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Effect of Silver Nitrate Concentration on Production of Silver Nanoparticles Using *Polygonum Minus* Extract and Analysis of Their Antimicrobial Property

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Abstract Green synthesis is the ability of organisms and organic compounds to reduce metal ions and stabilise them into nanoparticles (NPs). Among all metal nanoparticles, silver nanoparticles (AgNPs) have much attention due to the surface plasmon resonance (SPR), which can be easily observed by UV–visible spectrophotometer. In the present study, AgNPs were synthesized using *Polygonum minus* extract as a reducing agent and aqueous silver nitrate as a precursor. This study aims to investigate effect of silver nitrate ($AgNO_3$) concentrations (0.001 M, 0.01 M and 0.1 M) on the production of AgNPs as well as an antimicrobial activity of silver nanoparticles (AgNPs). Based on the observation, the colorless reaction mixture slowly changed from yellowish green to reddish brown and further confirmed by surface plasmonic resonance (SPR) band at 420 - 440 nm using UV–visible spectroscopy indicating of reduction of silver ion after several minutes of reaction. It was found that increased concentration of $AgNO_3$ resulted in increasing reaction time, production and decrease size of AgNPs. The morphological changes of bacterial cells treated with AgNPs were observed by FE-SEM and showed that the AgNPs has good antimicrobial properties against microorganisms. Thus, the ability of AgNPs to release Ag ions is a key to their antimicrobial.

Keywords Silver Nanoparticles; *Polygonum minus*; green synthesis, antimicrobial

1. Introduction

Nanotechnology, which deals with biology, chemistry, physics and engineering, is a versatile subject. Being very small in size, nanoparticles have a high surface area to volume ratio, which means that nanoparticles exhibit very different properties than their bulk content, such as electrical , magnetic and optical properties (Lakkappa *et al.*, 2017). Noble metal nanoparticles (NPs) have been studied extensively for applications such as optoelectronics, catalysis, sensing, medicine, etc. (Htwe *et al.*, 2019). Silver nanoparticles (AgNPs) have been widely investigated among various metal nanoparticles. Silver nanoparticles are reported to possess anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis and antiplatelet activity (Benakashani *et al.*, 2016). Owing to their high antibacterial properties, silver nanoparticles is the most commonly used nanoparticles in antimicrobial studies. Metallic nanoparticles provide an attractive alternative to antibiotics in the pharmaceutical field by developing novel applications and the synthesis of nanomaterials of specific composition and size is a burgeoning area of materials science research.

AgNPs can be produced using conventional or unconventional methods, by two different approaches: “top-down” and “bottom-up”. The top-down approach is a process that breaks up bulk materials using milling, nanolithography, or precision engineering to generate nano-level structures, while the bottom-up approach is a process in which nanoparticles are built from individual atoms or molecules that are capable of self-assembly (Figure 1).

Physically and chemically, nanoparticles have been formed for a long time, but recent findings show that microorganisms and biological systems play a major role in the production of metal nanoparticles. Due to their increasing success and ease of forming nanoparticles, the use of organisms in this field is rapidly expanding. In addition, metal nanoparticles biosynthesis is an environmentally friendly process (green chemistry) without the use of harsh, toxic and costly chemicals. For example, chemical reduction processing of silver nanoparticles such as hydrazine hydrate, sodium borohydride, DMF, and ethylene glycol can lead to the absorption of harsh chemicals on nanoparticles' surfaces that increase toxicity problem. Thus, researchers have used biological synthesis, since this technique is environmentally friendly, low cost and shaped silver nanoparticles are stable and well distributed with minimal aggregation and good control of size [5,6] as well as compatible with the environment and its economic advantages (Patil *et al.*, 2018 ; Seifipour *et al.*, 2020).

In search for new green method, plants, algae, fungi, yeast, bacteria and viruses has attracted great interest for production of nanoparticles due to its compatibility with the environment (Sana & Dogiparthi, 2018; Rasheed *et al.*, 2017; Abdelghany *et al.*, 2017). Compared to the chemical and physical process, the use of plant parts such as seed, fruit, bark, stem, leaf, etc. for the synthesis of nanoparticles is a very novel method. There are numerous natural plants and plant products that can be easily used to promote the biosynthesis of nanoparticles since alkaloids, tannins, steroids, phenols, saponins and flavonoids in their structure are compounds that can help reduce silver ions by different functional groups such as hydroxyl, ketone and aldehydes to silver nanoparticles (Gurunanthan *et al.*, 2014; Rasheed *et al.*, 2017; Marslin *et al.*, 2018). Many groups have recently succeeded in synthesising Ag, Au and Pd nanoparticles using extracts from single-celled organisms such as bacteria (Iravani & Varma, 2020) and fungi (Guilger-Casagrande & Lima, 2019), as well as plant extracts such as olive leaves (Khalil *et al.*, 2014), Dharbai (Htwe *et al.*,, 2019) lemon grass (Ajayi & Afolayan, 2017), neem leaves (Verma & Mehata, 2016), apple extract (Ali *et al.*, 2015) and several others.

The rate of reduction of metal ions using plants has been found to be much faster as compared to micro-organisms and stable formation of metal nanoparticles has been reported. The size of the nanoparticles synthesized using plants can be controlled by changing the concentration of silver nitrate as a precursor (Anigol *et al.*, 2017). While for Ag metal, since ancient times, the antibacterial effects of Ag salts have been noted and Ag is currently used in a number of applications to regulate bacterial growth, including dental work, catheters, and burn wounds. In general, Ag ions and Ag-based compounds are well known to be highly toxic to microbes, demonstrating strong biocidal effects.

In this study, a green method for the synthesis of AgNPs was produced from the plant extract of *Polygonum minus* (*kesum*) as a reducing agent. Widely known in Malaysia, *kesum* is used as spice condiment, and it is one of the herbs that were identified potentially as a source of essential oils, especially in the fragrance industry (Hassim *et al.*, 2014). Traditionally, the leaves have been used to treat maladies such as skin fungal infection, indigestion, dandruff, postnatal tonic, sprains, and body aches (Nurain *et al.*, 2012; Mohamad *et al.*, 2017; Ahmad *et al.*, 2018). *Polygonum minus* has been demonstrated to possess cytoprotective, antibacterial, antifungal, antiulcer, antiviral, and antioxidant activities. Several works also claimed that *Polygonum minus* promotes high levels of free radical scavenging activity and reducing power as well as antimicrobial properties (Qader *et al.*, 2012; Hassim *et al.*, 2014, 2015). Different concentration of silver nitrate solution were investigated and the produced nano particles were characterized by UV–Vis spectrophotometry, FESEM and TEM analyses. Furthermore the achieved silver nano particles was tested for antibacterial activities as well.

2. Material and Methods

2.1 Collection of Leaves

Polygonum minus was purchased from a local wet market in Shah Alam, Selangor. The plant sample was washed and rinsed with running tap water to remove dirt and contaminants. The cleaned sample was dried in the oven at 60 °C for two days. Then, the dried plant sample was weighed and ground with a high-speed blender and stored at room temperature for further analysis.

2.2 Preparation of Leaf Extract

An amount of 10 g *Polygonum minus* powder was weighed and added to 100 mL of double-distilled water. The mixture sample was boiled for 15 min at 100 °C and left to cool. The extract solution was filtered using a vacuum pump, and the filtrate was used as the reducing agent for the preparation of AgNPs.

2.3 Green Synthesis of Plant Silver Nanoparticles and Characterization

$AgNO_3$ (99.98%), which was applied as a silver precursor, was obtained from R & M Chemicals (UK). A 0.1 M $AgNO_3$ was prepared by dissolving 3.058 g of $AgNO_3$ in 180 mL of double-distilled water and stored in an amber-coloured bottle to prevent auto-oxidation of silver. The different concentration of silver nitrate aqueous solution also prepared using 0.01 M and 0.001 M in order to optimize the concentration of silver nitrate solution and yields of AgNPs.

The synthesis of AgNPs was carried out by added about 20 ml *Polygonum minus* extract in 180 ml of aqueous $AgNO_3$ solution. The mixture solution was stirred and heated at 80˚C. The color change of the solution was observed and recorded. UV-Vis spectrophotometer was used for the spectrometric analysis to confirm the formation of AgNPs. To determine the time point of maximum production of AgNPs, the absorption spectra of the sample was taken 300 – 700 nm using a UV-vis spectrophotometer (Thermo Fisher Scientific, Model Biomate 3 spectrophotometer). The deionized water was used as the blank. Proceed with collected of AgNPs, the solution was centrifuged at 10,000 rpm for 30 minutes. The separated nanoparticles settled at the bottom was collected and washed for three time with double distilled water at 10,000 rpm for 10 minutes of each sample. The collected AgNPs was dried in the oven at 60^{o}C for until achieve to the constant weight. The stabilized powder forms of the nanoparticles were stored for further characterization.

2.4 Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray (EDX)

The TEM analysis also perform to determine the morphology and size of AgNPs on the CM12 Phillip Model transmission electron microscopy (Holand). For this purpose , one drop of dispersed AgNPs was placed on a copper grid and allowed to evaporate completely under infrared light before investigation. Meanwhile, the analysis of EDX was used to find the elemental composition of the reaction mixture.

2.5 Observing Bacterial Cells through Field Emission Scanning Electron Microscope (FE-SEM)

The FESEM used is a SUPRA 55VP Carl Zeiss Model field emission scanning electron microscope (Germany). Analysis was carried out by observing the morphology of bacterial cells four selected bacteria, i.e. *Staphylococcus aureus* (ATCC 43300), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442) and *Bacillus Subtilis* (*ATCC 11774)*.

FE-SEM was used to directly observe the surface morphological changes of untreated or treated (with AgNPs) bacterial cells. The sample was cut to 1 cm^3 dimension and fixed in 4 % glutaraldehyde for 12–24 hours at 40 °C. The fixed cells were washed three times with phosphate-buffered solution (PBS) for 10 min of each sample. After washing with PBS, the dehydration process was conducted with 30, 50, 70, 80, 90, and 100 % of ethanol. The fixed cell was dried and gold-coated using an ion sputter. The pre-treated samples were observed by FE-SEM.

2.6 Antimicrobial Property of Silver Nanoparticles

2.6.1 Culture Condition and Cell Inoculum

Four bacteria, i.e. *Staphylococcus aureus* (ATCC 43300), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442), and *Bacillus subtilis ATCC®11774*™ were chosen to be tested in this study. The pure cultures of these bacteria were obtained from the Microbial Culture Laboratory, Institute of Bio-IT Selangor, Universiti Selangor (UNISEL). Each bacteria strain was respectively cultured on Mueller Hinton Agar (MHA) media (Oxoid, UK) at 37 °C for 24 h. The turbidity of the suspension was adjusted to an optical density (OD550nm) of 0.144 which is equivalent to 1 × 106 cells/mL. A stock solution of 1 mg/mL 0.1, 0.01 and 1 mM silver naniparticles (Ag NPs) was prepared in sterile distilled water (sdh20) and stored at 4 °C until used.

2.6.2 Well Diffusion

The well diffusion assay was conducted to determine which antibiotic or sample given are the most successful in treating bacterial infections. Wells with the diameter of 6 to 8 mm were punched into the agar medium on Mueller-Hilton (MH) plates, and a volume of 40 µL of the antimicrobial agent and *P. minus* extract solution at the desired concentrations were introduced into the wells. The microbial culture was standardised according to 0.5 McFarland standards (1×10^8 CFU/mL), and the streptomycin standard was used for each bacterium. All plates were incubated at 30–37 °C for 18–24 h. The diameters of the zones of complete inhibition were measured using a vernier calliper and interpreted according to the National Laboratory Standard Institute (NLSI, 2010).

3. Result and Discussion

3.1 Silver Nanoparticles Analysis

It is well known that AgNPs exhibit brown color in aqueous solution due to excitation of surface Plasmon vibrations in AgNPs (Bonnia *et al.*, 2016). In this experiment AgNPs were successfully synthesized from the aqueous $AgNO_3$ solution using *Polygonum minus*

extract in a continuously heated and stirred mixture. Figure 1 shows the colourless reaction mixture slowly changed from yellowish green to reddish brown indicating of reduction of the Ag ion after several minutes of reaction.

Polygonum minus has been reported to have a large group of flavonoid content in its polyphenolic compound, which can actively chelate and reduce metal ions into nanoparticles. Various functional groups of flavonoids can also form nanoparticles (Makarov *et al.*, 2014). The formation of AgNPs was confirmed by the change in the colour of the solution mixture by the bioreduction of Ag^{+} to Ag^{0} (Khan *et al*., 2017). The possible reduction mechanism of silver ion in this reaction can be carry out by the involvement of phenolic compound through oxidation of aldehyde groups (H-C=O) in the *P.* minus to carboxylic acids (-COOH). It's probably happening with the dealings of carbonyl groups or pi-electrons in the absence of other powerful chelating agents, create flavonoids and terpenoids adsorbed on the surface of AgNPs and act as capping agents (Anjum *et al.*, 2016).

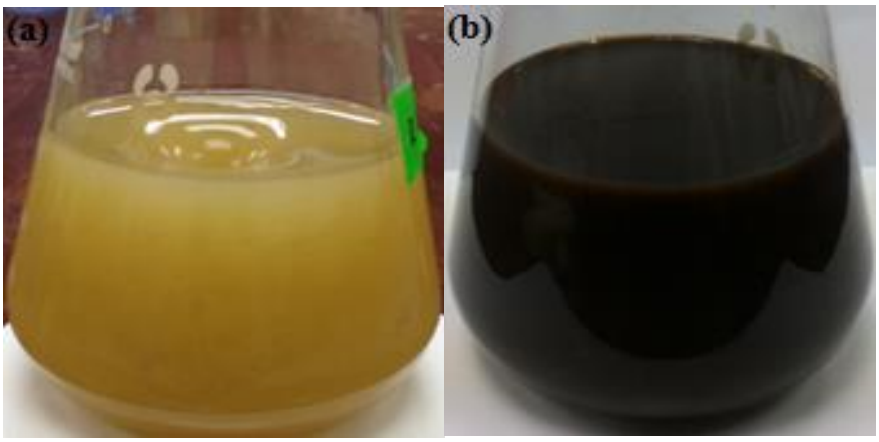


Figure 1. The colour changes of (a) *P. minus* extract and (b) synthesized AgNPs

3.2 Effect of Silver Nitrate ($AgNO_3$) Concentration

UV-Vis spectroscopy has been widely used to detect the presence of AgNPs during synthesis (Logeswari *et al*., 2015; Ali *et al.*, 2016). Surface plasmon absorption peaks in the range from 420 to 470 nm have been used as an indicator to confirm the reduction of Ag^{+} to metallic Ag in AgNPs (Hyllested *et al.*, 2015; Motitswe *et al.*, 2019). The absorption of silver nanoparticles at 0.001 M shows the occurrence of the peak at approximately 420 nm and this peak slightly changed to 440 - 450 nm at 0.01 M and 0.1 M of silver nitrate (Figure 2). This redshift of 440 nm to 450 nm indicated the increasing particle size of silver nanoparticles. Interestingly, 0.1 M and 0.01 M concentration supported rapid formation compared to other concentrations on the basis of UV-Vis studies. The synthesis of silver nanoparticles increased with the increase in silver nitrate concentration. Increase in yield of silver nanoparticles was observed when metal salt concentration was increased from 0.001 M – 0.1 M as well as the intensity of SPR peak increases with increasing concentration of $AgNO_3$ due to the increase of AgNPs concentration. Hence, 0.1 M concentration of $AgNO_3$ was chosen for best concentration.

Another important factor influencing the growth of silver nanoparticles is the contact time which is also known as reaction time. In this study, the formation of AgNPs was monitored by measuring UV-Vis spectra at different time intervals. The UV-Vis spectra showed a strong peak absorbance at 420 nm – 450 nm for all samples corresponding to the

surface plasmon resonance (SPR) of AgNPs, which increased with the time of incubation of $AgNO_3$ for 5 min, 10 min, 15 min, 20 min, 30 min, 60 min (0.1 M and 0.01 M) and 24 hours (0.001 M) with the plants extract indicating increased amount of AgNPs produced from the mixture (Figure 3 (a)- (c)). An increase in absorbance was noted with an increase in interaction time of silver ions with extract, and the best synthesis was observed at 60 minutes of incubation (0.1 M and 0.01 M) while 7 hours of incubation (0.001 M). On the contrary, the control experiment ($AgNO_3$) and the time point of 0 min showed no colour, indicating the absence of AgNPs but formation rate start gradually after 5 minutes (0.1 M, 0.01 M) and continued till 60 minutes (0.1 M, 0.01 M) while 7 hours (0.001M). After that no significant change in SPR means that the stability of the AgNPs colloidal solution within the reaction period.

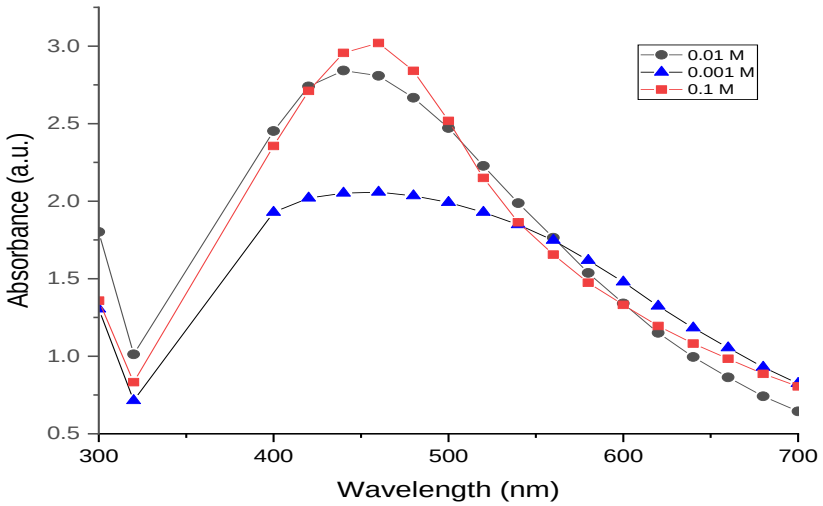
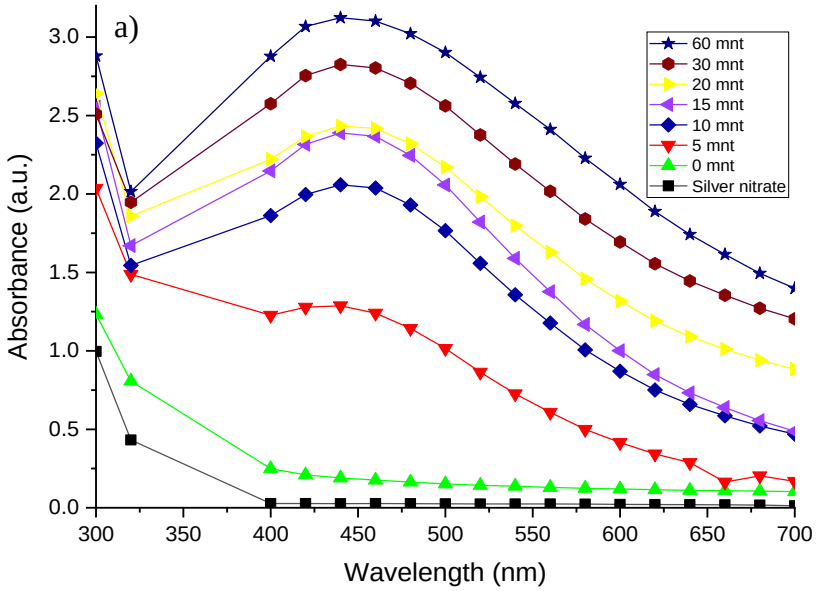


Figure 2. UV-Vis spectrum of silver nanoparticles with different silver nitrate concentration



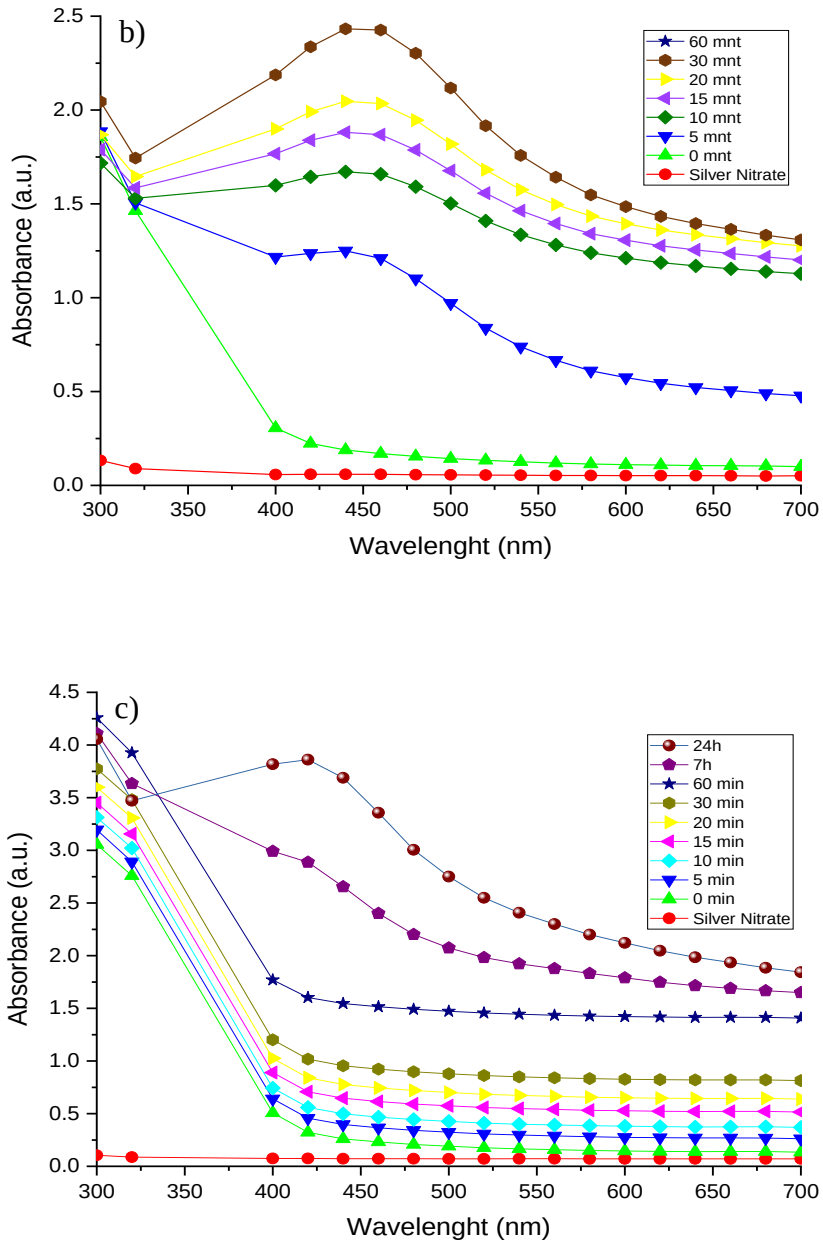


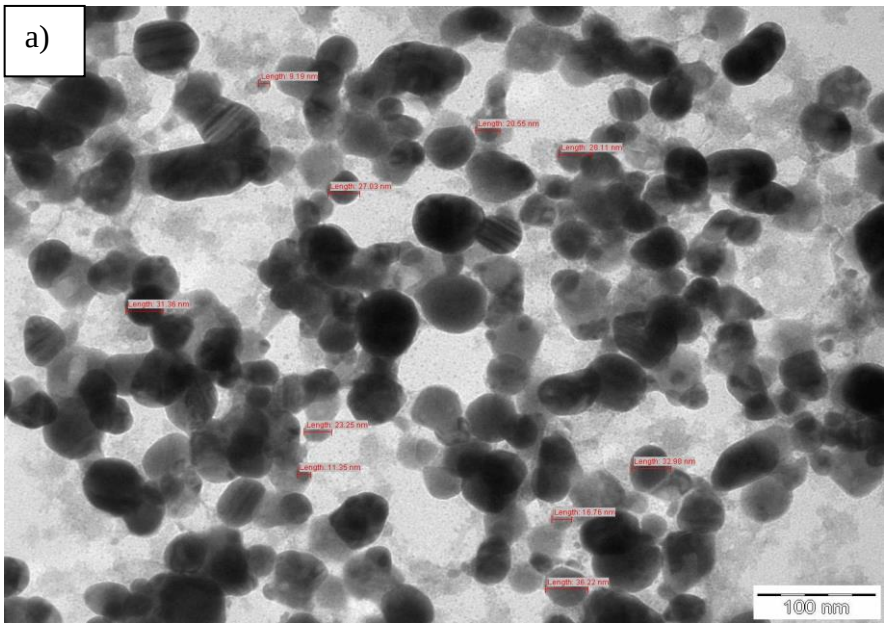
Figure 3. UV-Vis spectrum of AgNPs by *Polygonum minus* extract with a) 0.1 M, b) 0.01 M and c) 0.001 M silver nitrate concentration

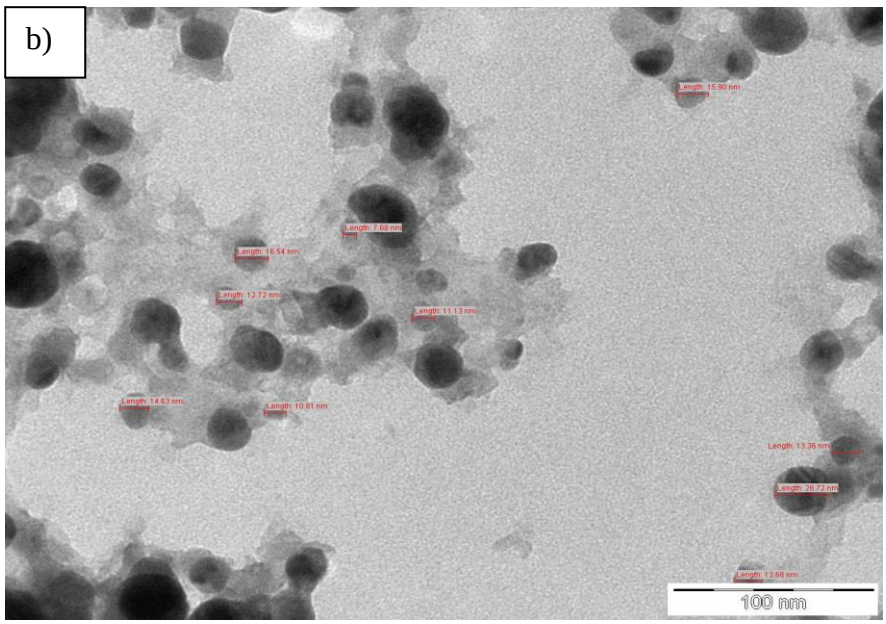
3.3 TEM Analysis

Transmission electron microscope (TEM) study was performed to estimate the shape and size of the nanoparticles. The TEM (Figure 4) revealed that with increase in silver nitrate concentration up to 0.1 M, the particles sizes decreased. The rationale for the reduction in particle size with an increasing concentration of $AgNO_3$ is not clear. The reasoned that it could be due to $AgNO_3$ forming a coat on rising particles and preventing their aggregation and thus, yielding particles of nanoscale size. The image shows that the particles are

predominantly spherical shape with smooth surface morphology. Some of the AgNPs were found to be oval and the scenario in variation of shape and size of nanoparticles is common when using a biological system (Rao *et al.*, 2016).

The particle size of AgNPs was found smaller at higher metal ion concentration. Based on the measurement, it can be observed that the particle size of silver nanoparticles decreased in ranging size from 9.19 nm – 36.22 nm, 7.68 nm – 26.72 nm, 6.49 nm – 25.95 nm at 0.001 M, 0.01M, and 0.1 M concentration of silver nitrate, respectively. The size was clearly shown to be within 100 nm, thereby confirming the presence of nanoparticles. The availability of secondary materials, which could be biocomponents present in Polygonum minus, was represented by dark shades on the AgNPs. In the promotion of the reduction of Ag^+ to AgNPs, these biocomponents are crucial. In addition, these particles serve as capping agents that work to prevent agglomeration (Benakashani et al., 2016; Rai et al.,2012).





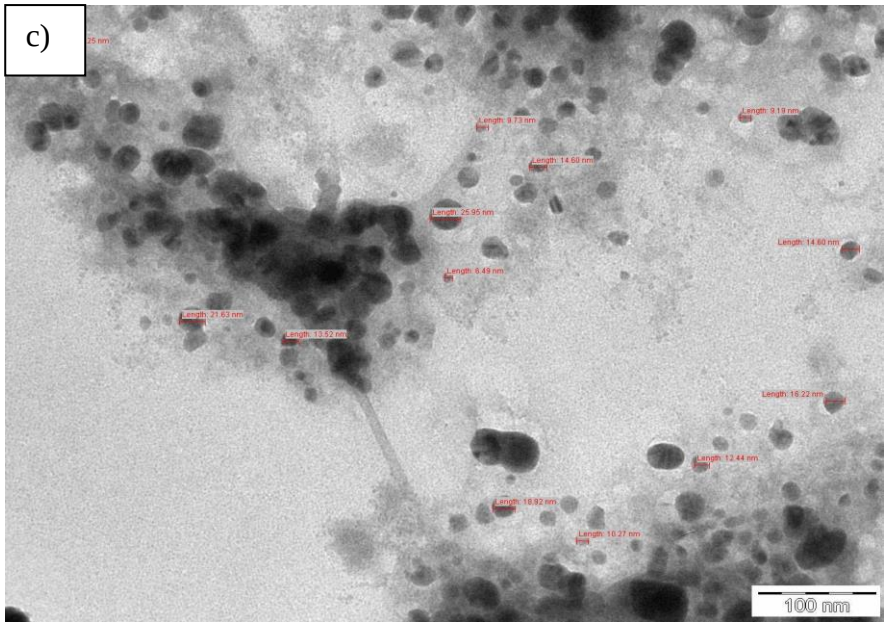
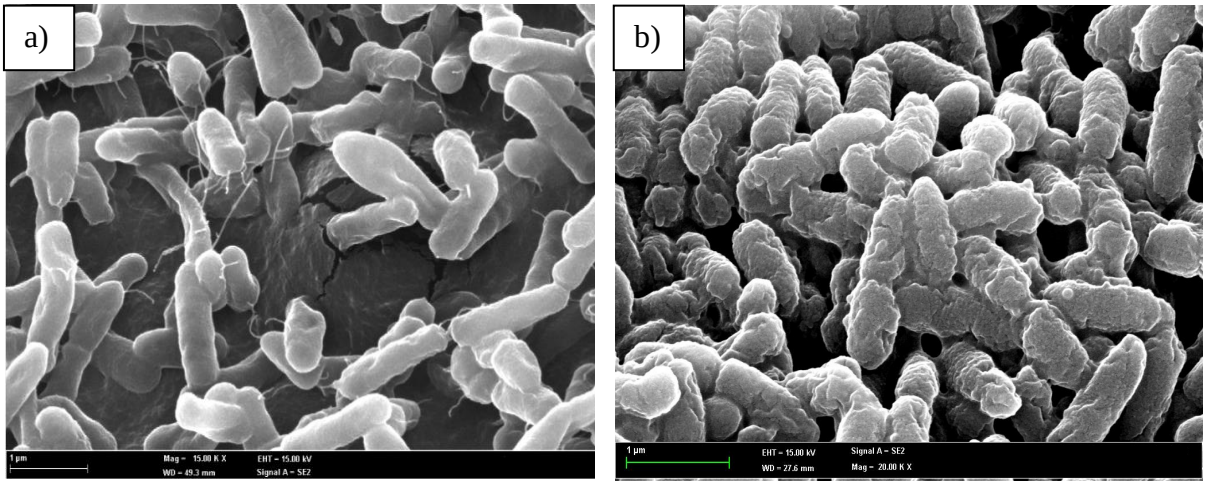


Figure 4. TEM micrographs of AgNPs by *Polygonum minus* extract with different concentrations of $AgNO_3$: (a) 0.001 M, (b) 0.01 M, (c) 0.1 M

3.4 Morphological Changes of Bacterial Cells Treated with Silver Nanoparticles

The morphological differences between untreated and treated bacterial cells with AgNPs were observed by FE-SEM and Figure 5, respectively. Cells of the control group were usually rod-shaped in the *E. coli*, *P. aeruginosa* and *B. subtilis* cultures. Each cell size was about the same and there was no detection of damage on the cell surface. However, in *E. coli*, *P. aeruginosa* and *B. subtilis* treated with AgNPs, irregular fragments appeared on the cell surface that suggested damage to the cell surface instead of the usual rod-shaped cells. Meanwhile, control cells were normally grape-shaped in the *S. aureus* culture, with an intact cell surface, and no damage was seen. However, fragments have been found on the cell surface after treatment with AgNPs, and they have become agglomerated, suggesting a weakened cell surface. The reactive oxygen species (ROS) could cause increased cell membrane permeability or leakage of cell content (Kim et al., 2011).



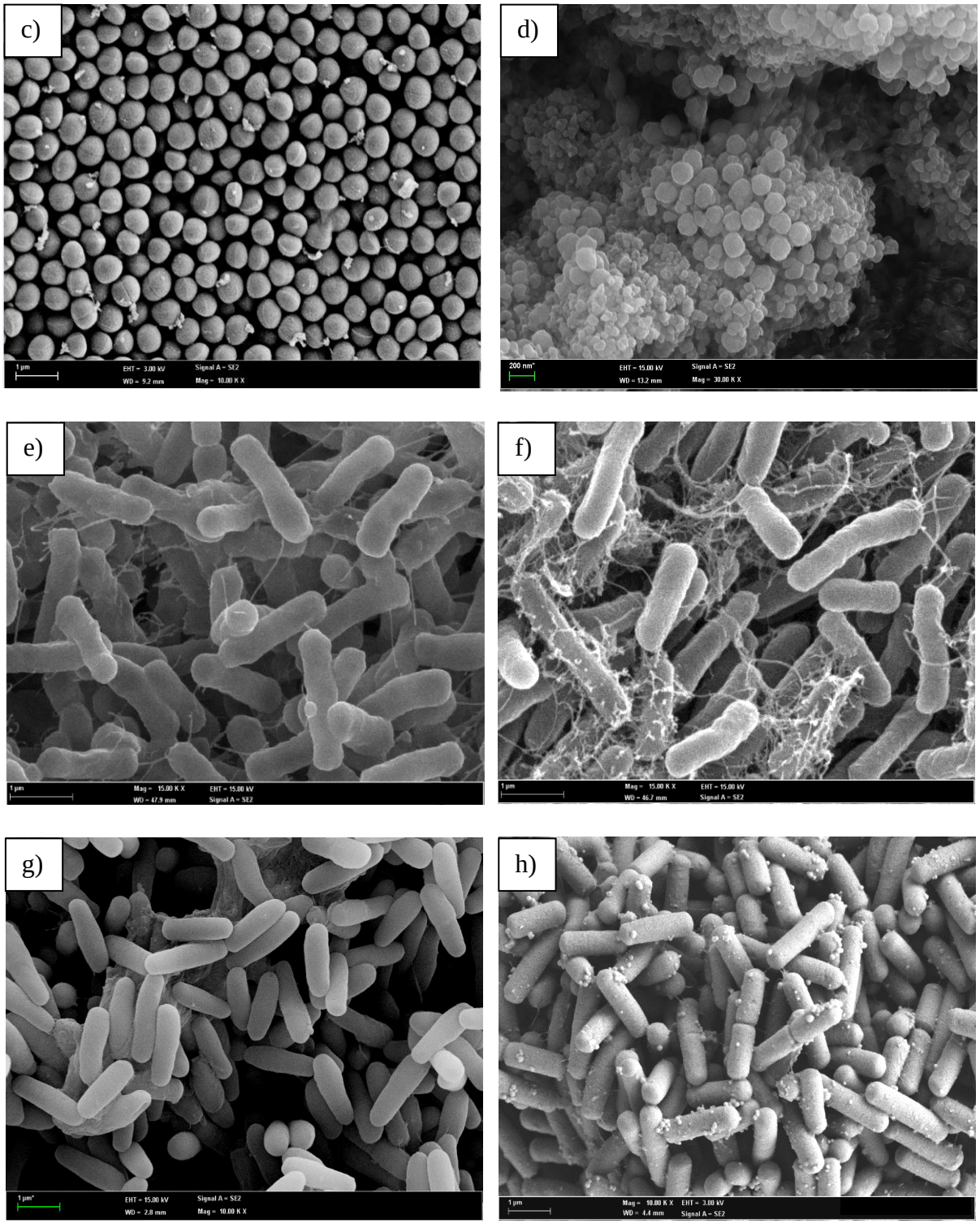


Figure 5. FE-SEM micrograph of *P. aeruginosa*, *S. aureus*, *E. coli*, and *B. subtilis* control (a, c, e, g) and *P. aeruginosa*, *S. aureus E. coli*, and *B. subtilis* treated with silver nanoparticles (b, d, f, h).

Compared to *E. coli* and *P. aeruginosa*, FE-SEM morphological micrographs showed that the destruction of the bacterial cells of *S. aureus* and *B. subtilis* was weaker. This may be due to the distinction between Gram-positive (*S. aureus, B. subtilis*) and Gram-negative (*E.*

coli, P. aeruginosa) bacterial peptidoglycan layers, where the basic role of the peptidoglycan layer is to defend the bacteria from antibacterial agents such as antibiotics, toxins, chemicals and decaying enzymes (Silhavy et al., 2010; Kim et al., 2011). The Gram-positive cell envelope usually consists of a thick peptidoglycan layer and cell membrane containing lipoteichoic acid, while the Gram-negative cell envelope consists of the outer membrane, thin peptidoglycan layer, and cell membrane.

3.5 Antimicrobial Activity of Silver Nanoparticles

After incubation for 24 h, growth inhibition was observed in samples impregnated with AgNPs and the positive control (streptomycin) with the inhibition zone of 30 mm, 28 mm, 30 mm and 28 mm, respectively (Table 1), while the negative control (sterile distilled water) showed no inhibition zone. AgNPs exhibited strong antimicrobial activity against both Gram-positive, (*S. aureus*, *B. subtilis*) and Gram-negative bacteria, (*E. coli* and *P. aeruginosa*) and formed inhibition zones of 13 mm, 13 mm, 14 mm and 17 mm, respectively (Table 1). At 0.1 M of AgNPs, the well diffusion method showed the highest inhibition zone against *E. coli* followed by *P. aeruginosa* (16.0 and 14.0 mm). While, *S. aureus* and *B. subtilis* exhibited 13.0 mm of inhibition zone diameter. While for 0.01 M of AgNPs, *E. coli* showed the largest zone of inhibition with 16.0 mm. *S. aureus* and *P. aeruginosa* exhibited 15.0 mm. The smallest zone of inhibition were observed on *B. subtilis* with 14.0 mm. *E. coli* showed the highest antibacterial activity when treated with 0.001 M AgNPs with 16.0 mm of inhibition zone, followed by *P. aeruginosa*, *S. aureus* and *B. subtilis* with 14.0, 13.0 and 12.0 mm respectively. Higher inhibition zones were noticed for *E. coli* and *P. aeruginosa* compared to *S. aureus* and *B. subtilis*.

Table 1. Well diffusion assay of AgNPs against bacteria

	Target Microbes			
Sample	*S. aureus* (ATCC 43300)	*P. aeruginosa* (ATCC 15442)	*E. coli* (ATCC 25922)	*S. subtilis* (ATCC 11774)
Zone of inhibition (mm)				
0.1 M	13 ± 1.00	14 ± 1.53	16 ± 1.53	13 ± 1.15
0.01 M	15 ± 1.00	15 ± 0.58	16 ± 1.73	14 ± 1.15
0.001 M	13 ± 1.00	14 ± 0.58	16 ± 1.15	12 ± 1.15
-ve control	-	-	-	-
+ve control	30 ± 0.58	28 ± 0.58	30 ± 0.58	28 ± 0.58

From the results obtained, Gram-negative bacteria showed a larger inhibition zone compared to Gram-positive bacteria (Figure 6). The Ag mode of action is presumed to be dependent on Ag^+ ions, which strongly inhibit bacterial growth through the suppression of respiratory enzyme and electron transport components, as well as the interference with DNA functions (Hassan *et al.*, 2014; Cedillo-Alvarez *et al.*, 2017). Thus, AgNPs have been demonstrated to exhibit antimicrobial properties against bacteria with a close attachment of the nanoparticles with the microbial cell.

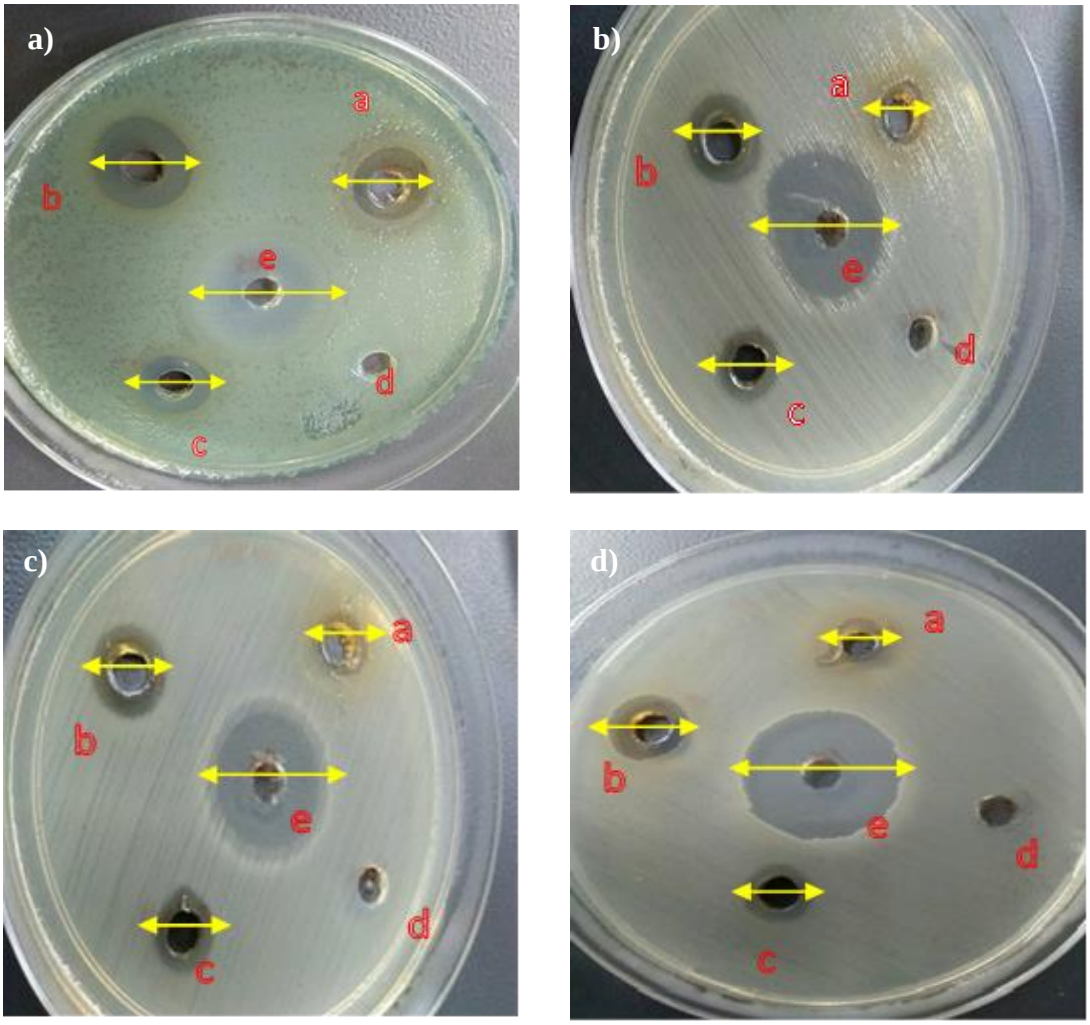


Figure 6. The observations of growth inhibition zone on each bacterial plate (a)) *P. aeruginosa* (b) *E. coli*, c) *S. aureus* and d) *B. subtilis*

Well **a was impregnated with 0.01 M AgNPs*
*Well **b** was impregnated with 0.1 M AgNPs*
*Well **c** was impregnated with 0.001 M AgNPs*
*Well **d** was impregnated with sterile distilled water as a negative control*
*Well **e** was impregnated with streptomycin as a positive control*

4. Conclusion

This study demonstrated that AgNPs can be synthesized using *Polygonum minus* plant extract, as a reducing agent by using green method. The green approach has proven to be environmentally sustainable and successful in synthesising AgNPs. The UV-vis spectroscopy confirmed the formation of silver nanoparticles. TEM analysis showed the particles sizes decreased with increasing silver nitrate concentration. Synthesis of AgNPs is enhanced with time at increased silver nitrate solution. Green synthesized AgNPs are found to inhibit bacterial activity against bacterial colony. Due to the antimicrobial activity of AgNPs it can concluded that plant extract being very ecofriendly, cost effective, promising a small size of

molecules and as effective antibacterial materials against various microorganisms and this method is potentially exciting for the large-scale synthesis of nanoparticles.

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