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Green Synthesis of Zinc Nanoparticles Using Bioreductor from *Azadirachta indica* Extract and its Characteristics

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ABSTRACT

Zinc nanoparticles represent a form of nanotechnology utilizing zinc, which can function as a drug delivery system. This study aims to investigate the role of *Azadirachta indica* extract as a bioreductor in influencing the characteristics of zinc nanoparticles, including functional groups, size, stability, crystal phase, composition, and shape. The zinc nanoparticles were synthesized by mixing the extract, which was prepared using distilled water (aquadest). The nanoparticles were characterized using various techniques, including ultraviolet-visible spectrophotometry (UV-Vis), Fourier transform infrared spectroscopy (FTIR), particle size analysis (PSA), X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDX), and transmission electron microscopy (TEM). A trial-and-error approach was employed, and the qualitative data were analyzed descriptively. The results indicated that the absorption peak of the zinc nanoparticles occurred at 365 nm. FTIR analysis revealed the presence of functional groups with high integrity. The particle size of the zinc nanoparticles was determined to be 368.8 nm, with a polydispersity index (PDI) of 0.472. The XRD pattern exhibited a typical phase plot with good quality and no interference. The zeta potential of the nanoparticles was found to be -43.6 mV, with a standard deviation of 18.8 mV. XRD analysis further confirmed that the zinc nanoparticles exhibited spherical to hexagonal crystalline phases with high crystallinity, as supported by the TEM images. Additionally, the EDX profile confirmed the presence of only zinc (Zn), carbon (C), oxygen (O), and silicon (Si), with no evidence of contamination from other elements.

Keywords: *Azadirachta indica*, Characteristics, Green Synthesis, Zinc Nanoparticles

INTRODUCTION

Nanotechnology refers to the process of reducing larger molecules to the nanoscale (1–1000 nm), which alters the physicochemical properties of the cell matrix (Jeevanandam et al, 2018; Wen et al, 2015; Martien et al, 2012). It involves the production of nanoparticles with varying sizes, shapes, and chemical compositions for applications in fields such as agriculture, animal husbandry, and health. Nanoparticles, especially in drug delivery systems, have gained significant attention in recent years (Martien et al, 2012). One type of nanoparticle that is being developed is nanominerals, utilizing organic minerals such as zinc (Zn) as a biosynthetic precursor. Zinc nanoparticles are additive agents that have been widely used in various applications. as the antibacterial, anti-inflammatory, antiviral, and anticancer agents, respectively. (Lopez et al, 2023; Gupta et al, 2022; Bisht & Rayamajhi, 2016). However, conventional zinc has low bioavailability (Hall & King, 2023; Hidayat et al, 2021), making nanoparticle synthesis essential for enhancing its bioavailability. Nanoparticle synthesis can be achieved through three main methods: chemical (bottom-up), physical (top-down), and biological (biosynthesis) (Nyabadza et al, 2023). Among these, biosynthesis is a promising environmentally friendly (green synthesis) approach, utilizing living organisms to produce nanoparticles without toxic chemicals or costly organic solvents (Jayappa et al, 2020; Aminuzzaman et al, 2018). Similar to previous research, zinc nanoparticles were synthesized using *Nigella sativa* (Lail et al, 2023), bioreductor *Lactobacillus plantarum* (Yusof et al, 2022), and extract of *Euphorbia petiolata* (Mohammadi et al, 2017). These methods offer advantages such as control over particle size and shape, as well as environmental and cost benefits (Yuvakkumar et al, 2014). Green synthetic alternatives for enhancing Zn bioavailability with the use of phytobiotics are environmentally friendly compared to other methods, cheaper in process, take a short time, use simple equipment and precursors, and produce very pure products (Heinlaan et al, 2008; Lee et al, 2011).

A promising phytobiotic for this purpose is *Azadirachta indica*, a plant native to tropical and subtropical regions, known for its diverse biologically active compounds that contribute to its therapeutic role in health management (Moin et al, 2021). Based on antimicrobial studies, all parts of the neem contribute significantly to potential cell wall damage or growth inhibition of various microbes, including bacteria (Ahmed et al, 2023; Ali et al, 2021; Wylie & Merrell, 2022). Neem plants are considered a miracle tree, divine tree, or panacea tree of all diseases that are considered an ancient medicine for the modern world (Irinda & Pratiwi, 2018), and the results of phytochemical screening and infrared spectrophotometry show that the neem leaves contain 3.79 mg quercetin equivalent of flavonoid secondary metabolite compounds per 100 mL (Alfira et al, 2023) and 51.02 mg of total phenolic per/g dry crude extract (Al-Jadidi & Hossain, 2015). Other compounds, such as tannins, triterpenes, saponins, alkaloids, flavonoids, and steroids, can act as bioreductors and biostabilizers in nanoparticle synthesis (Aklilu et al, 2022). Flavonoid compounds can form complexes with metal cations through the hydroxyl groups that bind to Zn^{2+} ions. This complex can decompose upon heating, leading to the formation of nanoparticles (Yuvakkumar et al, 2014). The carbonyl and hydroxyl functional groups found in plant extracts can contribute to the synthesis of nanoparticles. Based on publication in the scopus database (https://scopus.com) with the reseach query ALL ("zinc nitrate hexahidrate") AND PUBYEAR > 2023 AND PUBYEAR < 2025, there are three publications articles related to zinc nanoparticles, while the study of zinc nanoparticles using neem leaf extract synthesis with zinc nitrate hexahydrate has never been done. Yusof et al (2022); Mohammadi et al (2017) demonstrated that zinc nanoparticles can damage the cell walls. Therefore, to obtain the correct concentration for the nanoparticle formation process, it is necessary to synthesize neem leaf extract and zinc nitrate hexahydrate. The purpose of this study was to determine the effect of *Azadirachta indica* extract as a bioreductor on the characteristics of zinc nanoparticles (functional groups, size, stability, crystal phase, composition, and shape).

MATERIALS AND METHODS

The main chemicals used in this research included zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) as precursor (MERCK, Germany), the source of extract came from from neem leaves taken from the Gadjah Mada University campus, NaOH (MERCK, Germany), and distilled water (Jaya Santosa, Indonesia).

Preparation of neem leaf extract

The neem leaves were thoroughly washed with water to remove dust and other particles, then were cut into small pieces, and then air-dried at room temperature (20-25°C) until reaching 60% of moisture content. Pulverization was carried out with an electric grinder (HK-8300) to obtain a fine powder after drying. Ten grams of neem leaf powder were soaked in 100 mL of distilled water, heated at 50°C for 45 minutes, and then stored at 20-25°C for 24 hours. The mixture was subsequently filtered using Whatman filter paper No. 1. The filtration process was repeated three times, yielding 80 mL of pure extract, which was stored at 4°C until further use.(Ajayan & Hebsur, 2020).

Synthesis of Zinc Nanoparticles

The synthesis of zinc nanoparticles was initiated by preparing a $Zn(NO_3)_2.6H_2O$ solution. The 0.2 mM $Zn(NO_3)_2.6H_2O$ solution was prepared by diluting 14.87 g of zinc nitrate hexahydrate in 250 mL of distilled water. Zinc solution 30 mL was poured into 5 mL of the prepared homogenized neem leaf extract, and the mixture was magnetically stirred on a hotplate (DLAB MS 7 -

H550-S, Indonesia) at 70 °C at 700 rpm for 10 min. Subsequently, the mixture was shaken at room temperature (25°C) for 2 hour, followed by pH adjustment (Mettler Toledo, FiveEasy TM F20, Switzerland) by adding 0.1 % NaOH. After the pH reached 7, further shaking (Eppendorf New Brunswick INNOVA 42 R, USA) was performed at 200 rpm for 2 hour. Next, the solution was centrifuged (Eppendorf 5804R, Germany) three times for 10 min each. The first centrifugation was followed by ethanol washing, and on 2nd and 3rd centrifugation the solution was washed with distilled water. The obtained precipitate was then heated in an oven (Memmert E07086, USA) at 100°C for 1 hour and calcined in a furnace (Thermoliyne FB 1410M-33, Finland) at 450°C for 3 hours, resulting in the formation of stable zinc nanoparticles.

Characterization Analysis of Zinc Nanoparticles

One gram of zinc nanoparticles was dissolved in 20 mL of distilled water for further characterization. Zinc nanoparticles were diluted and tested for characterization, such as nano formation, using a UV-vis spectrophotometer (Thermo Scientific SKY-S1119700DP, Finland). A 200 µ solution containing zinc nanoparticles was placed in a quartz cuvette and then measured at a wavelength of 200-600 nm. Functional group analysis was performed using Fourier-transform infrared spectroscopy (Series-4600, Jasco Corp, Japan) of zinc nanoparticles as much as 200 µ with readings of 400-4000 cm^{-1} wavenumbers. nano size analysis, stability and phase plot by Particle size analyze (Malvern Zetasizer Nano Series, Malvern Instrument, Malvern UK) Setting the refractive index of 1.0402 and extinction coefficient of 5.3176, put 1 mL of zinc nanoparticle solution into the cuvette with 3 readings. X-Ray Diffraction of the crystalline phase (XRD-D8 Advance Eco, Germany) 1 g zinc nanoparticles with three readings, component analysis by EDX-SEM with a scanning electron microscope (Thermo Scientific Phenom XL G2 Desktop SEM, Finland), and shape analysis using a transmission electron microscope (JEOL JEM 1400 Plus, Japan) testing 1 g of sample on the plate with three readings.

RESULTS AND DISCUSSION

The synthesis of zinc nanoparticles occurs through a reaction mechanism between $Zn(NO_3)_2.6H_2O$ solution and the extract of the neem leaf and NaOH. The formation of zinc nanoparticles is caused by the secondary metabolite content of the neem leaf extract, which acts as a bioreductor and stabilizing agent. Yusof et al (2022) reported the mechanism of zinc nanoparticles through process of nucleation, aggregation, stabilization, and capping of nanoparticles (Figure 1). Secondary metabolites contained in neem leaf extract can reduce Zn^{2+} ions into Zn atoms with the help of NaOH. Furthermore, the Zn atoms combine to form Zn° clusters. Secondary metabolites interacted with the zinc surface and enveloped the Zn° clusters so that there was no aggregation between the Zn° clusters and stable zinc nanoparticles were formed. Reported by Tarroum et al (2023); Wang et al (2023) secondary metabolites play an active role as bioreductors and stabilizers in the process of forming zinc nanoparticles.

Characterization of Zinc Nanoparticles

The zinc nanoparticles showed a Z-average of 368.6 d.nm with a low polydispersity index (0.0472), indicating uniform size distribution. The zeta potential was -43.6 mV, suggesting good stability. Conductivity measured 0.0650 mS/cm, and overall result quality was reported as good (Table I).

Table I. Characterization of zinc nanoparticles

Characterization	Description
Z-average	368.6 (d. nm)
Polydispersity index	0.0472 (PDI)
Intercept	0.953
Result quality	Good
Zeta potential	-43.6 (mV)
Zeta deviation	18.8 (mV)
Conductivity	0.0650 (ms/cm)
Result quality (zeta)	Good
Phase plot count rate	917.1 (Kcps)
Cell description	Good

UV-Vis Spectrum of Zinc Nanoparticles

An optical analysis was performed using UV-visible spectroscopy to examine the peak spectrum (wavelength) of the zinc nanoparticles. The spectrum was analyzed within the wavelength range of 300 to 600 nm, which corresponds to the typical range for nanomaterials (Singh et al, 2012) (Figure 2). The UV-Vis spectrum of the zinc nanoparticles exhibited a peak at 365 nm. This absorption peak aligns with the characteristic wavelength for zinc nanoparticles, consistent with the findings of Abomuti et al (2021), where zinc nanoparticles biosynthesized using *Salvia officinalis* showed a peak at 368 nm.

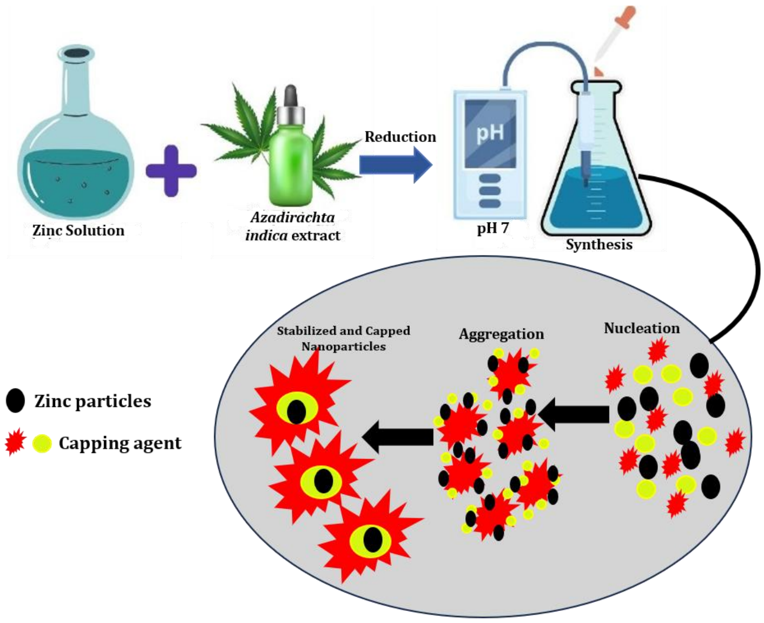


Figure 1. Mechanism zinc nanoparticles

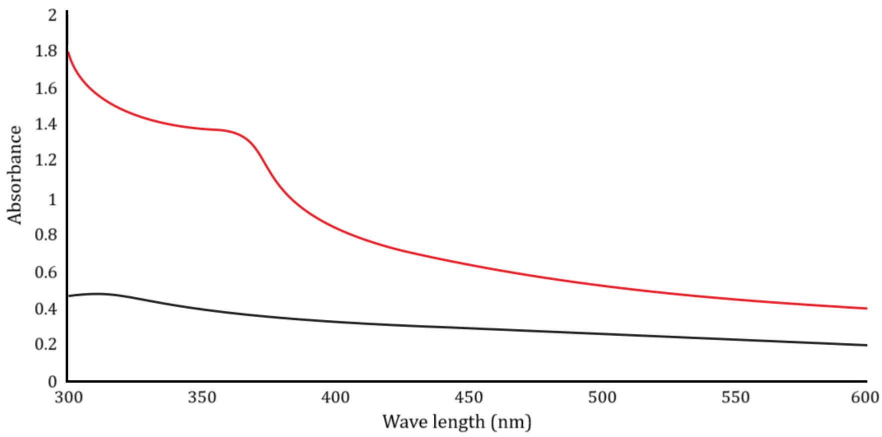


Figure 2. Spectrum of zinc nanoparticles and blank zinc (0.2 mm)

Similarly, Bhuyan et al (2015) observed a peak at 377 nm when zinc acetate dihydrate was biosynthesized using *Azadirachta indica*. Ghamsari et al (2017) reported that the formation of nano zinc nanoparticles has absorption peaks between wavelengths of 310 nm and 360 nm. The formation of zinc nanoparticles under neutral pH conditions is due to the ionization by electron transfer of zinc and secondary metabolites present in the aqueous solution. Absorption bands extending to higher wavelengths are caused by the movement of electron clouds throughout the zinc nanoparticles (Muhammad et al, 2019).

FTIR Spectroscopy Analysis

Phytochemicals analysis was performed to identify the functional groups present in Zinc nanoparticles, zinc nitrate hexahydrate and extract of neem leaf as shown in (Figure 3). The FTIR spectrum of zinc nanoparticles showed major absorption peaks at 3425, 2924, 2345, 1620, 1381 and 864 cm^{-1}, whereas the FTIR spectrum of zinc nitrate hexahydrate showed major absorption peaks at 3448, 2924, 2337, 1620, 1388 and 825 cm^{-1}, the FTIR spectrum of neem leaves showed major absorption peaks at 3425, 2924, 2376, 1604, 1442 and 1072 cm^{-1}.

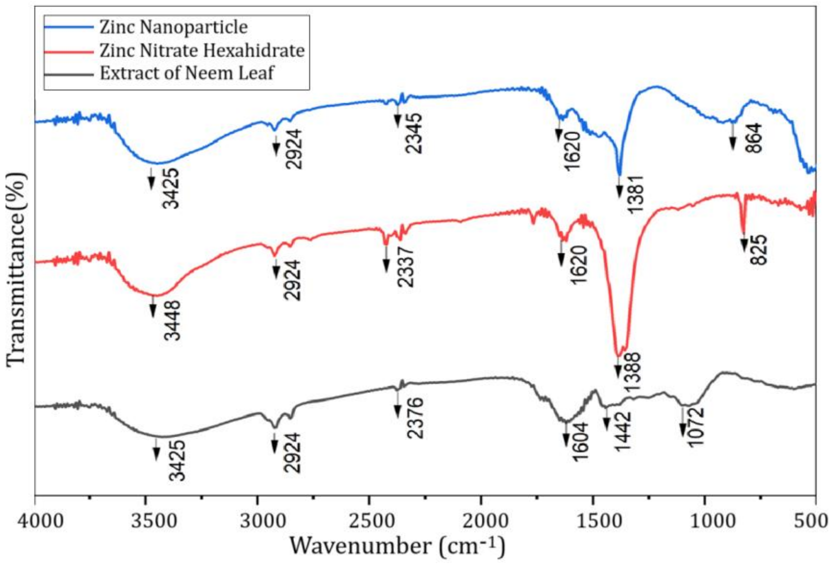


Figure 3. FTIR spectra of zinc nanoparticles, zinc nitrate hexahydrate and extract of neem leaf

Functional groups of the biosynthesized zinc using the extract of neem leaf (Skoog, 1998). The presence of O-H and CH- groups in zinc nanoparticles, zinc nitrate hexahydrate and neem leaf extract was not different compared to the results of Nurbayasari et al (2017), who obtained O-H groups (polyphenols) with a peak spectrum of 3420 cm^{-1} and CH- groups with a peak spectrum of 2922 cm^{-1}.

The results of the three samples showed differences in the C-N (amide) and CH- (alkene) groups, where the zinc nanoparticles and zinc nitrate hexahydrate did not contain amide compounds, whereas neem extract did not contain alkene compounds. The C-N band position of neem leaf extract was 1072 cm^{-1}. This finding is in accordance with the findings of Kathiraven et al (2014), who reported that the band position of C-N of marine algae *Caulerpa racemosa* are 1079 and 1027 cm^{-1}. The difference in the band shifts in the FTIR spectra of the samples indicates the involvement of secondary metabolites that contain functional groups such as alcohols, alkanes, alkenes, amines, and amides during the bioreduction process. Nurbayasari and Saridewi (2017) reported that the loss of absorption bands in the FTIR spectrum of the extract indicates a reaction that occurs during the bioreduction process whereas Al-Naamani et al (2016) noted that the shift of bands in the FTIR spectrum is due to the movement of the hydroxyl, amino, and amide groups towards the lower spectral range. The FTIR spectrum of the zinc nanoparticles showed an absorption band located at a lower wavenumber of 825 cm^{-1}. This result was in according to the results of Gilja et al (2018); Mutlaq et al (2021) who reported that FTIR spectrum of Zn-O-Zn and Zn-O were in the lower spectrum between 864-485 cm^{-1}.

Size Distribution Particles of Zinc Nanoparticles

The size distribution of the zinc nanoparticles was analyzed using the Zetasizer Nano ZS and the dynamic light scattering (DLS) technique. The average size of the nanoparticles was found to be 368.6 nm, with a polydispersity index (PDI) of 0.472 (Table I and Figure IVa). Particle size distribution analysis using a zinc precursor concentration of 0.2 mM at pH 7 resulted in an average nanoparticle size of 368.6 nm. Based on these results, the zinc nanoparticles were classified as colloidal nanoparticles. According to Vasquez et al (2016), particles with diameters less than 1000 nm are suitable as nano-sized carrier substances. The formation of these zinc nanoparticles is attributed to the stabilizing effects of secondary metabolites in the neem leaf extract, which prevent aggregation and minimize the formation of clusters, ensuring that the zinc oxide remains in the nanosized form. This stabilization occurs due to the repulsive forces between Zn^{2+} ions, which, upon reduction, form neutral zinc atoms that interact through inter-metal bonds, resulting in nanosized clusters.

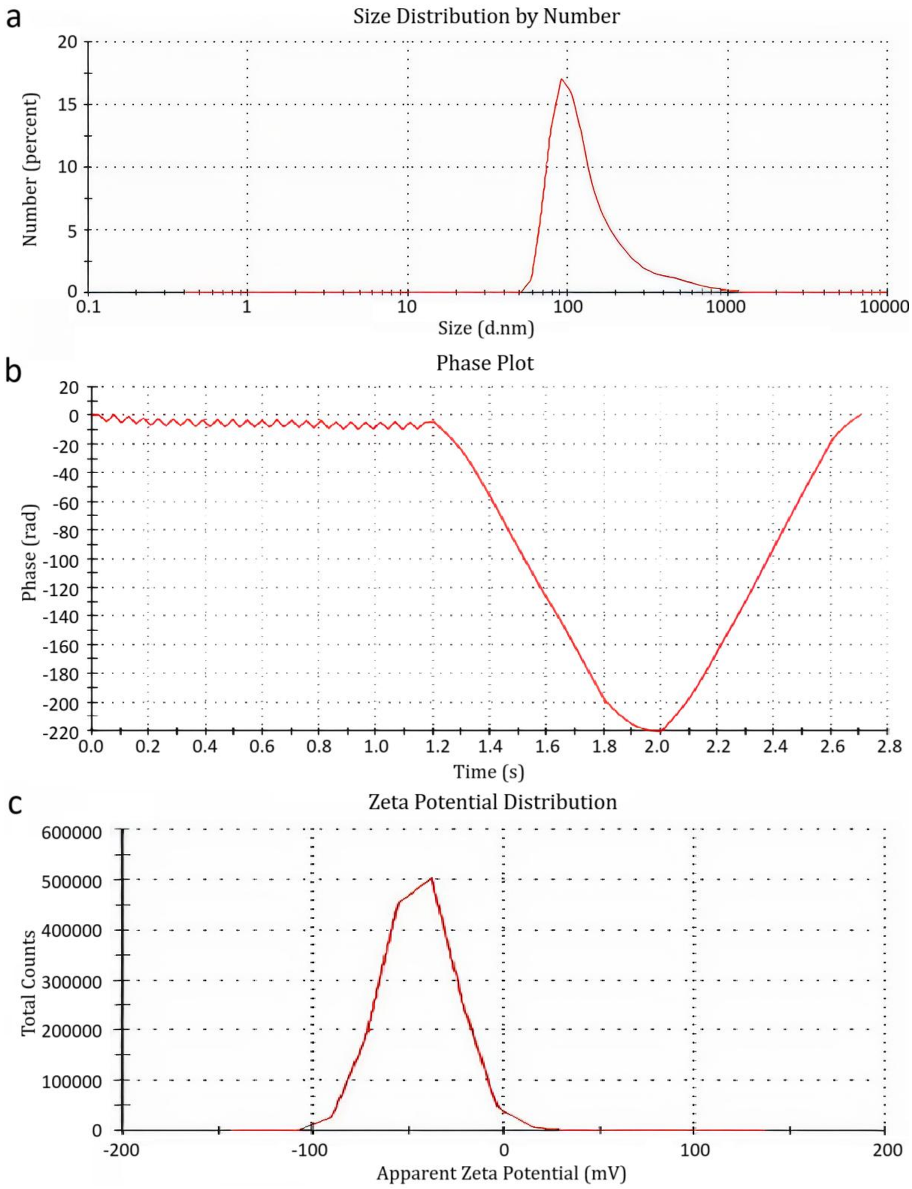


Figure 4. (a) Size and *polydispersity index* (PDI), (b) Quality of the phase plot. (c) Zeta potential

The result of this study is similar to the results of Nurbayasari and Saridewi (2017) who reported that biosynthesized of zinc oxide with *Caulerpa* sp has a particle size distribution of 370.72 nm. This finding is higher than the results reported by Yusof et al (2019) who reported a particle size of 327 nm with zinc oxide synthesis using chitosan bioreductor. Romadhan and Pujilestari (2019) synthesized zinc oxide nanoparticles with a calcination temperature of $100^{o}C$ to get a size of 452.7 nm. Based on this, the size of zinc nanoparticles meets the standard of nanoparticle size of 1-1000 nm. Tiyaboonchai and Technology (2003) stated that nanoparticles are solid colloidal particles with a diameter of 1-1000 nm. Abdassah (2017) noted that the particle diameter size of nanoparticles should be between 200 and 400 nm. In addition, the PDI value in this study was 0.472 which indicating a homogeneous colloidal particle size. According to Xu et al (2023), the PDI value is the value to measure the distribution of the particles formed. The PDI value indicates the level of confidence in the particle size of a substance dispersed in the colloidal nanoparticles. The PDI value is considered narrow if it is lower than 0.5, indicating a homogeneous and uniform particle size in the colloid; if the PDI value is higher than 0.5, it indicates a wide nanoparticle size distribution and tends to be heterogeneous or uneven.

Quality of the Phase Plot Zinc Nanoparticles

The results of the zinc nanoparticle phase plot analysis showed a difference in the phase between the measured frequency and time. A typical phase plot is of good quality, with no interference. The signal intensity is given by a stable count rate curve over time, which gives a photon count of 917.1 Kcps during the measurement (Table 1 and Figure 4b). The phase plot shows a well-defined and intermittent slope of the phase difference with time, which results from the fast-field reversal part of the measurement up to 1.2 seconds. The results of this study agree with those of Maiga et al (2020), who report similar firm and alternating graphs. However, if the quality of the zinc nanoparticles is poor, it is indicated by the absence of a well-defined alternating slope, and the fast-field measurement part will show noisy data or irregular waves (Varenne et al, 2015). The poor quality of zinc nanoparticles is caused by low or high sample concentration, high conductivity due to sample/electrode degradation, and manually set measurements.

Stability of Zinc Nanoparticles

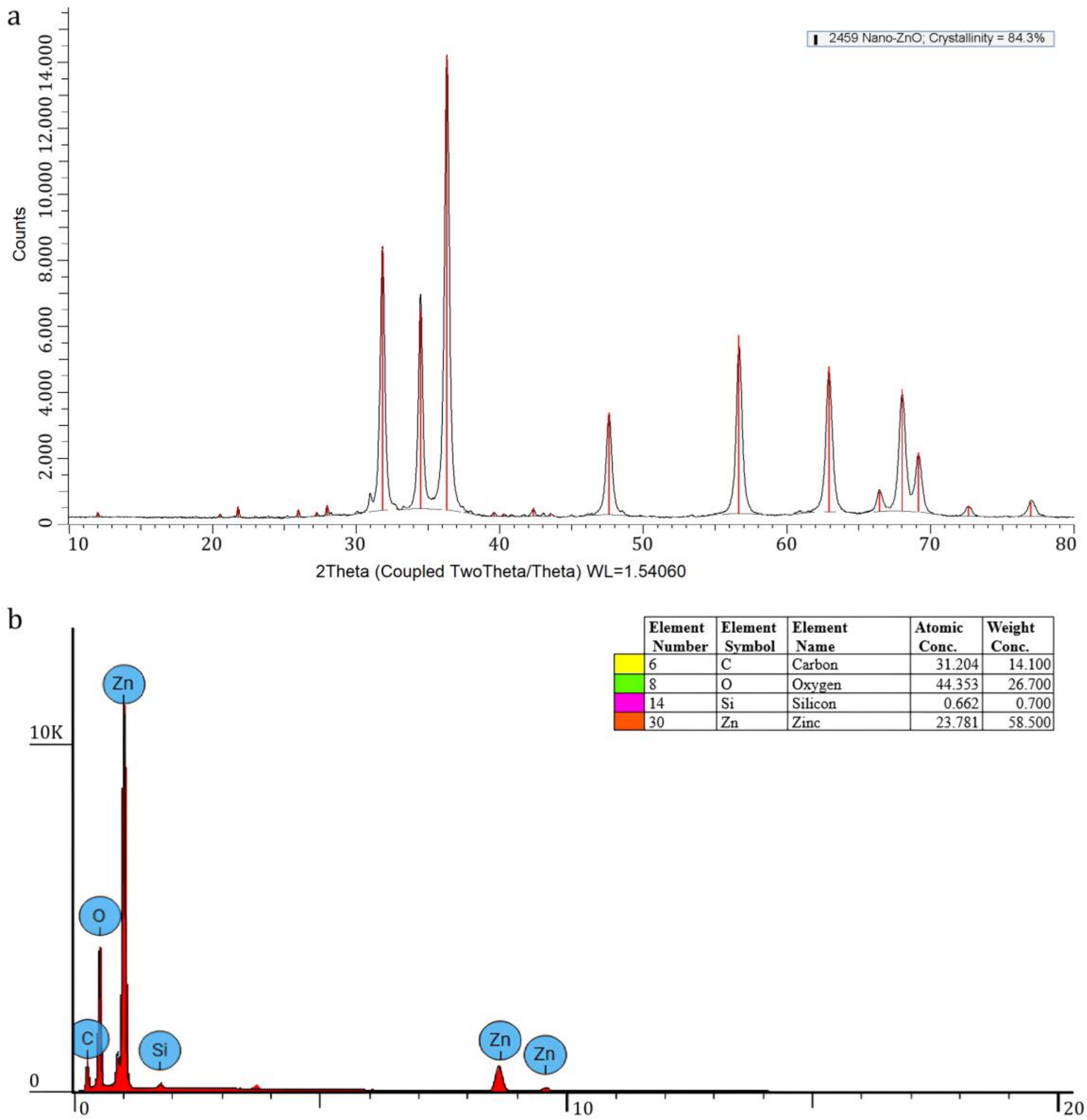
The stability of the zinc nanoparticles was determined using a zeta potential instrument. It is essential to know the parameters of stability of zinc nanoparticles, as the optimal range for high stability of zinc nanoparticles is greater than +30 mV and less than -30 mV (Melendrez et al, 2010). The negative zeta potential values of Zinc nanoparticles were -43,6 mV and the standard deviation was 18.8 mV, which categorized as good in suspension stability (Table I and Figure 4c). Yedurkar et al (2016) reported that the zeta potential of biosynthesized of zinc oxide using Ixora coccinea leaf extract a green approach was found to be −49.19 mV. Zeta min (ζ −) verified the dispersion capacity of the synthesized zinc nanoparticles. This indicates that the surface of the biosynthesized zinc nanoparticles is coated by molecules from negatively charged groups responsible for nanostability. The negative charge of the surfactant is due to the binding of secondary metabolites from neem leaf extract with zinc, thus providing stability to zinc nanoparticles. The results of this study are in accordance with the standard reported by Maiga et al (2020); Varenne et al (2015) stability of zeta min charged nano colloids.

X-ray Diffraction Analysis of Zinc Nanoparticles

The X-ray diffraction pattern of zinc nanoparticles shows definite line of the X-ray diffraction peaks, indicating that the prepared particles were in the nanoscale range, as show in (Figure 5a). The diffraction pattern shows 2θ values at 31.84° (100), 34.49° (002), 36.32° (101), 47.62° (102), 56.66° (110), 62.93° (103), 66.45° (200), 68.03° (112), 69.16° (201), 72.64° (004), 76.99° (202), which were indexed as the spherical to the hexagonal phase of zinc nanoparticles with high crystallinity. This finding corresponds to the JPCDS card number 36-1451. The indexation corfirms the standar hexagonal wurtzite structure of JCPDF no 00-036-1451. The results of this research are in accordance with those of Yedurkar et al (2016) who obtained the crystal structure of hexagonal wurtzite with biosynthesized zinc oxide nanoparticles using *Ixora coccinea* leaf extract as a green approach.

Energy Dispersive X-ray Diffractive (EDX) of Zinc Nanoparticles

EDX analysis confirmed the presence of zinc and oxygen signals in the zinc nanoparticles, as shown in (Figure 5b). EDX analysis was carried out for the green synthesis of zinc nanoparticles produced using zinc nitrate hexahydrate and the extract of neem leaves to determine the elemental composition. The elemental analysis of the zinc nanoparticles revealed that the atomic concentrations of carbon, oxygen, silicon, and zinc were 31.204%, 44.353%, 0.662%, and 23.781%, respectively, while the weight concentrations of carbon, oxygen, silica, and zinc were 14.100%, 26.700%, 0.662%, and 58.500%, respectively. This proves that the nanoparticles were in the highest-purified form. The presence of silica and carbon elements is due to the carbon bands (organic molecules) of the neem leaf extract, which are involved in the stabilization of zinc nanoparticles. Zhu et al (2021) reported that the presence of carbon element in zinc nanoparticles is due to the stabilization process during the heating process caused by organic molecules from *C. camphora* leaf extract. In conclusion, the neem extract has a significant capacity to produce zinc nanoparticles. The shape of zinc nanoparticles is especially important for their antibacterial effectiveness.



Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	31.204	14.100
8	O	Oxygen	44.353	26.700
14	Si	Silicon	0.662	0.700
30	Zn	Zinc	23.781	58.500

Figure 5. (a) Diffraction crystal phase*, (b)* Component of *zinc nanoparticles*

Since they are spherical in shape, zinc nanoparticles tend to be quite strong in anti-bacterial performance and can easily penetrate the pathogen cell wall. Pasquet et al (2021) reported that zinc nanoparticles with a spherical shape can be antibacterial with a mechanism of action that damages the bacterial cell wall, inhibits cell synthesis, and disrupts cell metabolism.

Morphology of Zinc Nanoparticles

A morphological study of the green synthesis of zinc nanoparticles using an extract of neem leaf was performed by TEM (Figure 6). The morphology of the zinc nanoparticles indicates that the particles exist in a homogeneous form, with spherical to hexagonal shapes and relatively small sizes. Previous studies have reported that the morphology of zinc nanoparticles determined by TEM analysis is spherical (Khoshhesab et al, 2011; Wahab et al, 2014; Ghoshal et al, 2009). The round shape of nanoparticles significantly influences the activity of pathogenic bacteria. Zinc nanoparticles with spherical morphology showed bacterial activity that was significant for *Escherichia coli* and *Staphylococcus aureus* (Ramadanti & Maharani, 2022).

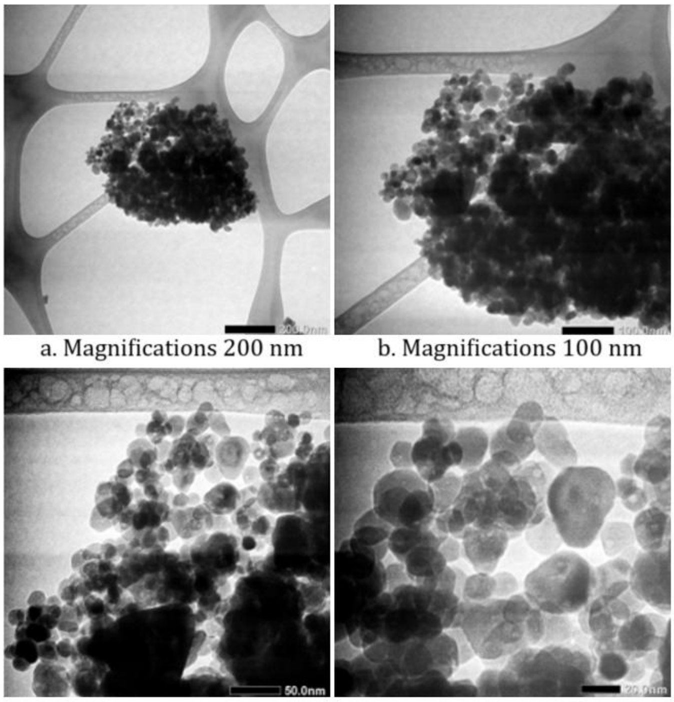


Figure 6. Morphology of zinc nanoparticles

The prohibition of the use of antibiotics in poultry in Indonesia is stated in Law No.41/2014, Article 22, paragraph 4c. This ban is due to the use of antibiotic growth promoters that can increase residues in the meat and excreta of broiler chickens; therefore, an alternative to antibiotic growth promoters is needed. This research has excellent prospects in the field of poultry farming, because zinc nanoparticles can be a substitute for antibiotic growth promoters.

CONCLUSION

The results showed that synthesis of 30 mL zinc nitrate hexahydrate with 5 mL neem leaf extract got a wavelength of 365 nm, Based on FTIR analysis, the spectrum, showed the presence of functional groups in high intencity. The size of Zinc nanoparticles is 368.8 nm with polydispersity index of 0.472. A typical phase plot was good in quality with no interference. The zeta potential value of zinc nanoparticles is -43.6 mV and the standard deviation is 18.8 mV. According to the morphology analysis, the zinc nanoparticles were indexed as the spherical to hexagonal phase with high crystallinity, which was confirmed by TEM images.

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CONFLICT OF INTEREST

"The authors declare no conflict of interest".

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