An Ethanolic Extract of *Psidium guajava* L. as Media for Biosynthesis of ZnO-CuO Nanoparticles: Characterization and In Vitro Study to *Staphylococcus aureus* Bacteria

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Abstract. ZnO and CuO nanoparticles have attracted a lot of attention lately because of their special qualities. In terms of structure and characteristics, this group of nanostructures is the most preferred one. The research was done on biosynthesis ZnO-CuO nanoparticle. The objective of this study was to synthesize ZnO-CuO nanoparticles, characterize them, and use them as an antibacterial agent against *Staphylococcus aureus*. The media of ethanolic extract of *Psidium guajava* L. in alkaline solution were used to biosynthesize ZnO-CuO nanoparticles. The tools used to examine the functional groups and physical structure of ZnO-CuO nanoparticles include FTIR spectroscopy and X-ray diffraction. Using the well agar diffusion method, ZnO-CuO nanoparticle suspension was used as an antibacterial agent. Wavenumbers of the functional groups of Zn-O and Cu-O groups are 507.28 and 617.22 cm^{-1}, respectively. ZnO-CuO nanoparticles' crystallite size was calculated to be 2.33 nm. *Staphylococcus aureus* may be resistant to the ZnO/CuO nanoparticles' antimicrobial effects. ZnO-CuO nanoparticles have a greater inhibitory zone than ZnO or CuO nanoparticles.

Keywords: ZnO-CuO nanoparticles, characterization, antibacterial Staphylococcus aureus

1 Introduction

Nanotechnology is a novel area of research in modern material science. Metal oxide nanoparticles are one of the classes studied in nanotechnology. Several methods (physical, biological, and chemical) are used to synthesize metal oxide nanoparticles [1]. There is a difference between physical, biological, and chemical methods. The main point in the physical method is the evaporation and condensate process. Physical method is needing the instruments modernly, highly chemicals and radiative heating. In the main point of the chemical method, a

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chemical such as hydrazine, sodium borohydride, and hydrogen is used for the reduction of a metal ion, and the small clusters of metals form through a process of nucleation or aggregation. The disadvantage of the chemical method is toxic and nonbiodegradable [2]. An alternative to the synthesis of metal oxide nanoparticles is using the biological method. This method is eco-friendly, not expensive, and easy because the process of synthesis is need low temperature, pressure, and a toxic chemical can be minimized [3]. The role of the biomolecules as reducing and stabilizing agents is the main point during biosynthesis [2].

The biological method (green synthesis or biosynthesis) can use a plant or not plant as a basic material and it was explored by previous literature for biosynthesis of ZnO or CuO nanoparticles, such as *Pseudomonas aeruginosa Rhamnolipids* [5], *aspergillus niger* [6], *ruta graveolens* (L.) [7] and *myrtus communis* L [8]. A basic material of sweet star fruit can be used to biosynthesis of CuO nanoparticles [9]. Biosynthesis with plant extract is more explored rapidly than not plant extract because in this biosynthesis with should be in a certain condition [2].

Non-alcoholic or alcoholic are the common solvent to prepare the aqueous extract of the plant. Previous literature reported that non-alcoholic solvent was used in the extraction process of metabolite compounds contained in the plant. This extract can be used as medium for biosynthesis process of Cu, ZnO and others nanoparticles [8,10,11]. The extraction process of metabolite compounds contained in the plant can use an alcoholic solvent such as ethanolic. The result of this process can be used for the biosynthesis of metal oxide nanoparticles [12-15].

There is a difference between non and alcohol as the solvent. Aquadest / water (non-alcoholic) and pure ethanol (alcoholic) have the polarity index 9 and 5.2 respectively [16]. The polarity of aquadest is the best than pure ethanol and this consequence, aquadest is chosen than ethanol in the extraction process of the biological compound. On another hand, universal solvent of ethanol [17] because it has a hydroxyl and an alkyl group. A hydroxyl and an alkyl group have polar and nonpolar properties so ethanol can be used to extract the compound which has non or polar properties [18].

Biosynthesis of ZnO nanoparticles can be done by aqueous or ethanolic extract a part of plant [15,19]. There is a difference between water and ethanolic as a solvent in the extraction process but both solvents can be used as a solvent through decoction or maceration method respectively. The maceration method with ethanol as a solvent is a very simple extraction method than a decoction [17]. The result of metabolite compounds through the decoction method with water as a solvent is a polar compound [17] and low levels of metabolite compounds such as flavonoids, alkaloids, and terpenoids [20]. On another hand, the maceration method with ethanol solvent can be done at room temperature than the decoction method [17] and can be used to extract polar or non-polar metabolite compounds [18]. This extracts contained metabolite compounds as bioactive phytochemicals [21] for natural reducing agents in the preparation of metal nanoparticles and they can help to control physical and chemical properties of metal nanoparticles product [22].

Based on our previous study, the higher the concentration of CuO nanoparticles, the larger the inhibition zone [9] and likewise with ZnO nanoparticles [23]. In this paper, we prepared the ZnO-CuO nanoparticles. Biosynthesis ZnO-CuO nanoparticles are using Zn^{2+}, Cu^{2+} ion, sodium hydroxide, and ethanolic extract of *Psidium guajava* L. ZnO-CuO nanoparticles are studied as antibacterial of *Staphylococcus aureus.*

2 Research Methods

2.1 Tools and Materials

All reagents are in high purity from Merck, such sodium hydroxide, acetic acid, zinc acetate dihydrate, copper sulfate pentahydrate, absolute ethanol, and nutrient agar. *Staphylococcus aureus* and distilled water (aquadest) from our laboratory. Guava leaves from local area (Palembang). Spectrophotometer (FTIR, Shimadzu Prestige-21) and X-Ray diffraction (Shimadzu 6000).

2.2 Preparation of ethanolic extract of *Psidium guajava* L

The maceration process of dry leaves of guava seed uses an ethanol solution (70 %, v/v). A 25 g of dry leaves of guava seed were dipped at ethanol solution (70 %, v/v, 250 mL) in the black bottle. This process is carried out for one night. After one night, the mixture was separated and the macerate was taken for further use [24]. The maceration process is carried out again for the residue with ethanol 70 % (v/v, 250 mL) overnight and filtered, the filtrate (macerate) is combined with the first filtrate for the further experiment.

2.3 Biosynthesis ZnO and CuO nanoparticles.

Zinc acetate dihydrate solution (25 mL, 0.55 g) was added into 250 mL a glass beaker (75 mL of ethanolic extract). The mixture was boiled at 80°C for 1 hour. The mixture was allowed to cool at room temperature and the pH was adjusted to 10 by adding 15 ml of sodium hydroxide 0.1 M drops by drops. The mixture was left overnight until a precipitate appeared. The solid precipitate was separated and rinsed with distilled water. The solid precipitate was dried in an oven until dry (60^{o} C) [25]. The product was named ZnO nanoparticles. For the biosynthesis of CuO nanoparticles, use the above procedure (copper sulfate pentahydrate, 25 mL, 0.625 g).

2.4 Biosynthesis ZnO-CuO nanoparticles [25]

75 mL of ethanolic extract and 25 mL of the solution of Zn^{2+} and Cu^{2+} (0.550 g of zinc acetate dihydrate and 0.625 g of copper sulfate pentahydrate) are mixed in 250 mL of beaker glass. The mixture is heated on a hot plate and the temperature is set at 80°C while stirring continuously (1 hour). After 1 hour, with continuous stirring, NaOH solution (0.1 M) was dripped drop by drop (total 24 mL) to the above mixture until the pH was 11. The mixture was left overnight until a precipitate appeared. The solid precipitate (ZnO-CuO nanoparticles) was separated and rinsed with 15 mL of distilled water followed by 15 mL of absolute ethanol. The obtained ZnO-CuO nanoparticles were dried in an oven (60°C) to a constant weight.

2.5 Characterization

The product (ZnO, CuO and ZnO-CuO nanoparticles) is characterized by its functional groups (FTIR Spectrophotometer, Shimadzu Prestige-21, the wavenumber (cm^{-1}) was chosen at a range of 4500–500) and physical structure (Shimadzu 6000 spectrophotometer) and the crystallite size is calculated from the XRD pattern of product.

2.6 The antibacterial evaluation of ZnO, CuO and ZnO-CuO nanoparticles

The solutions of CuO nanoparticles (10^4 ppm), ZnO nanoparticles (10^4 ppm), ZnO-CuO nanoparticles (10^4 ppm), acetic acid solution (1%, v/v) and chloramphenicol (200 ppm) as samples. The media preparation procedure for antibacterial evaluation refers to previous research [26] and prepared three petri dishes for evaluating samples as antibacterial. A paper disc (6 mm) containing 10 µL of each sample were placed on the prepared antibacterial test medium. Incubation process for 24 hours at a temperature of 37 °C. After 24 hours, the clear zone formed was calculated (mm) from each test sample.

3. Results and Discussion

3.1 Biosynthesis

Infuence of pH on biosynthesis of ZnO or CuO and others nanoparticles are reduction of metal ions. Higher pH (pH 8) was shown to accelerate the rate of reduction since the solution's color changed colloidal brown faster than it would in a lower pH solution [27]. Based on the statement above, ZnO, CuO and ZnO-CuO nanoparticles were biosynthesized in an alkaline medium. The previous researcher stated clearly that the biomolecules are not active in acidic conditions [28], the Zn^{2+} or Cu^{2+} ion and biomolecules in ethanolic extract of leaves guava seed were added by sodium hydroxide.

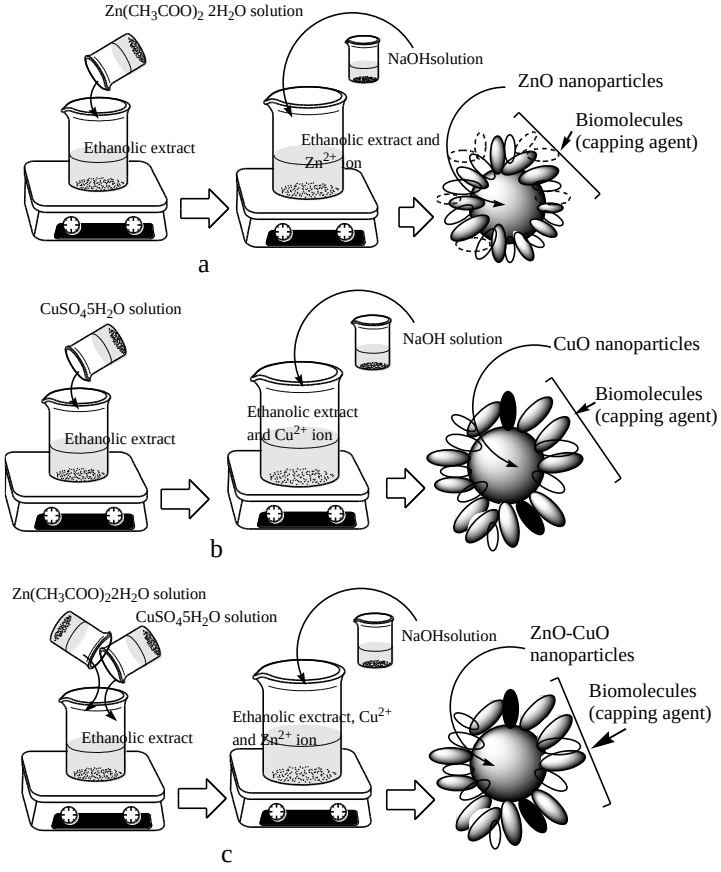

Figure 1. The description of biosynthesis ZnO, CuO and ZnO-CuO nanoparticles [25]

The addition of sodium hydroxide solution changes the electrical charge of the biomolecules and this biomolecule can act as capping and stabilizing agent [29]. After adsorption of OH^- ion, nanoparticles have stable properties because of the existence of the electrostatic repulsion [30]. Primary and secondary metabolites such as carbohydrates, flavonoids, alkaloids, glycosides, tannin, vitamins, and steroids [21] are found in ethanol extract. These metabolites may be act as a reducing and stabilizing agent during the biosynthesis of nanoparticles [2]. The biosynthesis process (ZnO, CuO and ZnO/CuO nanoparticles) is illustrated with a chemical reaction mechanism as depicted in Figure 1**.**

Products (ZnO, CuO and ZnO-CuO nanoparticles) are documented and can be seen in Figure 2. Figure 2 shows, the colors of this products are black as reported previous researchers [31-32].

Figure 2. The photograph from left to right, ZnO (a), CuO (b) and ZnO-CuO nanoparticles (c).

3.2 Analysis of functional groups

The stretching vibration of the O-H and N-H groups is shown in Figure 3 at a wavenumber of 3423–3425 cm^{-1} [33]. This wavenumber was first identified as a bioactive component in a secondary metabolite from the ethanolic extract of *Psidium guajava* L. At 929–1620 cm^{-1}, the C=C, C=N, and C=O group can be seen. Because of interatomic vibration, metal oxide absorption is characterized by a band below 1000 cm^{-1} [33]. At 619 cm^{-1}, the Cu-O group (stretched vibration) was observed [34–36]. The stretching vibration Zn-O group is represented by the band at 433-673 cm^{-1} [8,37–38] and at 507 and 617 cm^{-1}, the functional groups of Zn-O-Cu-O (stretching vibration) were seen [25].

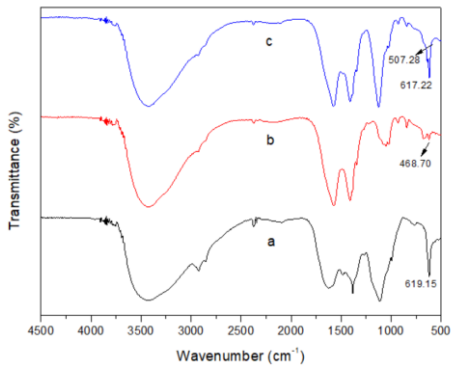

Figure 3. FTIR spectrum from bottom to top : ZnO nanoparticles (a), CuO nanoparticles (b) and ZnO-CuO nanoparticles (c)

3.3 Physical structure analysis

The ZnO, CuO and ZnO-CuO nanoparticles have difrractograms and are presented in Figure 4.

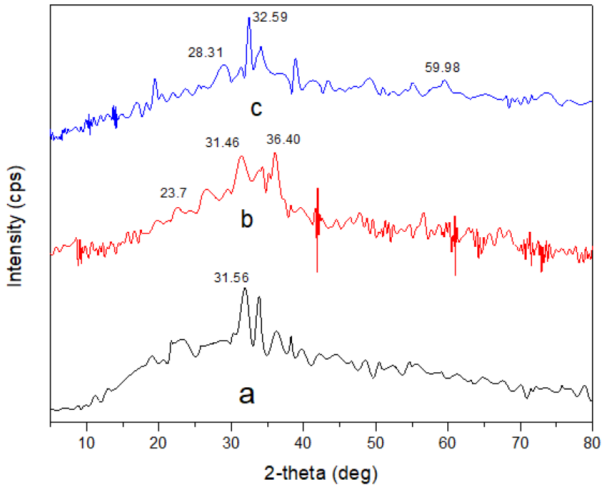

Figure 4. The XRD pattern of CuO (a), ZnO (b) and ZnO-CuO nanoparticles (c).

In the XRD pattern of CuO nanoparticles (Figure 4a), the diffraction peak appeared as a high-intensity peak at 2θ = 31.56° [39]. Diffraction peaks of ZnO nanoparticles at 2θ = 23.7°, 31.46°, and 36. 40° (Figure 4b). These peaks may be associated with the (100) and (101) crystal planes [11]. Diffraction peaks at 2θ = 28.31°, 32.59°, and 59.98° (Figure 4c) are the diffraction peaks of ZnO-CuO nanoparticles [39]. The peak at 2θ = 32.59° is the peak of CuO nanoparticles with high intensity. The crystallite size of ZnO, CuO, and ZnO-CuO nanoparticles was calculated according to the equation of Debye Scherrer [40].

$$D = (0.9.\lambda / \beta.cos\ \theta) \qquad (1)$$

Where D is the crystallite size, λ is the wavelength of X-ray used, β is the full width at half maximum (FWHM) and θ is Bragg's angle. The calculation results of the crystallite size of ZnO, CuO and ZnO-CuO nanoparticles are 1.06, 6.63 and 2.33 nm respectively. There is an effect of NaOH in this biosynthesis process and the effect is the change of the bond length and related to crystallite size [41].

3.4 Application of the product as antibacterial (ZnO, CuO and ZnO-CuO nanoparticles)

ZnO, CuO, ZnO-CuO nanoparticles, acetic acid solution and chloramphenicol solution were used as the antibacterial study. The result of the antibacterial study is displayed in **Figure 5** and the diameter of zone inhibition is summarized in **Table 1**.

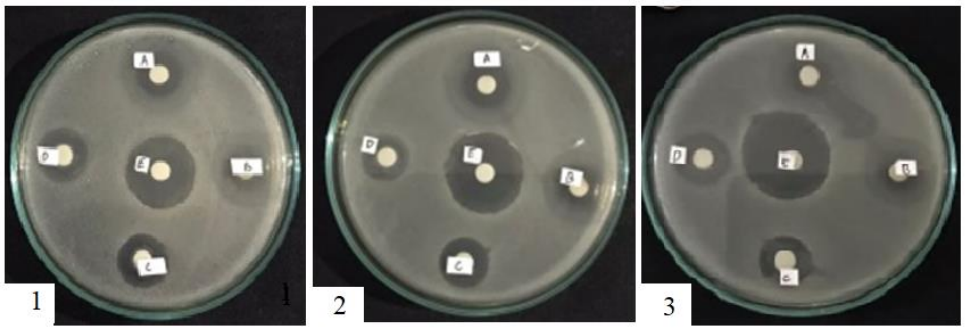

Figure 5. The result of the antibacterial study ZnO nanoparticles (A), acetic acid solution (B), ZnO-CuO nanoparticles (C), CuO nanoparticles (D) and chloramphenicol solution (E).

Table 1. Diameter of zone inhibition ZnO, CuO, ZnO-CuO nanoparticles, chloramphenicol and acetic acid solution.

No.	The Petri dish	Diameter of zone inhibition (mm)				
		ZnO Nanoparticles	**CuO Nanoparticles**	**ZnO-CuO Nanoparticles**	**chloramphenicol**	**Acetic acid**
1	1	14.21	15.12	15.67	22.57	11.88
2	2	16.33	13.68	15.18	24.83	12.25
3	3	13.92	16.51	17.14	28.07	11.45
Average ±		14.82	15.10	15.99	25.16	11.86
SD		± 1.10	±1.10	±0.80	±2.80	±0.30

Figure 5 and Table 1 show a clear zone of inhibition around ZnO-CuO is greater than ZnO or CuO nanoparticles. The effect of combining ZnO and CuO nanoparticles can inhibit bacterial growth in the growth phase [42]. Based on previous study [9,23], the growth of *E coli* bacteria can be inhibited by ZnO and CuO nanoparticles. However, the growth of *S. aureus* bacteria can be inhibited by the combination of ZnO and CuO nanoparticles, even though the inhibition zone is not as good as that of ZnO and CuO nanoparticles. It is possible that there are differences in cell wall structure between *E. coli* and *S. aureus* bacteria.

The antimicrobial activity of ZnO or CuO nanoparticles is related to the reactive oxygen species (ROS) [38]. Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO●), and organic hydroperoxides (OHP), accumulation of NPs on the bacterial surface, and accumulation of NPs in the cytoplasm / periplasmic region can cause bacterial death. ROS can damage cellular components (lipids, peptidoglycan, proteins, and DNA) by being released from NPs and then entering bacteria [3]. Acetic acid has a weak acid property and can penetrate bacterial membranes more easily than acids that are classified as strong acids. Acetic acid (ionized and non-ionized form) readily diffuses through

the hydrophobic membranes of bacteria and inhibits protein synthesis, resulting in inhibition of bacterial growth [39].

4. Conclusions

Interaction between ethanolic extract, Zn^{2+} and Cu^{2+} ion in alkaline solution can form of ZnO-CuO nanoparticles. The functional groups of Zn-O and Cu-O in ZnO-CuO nanoparticles was detected at 507 and 617 cm^{-1} on FTIR spectra respectively. The ZnO, CuO and ZnO-CuO nanoparticles have crystallite size of 1.06, 6.63 and 2.33 nm respectively. The inhibition zone of ZnO and CuO nanoparticles is lower than ZnO-CuO nanoparticles. ZnO-CuO nanoparticles can be antibacterial candidates.

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