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Original Research

Green synthesis of silver nanoparticles from papaya seed extracts with alkaloid content for antibacterial application

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Abstract: This research aims to develop a silver nanoparticle (AgNPs) using several plant extracts for antibacterial application. The papaya seed extract has been fractioned by n-hexane, ethyl acetate, water, and ethanol. The n-hexane fraction was the only fraction that succeeded in synthesizing AgNPs. The characterization methods showed AgNPs marked at 430 nm with UV-Vis and 1640 cm^{-1} with FTIR. SEM observed the aggregation of spherical AgNPs at the 200 nm scale. The particle size of 91.3 nm was measured with PSA that confirmed the nanoscale of the synthesized material. All fractions contained alkaloid compound, and ethyl acetate fraction showed a group of indole with specific wavenumber at 2623 cm^{-1}, 1737 cm^{-1}, and 1237 cm^{-1} representing N-H, C=O, C-N, respectively. All fractions at every concentration (25%, 50%, 75%, 100%) have been tested and showed the medium effect on bacterial growth inhibition. Among all fractions, the AgNPs n-hexane fraction has the highest bacterial effect, which was indicated by mean values of inhibition zone 7.2 mm against *S.aureus*, as well as 6.6 mm against *E.coli*. ANOVA analysis showed that AgNPs n-hexane fraction has a significant inhibition zone compared to other fractions against *S.aureus* ($p=0.002$), but not significant to *E.coli* ($p=0.128$). The insignificant results on *E.coli* because of gram-negative bacteria's biophysical characteristics, such as membrane cell wall and flagellin. This research emphasized that AgNPs could be synthesized via a green process of nucleation by using plant extract that effectively inhibits the growth of *S.aureus* and *E.coli*. Further studies on the mechanism of the antibacterial effect at the molecular level might be investigated soon.

Keywords: Silver Nanoparticle, Papaya seed, n-Hexane, Alkaloid, S.aureus.

INTRODUCTION

The decrease of the potential of the antibiotic against bacteria, especially Staphylococcus Aureus and Escherichia Coli, has triggered the development of new antibiotic agents[1]. The research on herbal extracts has been extensively reported for decades. However, the current progress on using organic compounds for the green synthesis of nanoparticles is still highlighted worldwide.

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One of the most widely investigated nanoparticles in health and medical applications is silver nanoparticles (AgNPs). Moreover, AgNPs have been reported to cause acute toxicity and damage the structure of bacterial cells, so that silver nanoparticles were proposed as a potential antimicrobial agent[2].

A natural product that can be antibiotic is the papaya plant[3] because of bioactive compounds such as alkaloids that play the role of antibacterial effect. Today, alkaloid compounds draw the attention of researchers on antibacterial activity. Alkaloids have different levels of antibacterial effect, depending on the place and origin of the alkaloids obtained[4]. There are differences in antibacterial activity in gram-positive and gram-negative bacteria, whereas the efficacy depends on the extraction solvent used in the extraction and isolation[5]. Research by Juliantina et al., 2009[6] reported the mechanism of alkaloids as an antibacterial by interfering with the peptidoglycan components in bacterial cells. The bacterial cell wall defects and causes cell death[7].

This research reports the synthesis of silver nanoparticles (AgNPs) using plant extracts followed by antibacterial tests. Before synthesizing nanoparticles, papaya seeds were extracted by graded fractions of water, ethanol, ethyl acetate, and n-hexane. Then the silver nanoparticles were synthesized by a nucleation process supported by plant extract. The characteristics of the nanoparticles were analyzed using UV-Vis, FTIR, SEM, and PSA. All fractions and AgNPs were tested for antibacterial activity using disc diffusion by calculating the inhibition zone diameter. Data obtained were evaluated by ANOVA Analysis.

MATERIAL AND METHOD

The papaya seeds (Carica papaya)[8] were collected from the traditional market, washed and dried, then identified in the herbarium of the University of North Sumatra. Solvents used are aqua dest, ethanol 96%, ethyl acetate, and n-hexane. Escherichia Coli, Staphylococcus Aureus, Nutrient Broth (NB), Mayer reagent, Dragendorff, DMSO 10%, Tween 80, and Liberman Burchad reagent have been used for antibacterial tests.

The instruments used for AgNPs synthesis are heating mantle (electrothermal), soxhlet apparatus, oven (Memmert), microwave (LG), analytical balance (Denver Instruments), 50 mesh sieve, magnetic stirrer (Daihan LabTech), UV-Vis Spectrophotometer (Shimadzu 1601), Particle Size Analysis (PSA) (Malvern 1.20), Scanning Electron Microscope (SEM) and FTIR Spectrophotometer.

About 1.7kg of dried and roughly crushed papaya seeds were macerated by ethanol with a ratio of 1:6. Samples were macerated for three days with stirring one time per day. The crude extract yield was about 48.1924 g (2.83%). About 5 g crude extract dissolved in 80 ml of aquadest at 70oC then moved into a separating funnel. Repeatedly, the graded fractionation was conducted three times by 80 ml of each n-hexane, ethyl acetate, and ethanol.

The synthesis of AgNPs was carried out by mixing 10 ml of AgNO3 solution (1 mMol) and 200uL of n-hexane fraction. The mixture was stirred for about 30 min until homogeneous. The formation of AgNPs was evaluated by a UV-Vis spectrophotometer (Bakir, 2011). The characterization of AgNPs was analyzed by FTIR (Nessa, 2010), SEM [9], and PSA (Manual Book LB-550).

The Muller Hinton Agar (MHA) media was poured aseptically and solidified. A bacterial suspension was taken by cotton swab and then scratched on the surface of the MHA media. Divided into four quadrants and labeled outside the petri dish. A paper disc with AgNPs was placed on the marked part then Incubated for 24. The clear zone was measured around the paper disc.

Statistical analysis has been employed to determine the normality of data obtained using Kolmogorov Smirnov and the homogeneity by Levene methods. Then one-way ANOVA was applied to evaluate the significances of each sample

RESULTS AND DISCUSSION

3.1 Synthesis of Silver Nanoparticles (AgNPs)

The n-hexane fraction was the only fraction that succeeded in synthesizing AgNPs. We could not get any AgNPs formed in water, ethanol, and ethyl acetate fractions. The content of flavonoid compounds in the n-hexane fraction might affect the nucleation process of the nanoparticles since the flavonoid compound has been known as natural reducing agents and role in the nucleation process. As a bio-reducing agent, flavonoid converted Ag^+ to Ag^0. As a capping agent, flavonoid stabilized the nano size of the synthesized nanoparticles[10]. Several studies have reported a significant effect in the synthesis of nanoparticles on changes in shape, size, and morphology depending on pH, temperature, extract concentration, metal salt concentration, and reaction time [11], [12], [13].

3.2 UV-vis spectra analysis

All fractions were measured by UV-vis spectra, as shown in Figure 1. The literature [14] described that the wavelength of alkaloids ranged from 203 nm to 285 nm. Figure 1(b) showed absorbance at 240 nm, and 250 nm indicated a group of alkaloids named indole [15]. Figure 1(e) showed absorbance at 280 nm of AgNPs synthesized with n-hexane fraction, this indicated carbonyl which usually ranged from 270 nm to 300 nm[15] . Our results showed that AgNPs in a colloidal system have contributed to a strong absorbance ranging from 400 nm to 500 nm, which is a typical absorbance of AgNPs[16] .

3.3 FTIR spectra analysis

In general, strong absorbances at 3745 cm^{-1} and 3318 cm^{-1} indicated a stretching vibration of hydroxyl groups. The peak at 1640 cm^{-1} of Figure 2(d) represented stretching vibration of C-N amines or amines aliphatics, whereas 1406 cm^{-1} indicated amides[17] . Alkaloid compound was indicated at 2623 cm^{-1}, 1737 cm^{-1}, and 1237 cm^{-1} of N-H, C=O, and C-N, respectively[18]. Another study on the characterization of AgNPs and papaya extract by (Jain 2011)[19] showed absorbances at 1697 cm^{-1}, 1618 cm^{-1}, 1514 cm^{-1}, 1332 cm^{-1}, and 1226 cm^{-1} that indicated vibrations of -C-C, C-O, -C-C- aromatics, C-O ether, C-O polyols such as hydroxy flavone and catechin. In our study, especially in ethanol and ethyl acetate fractions, we found specific absorbance at 1244 cm^{-1} and 1237 cm^{-1} that represented polyol groups that role the bioreduction of Ag^+ to Ag^0. In contrast, the polyol was oxidized to an unsaturated carbonyl that caused vibration at 1640 cm^{-1}.

3.4 SEM Images analysis

SEM images showed a spherical morphology with a scale of 200 nm, which indicates a high density of AgNPs in Figure 3(a). At the same time, Figure 3(b,c,d) showed that AgNPs were evenly distributed and aggregated at different magnifications. A study by (Balavijayalakshmi and Ramalakshmi 2017)[8] explained that aggregation was made of a high concentration of plant extracts, and the agglomeration may cause destabilization of AgNPs. UV-Vis data also supported these phenomena showing that absorbance at 400 nm indicated spherical nanoparticles [9].

3.5 Particle Size Analysis

The Polydispersity Index (PI) in figure 4 showed a broad distribution range of the sample, more than one peak, and the particle size varies (heterogeneous). The PSA data showed that the particle size of AgNPs is 91.3nm; this result has strongly confirmed the definitive nanoscale of the synthesized materials that should be ranged from 1 nm to 100 nm [20].

3.6 Antibacterial Analysis

The antibacterial effect of each fraction and AgNPs have been tested against *S. aureus* and *E.Coli* with the variation of concentration 25%, 50%, 75%, and 100%. This measurement parameter is the mean diameter of the inhibition zone.

Table 1. Antibacterial effects on *S. aureus.*

Bacteria	Sample (Fraction)	Concentration (%)	Inhibition Zone (mm)			Mean	Mean of each fraction	SD	*p*-value
			Test-1	Test-2	Test-3				
Staphylococcus aureus	Water	25	6.9	6.2	6.7	6.6	6.620	0.236	p= 0.002 < 0.05
		50	7.6	6.4	6.9	6.9			
		75	6.4	6.3	6.7	6.4			
		100	6.5	6.2	6.7	6.4			
	Ethanol	25	6.4	6.1	6.5	6.3	6.715	0.315	
		50	7.0	6.2	6.9	6.7			
		75	6.9	6.2	7.1	6.7			
		100	7.9	6.2	7.2	7.1			
	Ethyl Acetate	25	6.1	6.2	6.2	6.1	6.370	0.143	
		50	6.4	6.2	6.6	6.4			
		75	6.3	6.5	6.6	6.4			
		100	6.3	6.9	6.2	6.4			
	AgNPs	25	6.9	6.9	7.0	6.9	7.205	0.219	
		50	7.0	7.3	7.1	7.1			
		75	7.2	7.4	7.5	7.3			
		100	7.6	7.0	7.6	7.4			

In the water fraction of papaya seed extract, antibacterial effects against *S. aureus* were found at all concentrations, namely 25%, 50%, 70%, and 100%. The highest effect was at a concentration of 50%, where the inhibition zone was 6.96 mm. The result of this study was in line with (Kusumawati's 2020)[21] reported that the water fraction of papaya seeds has antibacterial effects against *S. aureus* with an inhibition zone of 8.43 mm to 12.98 mm. This effect is due to metabolite compounds, namely flavonoids, alkaloids, carbonyls, and terpenoids.

The ethanol fraction of papaya seed extract has antibacterial effects at all concentrations. The highest effect was at a concentration of 75%, and the inhibition zone was 6.73 mm. Research by (Roni, Maesaroh, and Marliani, 2019) [22] reported that ethanol fraction of papaya seed has antibacterial effects against *S. aureus* with an inhibition zone diameter of 12.2 mm. (Kusumawati's 2020) [21] also found that the ethanol fraction of papaya seeds has an antibacterial effect against *S. aureus* with inhibition zone diameter of 5 to 9 mm. This is presumably due to the presence of alkaloids and flavonoid compounds that can damage the bacterial membrane structure, resulting in the growth inhibition of bacteria [4].

The ethyl acetate fraction of papaya seed extract was effective against *S. aureus* at all concentrations. A significant effect was found at a 75% and 100% concentration, with an inhibition zone of 6.46 mm. The research results by (Roni, Maesaroh, and Marliani, 2019) [22] found that the ethyl acetate fraction of papaya seed extract could act as an antibacterial for *S. aureus* with an inhibition zone of 11.6 mm. However, this study did not clearly explain the mechanism of bacterial inhibition.

The synthesized AgNPs were found to act as an antibacterial against *S. aureus* at all concentrations, and the best seen at 100% concentration, with an average inhibition zone value of 7.4 mm. The obtained AgNPs have the best antibacterial effect among all treatments, with an average inhibition zone of 7.205 mm. The effect of AgNPs on the leakage of membrane proteins and reducing agents of the bacteria by increasing the permeability of the cell membrane of *S. aureus*. The presence of high plasma membrane leakage causes damage to the bacteria, which makes the bacterial structure decompose and causes death [23]. The n-hexane fraction of papaya seed extract has been reported to contain alkaloids, flavonoids, terpenoids, and saponins [24]. Alkaloids can disrupt peptidoglycan and inhibit bacterial topoisomerase enzymes[25]. Flavonoids could inhibit bacterial growth by reducing cell membrane permeability due to protein and membrane complex binding [26]. Terpenoids, the inhibitory ability, involves a reaction that forms a robust polymeric bond with the outer membrane of the bacterial cell wall, thereby causing porin infection[27] .Saponins are thought to cause leakage of proteins and enzymes in [28].

Based on Table 1, that all fractions have antibacterial activity against *S. aureus* at all concentrations with an average inhibition zone of 6.2-7.2 mm. This activity belongs to a medium-strength antibacterial[1]. The AgNPs showed a significant antibacterial effect compared to other fractions with the *p-value* < 0.05.

Table 2. Antibacterial effects on *E.coli.*

Bacteria	Sample (Fraction)	Concentration (%)	Inhibition Zone (mm)			Mean	Mean of each fraction	SD	*p*-value
			Test-1	Test-2	Test-3				
		25	6.1	6.2	6.3	6.2			
		50	6.1	6.2	6.3	6.2			
	Water	75	6.1	6.4	6.3	6.2	6.246	0.062	
		100	6.2	6.3	6.5	6.3			
		25	6.2	6.4	6.3	6.3			
		50	6.6	6.8	6.7	6.7			
	Ethanol	75	6.9	6.6	6.8	6.7	6.590	0.204	
		100	6.8	6.8	6.2	6.6			
Escherichia coli		25	6.1	6.1	6.2	6.1			p= 0.128 > 0.05
		50	6.4	7.0	6.5	6.6			
	Ethyl Acetate	75	6.3	6.1	6.2	6.2	6.348	0.228	
		100	6.3	6.6	6.4	6.4			
		25	6.1	6.1	6.3	6.1			
		50	6.2	6.2	6.6	6.3			
	AgNPs	75	6.5	6.2	7.1	6.6	6.623	0.218	
		100	6.6	6.3	6.9	6.6			

In the water fraction of papaya extract, the highest effect against *E.coli* was found at a concentration of 100%, where the inhibition zone was 6.33 mm. (Jenab's, 2017)[29] found that the water fraction of papaya seed extract had an antibacterial activity with an inhibition zone of 7.13-12.20 mm. (Hidayati, 2019) [24] also found that highest effect at 100% with an inhibition zone of 6.50 mm. This effect might be due to flavonoids and saponins in the fraction of water.

The ethanol fraction of papaya seed extract showed that all concentrations have antibacterial against *E.coli*. The best seen was at a concentration of 75% with inhibition zone of 6.76 mm. In addition, the ethanol fraction was obtained as the highest antibacterial among all treatments, with inhibition zone of 6.590 mm. These results were supported by (Taufiq 2015) [30] reported that ethanolic papaya extract can inhibit the growth of E. coli which is related to the active compound in each fraction.

The ethyl acetate fraction of papaya seed extract has an antibacterial effect against *E.coli* at all concentrations. The highest was found at a concentration of 50%, with inhibition zone of 6.63 mm. This result was in line with (Roni, Maesaroh, and Marliani, 2019)[22] that the presence of antibacterial activity with inhibition zone was 11.96 mm. They suggested that a terpenoid class can inhibit the growth of *E.coli* bacteria. Similar results showed that the ethyl acetate fraction of papaya seed extract was antibacterial against *E.coli* with an inhibition zone of 11.13-18.13 mm[29]. Moreover, (Hidayati 2019)[24] also mentioned antibacterial activity in the ethyl acetate fraction of papaya seeds with an inhibition zone of 13.83 mm, which was related to alkaloids.

The AgNPs extract has antibacterial against *E.coli* at all concentrations, and the best was found at concentrations of 75% and 100%, with inhibition zone of 6.6 mm. The study by conducting an antibacterial test of AgNPs using dried and mashed papaya seeds without extraction found that the synthesized AgNPs could inhibit the growth of *E. Coli* with an average inhibition zone of 9.1 mm. Several studies reported that various pathogenic organisms were inhibited by effectively biosynthesized AgNPs [31,32,33,34,35].

Based on Table 2 that all fractions have antibacterial activity against *E. coli* at all concentrations with an average inhibition zone of 6.3-7.2 mm, which belong to a medium-strength antibacterial. Unfortunately, the AgNPs did not show a significant antibacterial effect compared to other fractions with the *p-value* > 0.05. Several previous studies also reported insignificant results, which may be related to a similar resistance to AgNPs. It was explained that the large numbers of flagellin in bacteria could repel the AgNPs, which could reduce the antibacterial effectiveness, and test results will be varied [35].

3.7 Mechanism of Antibacterial Effect

Limited studies regarding the AgNPs synthesized by n-hexane fraction for the antibacterial application. However, the inhibition zone in the antibacterial test was known reported that the shape of the nanoparticles influenced it. In the previous discussion, the SEM results showed that AgNPs formed tended to be round or spherical. An explanation by (Kim, 2011)[36] stated that the shape of AgNPs affects its ability as an antibacterial. Triangular AgNPs with lattices on the base plane showed the most potent biocidal properties against bacteria than spherical and rod-shaped nanoparticles. This is because the triangular AgNPs can inhibit the growth of bacteria at a total silver of 1µg. The silver ions will cause the loss of K^+ ions from the bacteria, which will cause a potassium deficit, so that cell membrane leakage occurs, and this does not occur in spherical or rod-shaped nanoparticles.

Nevertheless, the target of silver ions in the plasma or cytoplasmic membrane of bacteria is associated with several enzymes and DNA. When bacterial growth is inhibited, silver ions will be deposited into vacuoles and cell walls such as granules. Silver ions inhibit cell division, damage cell membranes and bacterial cell organelles. As an additional explanation, silver ions can associate with nucleic acids, preventing association with DNA bases rather than with phosphate groups[36].

CONCLUSION

The AgNPs have been successfully synthesized using the n-hexane fraction of papaya seed extract. The colloidal system of AgNPs formed in this study was spherical and tended to be aggregated observed by SEM. UV-vis spectra have indicated the presence of AgNPs at 280 nm and 430 nm. FTIR Spectra also showed a typical absorbance of AgNPs at 1640 cm^{-1}. PSA has detected broad distribution and heterogeneous particle size. All of the papaya seed extract fractions showed the presence of alkaloid metabolites, especially indole alkaloids found in the ethyl acetate fraction. All fractions and AgNPs colloidal system have a medium antibacterial effect against *S. aureus* and *E.Coli* ranging from 6 mm to 7.2 mm. However, AgNPs were found to be more significant against *S. aureus* instead of *E.Coli* that could be explained by the biophysical characteristic of bacteria and the agglomeration of the AgNPs.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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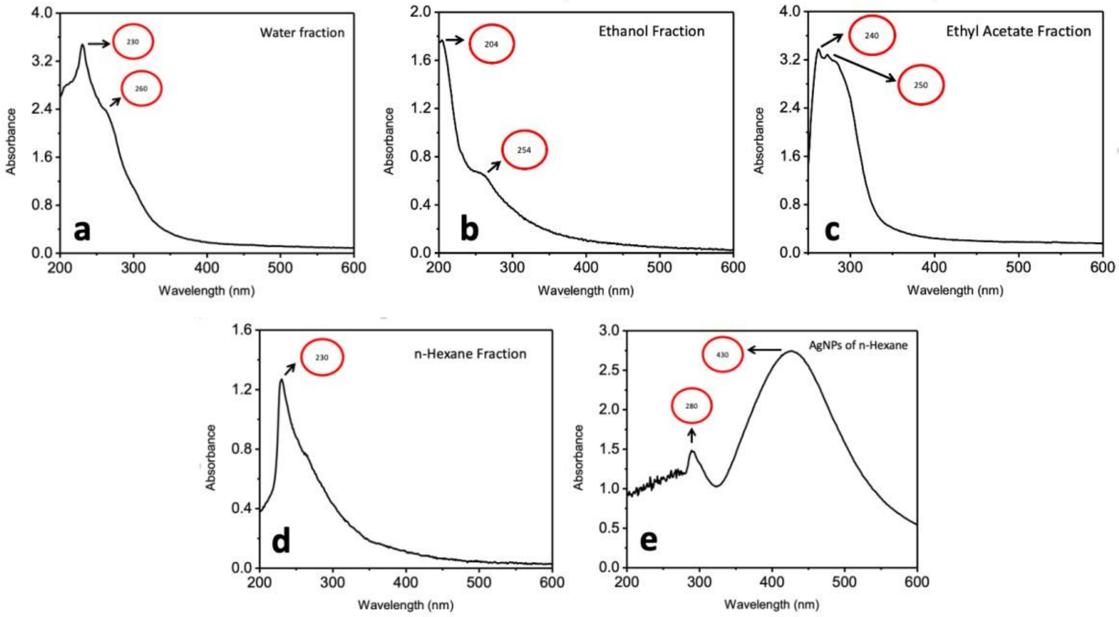

Fig.1 UV-vis data of each fractions; (a) water, (b) ethanol, (c) ethyl acetate, (d) n-hexane fractions, and (e) AgNPs

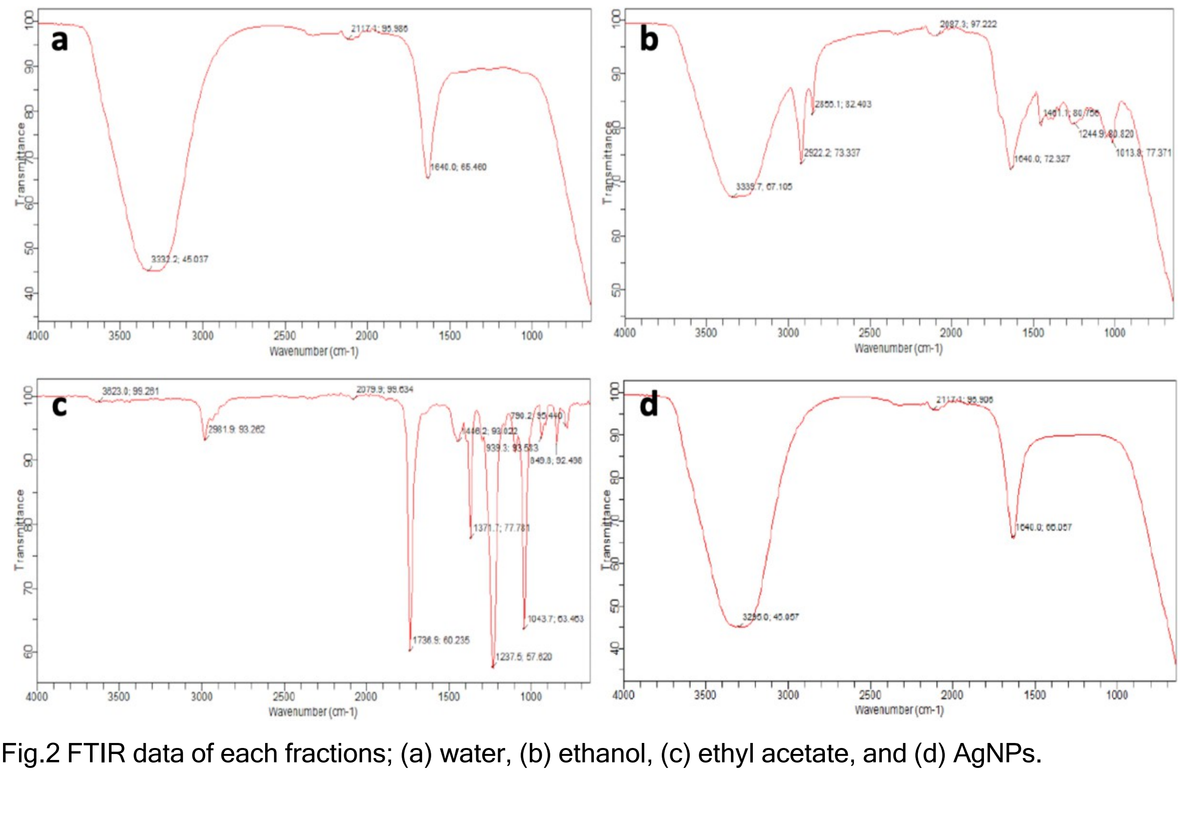

Fig.2 FTIR data of each fractions; (a) water, (b) ethanol, (c) ethyl acetate, and (d) AgNPs.

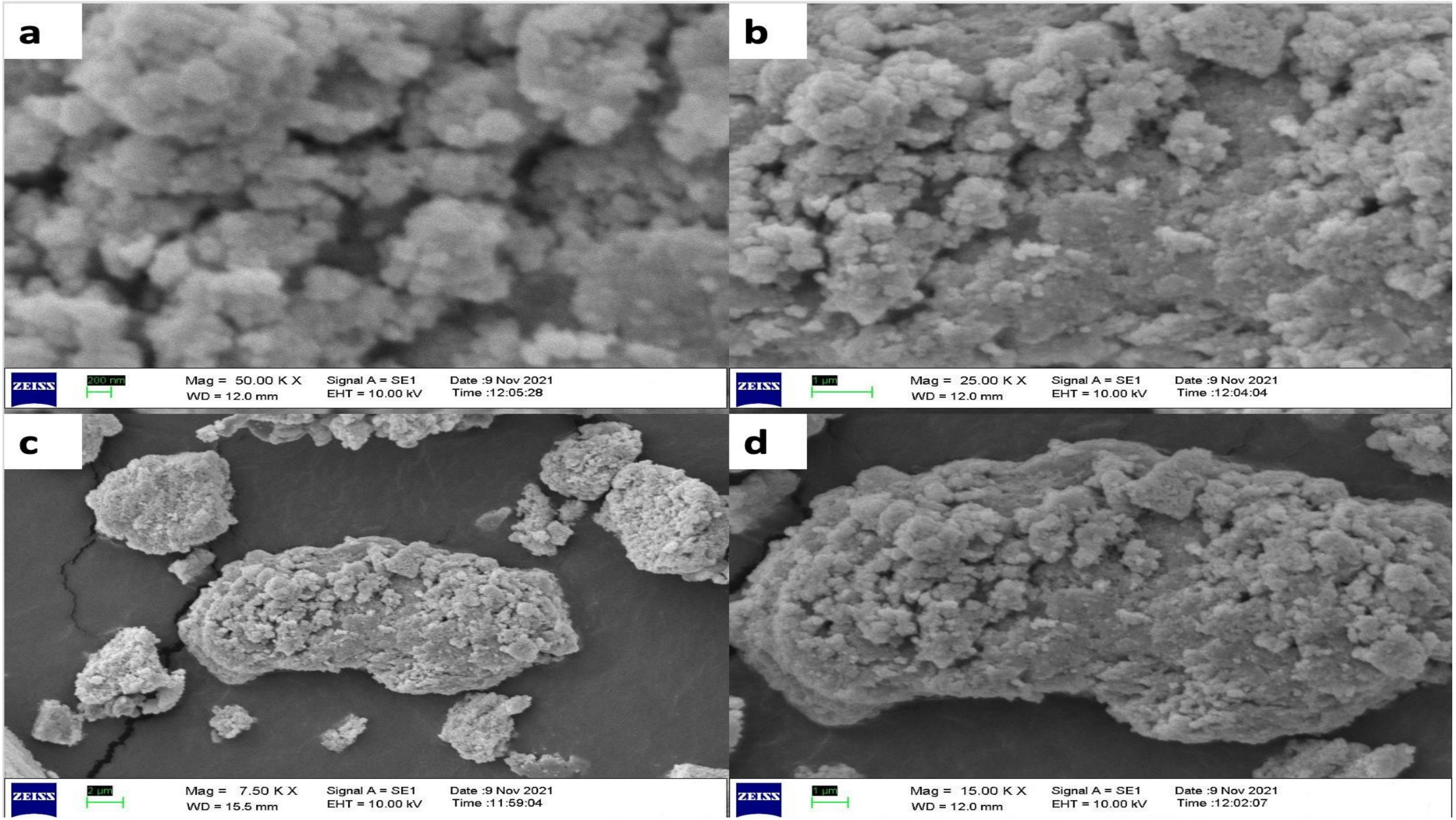

Fig.3 SEM images showed agglomerated and spherical AgN

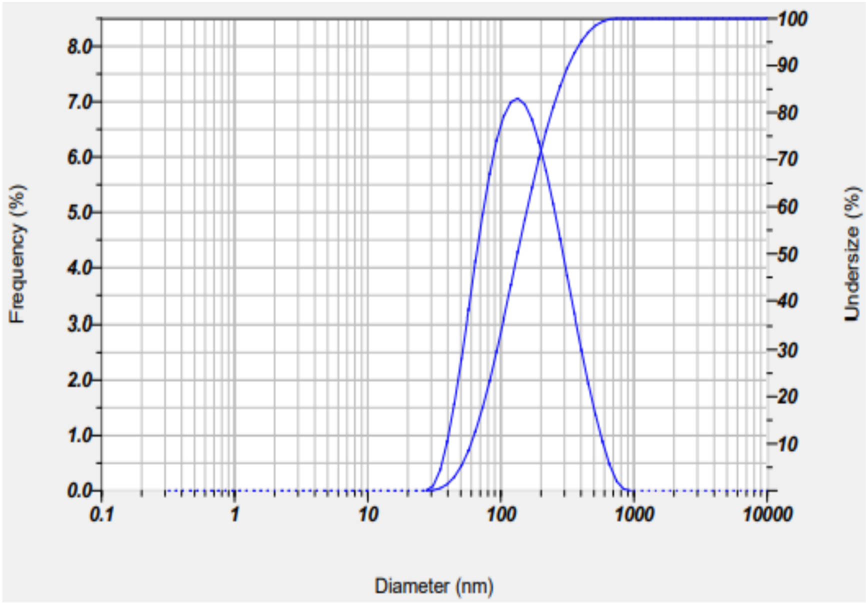

Fig.4 Particle Size Distribution of AgNPs.