Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 12, Issue 2, 2023, 59
https://doi.org/10.33263/LIANBS122.059

Synthesis of Silver Nanoparticles from *Vitex negundo* Plant by Green Method and their Bactericidal Effects

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Received: 10.02.2022; Accepted: 13.03.2022; Published: 23.03.2022

Abstract: Emerging nanotechnology has a significant impact on diverse fields such as medical, information technology, energy, advanced materials, textile, and many new upcoming areas. Out of all methods to produce nanoparticles, the green synthesis is the best one as it is cost-effective, non-toxic in nature, simple at feasible conditions. In our study, we are fabricating silver nanoparticles by a single-step green synthesis method using the water extract of the leaves of the *Vitex negundo* plant. The plant is well known for its rich supply of biologically active compounds. The plant consists of flavonoids, polyphenols, terpenoids, and many more metabolite compounds that are used to prevent many diseases. These help as a capping agent and stabilizing agent in the process of forming silver nanoparticles from silver nitrate solution. Thus, fabricated silver nanoparticles are subjected to characterization techniques such as UV-Visible spectroscopy, FTIR, DLS, SEM with EDX, and XRD to determine silver nanoparticles' size, structure, and shape. Further, the antimicrobial activity of these silver nanoparticles is evaluated against four bacterial strains. Two of them are gram-positive, and the other two are gram-negative. The gram-positive bacteria, namely *M. luteus* and B. subtilis have shown good activity compared to gram-negative bacteria, namely E. coli and *Salmonella. typhi*.

Keywords: silver nanoparticles; green synthesis; antibacterial activity; *Vitex negundo* plant.

1. Introduction

Nanotechnology is widespread in many fields of science. The present global market value of nanotechnology products is estimated at around 31.8 billion dollars, whereas it was 22.9 billion dollars in 2013 [1]. This shows the tremendous increase in the demand for nanotechnological products. Nanotechnology is applied in diverse fields such as medical (30%), advanced materials (29%), information technology (21%), energy (10%), automotive industries (5%), aerospace (2%), and textile (2%) and many other emerging hybrid fields [2]. There are various uses of nanoparticles within the medical field, such as drug delivery systems, biosensors, vaccines, disinfectants, and combination therapy [3]. In the world market of nanotechnology products, Asia stands first place comprising 52.3%, Europe precedes next with 28.4%, followed by North America with 14.7%, Latin America with 2.7%, and Africa with 1.9% [4].

Over the years, metal nanoparticles have been significantly explored due to their cost efficiency, eco-friendly nature, size-dependent, and electronic properties [5,6]. The field of nanotechnology is defined as the application of structures by controlling shape and size at the nanoscale (1-100nm) level [7,8]. Nanoparticles are divided into two types organic nanoparticles and inorganic nanoparticles. The organic nanoparticles include carbon nanoparticles, whereas inorganic nanoparticles include metallic and magnetic nanoparticles such as zinc (Zn), cadmium (Cd), gold (Au), silver (Ag), copper (Cu), cobalt (Co), titanium (Ti), iron (Fe), nickel (Ni) [9]. Out of all nanoparticles, the gold and silver nanoparticles have a popular demand because of their flexibility [10,11] and characteristics such as remarkable biochemical activity, catalytic activity, and atomic behavior [12]. There are many uses of silver nanoparticles for treating infections caused by burns, producing stainless steel materials, water treatment, preventing microorganisms on textile fibers, and dental materials [13].

Due to the potent bactericidal activity, silver nanoparticles are also being used in consumer products, cosmetics, antibacterial sprays, antibacterial detergents, cutting boards, socks, shoes, cell phones, laptop keyboards, and children's toys [14]. The new emerging branch of technology in the production of nanoparticles is green biosynthesis, which involves plant extracts, plant biomass, and microorganisms. This green biosynthesis is an eco-friendly and efficient alternative to physical and chemical methods, which usually is toxic [15,16].

The *Vitex negundo* is one such efficient plant. It is an aromatic shrub available in Asian countries. In the species of *Verbenaceae* one of the prominent genera is *Vitex*. The *Verbenaceae* genus comprising of 250 species distributed all over the world [17]. *Vitex negundo* L. (Verbenaceae) is richly available in the wastelands of tropical regions to temperate regions. This plant is a native of Japan, East Africa, South Asia, China, Indonesia, and South America. The seeds have been claimed to possess anti-inflammatory, analgesic, antioxidant, and anti-androgenic activity. The presence of lignans, flavonoids, and terpenoids has been previously reported [18].

Synthesis of silver nanoparticles is carried out through various methods. The main classification for the production of nanoparticles is broadly divided into two methods: the top-down approach and the bottom-up approach [19]. There are three categories in the sub-classification of the bottom-up approach: physical, chemical, and green synthesis [20,21]. In the concern with green synthesis, it can be defined as the process to produce nanoparticles from microbes and plant-mediated fabrication of nanoparticles [22,23]. A plant-mediated process is desired for us, which has three methods: phytochemical, living plant, and plant extract [24] again. This *Vitex negundo* plant extract facilitated the green synthesis of nanoparticles is adapted for the studies in a beneficial way due to their non-toxic nature [25,26].

Recently green synthesized silver nanoparticles are gaining huge attention for their anti-cancer activity and cytotoxic effect in the cell cycle. Besides these benefits, green synthesis is an efficient way of producing nanoparticles [27,28]. The latest review of the literature assumes silver nanoparticles' role in defeating P53 –positive and P-53 negative cancer cells. This indicated silver nanoparticles' potential to be anti-cancer drugs [29]. A few studies have been done to reveal the potential activities of the silver nanoparticles synthesized from *Vitex negundo* plant. It was applied to treat effluent water released from cracker industries [30].

The methanol extract of the plant was applied to produce silver nanoparticles and applied in treating nephrotoxicity in albino rats [31]. A neural network model is prepared to analyze the size of silver nanoparticles synthesized from the *Vitex negundo* plant [32,33]. The larvicidal activity of metal nanoparticles was investigated in a study to reveal that silver nanoparticles are potential in mosquitoes' destruction [34].

One of the recent studies has explored the anti-cancer activity of silver nanoparticles from this plant and recognized that colon cancer cell line (HCT15) is inhibited with the help of silver nanoparticles [35]. The gap to be filled in the production through an efficient, non-toxic green synthesis mechanism and functions of silver nanoparticles are yet to be revealed [36].

Our study intends to reveal the antibacterial activity of these silver nanoparticles in a simple and fast way. In the present study, we are concentrating on the synthesis of silver nanoparticles from the aqueous extract of leaf powder of plant *Vitex negundo*. The synthesis and characterization of silver nanoparticles are analyzed in the current report, which was not published earlier. The bactericidal activity of silver nanoparticles synthesized is evaluated with four different microorganisms.

2. Materials and Methods

The *Vitex negundo* plant is identified and collected from a nearby botanical garden at Warangal, Telangana, India. Silver nitrate purchased from Sigma Aldrich is used. All experiments through the study are conducted through double distilled water.

2.1. Synthesis of nanoparticles.

2.1.1. Plant extract preparation.

The silver nanoparticles are synthesized by reduction of silver nitrate ($AgNO_3$) solution with the help of biologically active compounds present in the *Vitex negundo* plant leaves. The plant is found in authorized botanical gardens nearby the forests of Warangal. The plant leaves are separated washed thoroughly under running tap water 3 times, followed by washing with double distilled water. The washed and cleansed leaves are left for drying at room temperature at room humidity for 10 days. Thus, dried leaves are ground into powder form with the help of an electric grinder. This leaf powder is stored and further used for extraction.

The *Vitex negundo* plant powder is measured for 10 grams and dissolved in 100ml of double distilled water stirred continuously at room temperature for 2 hours with the help of a magnetic stirrer. Thus, the formed aqueous extract is filtered with the help of Whatman filter paper. The obtained plant extract is stored.

2.1.2. Silver nitrate solution.

A 0.1mM silver nitrate solution is formed by adding 85mg of silver nitrate in 100ml of double-distilled water, scaled up for 400ml further. The solution is mixed by constant stirring at 120rpm speed on a magnetic stirrer for uniform silver nitrate solution.

2.2. Characterization of nanoparticles.

2.2.1. UV-Visible spectroscopy.

In this study, the plant *Vitex negundo* aqueous extract acts as a capping agent and reducing agent in synthesizing silver nanoparticles. Samples were taken at different time intervals as 2mins, 4mins, 6mins, 8mins, 10mins, 15mins, and 20mins to study the UV visible spectrum readings under

Shimadzu UV1800 spectrophotometer between 200-800nm wavelength. UV readings were also noted for the plant extract alone prior to forming the nanoparticles.

2.2.2. DLS.

The DLS (Dynamic Light Scattering) was performed under The Malvern – Zetasizer ZS90 instrument, confined to the refractive index of 0.135 for the observance of silver nanoparticles, maintained at room temperature, and time duration was set to 60seconds. Three trials under these conditions were conducted for each sample to obtain size distribution data of green synthesized silver nanoparticles.

2.2.3. FTIR.

The FTIR analysis is done by The Spectra100optica FTIR spectrometer, where approximately 5 grams of K-Br pellet is taken in a mortar and pestle. Addition of the few drops of sample to be analyzed. This is further pressed in a pellet maker to form a K-Br-sample pellet. This is installed in the spectrometer to run the sample for the readings. In our study, four samples are prepared and analyzed to estimate silver nanoparticles, the bio-compounds in the leaf extract, and the bio-compounds responsible for the production of silver nanoparticles. The readings are observed in between the 400-4000cm^{-1} Transmission graph.

2.2.4. SEM with EDS.

Scanning Electron Microscope (SEM) analysis was performed using the Hitachi S-3700N electron microscope. Conditions used were: extra high voltage 15 kV, working distance 11.3 mm, Magnification at x10.0k, display mode secondary electrons, high vacuum, and room temperature (30 ^{0}C). The silver nanoparticles are subjected to EDS (Energy Dispersive Xray Spectroscopy) by sprinkling the particles in a thin layer upon a gold-palladium coated copper grid and executed with SEM instrument with Thermo EDS attachment.

2.2.5. XRD.

The Pan analytical Xpert PRO diffractometer instrument is used to hold out XRD (X-Ray Diffraction) for the biologically synthesized silver nanoparticles. The operating conditions of the diffractometer are set to 40kV, Current (i)=30mA, to determine purity and crystalline nature of nanoparticles CuKα is used. The obtained graph is compared with JCPDS chart for evaluating the geometry of silver nanoparticles.

2.2.6. Antimicrobial activity.

We have tested the silver nanoparticles for antimicrobial activity with the help of four microorganisms, namely *Bacillus subtills, M. luteus, E. coli, S. typhi*. The Agar well diffusion method was used to proceed with the process as it is well known as an efficient method. The microorganisms were inoculated in the nutrient broth medium for 24 hours. The turbidity is visible there is confirmation of microorganisms' subculture growth in fresh broth; these are further used as test organisms. The agar plates are prepared with the help of nutrient agar media, the test organisms in the volume of 50 µl are spread over the solidified agar plates using the spread plate technique.

As the agar plates are solidified, wells of diameter 1cm are formed evenly in all the agar plates., in which the sample of silver nanoparticle, standard streptomycin antibiotic, and plant extract as control is added in the same amount as 100µl remained untouched in Laminar Air Flow unit (LAF). These plates are further incubated in a BOD incubator for 18-24 hours to evaluate antimicrobial activity. Many potential mechanisms exist to establish the antibacterial activity of silver nanoparticles. Reactive oxygen species (ROS) are generated from the silver nanoparticles that kill the bacteria through electrostatic forces [37]. Thus, silver nanoparticles are responsible for inhibiting bacterial growth; potential inhibitory activity is due to the surface area of nanoparticles [38]. The results show that bactericidal activity is due to the silver nanoparticles when compared to control and test groups. Thus, eco-friendly silver nanoparticles synthesized from *Vitex negundo* plant are potential bactericidal agents.

3. Results and Discussion

3.1. Synthesis of silver nanoparticles.

The pre-prepared 100 ml of $AgNO_3$ (10^{-1}M) solution is added to the aqueous plant extract and left for continuous stirring at room temp for 2 hours, followed by the incubation period of 24 hours. The color change from pale yellow to dark brown indicates the formation of nanoparticles from the figure 1.

Figure 1. Observation of color change indicating silver nanoparticles synthesis.

3.2. Characterization.

3.2.1 UV-visible spectrum analysis.

The UV visible spectrum was taken in a range of 300 – 700nm wavelength. For the plant extract alone, before adding silver nitrate solution, a peak was observed at 325nm as suggested from the reference as 343nm. The observed peak values of silver nanoparticles produced from the whole aqueous extract are noted at regular time intervals, as shown in figures 2 and 3. It was observed that the absorbance wavelength of the mixture shifted to 420 nm due to the reaction between plant extract with silver nitrate solution [39].

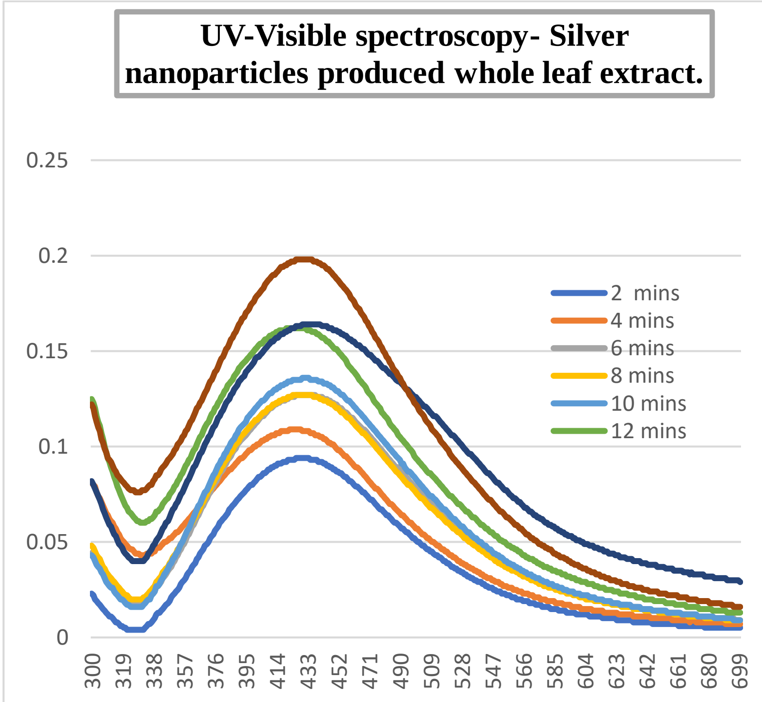


Figure 2. UV- Visible spectroscopy readings of silver nanoparticles produced from water extract of *Vitex negundo* leaf.

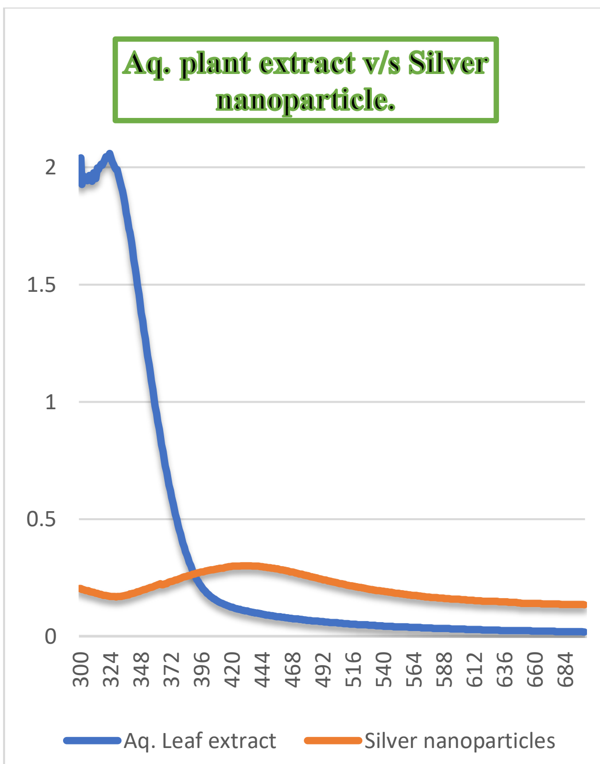
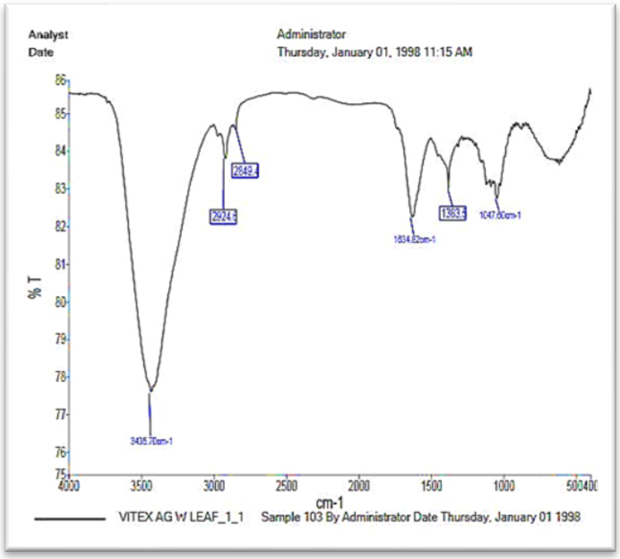


Figure 3. UV- Visible readings of plant extract v/s silver nanoparticles.

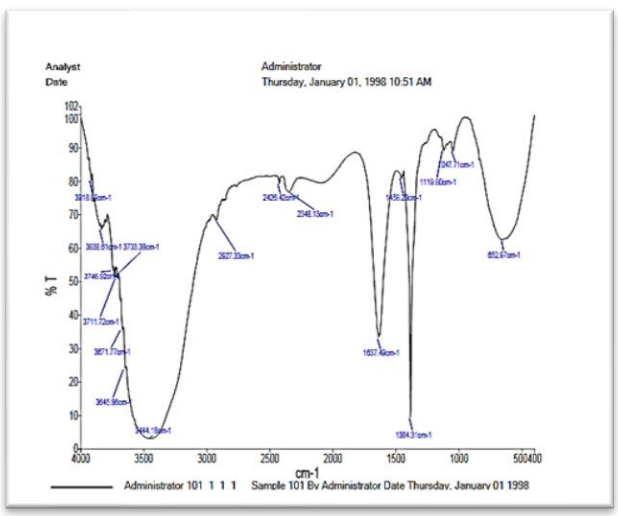
3.2.2. FTIR analysis.

The Spectra100optica FTIR spectrometer of PerkinElmer is used to measure FT-IR spectrum readings. The two samples are analyzed. From the inference of figure 4 it is observed that the aqueous extract of leaf sample alone had peaks at 1049 cm^{-1}, 1384.77 cm^{-1}, 1628.97 cm^{-1}, 2924.6 cm^{-1}, and

3435.39 cm^{-1}. Whereas the silver nanoparticles produced from the leaf powder aqueous extract many peaks are observed such as 652.97 cm^{-1}, 1047.71 cm^{-1}, 1119.80 cm^{-1}, 1384.31 cm^{-1}, 1458.28 cm^{-1}, 1637.49 cm^{-1}, 2348.13 cm^{-1}, 2426.42 cm^{-1}, 2927.33 cm^{-1}, 3444 cm^{-1}, 3645 cm^{-1}, 3671 cm^{-1}, and 3711 cm^{-1}. This implies that the biologically active compounds from the plant extract are responsible for the formation of silver nanoparticles. These peaks indicate the presence of functional groups such alkanes(C-H) at 1384 cm^{-1}, sulfoxide(S=O) at 1046 cm^{-1}, alkene (C=C) at 1634 cm^{-1}, and hydrogen (O-H) bonds at 3400 cm$^{-1.}$



(**a**)



(**b**)

Figure 4. The FTIR analysis of water extract of (**a**) *Vitex negundo* leaf; (**b**) silver nanoparticle formed.

3.2.3. DLS.

Silver nanoparticles with aqueous extract of whole leaf and leaf powder were characterized using DLS (Dynamic Light Scattering) analysis. From figure 5 it is observed that the particle sizes start from 10nm to 100nm, with an average size of 40nm. Relatively good symmetry of size distribution in DLS analysis indicates nanoparticles uniformity. The present analysis data shows an increase in average particle distribution.

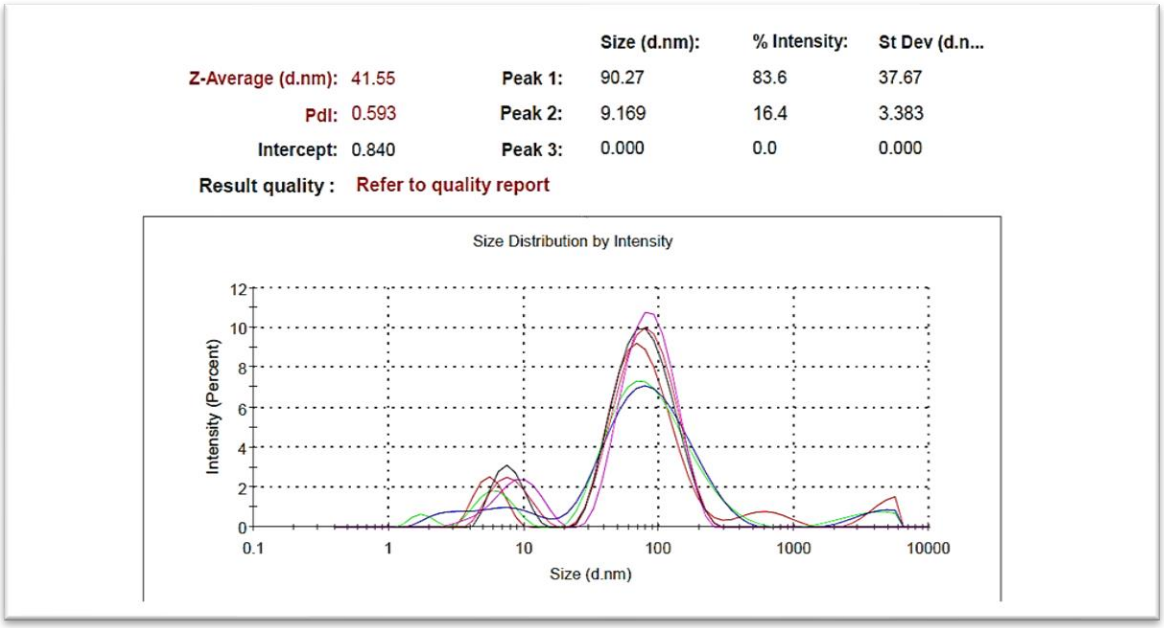


Figure 5. DLS analysis of silver nanoparticles.

3.2.4. SEM analysis.

Scanning Emission Microscopy (SEM) image was used to study the surface morphology, size, and shape of silver nanoparticles formed with an aqueous extract of *Vitex negundo* leaf. It was observed in figure 6 that nanoparticles are spherical with approximately 90-120nm particles. SEM Condition was measured at SEM hv=15kV Magnification=x10.0k WD=11.3mm. From figure 7 The EDS observation helps in the elemental proof of the silver element. The EDS conditions were maintained at Kv=15, magnification=8633, Amplitude time(μs)=7.68, and resolution at 1334.

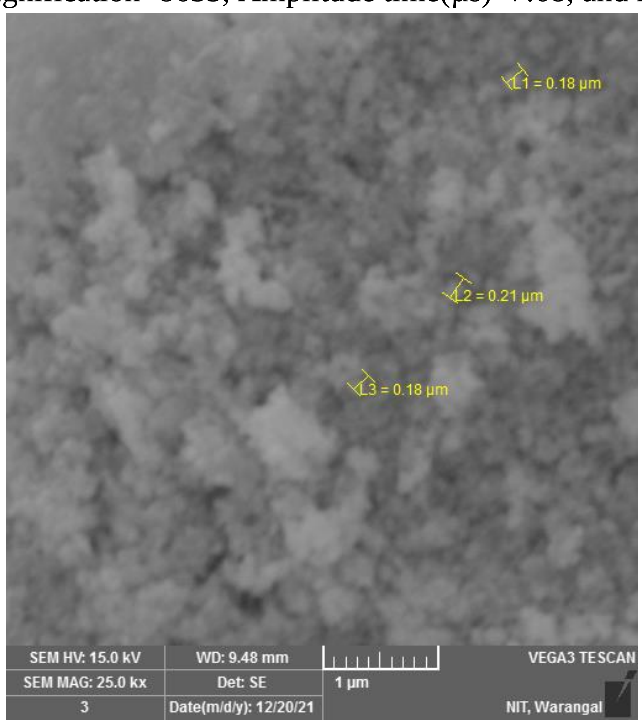
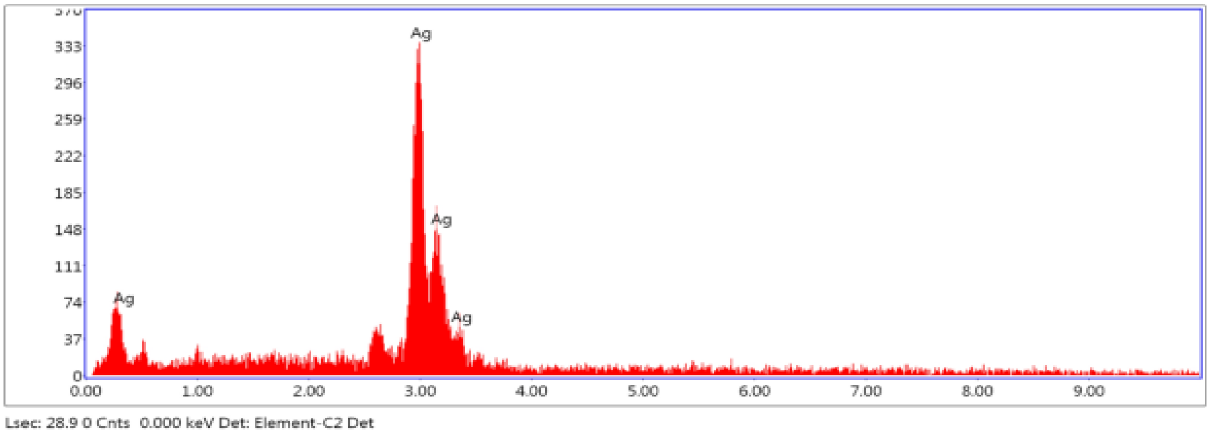


Figure 6. SEM analysis of silver nanoparticles formed from aqueous extract of *Vitex negundo* leaf.



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	R	A	F
AgL	100.00	100.00	126.23	4.47					

Figure 7. EDS results show the presence of the silver element.

3.2.5. XRD.

The XRD (X-Ray Diffraction) pattern recorded for biosynthesized silver nanoparticles from the plant leaf and aqueous powder extract clearly shows the intense peaks in the whole angle spectrum ranging from 10 to 80. XRD patterns of nanoparticles exhibit several size-dependent features leading to peak position, heights, and widths. The present study shows that the silver nanoparticles formed by reducing Ag+ ions by the *V. negundo* leave extract are of crystalline nature by observing the peaks in figure 8 at 38.12º,44.3º,64.4º,77.4º. The number of Braggs reflections obtained in the current study (1 1 1), (2 0 0), (2 2 0) corresponded to the diffraction facets of silver and indexed for the presence of crystalline silver nanoparticles.

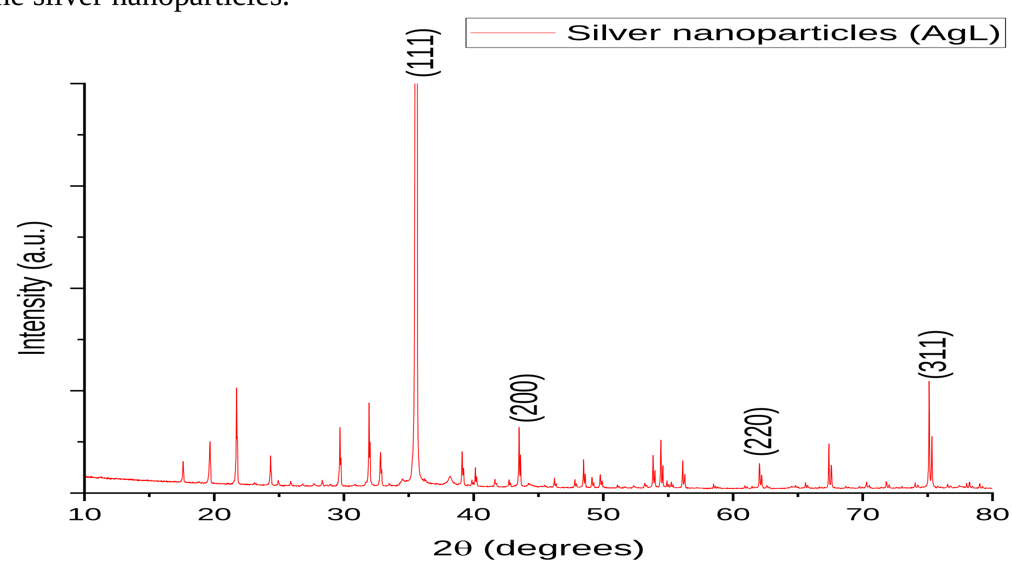


Figure 8. XRD plots for silver nanoparticles produced from Aq. Extract of *Vitex negundo* Leaf.

3.3. Antimicrobial activity.

As shown in the below figure 9, the silver nanoparticles had shown effective antibacterial activity against the four microorganisms, namely *E. coli, M. luteus, B. subtills*, and *S. typhi*, in comparison with the standard as streptomycin. The silver nanoparticles were suspended in the double-distilled water, and 100µl of the samples were added to the agar wells to evaluate the bacteriostatic activity. A zone of inhibition is the diameter of the area where bacteria do not grow because of the sample's bacteriostatic effect against the microorganism. The zone of inhibition (ZOI) is noted as 6mm for silver nanoparticles whereas 8mm for the streptomycin against *S. typhi* (gram-negative).

The other gram-positive microorganism Subtills has shown ZOI of 7mm, which is half of the standard with a ZOI of 14mm. For the gram-positive microorganism, *M. luteus*, silver nanoparticles showed around 6mm ZOI, whereas 9mm for the streptomycin. The bacteriostatic activity of silver nanoparticles against other gram-negative microorganisms *E. coli* showed ZOI of 5mm, which is half of the standard with ZOI 10mm. Thus by comparative analysis, it is clear that silver nanoparticles produced from *Vitex negundo* have an effective bactericidal activity against *S. typhi.* It is observed clearly from the results that the green synthesis of silver nanoparticles from *Vitex negundo* is antibacterial.

Table 1. Antibacterial evaluation by agar-well method indicating zone of inhibition.

Type of microorganism	**Name of microorganism**	**Zone of inhibition (measured in mm)**		
		Negative control (Plant extract)	**Silver nanoparticles**	**Positive control(streptomycin)**
Gram-negative	E. Coli	0	5	10
	S. Typhi	0	6	8
Gram-positive	M. Luteus	0	7	9
	B. Subtilis	0	7	14

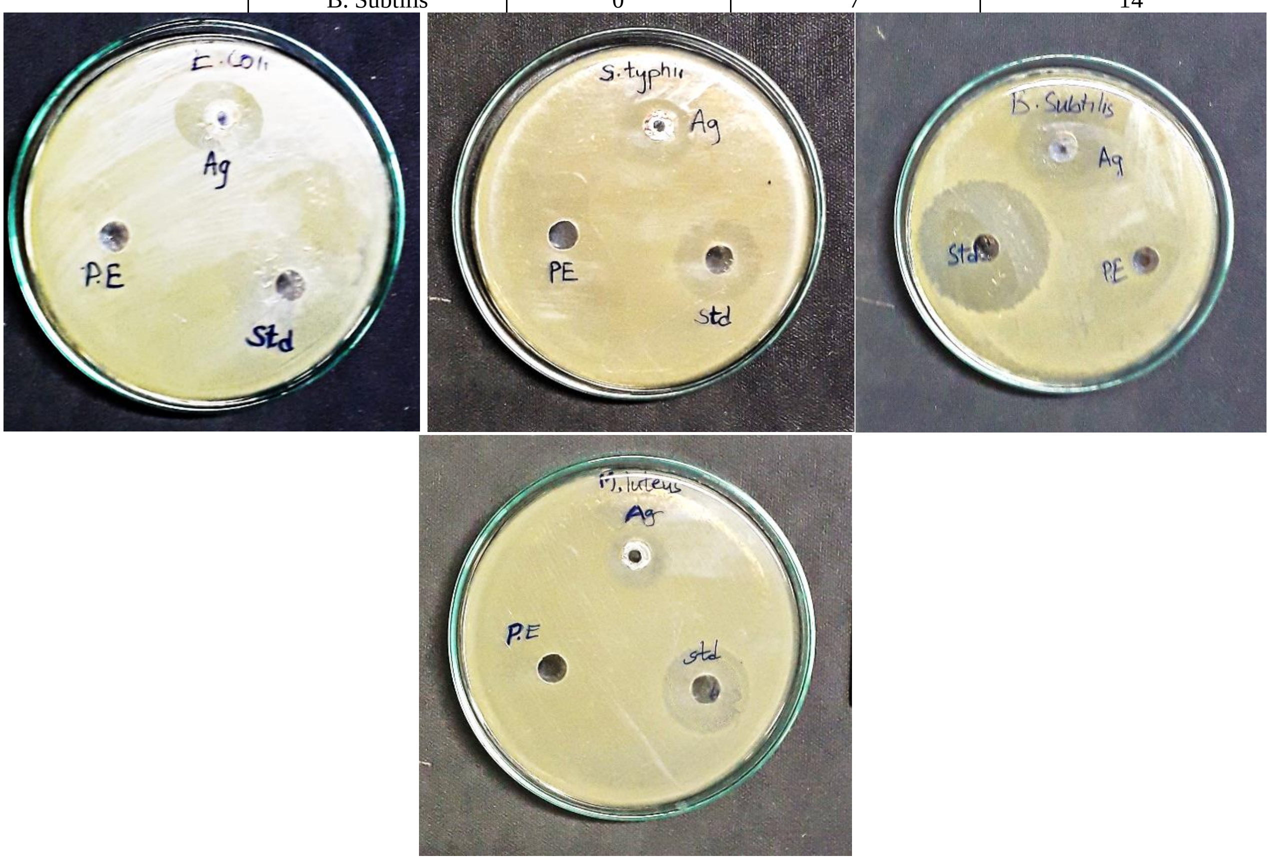


Figure 9. Antimicrobial activity of the plant extract in comparison with standard antibiotic streptomycin.

4. Conclusions

The method of green synthesis is a novel method to form silver nanoparticles. Green synthesis is adapted rather than highly toxic synthetic methods of synthesizing nanoparticles. This study is focused on the simple green synthesis of nanoparticles using an aqueous extract of *Vitex negundo* leaf. The synthesized nanoparticles were characterized by biophysical methods such as UV-Visible Spectrophotometer, FTIR, DLS SEM, EDS, and XRD. Initially, the color change appears from colorless in the silver nitrate solution to pale yellow on the addition of plant extracts, then eventually to dark brown color on vigorous mixing, indicating the formation of silver nanoparticles. Thus, the formed silver nanoparticles were subjected to UV-visible spectroscopy, and at 420 nm, a peak is observed confirming the formation of silver nanoparticles. The FTIR graph analysis stipulates the reaction of bioactive compounds in the plant help in the oxidation of silver nitrate solution into silver nanoparticles. The DLS study of the nanoparticle estimated size to be around 40nm evenly distributed. Further through SEM images, it is confirmed that the shape of the particle is spherical, and the size of the particle was approximately 40nm, whose diameter is scaled to 90-110nm. The EDS study is done along with SEM that summarizes the presence of silver as a major element in the nanoparticles. The XRD study supports the shape of nanoparticles as spherical and fcc shape unit cells. The overall results interpret those silver nanoparticles are spherical with 40-100 nm size. The antimicrobial activity is evaluated for the silver nanoparticles. The observations from antibacterial studies reveal that the bactericidal nature of silver nanoparticles is more effective in gram-positive bacteria compared to gram-negative bacteria. Further, the green synthesized silver nanoparticles can be subjected to develop into efficient drug delivery systems as they are smaller in size. The nanoparticles reveal their true potential as a clinical drug.

Funding

This research hasn't received any kind of funding from external sources.

Acknowledgments

The authors declare no acknowledgments.

Conflicts of Interest

The authors declare no conflict of interest.

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