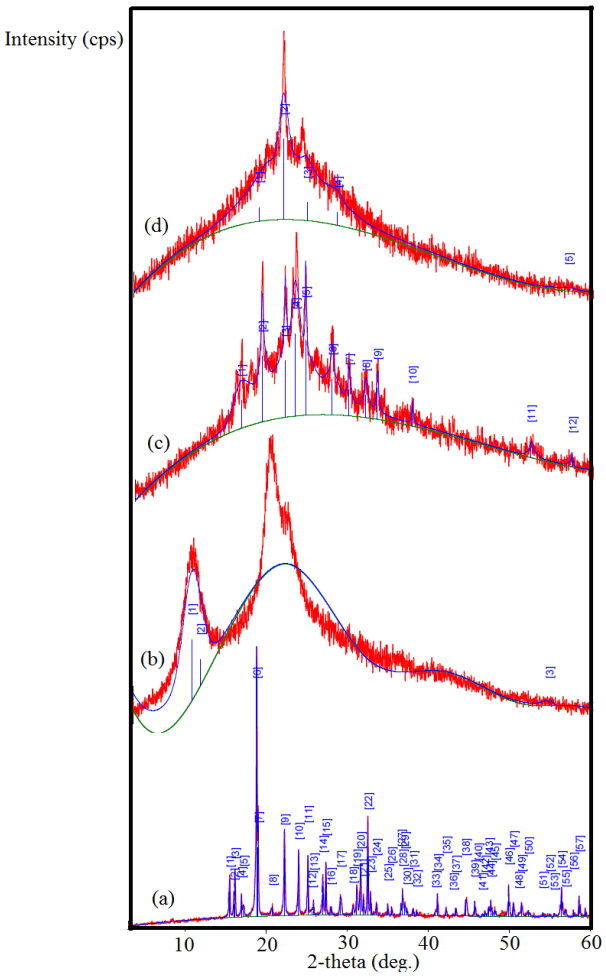

Figure 6. Diffractogram of copper (II) sulfate pentahydrate (a), chitosan (b), CuO nanoparticles (c), and chitosan-CuO nanoparticles (d)

3.5. The *in vitro* study of chitosan-CuO nanoparticles

The antibacterial activity of chitosan solution (A: 2.500 ppm), acetic acid solution 1% (v/v, B), and suspension of chitosan-CuO nanoparticles (C: 10.000, 7.500, 5.000, and 2.500 ppm, respectively) were investigated as shown in Figure 7 and tabulated in Table 1. The average inhibition zone of chitosan solution and acetic acid 1% (v/v) against *Shigella dysenteriae* bacteria were 15.73±0.35 and 10.37±0.64 mm, respectively. The average inhibition zone of the suspension of chitosan-CuO nanoparticles to *Shigella dysenteriae* bacteria (from lower to upper concentration) was 13.57±1.55; 14.90±1.20; 15.97±0.76 and 17.03±1.80 mm, respectively.

The clear zone of chitosan at a concentration of 2.500 and 5.000 ppm is higher than the chitosan-CuO nanoparticle in the same concentration. Allaker [43] reported that CuO nanoparticles in a 100-5000 ppm concentration had no toxic effect on cells wall or minimum bactericidal. This fact showed the transparent zone chitosan-CuO nanoparticles is lower than chitosan. On the other hand, the clear zone chitosan-CuO nanoparticles at a 7.500 and 10.000 ppm concentration are higher than chitosan. Chitosan-CuO nanoparticles at a concentration of 7.500 and 10.000 ppm maybe had a toxic effect, and the CuO nanoparticles released from the chitosan solution are responsible for the antibacterial activity [18] with the increase of the concentration of chitosan-CuO nanoparticles.

Chitosan-CuO nanoparticles can act as an antibacterial is described by many theories, such as the theory explained by Benhabiles *et al.* [22]. This theory described an interaction between the positive charge of NH_3^+ group chitosan and the negative charge of surface cell bacteria. Interaction of Cu nanoparticles with cell membrane bacteria, and its effect is a decrease of transmembrane electrochemical potential, accumulation on the cell surface, and DNA bacteria causes to destroy cell surface and DNA damage [44]. Acetic acid has an H^+ ion, enters the cell cytoplasm of bacteria, and becomes more acidic. The increasing H^+ ion in the cytoplasm then causes a decrease in the cell's local pH and disturbs bacteria's growth [45].

Table 1. The calculation of the inhibition zone diameter

No.	The Petri dishes	The diameter of the zone of inhibition (mm)					
		Chitosan (2.500 ppm)	Acetic acid (1% v/v)	Chitosan-CuO nanoparticles (ppm)			
				10.000	7.500	5.000	2.500
1	I	16.01	10.10	18.80	16.30	14.90	13.10
2	II	15.07	11.10	17.10	16.50	16.01	15.03
3	III	15.40	9.90	15.20	15.01	13.70	12.30
Average		15.73	10.37	17.03	15.97	14.90	13.57

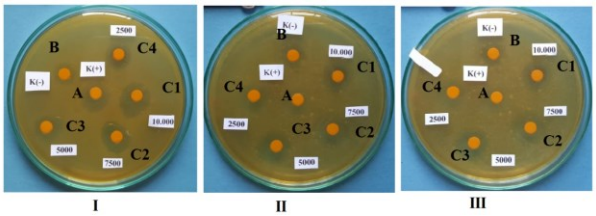

Figure. 7. Photograph of a clear zone of chitosan solution (A), acetic acid 1% v/v (B), and suspension of chitosan-CuO nanoparticles (C) to *Shigella dysenteriae,* respectively

4. Conclusion

CuO nanoparticles resulted from an interaction between copper (II) sulfate pentahydrate and aqueous leaf extract of sweet star fruit. The average crystallite size of the CuO nanoparticles was 4.25 nm. The Cu-O group was detected at 721 and 783 cm^{-1} on the FTIR spectra of chitosan-CuO nanoparticles. The Cu-O group was bonded by the N-H and O-H group of the chitosan framework. The concentrations of chitosan-CuO nanoparticles at 10.000, 7.500, 5.000 and 2.500 ppm had inhibition zone 13.57±1.55; 14.90±1.20; 15.97±0.76 and 17.03±1.80 mm respectively.

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