BIOSYNTHESIS OF SILVER NANOPARTICLES USING EXTRACTS OF NEEM LEAVES

Yoon Noe Aung[1], Ye Myint Aung[2], Nilar Kyi[3]

Abstract

The biosynthesis of silver nanoparticles from extracts of neem leaves was studied. In this research, silver nitrate was used as a metal precursor and nee[1]m leaves extract was taken as a reducing agent for synthesis of silver nanoparticles, AgNPs. The characteristics of silver nanoparticles were mainly confirmed by UV-Visible spectroscopy, Energy Dispersive X-ray Fluorescence (EDXRF) spectroscopy, X-ray Diffraction (XRD) analysis, Fourier Transform Infra-red (FT IR) spectroscopy and Scanning Electron Microscopy (SEM). In the biosynthesis process, effect of neem leaves extract and effect of stirring time on synthesized AgNPs were also studied. It was observed that the maximum wavelength of synthesized AgNPs was 410 nm that agreed with the literature value of the wavelength range of 400-500 nm for silver nanoparticles. The EDXRF data showed a percent relative abundance of Ag (50.108%) that confirmed the presence of silver in the suspension. From XRD data, the average crystallite size was calculated by Scherrer equation and found to be 37.14 nm. FT IR analysis showed the presence of the protein or amino acid group in neem extract which reduced Ag^+ ion into metallic Ag nanoparticles. In SEM analysis, it was found that the sample was likely to be well dispersed agglomerates of grains with narrow size distribution. As other evidence, the characteristic of AgNPs was observed by Tyndall effect. The antimicrobial activity of prepared AgNPs was also studied by the six microorganisms - *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli.* It was observed the AgNPs suspension was more potent than neem extract only especially in *Bacillus pumilus* and *Escherichia coli* with the zone diameters of 15 mm.

Keywords: neem leaves extract, reducing agent, silver nitrate, antimicrobial activity

Introduction

Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials (Saifuddin *et al.*,2009). Nanoparticle synthesis and the study of their size and properties is of fundamental importance in the advancement of recent research. It is found that the optical, electronic, magnetic and catalytic properties of metal nanoparticles depend on their sizes, shape, and chemical surroundings.

Nanoparticles can be broadly grouped into two: namely organic and inorganic nanoparticles. Inorganic nanoparticles (such as metallic and semiconductor nanoparticles) exhibit intrinsic optical properties which may enhance the transparency of polymer-particles composites. For such reasons, inorganic nanoparticles have found special interest in studies devoted to optical properties in composites (Caseri, 2009). Green synthesis methods employing either biological microorganism or plant extracts have emerged as a simple and alternative to chemical synthesis. Generally, the green synthesis method involves three main steps, (1) solvent medium selection, (2) environmental benign reducing agent selection, and (3) non-toxic substances for nanoparticles stability selection. The synthesis of nanoparticles by using plant extracts can be advantageous over other biological processes because it eliminates the elaborate process of maintaining cell cultures and can be suitably scaled up for large scale production under non-aseptic environments (Loo *et al.*, 2012).

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Azadirachta indica commonly known as Neem belongs to Meliaceae family, and is well known in India and its neighbouring countries for more than 200 years as one of the most versatile medicinal plant having a wide spectrum of biological activity. Every part of the tree has been used as a traditional medicine for house-hold remedy against various human ailments, from antiquity (Koul *et al.*, 1990). Antimicrobial capability of AgNPs allows them to be suitably employed in numerous household products such as textiles, food storage containers, home appliances and in medical devices (Marambio-Jones and Hoek, 2010). The most important application of silver and AgNPs is as topical ointments to prevent infection against burns and open wounds (Ip *et al*., 2006). The aim of this research is to study the biosynthesis of silver nanoparticles by using aqueous neem leaves extract and to determine the antimicrobial activities of synthesized AgNPs.

Materials and Methods

Sample Collection

In this experimental work, the leaves of *Azadirachta indica* A. Juss (Tama-kha) were collected from Pathein Township, Ayeyarwady Region, Myanmar. After collection, the scientific name of the plant was identified at Botany Department, Pathein University. The collected fresh samples were washed and air dried at room temperature for about one week and then they were ground into powder by a grinder. The dried powder samples were stored in air-tight containers.

Preparation of Neem Leaves Extract

The leaves were ground to get a fine powder and 30 g of dried powder was weighed in an electric balance and put into a beaker. A 100 mL of phosphate buffer, pH 9.0 and a 100 mL of distilled water were added into the sample, and then were boiled for 30 min. After cooling at room temperature, it was filtered with filter paper into the conical flask and then centrifuged at 6,000 rpm for 10 min and filtered. The filtrate was stored at 4 ºC for further experiments. The filtrate was used as reducing and stabilizing agent for 1 mM of $AgNO_3$ (Roy *et al.,* 2017).

Preparation of Silver Nanoparticles (AgNPs) Using Neem Leaves Extract

The extract of neem leaves (20 mL) was mixed with 50 mL of 1 mM silver nitrate ($AgNO_3$) solution in a conical flask under aseptic condition. The flask was magnetically stirred while heating at 50 ºC in dark for 5 h. A change in the colour was observed indicating the formation of silver nanoparticles. The solution was centrifuged at 6,000 rpm for 30 min to obtain the silver nanoparticles and the supernatant was discarded. The particles were repeatedly washed to ensure purity and dried at 100 °C in an oven (Roy *et al.*, 2017).

Effect of Different Volumes of Neem Leaves Extract

Each of the extract of neem leaves (10, 20, 30) mL was mixed with 50 mL of 1 mM silver nitrate ($AgNO_3$) solution in a conical flask under aseptic condition. The flask was magnetically stirred while heating at 50 ºC in dark for 5 h. A change in the colour was observed indicating the formation of silver nanoparticles. The solution was centrifuged at 6,000 rpm for 30 min to obtain the silver nanoparticles and the supernatant was discarded. The particles were repeatedly washed to ensure purity and dried at 100 °C in an oven.

Effect of Stirring Time

The extract of neem leaves (20 mL) was mixed with 50 mL of 1 mM silver nitrate ($AgNO_3$) solution in a conical flask under aseptic condition. The flask was magnetically stirred while heating at 50 ºC in dark for each about (3, 4, 5) h. A change in the colour was observed indicating the formation of silver nanoparticles. The solution was centrifuged at 6,000 rpm for 30 min to obtain the silver nanoparticles and the supernatant was discarded. The particles were repeatedly washed to ensure purity and dried at 100 °C in an oven.

Characterization of Silver Nanoparticles

The silver nanoparticles prepared from neem leaves extract and silver nitrate ($AgNO_3$) was characterized by UV-Visible EDXRF, XRD, FTIR, and SEM techniques.

Determination of λ_{max} of AgNPs by UV-Visible Spectroscopic Method

The bio-reduction of the Ag^+ ions by the supernatant of the test plant extracts in the solutions and formation of silver nanoparticles were monitored by UV-1800 (Shimadzu) spectrophotometer operated at a resolution of 1 nm. The UV-Vis spectra of the samples were measured in the wavelength range of 300 - 600 nm. Distilled water was used to adjust the baseline.

Screening of Antimicrobial Activities

The antimicrobial activity of the crude extracts was performed by the agar well diffusion assay. The pathogenic test organisms were incubated in trypticase soy broth at appropriate temperature for 24 h. Nutrient agar medium containing meat extract (0.5 g), peptone (0.5 g), sodium chloride (0.25 g), agar (1.5 g) and 100 mL of distilled water were placed in a beaker and the contents were heated for 30 min. The nutrient agar medium was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121 ºC for 15 min. After cooled down to 40 ºC, one drop of suspended strain was inoculated to the nutrient agar with the help of a sterilized disposable pipette near the burner. About 20 mL of medium was poured into the sterilized petri dish and allowed to set the medium. Once solidified the dishes were stored for 2 h in a refrigerator. Two wells of 10 mm diameter each were cut out in the inoculated agar to place extract samples to be tested. The volume of each extract sample placed in each well was 0.2 mL. Two samples, namely neem leaves extract and AgNPs suspension were tested. The petri-dishes were then incubated at 37 ºC for 24 h, and the diameters of clear inhibition zone around the wells were measured (Collin, 1965).

Results and Discussion

Silver Nanoparticles Using Neem Leaves Extract

In this work, silver nanoparticles were synthesized by using silver nitrate and neem leaves extract. The silver nitrate was used as a metal precursor and neem leaves extract was used as a reducing agent. The formation of silver nanoparticles was confirmed through visual assessment. The reaction mixture turned to dark brown colour from brownish-yellow colour within 20 min indicating the formation of silver nanoparticles in the solution. Reduction of silver ion into silver particles during exposure to the leaf extracts could be followed by colour change. Silver nanoparticles exhibited dark yellowish-brown colour in aqueous solution due to the surface plasmon resonance phenomenon (Azizi *et al.*, 2013). Figure 1 shows the aqueous extract of neem leaves before and after synthesis of AgNPs.

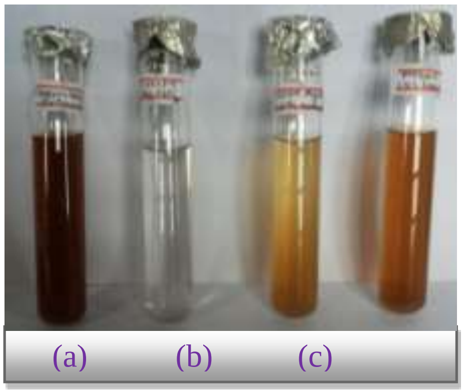

Figure 1 Photographs of (a) aqueous extract of neem leave extract (b) silver nitrate solution (1mM) (c) silver nanosuspension (after 20 min) and (d) silver nanosuspension (after 5 h)

Effect of Different Volumes of Neem Leaves Extract

The reducing agent gives the particles with narrower size distribution of the uniform size of AgNPs. The extracts of neem leaves (10, 20 and 30) mL were used in this work. The absorption spectra of AgNPs with different volumes of neem leaves extracts are shown in Table 1 and Figure 2. With the increasing reducing agent larger nanoparticle cluster were formed. The silver colloids exhibit absorbance in the visible region, and the wavelength at the maximum absorption highly shifts towards a longer one with the increase of leaf extracts (Dagmara *et al.,* 2012). Maximum absorbance of electromagnetic wave of visible range (400-500 nm) was observed in the literature where silver nanoparticles synthesized by using neem leaves extraction (De Silva *et al.,* 2013). In this study,the wavelength of maximum absorption was found at 410 nm which was in accordance with the literature value.

Table 1 Relationship between Absorbance of AgNPs Solution using Different Volumes of Neem Leaves Extract as a Function of Wavelength

Wavelength (nm)	Absorbance		
	10 mL	20 mL	30 mL
390	3.20	3.40	3.80
400	3.25	3.60	3.79
410	3.56	3.70	4.0
420	1.34	1.60	1.93
430	1.52	1.67	2.06
440	1.35	1.40	1.43
450	1.24	1.43	1.47
460	0.52	0.70	0.97
470	0.76	0.90	1.04

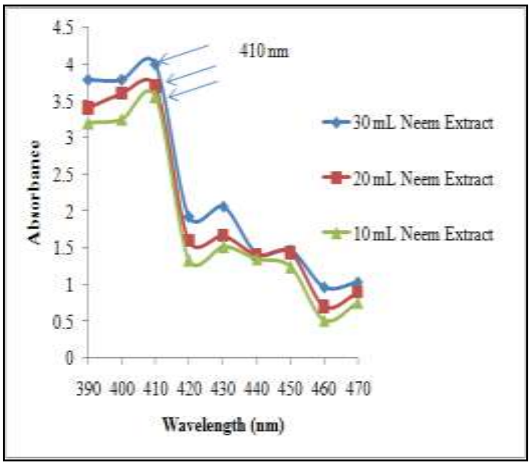

Figure 2 Changes of absorbance of silver nanoparticles with different volumes of neems leaves extract at different wavelengths

Effect of Stirring Time

The extract of neem leaves (20 mL) was mixed with 50 mL of 1 mM silver nitrate ($AgNO_3$) solution in a conical flask. The flask was kept in heat and magnetic stirrer at 50 ºC in dark for each about (3, 4, 5) h for stirring time. The colour intensity increased with the duration of stirring time. The position of absorption maxima does not change significantly during time interval of stirring (Table 2 and Figure 3).

Table 2 Absorbance of AgNPs Solution as a Function of Wavelength at Different Stirring Times

Wavelength (nm)	Absorbance		
	3 h	4 h	5 h
390	2.80	3.20	3.40
400	3.40	3.51	3.60
410	3.54	3.57	3.70
420	1.43	1.48	1.60
430	1.54	1.58	1.67
440	1.26	1.29	1.40
450	1.29	1.38	1.43
460	0.56	0.64	0.70
470	0.67	0.79	0.90

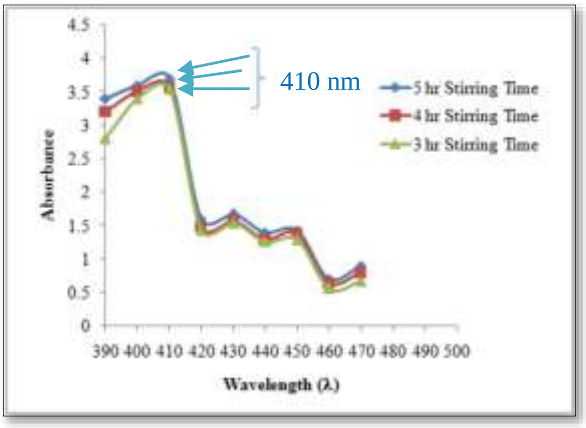

Figure 3 Changes of absorbance of silver nanoparticles with various stirring time at different wavelengths

EDXRF Analysis

EDXRF technique was used to verify the presence of silver in the resulting suspension. The percents of elements present in neem leaves extract and silver nanoparticles formed in the reaction media are shown in Figures 4 and 5. In the neem leaves extract, Ca (74.816%) and K (18.716%) were present as major constituents. Other elements: S (2.773%), Si (1.217%), Fe (1.018%), Sr (0.612%), Mn (0.443%), Br (0.214%) and Zn (0,191%) were present as minor constituents. By EDXRF data, Ag (50.108%), Ca (26.961%), K (17.323%), P (2.843%), S (1.065%), Mn (0.585%), Br (0.502%), Fe (0.340%), Sr (0.106%), Cu (0.079%), Zn (0.058%) and Rb (0.031%) were also present in AgNPs suspension.

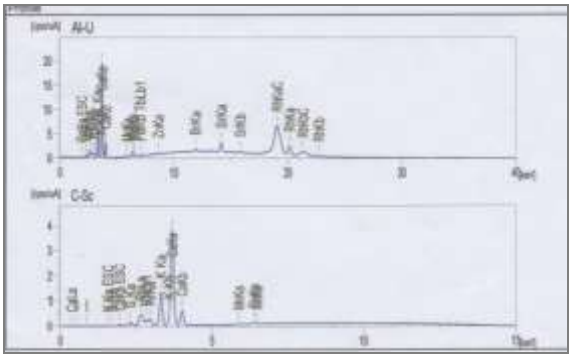

Figure 4 EDXRF spectrum of neem leaves extract

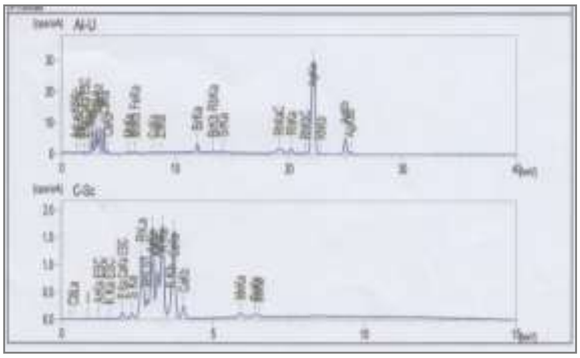

Figure 5 EDXRF spectrum of AgNPs suspension

XRD Analysis

The X-ray diffractogram of silver nanoparticles is shown in Figure 6. The synthesized AgNPs shows the crystalline nature. The average crystallite size of silver nanoparticles was calculated by using the Scherrer equation and found to be 37.14 nm.

FT IR Analysis

The FTIR spectra of neem leaf extract and silver nanoparticles are shown in Figures 7 and 8. The spectrum of neem extracts consist of the band at 3439 cm^{-1} attributed to the OH stretching vibration. The band appeared at 2933 cm^{-1} was due to the C-H stretching vibration in CH_2 and CH_3 groups. The absorption band corresponding to 1624 cm^{-1} was due to (NH) C=O stretching vibration of the characteristic of proteins. It may be concluded that protein or amino acid group present in neem extract reduced Ag^+ ion into metallic Ag nanoparticles. After reduction of $AgNO_3$, the additional peaks at 1383 cm^{-1} and 1066 $cm^{-1}$are related to AgNPs. Some peaks were disappeared in the reduction process of AgNPs (Silverstein, 1998).

SEM Analysis

The surface morphology of nanoparticles was examined by SEM micrograph. It was found that the sample was likely to be well-dispersed agglomerates of grains with narrow size distribution (Figure 9).

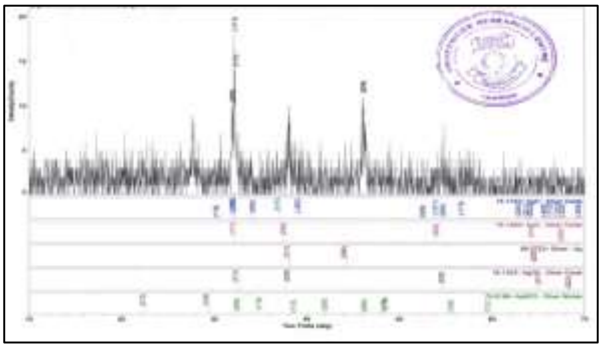

Figure 6 X-raydiffractogram of AgNPs suspension

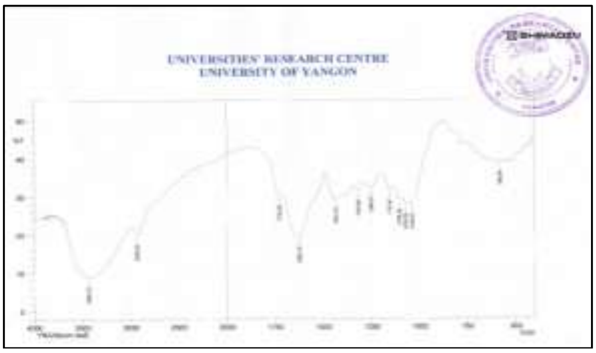

Figure 7 FT IR spectrum of neem leaves extract

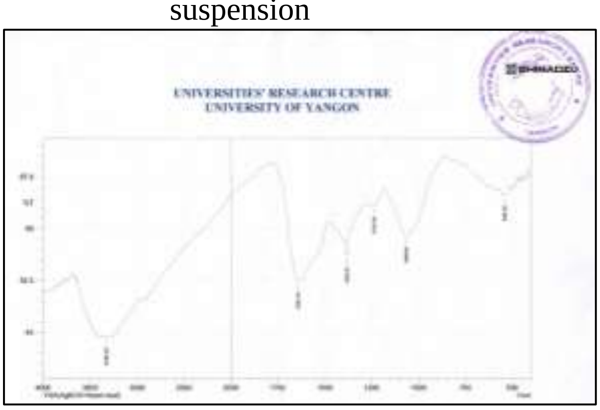

Figure 8 FT IR spectrum of AgNPs suspension

Figure 9 SEM micrograph of AgNPs suspension

Characteristic of AgNPs by Tyndall Effect

The presence of a colloidal suspension can be detected by the reflection of a laser beam from the particles. Because a laser pointer emits polarized light the pointer can be oriented such that the beam appears to disappear. If the colloidal particles are present, the laser beam can pass and if the particles are absent, the beam cannot pass through the medium. It was found that the laser beam passed through the AgNPs suspension but did not pass through in silver nitrate solution (Figure 10). The particles are large enough that they do scatter light, and the Tyndall effect is observed.

Screening of Antimicrobial Activities

The selected strains are used to perform the antimicrobial activities for the neem leaves extract and synthesized silver nanoparticles. The antimicrobial activity screening of neem leaves crude extract and AgNPs were carried out against six species of microorganism *viz*., *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* by employing agar well diffusion method. It was observed that both neem extracts and AgNPs suspension showed antimicrobial activity against all test organisms except *Pseudomonas aeruginosa*. The AgNPs suspension was more potent than neem extract especially in *Bacillus pumilus* and *Escherichia coli* with the zone diameters of 15 mm (Table 3).

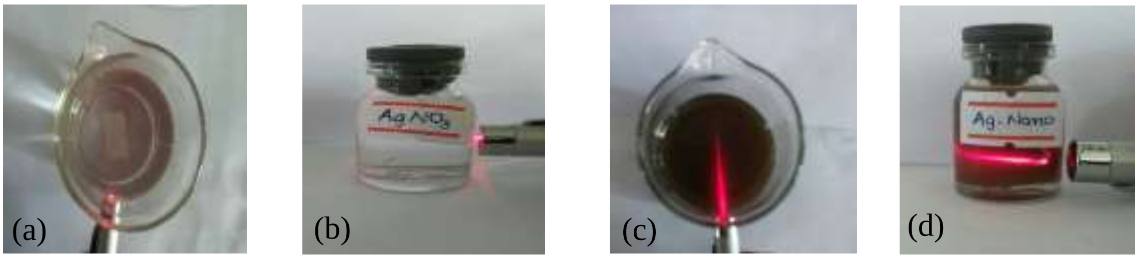

Figure 10 Photographs of Tyndall effect (a) $AgNO_3$ solutions (top view) (b) $AgNO_3$ solutions (side view) (c)Ag NPs suspension (top view) (d) Ag NPs suspension (side view)

Table 3 Antimicrobial Activity of Neem Leaves Extract and AgNPs Suspension

Samples	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*B. pumilus*	*C albicans*	*E. coli*
Neem leaves extract	11 (+)	11 (+)	-	12 (+)	11 (+)	13 (+)
Ag NPs suspension	12 (+)	14 (+)	-	15 (++)	13 (+)	15 (++)

Conclusion

Silver nanoparticles were prepared by biosynthesis, green synthesis. The green synthesis of AgNPs is alternative to chemical method. It is a cheap, pollutant free and eco-friendly method. In this research, silver nitrate was taken as the metal precursor and neem leaves extract as a reducing agent. In this work, AgNPs were prepared by different volumes of neem leaves extract (10, 20 and 30 mL) and stirring times (3, 4 and 5 h) for synthesis process. The AgNPs suspension has been detected by UV-Visible absorption spectroscopy and the wavelength of maximum absorption was observed at 410 nm. By EDXRF data, silver suspension as nanoparticles showed silver content of 50.108%. From the XRD result, the average crystallite size of AgNPs was 37.14 nm. In FT IR analysis, the protein or amino acid group was present in neem extract to reduce Ag^+ ion into metallic AgNPs. In SEM analysis, the sample was likely to be well-dispersed agglomerates of grains with narrow size distribution. In this study, the presence of a colloidal suspension was detected by the reflection of a laser beam from AgNPs particles showing the Tyndall effect. Neem extract and AgNPs suspension showed the inhibitory effects against six tested organisms except *Pseudomonas aeruginosa.*

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