Plant extract-assisted biosynthesis of zinc oxide nanoparticles and their antibacterial application

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Abstract. ZnO nanoparticles are multi-purposes materials that can be synthesized by several methods, including physical and chemical routes. A novel synthesis method of ZnO nanoparticles is the biological method using plant extracts as reducing and capping agents, such as the fruit extract of Averrhoa bilimbi. Plant extracts are superior agents for synthesizing nanoparticles because it provides essential phytochemical substances as reductor, capping agents, and free from toxicants. In this study, the effects of precursor concentrations and the amount of plant extract on the formation and morphology of nanoparticles were investigated. The characteristics of ZnO particles were studied by UV-Vis spectroscopy, XRD, FTIR, TEM, and DLS. The study showed that the formation of ZnO nanoparticles occurred after five hours reaction at 70°C, as indicated by color change of the solution. ZnO nanoparticle formation was confirmed by the maximum absorption at the wavelength of 372 nm and XRD analysis. FTIR analysis showed that the as-synthesized ZnO contained significant organic compounds on its surface, especially compared to commercial ZnO. Reduction reactions using A.bilimbi produce nanoparticles in the size from 35.4 to 59.5 nm with round shape and some agglomeration that were observed by TEM. The ZnO antibacterial property was tested against planktonic and biofilm Escherichia coli. The result showed that as-synthesized ZnO have comparable antibacterial antibiofilm property as the commercial ZnO nanoparticles at low concentration. Interestingly, this property was diminished when as-synthesized ZnO nanoparticles were used at high concentrations.

1. Introduction

ZnO nanoparticles are multi-purposes material with a wide range of applications, such as photocatalyst and antibacterial agents. These nanoparticles can be synthesized by several methods, such as physical and chemical methods. Physical methods for synthesizing nanoparticles that have been used are ball milling, melt mixing, physical vapour deposition, laser ablation, sputter deposition, electric deposition, and ion implantation. Due to the involvement of specific equipment, physical methods require expensive investment [1,2]. Researchers have developed nanoparticle synthesis processes through a chemical method that is more efficient and able to produce nanoparticles wit goof physicochemical characteristics [1,3]. This method requires the use of an organic and hazardous compound that acts as
reductor and capping agent, such as sodium borohydride, ascorbic acid [4], ethanol, NaOH [5], sodium citrate, dimethyl fumarate (DMF), Tollens reagent, and ethyleneglycol [6].

A novel method to synthesize ZnO nanoparticles is biological method that uses plant extracts as the reducing and capping agents. Plant extracts are superior agents for synthesizing nanoparticles because they are free from toxicants and provides essential phytochemical substances as reductor and capping agents. Enzyme, protein, flavonoid, polysaccharide, and phenol in the plant extract play an important role in the reduction process of particle [7]. Other studies also proved that polyphenol is the main substances that influenced the formation and stabilization of nanoparticles [8]. Compared to physical and chemical routes, biological method is an efficient and eco-friendly method because of the relatively mild operating condition [1,9].

Several factors influence the nanoparticles synthesis process and its resulting characteristics. Morphology and particle size are controlled by precursor concentration and volume ratio of precursor-extract which were used in the reaction [10]. Another study also confirmed that the volume ratio of precursor and reductor/capping agent influence the kinetics of reaction [11]. Increasing the extract volume led to an increase in absorbance intensity of the suspension, implying the increase in nanoparticle formation. After reaching a maximum number, absorbance intensity tends to decrease because of metal ions scarcity, which could provoke agglomeration of the smaller particles to form larger particles [12]. In line with the extract addition, the higher precursor volume led to the higher absorbance intensity of suspension [13].

Due to the variety of biomolecules composition and content in the plant extract, nanoparticle synthesis through biological method requires different process conditions from each other producing particle with different characteristics. The fruit of Averrhoa bilimbi is a potential plant used in synthesis ZnO nanoparticles. In this part, there are some phytochemical substances such as polyphenol as one of the major constituent, alkaloid, carbohydrate, flavonoid, saponin, and tannin [14]. Another study confirmed that the fruit extract of A. bilimbi is a complex matrix that contains bioactive components such as 4H-pyran-4-one, 2,3-dihydro 3,5-dihydroxy-6methyl, hexadecanoic acid, squalene, erucic acid, oleic acid, furfural, boron acid, and mannitol [15]. Therefore, this study intends to identify characteristics of ZnO nanoparticles synthesized by the fruit extract of A. bilimbi in different precursor concentration and volume ratio of precursor-extract, and also to explore its potential application as antibacterial/antibiofilm agents.

2. Material and Method

2.1 Materials
Fresh fruit of A. bilimbi was collected from Bandung city. Zinc nitrate tetrahydrate, (Zn(NO3)2.4H2O), and Folin-Ciocalteu reagent were purchases from Merck Indonesia. Commercial ZnO nanoparticles from Sigma-Aldrich was used as a comparison.

2.2 Extraction of phenol from A. bilimbi fruit
10 g fresh fruit were washed and crushed using blender. Fruit pulp was added to 300 mL aqua demineralization (Aqua DM) and shaked in the shaking incubator (200 rpm) at 70°C for 90 minutes. After cooling process, the mixture was filtered using filter cloth and the supernatant was stored at 4°C for total phenol content analysis.

2.3 Total phenolic content analysis
Total phenol content was determined using the Folin-Ciocalteu [16], 40 μl extract solution was mixed with 1.8 ml of the Folin-Ciocalteu reagent that was previously dissolved in water (1:10). After 5 minutes, 1.2 ml of Na2CO3 (7.5% w/v) was added to the mixture. The solution was allowed to stand at room temperature for 1 hour, then absorbance was measured using a UV-Vis spectrophotometer (Thermo Fisher Scientific 4001/4) at 765 nm. Calibration curve was prepared using standard solution of gallic acid at several concentrations (20, 40, 60, 80, and 100 mg/L r² = 0.999). The measured total
phenol was expressed on a fresh weight basis as mg gallic acid equivalents/100 gr sample (mg GAE/100 gr extract). Average measurement of the extract showed that total phenol of fruit extract that is available as reducing and capping agent was 124.285 mg GAE/100 g sample.

2.4 Synthesis of ZnO nanoparticles using A. bilimbi
Experiment was conducted using 2^nd full factorial design. The first factor was precursor concentration (0.025 M, 0.050 M, 0.100 M), while the second factor was volume ratio precursor-extract (2:1; 1:1; 1:2). Precursor solution in a certain volume and concentration was heated on hot plate until it reached 70°C. Then, the extract was added using a burette under constant stirring for up to 5 hours. ZnO nanoparticle formation was indicated by a color change of the solution to brownish-yellow. The solution was cooled to 30°C, then it was purified through centrifugation at 14,000 rpm for 5 minutes to get deep white powder. The powder was washed twice using aqua DM, then it was dried for 24 hours at 70°C. The dried solid was crushed with a mortar for analyzing.

2.5 Analytical methods
The characteristics of ZnO nanoparticles were analyzed using UV-Vis spectrophotometer, X-ray diffraction (XRD), Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), and Fourier Transform Infra-red (FT-IR).

2.6 Antibacterial assay

2.6.1 Inhibition of planktonic bacteria growth of Escherichia coli. 1 mL of E.coli overnight inoculum was added into 50 mL LB-Broth (10 gr/L tryptone, 5 gr/L yeast extract, 10 gr/L NaCl, pH 7). 0.9 mL of bacterial inoculum was poured into 24-well plates which contained 0.1 mL of ZnO nanoparticles solution in several concentrations (0, 50, 100, and 200 ppm). Then, the plates were incubated for 6 hours, 37°C, and constant agitation at 180 rpm in a shaker incubator. The bacteria population was measured using a spectrophotometer at 600 nm. The result was analyzed by ANOVA using statistical software.

2.6.2 Inhibition of biofilm formation of E.coli. 1 mL of E. coli bacterial culture was poured to 50 mL of M9 minimal medium (48 mM Na2HPO4, 22 mM KH2PO4, 8.6 mM NaCl, 19 mM NH4Cl, 2 mM MgSO4, 7H2O, 100 μM CaCl2, and 20 mM glucose). 0.9 mL of bacterial inoculum was poured into 24 well-plates containing 0.1 mL of ZnO nanoparticles to reach a certain concentration (0, 50, 100, and 200 ppm) and incubated in a shaker incubator at 37°C and 180 rpm. After incubation for 6 hours, the supernatants were removed from each well and the absorbances were measured at 600 nm to denote the planktonic bacteria concentrations. Then, the wells were washed with phosphate buffer solution (PBS) and stained using crystal violet 0.1 w/v and allowed to stand for 15 minutes. After the incubation process was complete, the leftover crystal violet was removed from the well. The remaining liquid at the well was redissolved using 1 mL ethanol and its absorbance was measured by a spectrophotometer at 540 nm. The result was analyzed by ANOVA using statistical software.

3. Result and Discussion

3.1 Formation of ZnO nanoparticles
The synthesis process is carried out at 70°C because it allowed faster water solvent evaporation at high temperatures. Throughout the synthesis process, the solution absorbance was measured to determine the formation of the nanoparticles. The formation of ZnO nanoparticles using plant extract indicated the presence of a peak UV-Vis absorption band at 375 nm [17]. Moreover, the reduction of precursors by plant extracts can also be identified by colour change of the suspension to brownish-yellow and the formation of deep white precipitate.
When 0.05 M Zn precursor was mixed with *A. bilimbi* extract at 1:1 v/v ratio, two peaks at 240 and 300 nm were observed (Figure 1). These peaks were still observable during the first 3 hours. However, when the reaction was continued up to 5 hours, the peak slowly shifted to 372 nm, which indicated the formation of ZnO nanoparticles. Therefore, 5 hours was chosen as the basis for reaction time for the remaining variation.

![UV-vis spectra recorded as a function of reaction time of 0.05 M Zn(NO$_3$)$_2$.4H$_2$O solution with *A.bilimbi* fruit extract (1:1 v/v)](image)

**Figure 1.** UV-vis spectra recorded as a function of reaction time of 0.05 M Zn(NO$_3$)$_2$.4H$_2$O solution with *A.bilimbi* fruit extract (1:1 v/v)

| Precursor concentration (M) | Precursor : Extract (v/v) | Solution color          | Precipitate formed | pH         |
|----------------------------|----------------------------|-------------------------|-------------------|------------|
|                             |                            |                         |                   | Initial    | Final |
| 0.025                      | 2 : 1                      | Brownish yellow         | No                | 3.5        | 2.5   |
|                            | 1 : 1                      | Brownish yellow         | Yes               | 3.5        | 1.5   |
|                            | 1 : 2                      | Brownish yellow         | Yes               | 3.5        | 1     |
| 0.050                      | 2 : 1                      | Brownish yellow         | No                | 4          | 2.5   |
|                            | 1 : 1                      | Brownish yellow         | Yes               | 3          | 1     |
|                            | 1 : 2                      | Brownish yellow         | Yes               | 3          | 1     |
| 0.100                      | 2 : 1                      | Brownish yellow         | No                | 4          | 2.5   |
|                            | 1 : 1                      | Brownish yellow         | No                | 3.5        | 1.5   |
|                            | 1 : 2                      | Brownish yellow         | Yes               | 3.5        | 1.5   |

Table 1. Physical characteristics of solution after 5 hours reaction

When the synthesis process variables established previously was tested at other precursor concentration and different precursor:extract volume ratio, some combination failed to yield any precipitate, even after centrifugation (Table 1). This especially happened when the precursor volume used is greater than the extract volume. For instance, the mixture of 0.100 M precursor and 1:1 ratio produced a brown cloudy layer on the solution surface which were suspected to be Zn(OH)$_2$. However, after the centrifugation process, the brown sediment could not be separated from the suspension. This implies that the [OH$^-$] ions needed to form Zn(OH)$_2$ (and subsequently ZnO) from the extract was not
sufficient to bind [Zn$^{2+}$] ion. On the other hand, when the 1:2 ratio was used, the recoverable precipitate was obtained (Table 1).

3.2 Characterization of as-synthesized ZnO nanoparticles

Confirmation of ZnO formation was analyzed using XRD. The result showed a slight shift in the curve peaks at several diffraction angles on ZnO nanoparticles compared to commercial ZnO. The diffraction peaks of commercial ZnO nanoparticles were found at 31.76°, 34.42°, 36.24°, 47.54°, 56.58°, 62.85°, 63.02°, 66.37°, 67.93°, 68.12°, 69.07°, 72.56° and 76.96°. The reaction using 0.025 M of precursor solution with ratio volume precursor-extract at 1:1 and 1:2 produce ZnO nanoparticle with the diffraction peaks found at 33.68°, 56.35°, 70.65° and 33.71°, 56.36°, 70.69°, respectively. These appearances of new peak diffractions could indicate that the particles contain some amorphous phases with high peak intensity which contributed to the noisy spectra [18].

Formed ZnO nanoparticles had average particle sizes ranging from 35 to 60 nm (Table 2). The size trend of ZnO nanoparticles synthesized from A.bilimbi fruit extract was almost close to the size of commercial ZnO nanoparticles (29 nm). However, an inconsistent trend of particle size in the treatment variation of volume ratio of precursor and extract was observed. For reaction using 0.025 M precursor, increasing of extract volume caused the reduction in particle size. However, on the reaction using 0.050 M precursor solution, increasing the ratio of precursor-extract led to increasing particle size from 49.0 nm to 57.1 nm, which have been observed before [19]. Furthermore, Song et al. (2009) argued that this increase in extract volume provides abundant reducing agents that can stimulate particle aggregation because of interaction between capping agent molecules to bind surface particles which cause a secondary reduction to form new nuclei of particle.

Generally, the formed ZnO nanoparticles had a round shape, showed some agglomeration, and had good crystallinity. Although the polyphenols contained in the plant extracts were able to reduce Zn to form nanoparticles (size of ± 50 nm), these compounds can form a mantle that covers the surface of the particles (Figure 3.a). Agglomeration of the nanoparticles was attributed to the nature of the synthesis process (Figure 3.b). Note that throughout the synthesis process, the pH of the solution dropped from 3-4 to 1-2.5 due to the evaporation of water molecules (Table 1). This can affect the size and crystallinity of the nanoparticles. In acidic medium, nanoparticles formation was more difficult due to the limited presence of [OH$^{-}$] ions in suspension compared to [H$^{+}$] ions, thus limiting the formation of alkoxide (Zn(OH)$_2$) which subsequently limit the formation of ZnO [20].

The pH strongly influences the degree of aggregation and particle settling, due to the change of surface charge and zeta potential of particles which allowed for a greater degree of particle interaction [21]. ZnO nanoparticle synthesis processed under acidic or neutral conditions tends to cause particle agglomeration [20]. The optimum pH for synthesizing ZnO nanoparticles and having lower agglomeration is 8 [22,23]. At low pH, the adsorption of metallic particles that were positively charged to the negatively charged organic matter from capping agent was enhanced thus resulting in a high level of aggregation [24]. The acidic process also would change chemical structures and activities of reductant to form alkoxide ions [25]. Other studies confirmed that the size and density of nanoparticles increase as a response to a decrease in the acidity of the medium. When the pH was continuously decreased to certain limits cause re-dissolution of Zn(OH)$_2$ [26]. Good crystallinity of the ZnO nanoparticles was found on synthesized particles with a concentration of 0.100 M precursors at a 1:2 precursor-extract ratio.
Figure 2. X-ray diffraction pattern of ZnO nanoparticle synthesized using 0.025 M of precursor solution; (a) 1:1 (v/v), (b) 1:2 (v/v), (c) commercial

Table 2. Physical characteristics of ZnO nanoparticle

| Precursor concentration (M) | Precursor : extract (v/v) | Average dried weight (g) | Average particle size (nm) |
|-----------------------------|----------------------------|---------------------------|---------------------------|
| 0.025                       | 2 : 1                      | -                         | -                         |
|                             | 1 : 1                      | 0.065                     | 51.7                      |
|                             | 1 : 2                      | 0.104                     | 35.4                      |
| 0.050                       | 2 : 1                      | -                         | -                         |
|                             | 1 : 1                      | 0.060                     | 49.0                      |
|                             | 1 : 2                      | 0.130                     | 57.1                      |
| 0.100                       | 2 : 1                      | -                         | -                         |
|                             | 1 : 1                      | -                         | -                         |
|                             | 1 : 2                      | 0.105                     | 59.5                      |

ZnO nanoparticle (commercial as control) 28.9

FTIR analyses were performed on the formed nanoparticles. ZnO nanoparticles synthesized using A. bilimbi fruit extract transmit significantly more peaks spectrum compared to commercial ZnO nanoparticles, implying the presence of various organic groups on the surface of the sample (Figure 4 and Table 3). The bond between M-O (Zn-O) would be found in areas ranging between 600 cm⁻¹ and 400 cm⁻¹ [27]. The FTIR spectrum confirms Zn-O bond on the peak area between 445.56 cm⁻¹ to 493.78 cm⁻¹. Bending vibrations of C-H from the alkyne compound derived from plant extract were found at 626.87 cm⁻¹ to 634.58 cm⁻¹. The peak area between 740.67 cm⁻¹ to 821.68 cm⁻¹ represented C-H bending vibrations derived from aromatic compounds (alkene groups). The peak area ranging from 1315.45 cm⁻¹ to 1363.67 cm⁻¹ confirmed vibrations of the C-N bond derived from aromatic amine compounds. Vibration of the C=C from alkene compound with strong to moderate intensity was found at 1633.71 cm⁻¹ to 1656.85 cm⁻¹. Stretching vibration of C-H derived from alkane in low intensity was
found at 2854.65 cm\(^{-1}\) to 2926.01 cm\(^{-1}\), while stretching vibration of O-H with strong intensity was found at 3369.64 cm\(^{-1}\) to 3379.29 cm\(^{-1}\).

**Figure 3.** TEM micrograph of ZnO nanoparticles, (a) 0.050 M of precursor solution, 1:1 (v/v), (b) 0.050 M of precursor solution, 1:2 (v/v), (c) 0.010 M of precursor solution, 1:2 (v/v), (d) SAED pattern of ZnO nanoparticles from 0.010 M of precursor solution, 1:2 (v/v)

**Figure 4.** FTIR spectra of ZnO nanoparticles (a) 0.025 M precursor solution, 1:1 (v/v), (b) 0.025 M precursor solution, 1:2 (v/v), (c) 0.050 M precursor solution, 1:2 (v/v), (d) 0.100 M precursor solution, 1:2 (v/v) (e) commercial
As the ZnO concentration increased to 100 ppm, the efficiency of commercial ZnO (40% inhibition) significantly decreased while significant increase in %inhibition (37%), which is more commonly observed in other studies, i.e., increasing the ZnO concentration resulted in increasing inhibition efficiency [28].

Similar trend of antibacterial activity of the as-synthesized nanoparticles the formation and morphology of the nanoparticles on biofilm inhibition assay. Initially, 50 ppm of ZnO resulted in 37% and 33% inhibition for as-synthesized and commercial samples, respectively. Increasing the ZnO concentration to 100 ppm significantly decreased the efficiency of as-synthesized ZnO (25% inhibition) while significantly increased the efficiency of commercial ZnO (40% inhibition). Further increment to 200 ppm ZnO saw a further decrease in efficiency for as-synthesized (2.5%) and similar efficiency (40% inhibition) was observed for commercial ZnO.

As mentioned previously, the as-synthesized sample showed a different trend than that normally observed. At this stage, the exact reason is not clear yet. Remembering FTIR result (Figure 4) showed before, it seems that the presence of significant organic compounds on the surface of as-synthesized ZnO could affect the trend observed here. The presence of higher ZnO could result in a higher organic compounds in the system. This organic compound could easily leach out of the ZnO surface and subsequently used by the bacteria as a carbon source for growth, therefore reducing the antibacterial activity of the nanoparticles. Moreover, the presence of organic compound together with metal oxide nanoparticles have been shown to reduce the antibacterial efficiency, especially when compared to its respective metal salts [29]. Meanwhile, TEM results (Figure 3) also suggest the agglomeration of as-synthesized ZnO. It has been argued before that agglomeration of nanoparticles which led to the deposition of the nanoparticles also could contribute to the low antibacterial activity of nanoparticles [30].

### Table 3. Functional groups capping on ZnO nanoparticles surface

| Wavenumber (cm⁻¹) | Functional groups | Compound |
|-------------------|-------------------|----------|
| 453.27            | Zn-O              | -        |
| 493.78            | Zn-O              | -        |
| 626.87            | C-H               | Alkyne   |
| 742.59            | C-H               | Aromatic |
| 821.68            | C-H               | Aromatic |
| 1317.38           | C-N               | Aromatic amine |
| 1363.67           | C-N               | Aromatic amine |
| 1633.71           | C=O               | Alkene   |
| 2854.65           | C-H               | Alkane   |
| 2926.01           | C-H               | Alkane   |
| 3379.29           | O-H               | Phenol   |

Note:

- a = 0.025 M precursor solution, 1:1 (v/v)
- b = 0.025 M precursor solution, 1:2 (v/v)
- c = 0.050 M precursor solution, 1:2 (v/v)
- d = 0.100 M precursor solution, 1:2 (v/v)
- e = Commercial

**3.3 Antibacterial and antibiofilm properties of the synthesized ZnO**

The antibacterial properties of ZnO nanoparticles were tested against *E.coli* grown in two different life mode, planktonic (free-swimming) form and biofilm form. Three different nanoparticles concentration (50, 100, and 200 ppm) were tested. Again, commercial ZnO was used as a comparison. At 50 ppm, around 30% of planktonic bacteria were inhibited, both for the as-synthesized and commercial ZnO. Increasing the ZnO concentration to 100 ppm saw a slight %inhibition increase for the commercial ZnO (34%), while similar %inhibition was observed for 100 ppm. Interestingly, when the ZnO concentration was further increased to 200%, the as-synthesized ZnO saw a decrease in antibacterial property, as the %inhibition was decreased to 25.5%. On the other hand, commercial ZnO saw a significant increase in %inhibition (37%), which is more commonly observed in other studies, i.e., increasing the ZnO concentration resulted in increasing inhibition efficiency [28].

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Figure 5. Inhibition activity of ZnO nanoparticles on planktonic bacteria of *E.coli*. Note: Mean of replications ± standard error (n=3).

Figure 6. Inhibition activity of ZnO nanoparticles on biofilm of *E.coli*. Note: Mean of replications ± standard error (n=3).

4. Conclusion
ZnO nanoparticles were successfully synthesized using *A. bilimbi* extract as the reducing and capping agent. A preliminary experiment showed that when synthesis was performed at 70°C, the reaction time needed for the formation of ZnO was 5 hours. However, the formation of ZnO was highly dependent on the precursor concentration and volume ratio of precursor-extract. The abundance of reductant in the solution increases non-binding ions accumulation that would bind to the capping agent molecules thus form particles aggregate in a larger size and stimulate a new compound growth that reduces the purity of the ZnO nanoparticles. The formation of ZnO nanoparticles was confirmed by XRD analysis. The as-synthesized ZnO nanoparticles were generally round with good crystallinity and some agglomeration, with sizes ranging from 35.4 to 59.5 nm. Due to the nature of the synthesis process, the as-synthesized ZnO showed a significant presence of organic compounds on its surface, especially when compared to commercial ZnO. Due to agglomeration, ZnO nanoparticles synthesized from *A.bilimbi* fruit extract have lower antibacterial activity compared to commercial ZnO. Antibacterial and antibiofilm testing showed that as-synthesized ZnO had comparable antibacterial/antibiofilm property to commercial ZnO at low concentration (50-100 ppm). However, the
antibacterial/antibiofilm property was reduced when high concentration (200 ppm) of as-synthesized ZnO was used.

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