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Research Article

GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM THE LEAF EXTRACT OF *ADHATODA VASICA* NEES. AND ASSESSMENT OF ITS ANTIBACTERIAL ACTIVITY

BHUMI G*, LINGA RAO M, SAVITHRAMMA N

Department of Botany, S.V. University, Tirupati, Andhra Pradesh, India. Email: Bhumi.gaddala10@gmail.com

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ABSTRACT

Introduction: Green synthesis of nanoparticles has been an exploring research topic in recent days due to their advanced uses in biomedical, chemical, and related fields.

Objective: The objective of the present study was to rapidly synthesize silver nanoparticles (AgNPs) using *Adhatoda vasica* leaf extract. The nanoparticles obtained have been characterized with various techniques like ultraviolet-visible spectrum, Fourier transform (FT) infrared spectrometry, scanning electron microscopy, energy dispersive spectroscopy, X-ray diffraction, atomic force microscopy, and FT-Raman spectroscopy.

Results: These techniques showed the formation of AgNPs with an average size of 21.1-29.1 nm. Phytochemicals present in the plant were responsible for the quick reduction of Ag^+ ions to Ag^0 nanoparticles. The synthesized AgNPs had the potential to mitigate the bacterial and fungal cell proliferation particularly *Staphylococcus aureus, Pseudomonas auroginosa, Bacillus thuringiensis, Escherichia coli, Klebsiella pneumoniae* and fungal species were *Aspergillus niger, Aspergillus flavous, Pencillium chrysogenum,* and *Trichoderm harizanum*. The results were compared with ciprofloxacin and fluconazole as positive controls.

Conclusion: *A. vasica* being a crucial medicinal plant widely used in the treatment of cancer, the present study shows that the plant can be used as best source for synthesis of AgNPs for further research in the cancer field.

Keywords: *Adhatoda vasica*, Phytochemials, Phenols, Fourier transform Raman spectroscopy, Atomic force microscopy.

INTRODUCTION

Nanotechnology can be defined as a research for the design, synthesis, and manipulation of structure of particles with dimension smaller than 100 nm. Bio-nanotechnology combines biological principles with physical and chemical approaches to produce nanosized particles with specific functions. It also represents an economic substitute for chemical and physical methods of nanoparticles formation. This method of synthesis can be divided into intracellular and extracellular [1] with three main steps, which must be evaluated based on green chemistry perspective. They are: (1) Selection of the solvent medium, (2) selection of environmentally being reducing agent, (3) selection of non-toxic substances for the silver nanoparticles (AgNPs) stability.

Metallic nanoparticles have a high definite surface area and a high fraction of surface atom. Extensive studies are done on this because of their exceptional physical and chemical characteristics including catalytic properties, optical properties, electron properties, etc. Nanoparticles fall in the transition zone between individual molecules and the corresponding bulk materials, which generate both positive and negative biochemical effects in living cells [2]. AgNPs were used in the broad range of applications like drug delivery [3], food industries [4] anti-inflammatory [5], antimicrobial [6], anti-cancer [7] and larvicidal [8]. Studies on the green synthesis of AgNPs have been recently reported using the extracts of plants such as *Svensonia hyderobadensis* [9], *Shorea tumbuggaia* [10] and *Allamanda catharatica* [11], etc.

Adhatoda vasica is an important medicinal plant belongs to the family Acanthaceae. The plant parts are reported to be an expectorant [12], abortifacient [13], antimicrobials [14], antitussive [15] and anticancer [16]. Several compounds had been isolated from this plant to treat cancer. The phytochemical research based on ethanopharmacological information is an effective approach in the discovery of new anti-infective agents from higher plants. The qualitative and quantitative analysis is very essential for identifying and quantification of active principles present in the medicinal plants, which is important for medicinal action and drug preparation. The phytochemicals responsible for the synthesis of nanoparticles are terpenoids, flavonoids, phenols, carbohydrates, saponins, alkaloids, and proteins [17]. The term phenolic compound embraces a wide range of plant substances that bear in common an aromatic ring with one or more hydroxyl substituents. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The present study aimed at the green synthesis of AgNPs from the leaves of *A. vasica* and the characterization of the so formed nanoparticles with scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDAX), X-ray diffraction (XRD), Fourier transform infrared (FTIR), atomic force microscopy (AFM), ultraviolet-visible (UV-Vis) spectroscopy, and FT-Raman spectroscopy. The synthesized AgNPs were further evaluated for antimicrobial activity against *Bacillus thuringiensis*, *Escherichia coli, Staphylococcus aureus, P. aeuroginosa* and *Klebsiella pneumoniae* and antifungal activity against *Aspergillus niger, Aspergillus flavus, Pencillium chrysogenum,* and *Trichoderm harizanum*.

METHODS

All the chemicals and reagents used in the present study were of analytical grade. Silver nitrate was purchased from Sigma-Aldrich Chemicals. The glassware were washed in dilute nitric acid and thoroughly washed with double distilled water and dried in hot air oven.

Preparation of plant extract

A. vasica leaves were collected from S.V. University, Botanical Garden, Tirupati, Andhra Pradesh, India. The leaves were washed thoroughly thrice with distilled water and were shade dried for 10 days. The fine powder was obtained from dried leaves using the kitchen blender. The leaf powder was sterilized at 121°C for 5 minutes. 5 g of powder were

taken into 250 ml conical flask and 100 ml of sterile distilled water was added and boiled for 15 minutes at 100°C. Then the leaf extract was collected in another conical flask by standard filtration method.

Phytochemical analysis

The phytochemical analysis of secondary metabolites like alkaloids, flavonoids, sterols, triterpenes, proteins, saponins, phenols, and glycosides were carried out according to the modern techniques of plant analysis of Harborne [18].

Synthesis of AgNPs

A volume of 1 mM $AgNO_3$ solution was prepared and stored in dark colored bottle. The leaf extract was added to 1 mM $Ag(NO_3)_2$ solution. The color change of the solution from yellow to brown indicated that the AgNPs were synthesized from the leaf. This was used for the characterization and antimicrobial activity.

Characterization of AgNPs

The synthesized AgNPs were characterized by UV-Vis spectroscopy. The AgNPs were confirmed by measuring the wavelength of reaction mixture in the UV-Vis spectrum of the Perkin Elmer spectrophotometer at a resolution of 1 nm (from 300 to 600 nm) in 2 ml quartz cuvette with 1 cm path length. SEM analysis was carried out by using Hitachi S-4500 SEM machine. Elemental dispersive analysis of the prepared samples was examined through energy dispersive analysis using Hitachi S-3400 SEM instrument equipped with thermo EDAX attachments. Surface topology of the formulated AgNPs was studied by AFM by using Nano Surf® AG, Switzerland, product: BTO 2089, BRO, Machine. The particle size and nature of the AgNPs were determined using XRD with the help of Shimadzu XRD 600/6100 model with CuK ∝ radians at 2θ angle. The functional groups of AgNPs identified by FTIR using Thermo Nicolet nexus 670 spectrometer, FT-Raman spectra were obtained on a Bruker RFS-100 Instrument.

Antibacterial activity

The clinical pathogenic strains of *S. aureus* ATCC 6538, *Pseudomonas aeuroginosa* ATCC 15442, *E. coli* ATCC 25922, *B. thuringiensis* ATCC 10792 and *K. pneumoniae* ATCC 4352 disc diffusion method was carried out by using standard protocol [19]. Overnight bacterial cultures (100 µL) was spread over Muller Hinton Agar (Hi Media Laboratories Private Limited, Mumbai, India) plates with a sterile glass L-rod. 100 µL of each extract was applied to each filter paper disc Whatman No. (5 mm dia) and allowed to dry before being placed on the agar. Each extract was tested to triplicate and the plates were inoculated at 37°C for 24 hrs after incubation. The diameter of inhibition zones was measured and tabulated.

Antifungal activity

Antifungal activity of AgNPs was determined using Agar-well diffusion method [20]. Potato dextrose agar (PDA) plates were prepared, and then the medium was kept for sterilization and solidification, after solidification fungal cultures were swabbed on these plates. Wells of 5 mm diameter were made on PDA plates using a cork borer and 50 µL of nanoparticles solution was poured on to each well on all plates. After incubation at 37°C for 3 days zone of inhibition was measured.

RESULTS

The phytochemical analysis of *A. vasica* leaf extract (Table 1) revealed that this plant contains biomolecules, including alkaloids, proteins, tannins, glycosides, steroids, phenols, reducing sugars, carbohydrates, proteins, and lignins. These phytochemicals are responsible for the immediate reduction of ions and formation of AgNPs [21] shown in Fig. 1 particularly phenols and alkaloids which could be used as reductant to react with silver ions and therefore used as Scaffolds to direct the formation of AgNPs in the solution [22]. The chemical reactions, which proceed in the aqueous extract may be as follows.

$$Ag(NO_3)_2 + Plant\ aqueous\ extract \longrightarrow Ag^+ + NO_3 \quad \text{..... (1)}$$

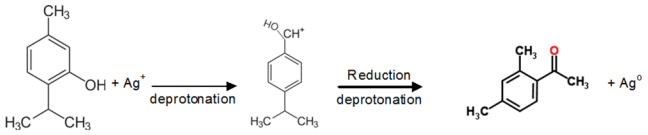
$$Biomolecule\ of\ phenols + Ag + e^- \xrightarrow{Reduction} Ag^0 \quad \text{..... (2)}$$

(E.g. Thymol – $C_{10}H_{14}O$)

The formation of AgNPs is Green method. It involves the formation of the atom by the nucleation process; which is followed by the formation of nanoparticles by the aggregation. In the nucleation process, the hydrated electrons (e^-_{aq}) behaves as strong reducing agent and can reduce Ag ions (Ag^+) into zero-valent Ag atoms (Ag^o) reaction-2.

$AgNO_3$ separated to Ag^+ and NO_3^- ions in the aqueous solution as shown in Equation-1. The solvated electrons, i.e., $e^-_{(aq)}$ and H atoms are strong reducing agents so that they easily reduced silver ions down to the zero valence state [2].

The term phenolic compound includes a wide range of plant substances that bear in common an aromatic ring with one or more hydroxyl substituents. The antioxidant activity of phenolic compounds is mainly due to their redox properties. This property in absorbing and neutralizing free radicals, quenching singlet, and triplet oxygen or decomposing peroxides. Thymol is a naturally occurring phenolic compound in *A. vasica* with antioxidant properties. Hydroxyl group bonded to benzene ring is much more acidic than the hydroxyl group to alcohol. This resonance in phenol, which reduces Ag quickly. Because of the resonance in phenol, the oxygen atom acquires a positive charge, which weakens the oxygen hydrogen bond and facilitates the release of a proton. The deprotonation of phenol forms phenoxide ion or the phenate which also exists as a resonance. Hence, both phenol and phenoxide ions are stabilized by resonance. However, the resonating structure of phenol involves the separation of negative and positive charges.



(Thymol) (Intermediate) (2, 4-Dimethyl acetophenone)

Visual inspection

When *A. vasica* leaf extract was exposed to Ag^+ ions [$(AgNO_3)_2$1 mM)], reaction mixture turned to yellowish brown and then to dark brown in color, which was in agreement with the previous studies, and was considered as the formation of AgNPs [23]. The appearance of dark brown seems to be due to excitation of surface plasmon resonance in the nanoparticles.

UV-Vis spectra analysis

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 1, 4, 8, and 24 hrs time course of the reaction was obtained.

Table 1: Phytochemical screening of *A. vasica* leaves

S. no	Secondary metabolites tested	Indicate of phytochemicals
1.	Flavonoids	–
2.	Tannins	+
3.	Glycosides	++
4.	Steroids	+
5.	Saponins	–
6.	Phenols	++
7.	Triterpenoids	–
8.	Reducing sugars	++
9.	Anthocyanins	–
10.	Carbohydrates	+
11.	Proteins	+
12.	Alkaloids	++
13.	Fatty acids	–
14.	Lignins	++
15.	Anthraquinones	–

+: Present, –: Absence, *A. vasica: Adhatoda vasica*

UV-Vis spectra show (Fig. 2) the peaks approximately at 417, 421, 424 and 438 nm clearly indicating the formation of AgNPs in *A. vasica*. By plotting UV-Vis absorption of the reaction mixture against time, time course of the reaction was obtained. The curve shows increased absorbance in various time intervals (1, 4, 8, and 24 hrs) and the peaks were noticed at 421 nm corresponding to the surface plasmon resonance of AgNPs. The observation indicated that during the reaction period, an increase in absorbance was observed in this wavelength, which can be due to the increase in production of colloidal AgNPs [24].

SEM analysis

In SEM analysis, a film beam of electron (10-40 KeV) is caused to scan the sample in a series of parallel tracks. These electrons interact with an electron in the sample, producing various signals that can be detected and that contain information about the samples. SEM analysis was used to determine the structure of the reaction products that were formed. A SEM was employed to analyze the shape of the AgNPs that were synthesized by green method. SEM image (Fig. 3) has showed individual AgNPs, as well as a number of aggregates relatively spherical in shape nanoparticles formed with a diameter range 22.1-29.1 nm.

EDAX analysis

The elemental composition of green synthesized AgNPs was analyzed through EDAX. From Fig. 4, the EDAX spectrum confirms the formation of elemental silver peak at 2.0-4 KeV, which is in congruence with the major emission peaks specified for metallic silver along with this, small peaks of C, O, Mg, Si, Au, and Ca were also arisen due to the capping of AgNPs by biomolecules of *A. vasica* leaf extract. The carbon and oxygen spots in the examined samples confirm the presence of stabilizers composed of alkyl chains. The spectra obtained during EDAX studies were used for carrying out the quantitative analysis. Qualitative analysis shows the presence of C, O, Mg, Si, Ag, Au, and Ca, the contents of which amounted to respectively 24%, 6%, 0.92%, 0.6%, 57%, 0.89%, and 8.82%.

Fig. 1: Synthesis of silver nanoparticles (color change) by using leaf extract of *Adhatoda vasica*, (a) plant extract, (b) treated with Ag $(NO_3)_2$

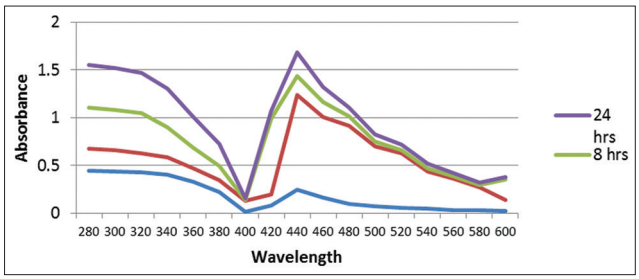


Fig. 2: Ultraviolet-visible images of silver nanoparticles synthesized by using leaf extract of *Adhatoda vasica*

AFM analysis

AFM is a unique technique for providing sub-nanometer resolution at a reasonable signal-to-noise ratio under physiological conditions. Surface topology of the formulated AgNPs was studied by AFM analysis (Fig. 5a and b). The micrographs clearly indicate that the formulated

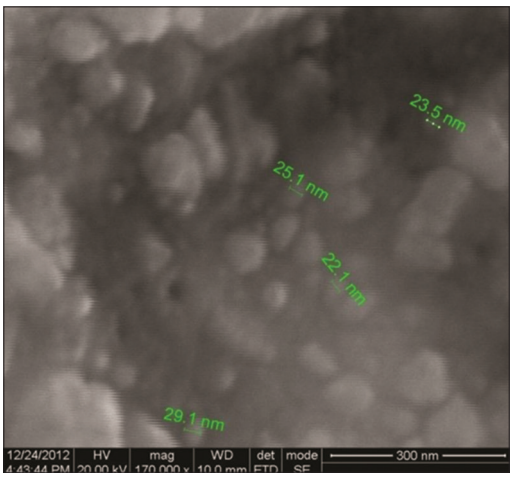


Fig. 3: Scanning electron microscopy images of silver nanoparticles synthesized by using leaf extracts of *Adhatoda vasica*

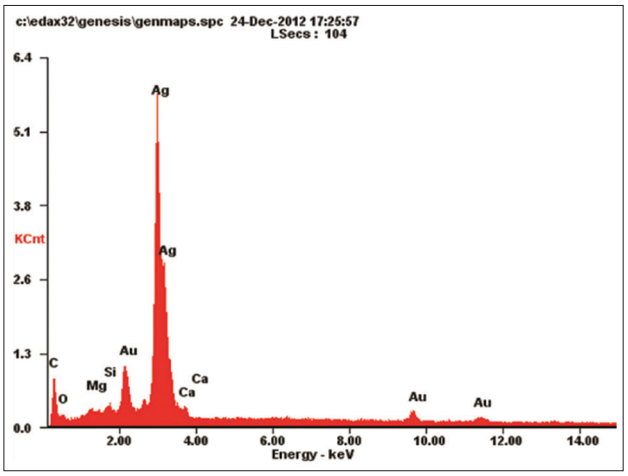


Fig. 4: Energy dispersive X-ray analysis images of silver nanoparticles synthesized by using leaf extracts of *Adhatoda vasica*

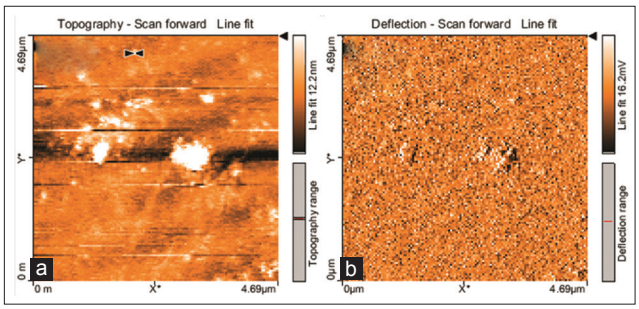


Fig. 5: Atomic force microscopy images of silver nanoparticles (AgNPs) synthesized by using leaf extracts of *Adhatoda vasica*, (a) Two-dimensional image of AgNPs, (b) three-dimensional view of the surface of AgNPs

AgNPs possess irregular shape and have the calculated size in the range of 58 nm.

XRD analysis

XRD analysis is used to determine the phase distribution, crystallinity, and purity of the synthesized nanoparticles. XRD spectra (Fig. 6) showed strong diffraction peaks at 30°, 33° and 36° degrees of 2θ which corresponds to 111, 200, 220, and 311 crystal planes. Which were significant agreement with the JCPDS data file No. 040783 it was concluded that the nanoparticles were crystalline in nature having cubic shape with no such impurities. The average particle size of AgNPs synthesized by the present green method can be calculated using the Debye–Scherrer equation $D=K\lambda/\beta \cos\theta$. It was found that the average size from XRD data and using the Debye–Scherrer equation was approximately 22 nm.

FTIR analysis

FTIR gives the information about functional groups present in the synthesized AgNPs for understanding their transformation from simple inorganic $AgNO_3$ to elemental silver by the action of the different phytochemicals which would act simultaneously as reducing, stabilizing and capping agent. Fig. 7 shows FTIR peaks were observed at 3316.98/cm shows O-H stretching vibration of the hydroxyl group [25], H-bonded alcohols, phenols or N-H stretching of I and II amines and amides. The peak at 2117/cm corresponds to C-H stretch alkanes and O-H carboxylic acids [26]. The peak at 1636/cm contribute to C=0 stretch α, β-unsaturated aldehydes and ketones, the peak at 1333/cm assigned to O-H bend indicates carboxylate [25]. The band at 1197/cm indicates the presence of C-N stretch aliphatic amines, the peak at 605/cm was assigned for C-Cl stretching vibrations of alkyl halides. The functional biomolecules are carboxylic and amine groups involved in the reduction of silver ions, as confirmed by FTIR spectrum.

FT-Raman spectroscopy analysis

In order to find out the possible functional groups of capping agents associate in the stabilization of AgNPs. FT-Raman spectrum of the nanoparticles was recorded. Fig. 8 gives the selective enhancement of Raman bands of the organic capping agents bound to the nanoparticles.

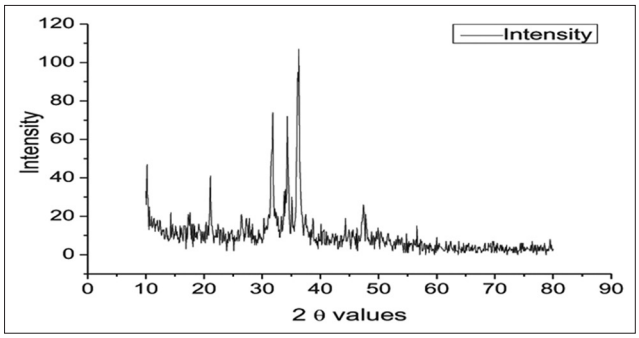


Fig. 6: X-ray diffraction images of silver nanoparticles synthesized by using leaf extracts of *Adhatoda vasica*

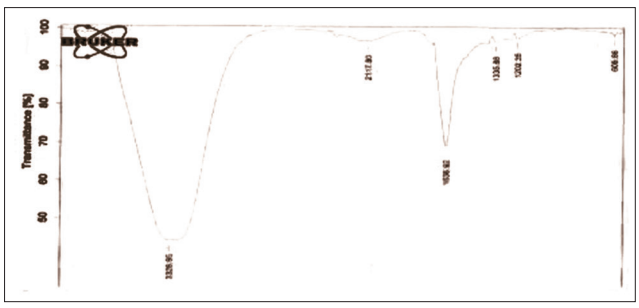


Fig. 7: Fourier transforms infrared images of silver nanoparticles synthesized by using leaf extracts *Adhatoda vasica*

The spectrum shows a strong and sharp band at 1500/cm. The following absorption bands at 3350/cm stretching of N-H or OH (COOH), 2970-2500/cm stretching of CH_3, 1720-1700/cm stretching vibration of CH_3 deformation, and the bond between 1500 and 1375/cm correlated to AgNPs.

Antimicrobial activity

Green synthesized AgNPs were analyzed against bacterial cell proliferation by using *E. coli*, *P. aeuroginosa*, *S. aureus* – ATCC 6538, *B. thuringiensis* – ATCC 10792 and *K. pneumoniae* ATCC 4352 by disk diffusion method. Fig. 9 revealed that the highest potential was observed against *P. aeuroginosa* while lowest zone was observed in *K. pneumoniae*. The results were compared with the ciprofloxacin as a positive control, Ag $(NO_3)_2$ as a negative control and plant extract as control. The exact mechanism behind the antimicrobial activity of nanoparticles in not clearly known, but it may by hypothesized that: (i) AgNPs may interfere with the bacterial cell membrane and bind with mesosome cell organelle and thereby reducing the mesosomal function

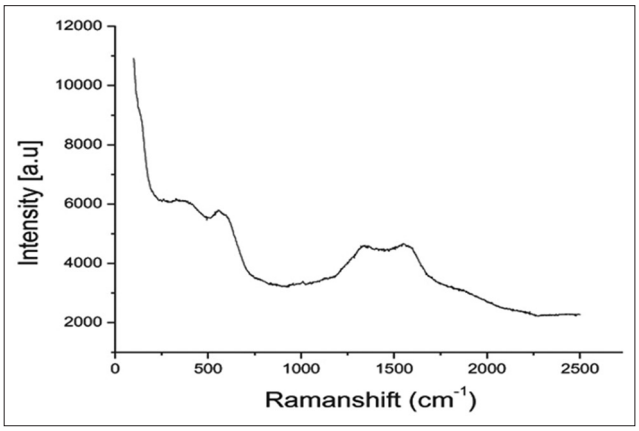


Fig. 8: Fourier transform-Raman spectrum images of silver nanoparticles synthesized by using leaf extracts of *Adhatoda vasica*

Table 2: EDAX of synthesized elements during the formation of AgNPs through the leaves of *A. vasica*

Element	Weight %	Atomic %
CK	24.96	66.59
OK	06.08	12.17
MgK	00.92	01.22
SiK	00.65	00.74
AgL	57.67	17.13
CaK	00.89	00.71
AuL	08.82	01.44
Matrix	Correction	ZAF

A. vasica: Adhatoda vasica, AgNPs: Silver nanoparticles

Table 3: *In vitro* antibacterial activity of AgNPs of aqueous leaf extract of *A. vasica*

S. no.	Organisms	*A. vasica*			
		Plant extract	$Ag(NO_3)_2$	SNPs	Positive control
1.	*E. coli*	-	18±2.6	19±1	39±1
2.	*Bacillus*	-	15±0.5	18.3±1.5	30±0.5
3.	*Klebsiella*	-	9±1.7	12±0.3	29±0.3
4.	*Psuedomonas*	-	22±1.5	24.3±1.5	40±1
5.	*Staphylococcus*	-	17±0.5	19±0.01	36±0.5

±: Standard error, *E. coli: Escherichia coli*, AgNPs: Silver nanoparticles, SNP: Single nucleotide polymorphism, *A. vasica: Adhatoda vasica*

Table 4: Anti-fungal activity of AgNPs of aqueous leaf extract of *A. vasica*

S. no.	Organisms	*A. vasica*			
		Plant extract	$Ag(NO_3)_2$	SNPs	Positive control
1.	*A. niger*	-	14±0.8	17±0.2	18±1
2.	*A. flavus*	-	18±0.5	19±0.9	21±1.5
3.	*Penicillium*	-	15±2.1	18±1.3	22±0.3
4.	*Trichoderma*	-	11±0.6	13±1.1	15±0.27

±: Standard error, *A. niger: Aspergillus niger*, A. flavus: Aspergillus flavus, *A. vasica: Adhatoda vasica*, SNP: Single nucleotide polymorphism

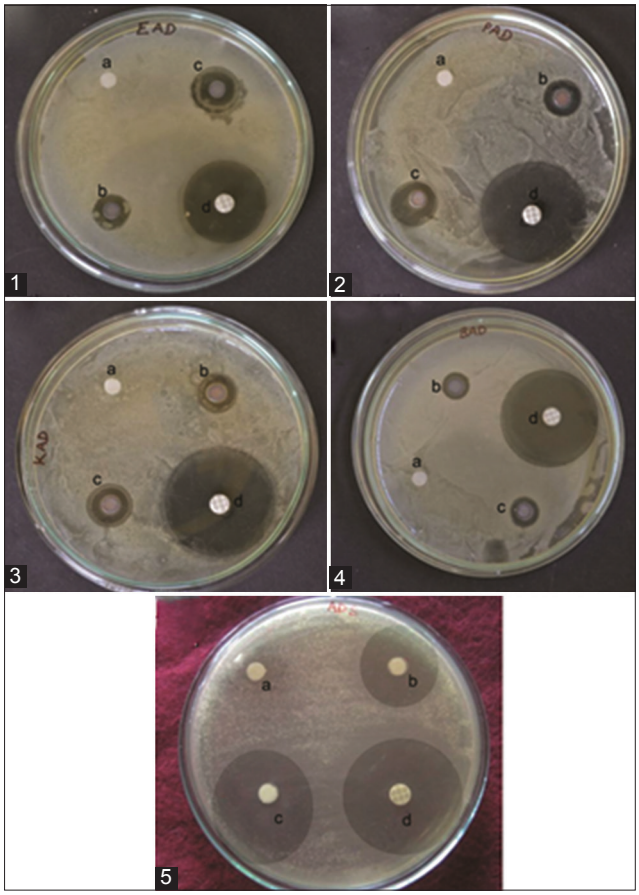


Fig. 9: Anti-bacterial activity of *Adhatoda vasica*, (1) *Escherichia coli* (2) *Psuedomonas* (3) *Klebsiella* (4) *Bacillus* (5) *Staphylococcus*, (a) Plant extract, (b) $Ag(NO_3)_2$, (c) single nucleotide polymorphism, (d) standard

and increasing the reactive oxygen species generation, (ii) AgNPs may interact with the protein that induces the inactivation of the bacterial protein, synthesis as well as DNA replication [27]. Similarly, oxygen associates with Ag and reacts with the sulfhydryl (-S-H) groups on cell wall to remove the hydrogen atoms (as water), causing the sulfur atoms to form on R-S-S-R bond, blocked the respiration, and causing the lethal effect of bacterial cells [28].

Antifungal activity of *A. vasica* plant extract capped AgNPs was studied using antifungal susceptibility test by four fungal pathogens such as *P. chrysogenum, T. harzianum, A. niger,* and *A. flavus.* Fig. 10 which showed promising antifungal activity was observed against *T. harzianum* followed by *A. flavus, P. chrysogenum,* and *A. niger* the results were compared with fluconozole as a positive control $AgNO_3$ as a negative control.

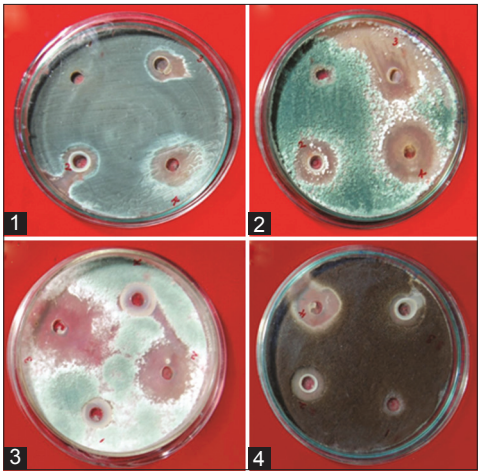


Fig. 10: Anti-fungal activity of *Adhatoda vasica,* (a) *Penicillium* (b) *Aspergillus flavus* (c) *Trichoderma* (d) *Aspergillus niger,* (1) Plant extract, (2) $Ag(NO_3)_2$, (3) single nucleotide polymorphism, (4) standard

CONCLUSION

A. vasica is a good medicinal plant for synthesis of AgNPs using the method of rapid reduction of Ag_+ ions. AgNPs showed strong potential against bacterial cell proliferation and can be used in the preparation of apoptotic agent and also in the synthesis of novel antibiotics.

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