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Green synthesis of silver nanoparticles from aqueous extract of leaves of *Azadirachta indica,* its phytochemical screening and antibacterial activity#

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Abstract

Silver nanoparticles are gaining importance on account of its antimicrobial properties and low toxicity to humans. The present study was conducted to synthesise silver nanoparticles (AgNPs) from aqueous extract of neem leaves. The phytochemical screening of the aqueous extract of neem leaves was also performed for determining its active principles. The qualitative phytochemical screening revealed that the aqueous extract of neem leaves contained phytochemicals like steroids, alkaloids, phenolic compounds, tannins, flavonoids, saponins and terpenoids. The AgNPs synthesised were characterised using X-ray diffraction analysis which revealed that synthesised AgNPs were face centred, cubic and crystalline in nature and the sample contained silver nanoparticles in a high concentration. The antibacterial activity of the green AgNPs against E. coli isolates including the reference strain was tested and observed that the AgNPs exhibited antibacterial activity though a significant zone of inhibition was not obtained. Based on the results obtained, it could be concluded that silver nanoparticles could be efficiently synthesised from neem leaves and could be utilised in various fields especially in nanotechnology.

Keywords: *Silver nanoparticles, neem leaves, phytochemical screening, X-ray diffraction analysis, antibacterial activity, E. coli*

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Nanotechnology is one of the most active areas of research due to its wide use in the fields of opto-electronics, biosensors, bio-nanotechnology, biomedicine and many others. Synthesis of nanoparticles with the desired shape and size requires numerous physical and chemical techniques. These techniques though developed and are being widely used are not cost-effective and ecofriendly. Considering the biocompatibility, low toxicity and environment friendly nature, green synthesis has been regarded as one of the potential methods for the synthesis of nanoparticles (Verma and Mehata, 2016).

Silver has been widely accepted for its oligodynamic effect. Silver ions (Ag+) and its compounds have a mild toxicity toward animal cells but are cytotoxic to microorganisms, with severe biocidal effects on numerous bacterial species. Considering all these factors, the green synthesis of silver nanoparticles could be advocated using common medicinal plants like Neem *(Azadirachta indica),* Tulsi *(Ocimum sanctum)* and Indian gooseberry (*Phyllanthus emblica)* (Gupta *et al*., 2013). Terpenoids and flavonoids make up the majority of the reducing phytochemicals in neem leaf (Shankar *et al.,* 2004). The flavonoids also function as capping and stabilising agents in addition to reducing properties (Tripathy *et al*., 2009). The main benefit of utilising neem leaves is that they are a widely accessible medicinal plant and the antibacterial activity of the biosynthesised silver nanoparticle is augmented by capping with neem leaf extract. The major chemical constituents in the neem extract are nimbin (triterpenoid) and a polyphenolic flavonoid, quercetin (Joany *et al.,* 2015). In light of all these factors, the present work was undertaken to study the antibacterial activity of AgNPs synthesised from neem extract.

Fig. 1. Fresh neem leaves

Materials and methods

Plant collection and identification

The neem leaves were collected from the households in Mannuthy, Thrissur, Kerala. The collected plant material was identified and the authenticity was confirmed by the Department of Botany, St. Thomas College, Thrissur, Kerala.

Synthesis of silver nanoparticles

The study was conducted at the laboratory of the Department of Veterinary Public Health, CVAS, Mannuthy. Matured fresh neem leaves (Fig.1) were thoroughly washed in running water to remove the dirt and dust on the surface of the leaves. Twenty-five grams of finely chopped neem leaves were added to 100 mL of double-distilled water and boiled at 100°C for one hour in a magnetic stirrer kept at 80 rpm speed. The extract was cooled and filtered to obtain aqueous extract of 20 per cent concentration and stored at 4° C for further use. This solution was used for green synthesis of silver nanoparticle (AgNP) or reducing the silver ions (Roy *et al.,* 2017). Similarly 40 per cent concentration of neem extract was also prepared and stored by using 25 g neem leaves in 50 mL of double distilled water. The prepared neem leaf extracts (20 and 40 per cent) were mixed with 0.01 M and 0.1 M silver nitrate at 1:2 and 1:4 ratio and observed for colour change (Fig. 2 and Table 1). The whole procedure was carried out in a dark chamber.

The qualitative analysis of aqueous neem leaf extract was carried out to find out the phytochemical principles present using methods (Aathira *et al*., 2021; Prakash *et al*., 2021).

Test for terpenoids

Five milligrams of the extract was mixed with three milligrams of five per cent copper acetate solution. Development of green colour indicated the presence of terpenoids.

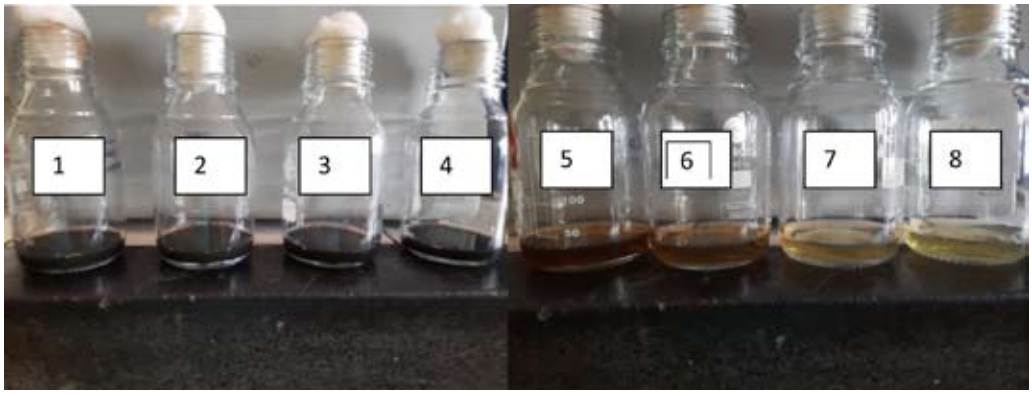

Fig. 2: Gradient of colour change for different concentration of AgNPs (Number indicate the serial number of Table 1)

Table. 1. Representation of gradient of colour change for different concentration of AgNPs as shown in Fig. 2

Sl. No.	Concentration of neem extract	Concentration of silver nitrate	Ratio of neem extract to silver nitrate
1.	40	0.1	1:4
2.	20	0.1	1:4
3.	40	0.1	1:2
4.	20	0.1	1:2
5.	40	0.01	1:4
6.	20	0.01	1:4
7.	40	0.01	1:2
8.	20	0.01	1:2

Mayer's test

To one millilitre of acid layer, a few drops of Mayer's reagent (1.358 g of mercuric chloride dissolved in 60 mL of water and poured into a solution of five g of potassium iodide in 10 mL of water and then made up the volume to 100 mL with distilled water) were added. Development of a creamy white precipitate indicated the presence of alkaloids.

Wagner's test

A few drops of Wagner's reagent (2.0 g of iodine and 6.0 g of potassium iodide dissolved in 100 mL of water) were added to one millilitre of the acid extract. Development of reddish brown precipitate indicated the presence of alkaloids.

Hager's test

To one millilitre of the acid extract, a few drops of Hager's reagent (one gram of picric acid dissolved in 100 mL of water) were mixed. Development of yellow precipitate indicated the presence of alkaloids About 5.0 mg of the filtrate was mixed with three millilitre of chloroform and then shaken with equal volume of concentrated sulphuric acid. Development of red colour indicated the presence of steroids.

Test for detection of phenolic compounds

Five mg of the filtrate was dissolved in one millilitre of water and five drops of 10 per cent ferric chloride was added to it. Development of dark blue colour indicated the presence of phenolic compounds.

Ferric chloride test

Two milligram of the filtrate was mixed with three millilitre of one per cent ferric chloride solution. Development of a blue, green or brown colour indicated the presence of tannins.

Gelatin test

About 0.5 g of the filtrate was mixed with a few drops of one per cent solution of gelatin containing 10 per cent sodium chloride. Development of a white precipitate indicated the presence of tannins.

Sodium hydroxide test

A small amount of the extract was mixed with one millilitre water and added five to six drops of sodium hydroxide solution (one per cent). Development of yellow colour indicated the presence of glycosides.

Foam test

A small amount of the extract was shaken with five millilitre of water. Development of the foam that persisted for ten minutes indicated the presence of saponins.

X-ray diffraction analysis of synthesised silver nanoparticles

The phase variety of the synthesised AgNPs were determined using the technique of X-ray diffraction spectroscopy (Rigaku X-ray diffractometer, Miniflex, UK) at the Centre for Materials for Electronics Technology (C-MET) Athani, Thrissur. The AgNPs were studied using CUK α radiation at a voltage of 30 Kilo Volt and a current of 20 milli ampere with a scan rate of 10° 2*θ*/min (*θ* is the Braggs angle in radians). Different phases present in the samples were determined by X' per high score software with search and match facility (Zhang *et al*., 2016).

Antibacterial activity of silver nanoparticles

The antibacterial activity of silver nanoparticles against standard culture of *E. coli* ATCC 13706 and three PCR confirmed isolates of *E. coli* isolated from water was tested using agar well diffusion method (Sajeshkumar *et al*., 2015). The Brain Heart Infusion Broth and Mueller Hinton (MH) agar plates were prepared according to standard protocol. The test organism culture was adjusted to 0.5 Mac Farland standard and was spread plated on the MH agar plates. A well of about six millimeter diameter was made with sterile cock borer by punching on each agar plate. A 30 µL volume of silver nanoparticles broth at different concentrations was added into the wells in the plates in triplicates. A negative control well was made and 30 µL of the sterile distilled water was added. A positive control was made by using 30 µL amoxycillin (10 mg/mL) in the agar plate. Plates were kept in a laminar flow cabinet for 30 min for pre diffusion of extract to occur and then incubated at 37ºC for 24 h. The resulting zone of inhibition was measured in three different directions keeping the midpoint of the well as the centre of the zone.

Results and discussion

Qualitative phytochemical screening of neem extract

The qualitative phytochemical screening of hot aqueous extract of neem leaves (Fig.3) revealed the presence of steroids, alkaloids, phenolic compounds, terpenoids and saponins (Susmitha *et al*., 2013) which supported this study (Fig. 3 and Table. 2). A study by Taiga and Friday (2009) on variations in phytochemical properties of aqueous extracts of neem leaves revealed that neem leaves extract contained alkaloid and flavonoid. Joshi *et al*. (2010) reported that phytochemical analysis of neem leaves extract revealed the presence of phenols, unsaturated sterols, triterpenes and saponins, respectively. Gupta *et al*. (2013) found that the aqueous extract of neem leaves contained flavonoids and terpeniods which is in agreement with this study. The biologically most active compound present in neem is azadirachtin which is a mixture of seven isomeric compounds labelled as azadirachtin A-G of which, azadirachtin E is the most effective. Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin (Hashmat *et al*., 2012).

X-ray Diffraction (XRD) analysis of silver nanoparticles

The XRD analysis of single replicate of the sample was performed to determine the crystal structure of green synthesised silver

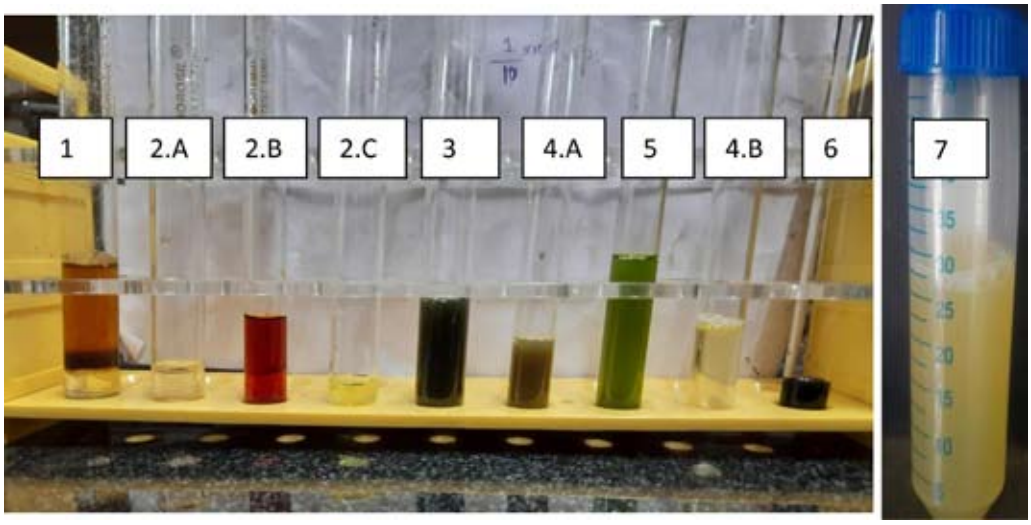

Fig. 3. Phytochemical screening of hot aqueous neem extract (Number indicate the serial number of Table 2)

Table. 2. Representation of phytochemical screening of hot aqueous neem extract

Sl.No.	Phytochemicals screened	Tests	Results
1	Steroids	Salkowski test	+
2.A	Alkaloids	Mayer's test	+
2.B	Alkaloids	Wagner's test	+
2.C	Alkaloids	Hager's test	+
3	Phenolic compounds	Ferric chloride test	+
4.A	Tannins	Ferric Chloride test	+
4.B	Tannins	Gelatin test	+
5	Glycosides	Sodium hydroxide test	-
6	Terpenoids	Copper acetate test	+
7	Saponins	Foam test	+

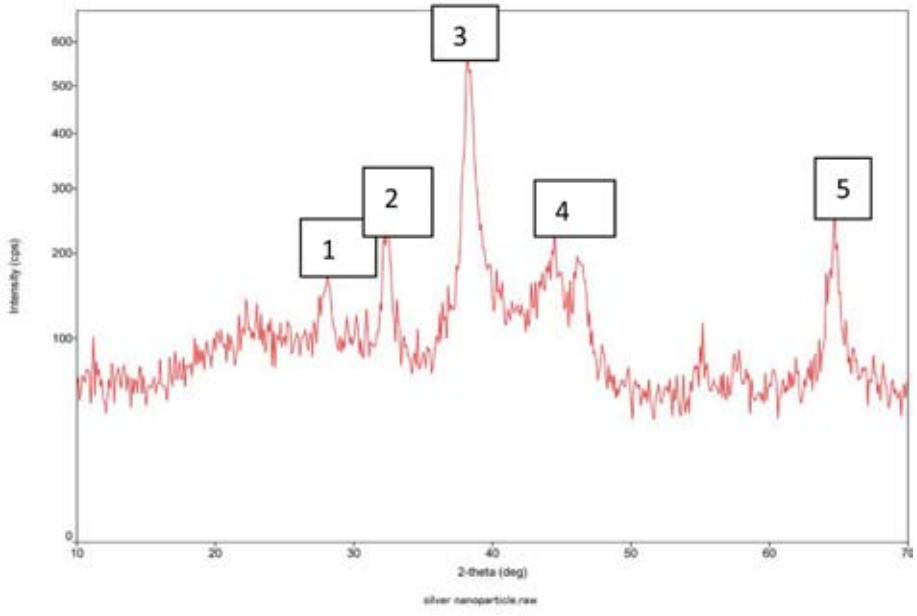

Fig. 4. XRD pattern of synthesised silver nanoparticles

Table: 3. Lists of peaks in XRD analysis

No.	2-theta (deg)	d (ang.)	FWHM (deg)	Int. I (cps deg)	Int. W (deg)	Chemical formula
1	28.10(12)	3.173(13)	0.77(13)	46(7)	1.0(3)	Unknown
2	32.28(3)	2.771(2)	0.67(7)	69(9)	0.74(19)	Unknown
3	38.27(2)	2.3502(13)	1.04(8)	522(16)	1.76(18)	Unknown
4	44.5(3)	2.035(13)	3.5(3)	248(22)	3.9(10)	Unknown
5	64.68(5)	1.4400(10)	0.87(10)	169(7)	1.4(2)	Unknown

nanoparticles. A number of Brag reflections with 2 theta values of 28.10, 32.28, 38.27, 44.5 and 64.68 corresponding to 1, 2, 3, 4 and 5 peaks, respectively (Fig.4 and Table. 3) were obtained using Rigaku X-ray diffractometer (Miniflex, UK). The peak corresponding to the plane 3 was more intense than the other planes (Fig. 4) which indicated that the silver nanoparticles synthesised were face centred, cubic and crystalline in nature and the sample contained silver nanoparticles in a high concentration (Keerthika *et al*., 2022).

The XRD pattern showed strong diffraction peaks at 37, 44, 66 and 77 degrees of 2 theta which indicated that the silver nanoparticles was formed by the reduction of Ag+ ions by the neem leaf extract. The sharp peaks indicate that nanoparticles synthesised were crystalline in nature. In addition to the Bragg peaks representative of face centred cubic silver nano crystals, no additional peaks and phase shift were observed which revealed that the synthesised material was pure. The average grain size obtained for synthesised nanoparticles was calculated by Debye-Scherrer formula and was found to be 8.625 nm (Shukla *et al*., 2010). Namratha and Monica (2013) performed XRD analysis of the green synthesised silver nanoparticles from neem leaves extract which showed the XRD data obtained was in the 2 theta range from 10° to 80° in step scan mode with 2 theta step of 0.02° and the diffractometer reading of their study indicated that the synthesised sample was silver nanoparticles and the smaller peaks contributed to impurities. This was in accordance with the diffractometer reading obtained in this study.

Antibacterial activity of silver nanoparticles

The results of the antibacterial effect of AgNPs on *E. coli* revealed that the green synthesised silver nanoparticles had antibacterial activity against the standard *E. coli* ATCC 13706 with zone of inhibition ranging from 6 to 16 mm diameter (Fig. 5). No zone of inhibition was visible around any of the wells created on the MHA plates that contained isolated strains of *E. coli* from water (Fig. 6). The AgNPs activity would depend not only on their concentration and size, but also on their shape which could probably decreased the antibacterial activity of AgNPs against isolated strains of *E. coli* from water (Gupta *et al*., 2013).

Fig. 5. Zone of inhibition for *E.coli* ATCC 13706

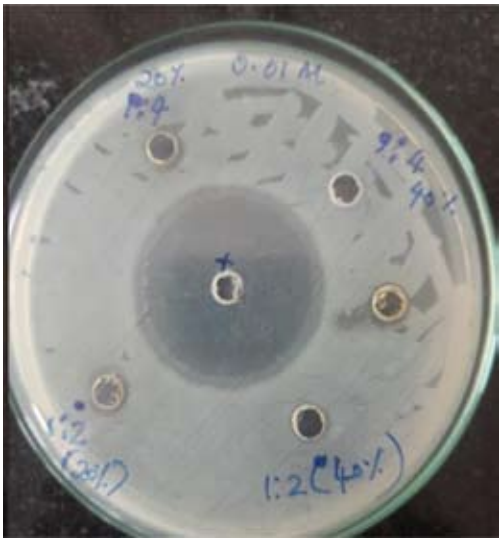

Fig. 6. Zone of inhibition for *E. coli* isolate from water

According to Ahmed *et al*. (2013), the antibacterial activity of the silver nanoparticles would be higher for the maximum interaction of silver nanoparticles with the bacterial cells at a grain size range of 1-10 nanometer diameter in XRD analysis of AgNPs. The XRD analysis of green synthesised silver nanoparticles had a grain size ranging from 20-100 nm (Namratha and Monica, 2013) which reduced the antibacterial effect of silver nanoparticles. Roy *et al*. (2017) evaluated the antimicrobial activity of the silver nanoparticles synthesised from neem leaves against *E. coli* where the inhibition zone of zero was obtained in the wells of 20µL and 50 µL of silver nanoparticles and a zone of 16.33 mm was observed in wells with 100 µL of silver nanoparticles. This concurred with the findings of this study that increase in concentration of silver nanoparticles increased its antibacterial activity. Tashi *et al*. (2016) reported that the antibacterial activity of silver nanoparticles was due to the formation of free radicals by the silver nanoparticles and these free radicals damage the bacterial cell wall resulting in death of bacteria. Morones *et al*. (2005) reported that the bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio which allows them to interact closely with microbial membranes and not merely due to release of metal ions in solution or in culture plates. The flavonoids and terpeniods in the neem extract were responsible for reducing silver salt to AgNPs which contributed to the antibacterial activity of silver nanoparticles (Shankar *et al.,* 2004).

Conclusion

Silver nanoparticles could be successfully synthesised from the commonly available neem plant. The XRD analysis revealed that the yield of silver nanoparticles obtained from the neem leaves was very high and also the particles were face centred cubic and crystalline in nature. The active principles present in the neem leaves like terpenoids and flavonoids were responsible for reducing the silver salts to silver nanoparticles which was responsible for the antibacterial activity of AgNPs. Green synthesised silver nanoparticles exhibited antibacterial activity against *E. coli*, though not at a considerable level. Hence, efficient utilisation of green synthesised silver nanoparticles if used at an appropriate concentration could be exploited in various fields of study.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Aathira, K.K., Kariyil, B.J., Dhanusha, G., Haima, J.S., Sujith, S., Shynu, M. and Nisha, A.R. 2021. Qualitative and quantitative analysis (GC-MS) of methanol extract of *Crataeva nurvala* stem bark. *J. Vet. Anim. Sci.* **52**(2): 135-141.

Ahmed, T., Imdad, S., Yaldram, K., Butt, N.M. and Pervez, A. 2014. Emerging nanotechnology-based methods for water purification: a review. *Desalin. Water Treat.* **52**(22-24): 4089-4101.

Gupta, A.K., Ahirwar, N.K., Shinde, N., Choudhary, M., Rajput, Y.S. and Singh, A. 2013. Phytochemical screening and antimicrobial assessment of leaves of *Adhatoda vasica*, *Azadirachta indica* and *Datura stramonium*. *J. Pharm. Biol. Sci.* **1**: 42-47.

Hashmat, I., Azad, H. and Ahmed, A. 2012. Neem (*Azadirachta indica* A. Juss)-A nature's drugstore: an overview. *Int. Res. J. Biol. Sci*. **1**(6): 76-79.

Joany, R.M., Shanthy, P., Mary, A.C.C. and Jeyasundari, J. 2015. Synthesis of silver nanoparticles using neem extract. *Int. J. Nano. Corr. Sci. Engg*. **2**(2): 1-6.

Joshi, B., Sah, G.P., Basnet, B.B., Bhatt, M.R., Sharma, D., Subedi, K., Janardhan, P. and Malla, R., 2011. Phytochemical extraction and antimicrobial properties of different medicinal plants: *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem). *J. Microbiol. Antimicrob.* **3**(1): 1-7.

Keerthika, V., Nisha, A.R., Nair, S.N., John, P., Ramnath, V. and Mohan, R.S.A. 2022. Phytochemical analysis and biosynthesis of silver nanoparticles from aqueous extract of seeds of *Sesamum indicum*. *J. Vet. Anim. Sci*. **53**(2): 262-268.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramirez, J.T. and Yacaman, M.J. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnol.* **16**(10): 2346-2353.

Namratha, N. and Monica, P.V. 2013. Synthesis of silver nanoparticles using Azadirachta indica (Neem) extract and usage in water purification. *Asian J. Pharm. Technol*. **3**(4): 170-174.

Prakash, K.P., Kariyil, B.J., Nisha, A.R. and Raji, K. 2021. Toxicity evaluation of aqueous soluble fraction of *Tamarindus indica* seed coat extract in albino wistar rats. *J. Pharmacog. Phytochem.* **10**(3): 94-99.

Roy, P., Das, B., Mohanty, A. and Mohapatra, S. 2017. Green synthesis of silver nanoparticles using *Azadirachta indica* leaf extract and its antimicrobial study. *Appl. Nanosci.* **7**(8): 843-850.

Sajeshkumar, N.K., Vazhacharickal, P.J., Mathew, J.J. and Joy, J. 2015. Synthesis of silver nanoparticles from neem leaf (*Azadirachta indica*) extract and its antibacterial activity. *CIB Tech J. Biotechnol.* **4**(2): 20-31.

Shankar, S.S., Rai, A., Ahmad, A. and Sastry, M. 2004. Rapid synthesis of Au, Ag and bimetallic Au coreAg shell nanoparticles using neem (*Azadirachta indica*) leaf broth. *J. Colloid Interface Sci.* **275**(2): 496-502.

Shukla, V.K., Pandey, S. and Pandey, A.C. 2010. Green synthesis of silver nanoparticles using neem leaf (*Azadirachta indica*) extract. In: Giri, P.K., Goswami, D.K., Perumal, A. and Chattopadhyay, A. (ed.), *AIP Conference Proceedings;* 9th to 11th December, 2009, Guwahati. American Institute of Physics. pp. 43-49.

Susmitha, S., Vidyamol, K.K., Ranganayaki, P. and Vijayaragavan, R. 2013. Phytochemical extraction and antimicrobial properties of *Azadirachta indica* (Neem). *Glob. J. Pharmacol.* **7**(3): 316-320.

Taiga, A. and Friday, E. 2009. Variations in phytochemical properties of selected fungicidal aqueous extracts of some plant leaves in Kogi state, Nigeria. *Am. Eurasian J. Sustain. Agric.* **3**(3): 407-409.

Tashi, T., Gupta, N.V. and Mbuya, V.B. 2016. Silver nanoparticles: Synthesis, mechanism of antimicrobial action, characterization, medical applications, and toxicity effects. *J. Chem. Pharm. Res.* **8**(2): 526-537.

Tripathy, A., Raichur, A.M., Chandrasekaran, N., Prathna, T.C. and Mukherjee, A. 2009. Process variables in biomimetic

synthesis of silver nanoparticles by aqueous extract of *Azadirachta indica* (Neem) leaves. *J. Nanoparticle Res.* **12**(1): 237-246.

Verma, A. and Mehata, M.S. 2016. Controllable synthesis of silver nanoparticles using Neem leaves and their antimicrobial activity. *J. Radiat. Res. Appl. Sci.* **9**(1): 109-115.

Zhang, X.F., Liu, Z.G., Shen, W. and Gurunathan, S. 2016. Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. *Int. J. Mol. Sci.* [online]. **17**(9): 1534. Available: https://www.mdpi.com/1422-0067/17/9/1534/pdf?version=1473764122 [13 Sept. 2016]. ■