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Bio-synthesis of silver nanoparticles from cotton plant extract and assessing its antibacterial activity

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Abstract
In the present study, we have explored the simple, cost-effective, non-toxic, biological approach for the green synthesis of silver nanoparticles (Ag NPs) utilizing aqueous extract from cotton plant. Phytochemicals present in the plant were employed as a bio-reductant to reduce silver ions into silver nanoparticles. The Ag NPs synthesized were visually observed for appearance of dark reddish-brown color and confirmed by UV-Vis spectra through surface plasmon resonance (SPR) at 440-450 nm. The TEM and PSA studies revealed cuboidal shaped NPs coated with organic layer. The Ag NPs were found to be polydispersed with some of them to be agglomerated. Optimizing the factors (pH, incubation temperature, time and silver nitrate concentration) influencing Ag NPs' formation, acidic pH (pH 5) was found to be more effective. The antibacterial activity of Ag NPs was investigated against *Pseudomonas aeruginosa*, *E. coli* and *Staphylococcus aureus*. Significant antibacterial activity was observed in dose dependent manner. This study demonstrates a simple, cost effective and eco-friendly method for synthesis of Ag NPs from cotton plant extract and its application in the field of nanomedicine as a potent antibacterial agent.

Keywords: silver nanoparticles, cotton, optimization, green synthesis, antibacterial activity

Introduction

Nanobiotechnology is the field of science involving study of nanoparticles (NPs) in the range of 1-100 nm. It involves manufacturing and application of NPs in various fields based on size and shape. These particles show different role owing to its unique structural properties, physical properties and high surface area to volume ratio [1]. NPs are used in diverse industries such as food, chemicals, space, Electronics, medical and in environment and agriculture, etc. There are numerous research articles emphasizing the role of different nanoparticles such as gold, silver, titanium, zinc, iron, copper, etc. in varied fields. Of these, silver NPs are widely studied due to extensive applications. It is used in biosensors, in bioimaging, as a photocatalyst etc [2]. This NP also illustrates important role in medical field. They have been found to show prominent anti-inflammatory, anti-oxidant [3] and antimicrobial activities [4]. It has also been used in diagnosis and treatment of cancer [5].

Synthesis of Ag NPs using physical and chemical method is mostly employed, however owing to its toxicity caused due to application of high pressure, temperature and chemicals, biological method is gaining importance as an alternative method [6]. Biological method involves synthesis of NPs from microbes (bacteria, fungi, yeast, actinomycetes, algae) and plants [5, 7]. However, synthesis of NPs using microbes is not practicable due to pathogenicity of microbes, tedious isolation process, Maintenance of cultures and requirement of skilled manpower [5]. Therefore, plant mediated synthesis of NPs emerged. This process is considered to be cheap, simple and eco-friendly [3, 7]. Plants contain various phytochemicals such as alkaloids, flavonoids, phenolics, terpenoids, saponins, aldehydes, ketones, polysaccharides, proteins, vitamins, quinones and acids (amino acids, carboxylic acids), etc. that act as agents reducing silver ions from silver nitrate to Ag NPs and stabilize them [8, 9]. Extensive study has been carried out for synthesis of Ag NPs using plant extracts *viz*. *Terminalia arjuna* bark extract [9], *Punica granatum* aqueous leaf extract [5], *Ocimum Tenuiflorum* [10], *Ganoderma applanatum* [11], *Euphorbia hirta* leaf extract [12], *Alcearosea* flower extract [13], *Morinda pubescens* [14], *Citrullus lanatus* fruit rind extract [15], Banana leaf extract [16], *Butea monosperma* bark extract [17], *Carica papaya* peel [18], etc. Different parts of plants such as leaves, seeds, stems, fruits, peels, and flowers are used in green synthesis of NPs [19].

Ag NPs act as a potent antimicrobial agent as it inhibits microbial growth and leads to cell death. Ag ions have been reported to bind to cell wall, disrupt it, diminish cell metabolism by hampering processes of replication, protein synthesis, affect membrane permeability and ultimately lead to cell death [20]. [21] Has reported release of inhibitory compounds from these NPs that inhibits bacterial growth. Likewise, [22] has recorded antifungal effect of Ag NPs where Ag ions were found to attack fungal cell membranes and disrupt membrane potential, inhibiting fungal growth.

Cotton (*Gossypium herbaceum* Linn.) is a perennial or annual fibre crop grown worldwide in temperate and tropical regions [23]. The plant grows around 3 m tall having thick stem and few branches with spirally arranged leaves. The plant parts such as leaves, seeds, bark and flowers are known to show medicinal properties [24]. Seeds are used to treat headaches, migraines, and fever, and as a snake venom antidote. Leaves, seeds, and roots are used to stimulate labour and placenta retention. Roots were used as a tonic to relieve heart palpitations and control vomiting, as well as a root infusion to treat a loss of appetite. Otitis was treated with stem juice [24]. This plant contains various phytochemicals such as macromolecules (sugars, proteins,

amino acids), flavonoids, tannins, steroids, terpenoids, saponins, resins, phenols, etc. [25]. It is also known to possess antibacterial, anti-depressant, anti-cancer, anti-ulcer, anti-oxidant, diuretic, anti-fertility, hepatoprotective, and woundhealing properties [26].

In the present study, formation of silver nanoparticles using cotton plant extract has been carried out. Further, optimization of Ag NPs production under different reaction conditions were done considering four parameters *viz*. time, incubation temperature, pH and $AgNO_3$ concentration. The biosynthesized Ag NPs were further characterized using UV-Vis spectroscopy, TEM analysis and PSA. The antibacterial activity was evaluated against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Results have shown that biosynthesized Ag NPs are bactericidal. To our knowledge, no studies on the biosynthesis of silver nanoparticles using cotton plant extract have been published to date.

Material and Methods

Materials

All the chemicals, reagents and culture media used in the present study were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. The cotton plant material was collected from Ahmednagar district, Maharashtra, India. Bacterial strains required for the antibacterial assay namely *Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli* were procured from Ingenious Biosciences, Consultancy Contract Services, Pune. Sterile de-ionized water was used for all the experiments.

Preparation of plant extract

Cotton plant was found in their natural habitat and on identifying using morphological characteristics; the plant material was collected and brought to laboratory. Plant material (leaves and peels) were washed initially with running tap water followed by de-ionized water several times to get rid of dust particles and other impurities [7]. The leaves and peels were further dried for 48 hrs at 40 ºC in an incubator and converted into fine powder by grinding in a mixer. 500 mg of fine powder was collected and mixed in 80% ethanol (20 ml). This mixture was agitated intermittently for 3 hrs to dry the mixture. The dried powder obtained was dissolved in 2 ml of deionized water after the ethanol had evaporated [27]. The extract was stored at 4 ºC till further use, for synthesis of Ag NPs as a bioreductant.

Synthesis of Ag NPs

For synthesis, 1ml of mixture was added to 14 ml of water to make final volume 15 ml. This solution was then mixed with 15 ml of 1 mM $AgNO_3$ solution in 1:1 ratio. Flask containing total 30 ml solution was covered with aluminium foil and incubated at 60 ºC for 5 hrs [28]. To validate formation of Ag NPs, color change of solution was recorded. The intensity of color was recorded by taking absorption spectra with UV visible spectrophotometer (Double beam UV-1, thermo scientific) in the range of 300-700 nm.

Optimization

Plackett-Burman design was used to optimize the synthesis of Ag NPs production. It includes use of various physical parameters to find optimum conditions for the preparation of AgNPs. Four physical parameters were considered for optimization specifically silver nitrate concentration, temperature of incubation, pH and incubation time as shown in following table. Based on the physical parameters, four experiments were designed. $AgNO_3$ Concentration was changed to 10mM from 1 mM, incubation temperature during synthesis of nanoparticles was changed to 37 ^{0}C from 60 ^{0}C, pH of the reaction mix was changed to 5.0 from 7.0 ± 0.5 and incubation time for the reaction was changed to 24 hrs instead of 5hrs. While changing one parameter, remaining parameters were kept constant.

Table 1: Plackett Burman design for optimization of AgNPs synthesis

Parameters	Exp 1	Exp 2	Exp 3	Exp 4
$AgNO_3$Conc	10mM	1mM	1mM	1mM
Temperature	60 °C	37 °C	60 °C	60 °C
pH	7	7	5	7
Incubation Time	5 hrs	5 hrs	5 hrs	24 hrs

Characterization of Ag NPs

UV spectrophotometric characterization of nanoparticles

The reduction of silver ions from silver nitrate to silver nanoparticles using bioreductant present in plant extract was monitored by taking the absorption spectra of sample using UV visible spectrophotometer (Double beam UV-1, thermo scientific). The samples were scanned in the range of 300-700 nm.

Transmission Electron Microscopy (TEM)

The samples for TEM were prepared by dissolving pellets in 2 ml de-ionized water such that 0.3 mg/ml concentration is attained and samples were examined using Libra 120 (Carl Zeiss make).

Particle Size Analysis (PSA)

NPs synthesized were diluted in 400 µl of de-ionized water and particle size analysis of synthesized NPs was performed on NANOPHOX (NX0088) (Sympatec GMBH make).

Antibacterial assay of synthesized Ag NPs

Antibacterial activity of synthesized Ag NPs was evaluated using well diffusion method. The assay was performed against *Staphylococcus aureus* (Gram positive bacteria), *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative bacteria). Aseptic conditions were maintained during the performance of assay. All glassware and media were sterilized using autoclave at optimum conditions (120ºC, 15 psi, 20 minutes).

Ag NPs were filtered aseptically through Whatman filter paper no. 2 (pore size 8 µm) and used [29]. Initially, bacterial stock for all three cultures were revived by inoculating loop full of culture in sterile Nutrient broth (10 ml) and incubated till bacterial growth attains optical density 0.5-1.0 after proper mixing. From this stock, 100 µl of bacterial inoculum was uniformly spread on sterile petri plates containing Nutrient Agar (NA) by sterile glass spreader separately. After that, 2 wells of 7 mm size were prepared on the same plate using sterile tips aseptically. In these plates, NP suspension of different volumes (100 µl, 200 µl, 300 µl, 400 µl and 500 µl) were added in wells for each culture separately. Ampicillin was used as positive control, whereas sterilized de-ionized water was used as negative control. After addition of NPs and controls, plates

were incubated at 37 °C for 24 hrs in incubator to evaluate antibacterial activity. The diameter of zone of inhibition was measured in millimeter (mm). The experiments were done in triplicates and data was statistically expressed as mean ± standard deviation [30].

Results and Discussion

Synthesis of Ag NPs

Formation of AgNPs was initiated on addition of plant extract to silver nitrate solution. This was indicated based on the color change of reaction mixture from colorless to dark reddish brown. This change in color is caused due to surface plasmon resonance (SPR) property of NPs. Silver ions from silver nitrate are reduced and converted into Ag NPs. There are reports suggesting presence of phytochemicals present in plant extract that act as bioreductant and reduce silver ions to Ag NPs [8, 9]. In this study, cotton plant extract is used which is rich source of various phytochemicals such as macromolecules (sugars, proteins, amino acids), flavonoids, tannins, steroids, terpenoids, saponins, resins, phenols, etc. [25], that may be utilized as a bioreductant. The reaction between plant extract and silver nitrate solution was monitored by UV– visible spectra of silver nanoparticles in aqueous solution. There was change in spectra with progress in reaction time and maximum absorbance was observed at 440 nm due to excitation of surface vibration of Ag NPs. Figure 1 illustrates the SPR band at 440 nm that goes in accordance with [31]. Determining the absorbance at 452 nm, optical density was found to be 1.0129 indicating maximum synthesis of NPs in the given range.

3.2. Optimization of factors affecting Ag NPs synthesis

Figure 2 represents the absorption spectra of SPR of Ag NPs synthesized using cotton plant extract at different reaction conditions such as pH, incubation time, Temperature and $AgNO_3$ concentration. Production of Ag NPs on addition of cotton plant extract to $AgNO_3$ solution was indicated initially by visual observation based on color change. Colorless solution was changed to dark brown color within 4 hrs on adding cotton filtrate to 1 mM $AgNO_3$. SPR vibrations in the Ag NPs resulted in color changes that were further confirmed by UV-Vis spectroscopy. The absorbance spectra for all the experiments conducted based on different factors were recorded in the range of 300-700 nm. Ag NPs synthesized under different reaction conditions showed a peak around 450 nm. A slight pointed SPR peak was detected that indicated spherical shape of NPs. Observing figure 2, it is clear that pH influences the synthesis of Ag NPs compared to other variables. Acidic pH favors the color change and intensity of SPR peak. Hence, this study proved that acidic environment influences synthesis of Ag NPs observed. However, the results are contradictory to [1] that shows acidic pH does not favor synthesis. But our results indicate acidic pH to promote synthesis of NPs. This is the first report observed yet on synthesis of NPs using cotton plant extract.

Characterization of Ag NPs

Transmission Electron Microscopy

TEM was used determination of morphological characters. Observing figure 3, it is clear that most of the Ag NPs synthesized are quite polydispersed, however, few are found to be agglomerated. These NPs are seen to be surrounded by an organic layer. Moreover, this organic layer has been found to separate the NPs from each other. The NPs were cuboidal shaped coated with an organic layer. Various polyphenolic compounds such as flavonoids and terpenoids are present in the plant extract that must have helped in the reduction of Ag+ ions from $AgNO_3$ in the reaction and stabilized NPs by forming an organic layer on the surface.

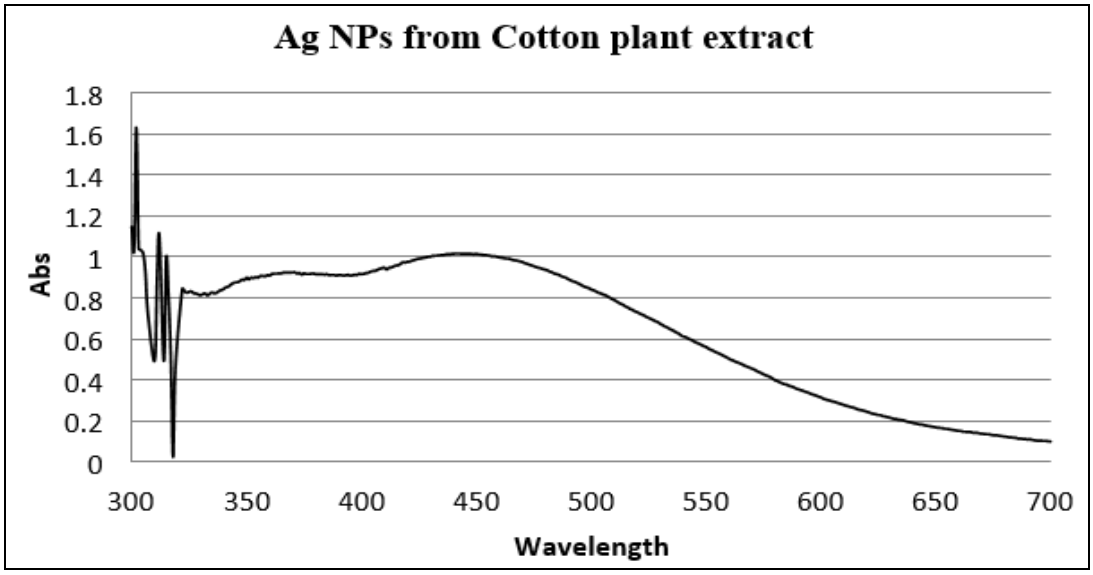

Fig 1: UV-Vis absorption spectra of synthesized silver nanoparticles using Cotton plant extract.

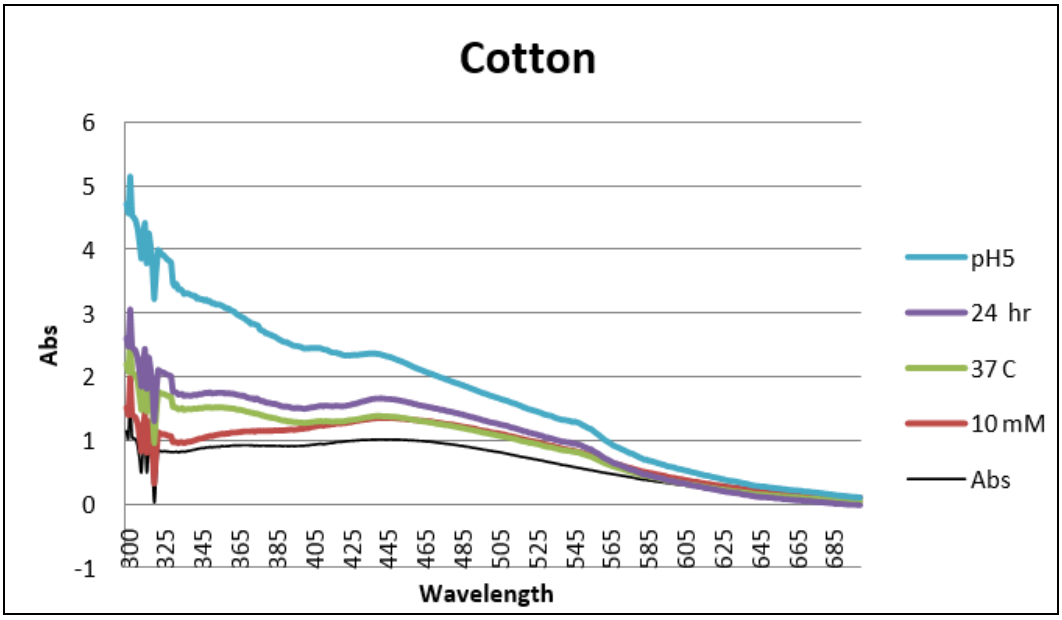

Fig 2: UV-Vis absorption spectra of synthesized Ag NPs by optimizing parameters

Particle Size Analysis

PSA determined the size and polydispersity index of NPs. Figure 4 depicts the average diameter and polydispersity index of Ag NPs obtained using cotton plant extract by green synthesis. The average diameter was observed to be 11.66 nm, however maximum NPs also showed average diameter of 13.74 nm. Polydispersity index was 0.18 that indicated the size distribution of NPs to be nanoscopic, uniform and polydispersed [4, 32].

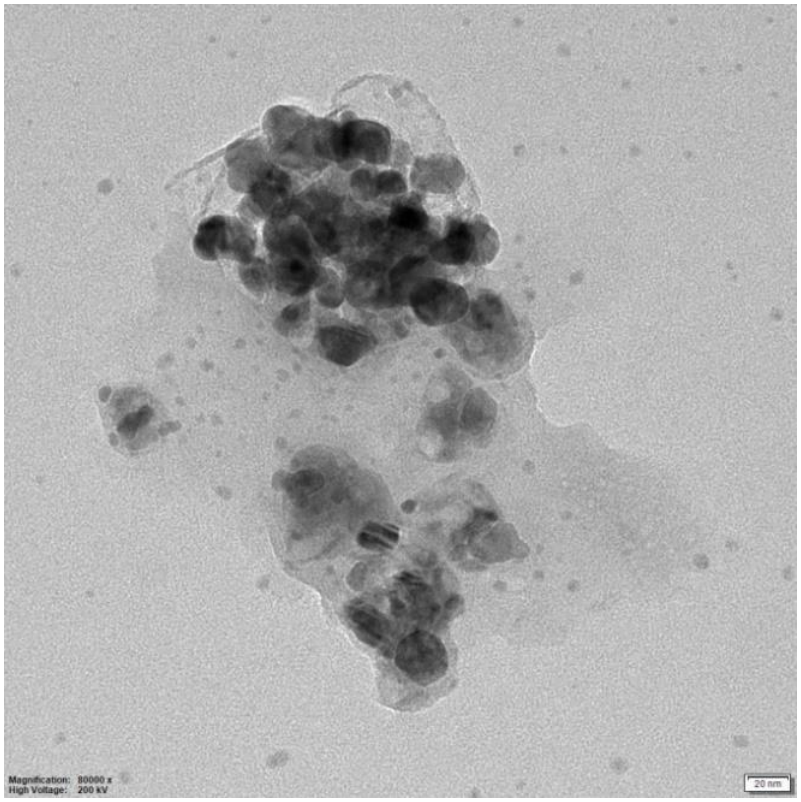

Fig 3: TEM images of synthesized Ag NPs

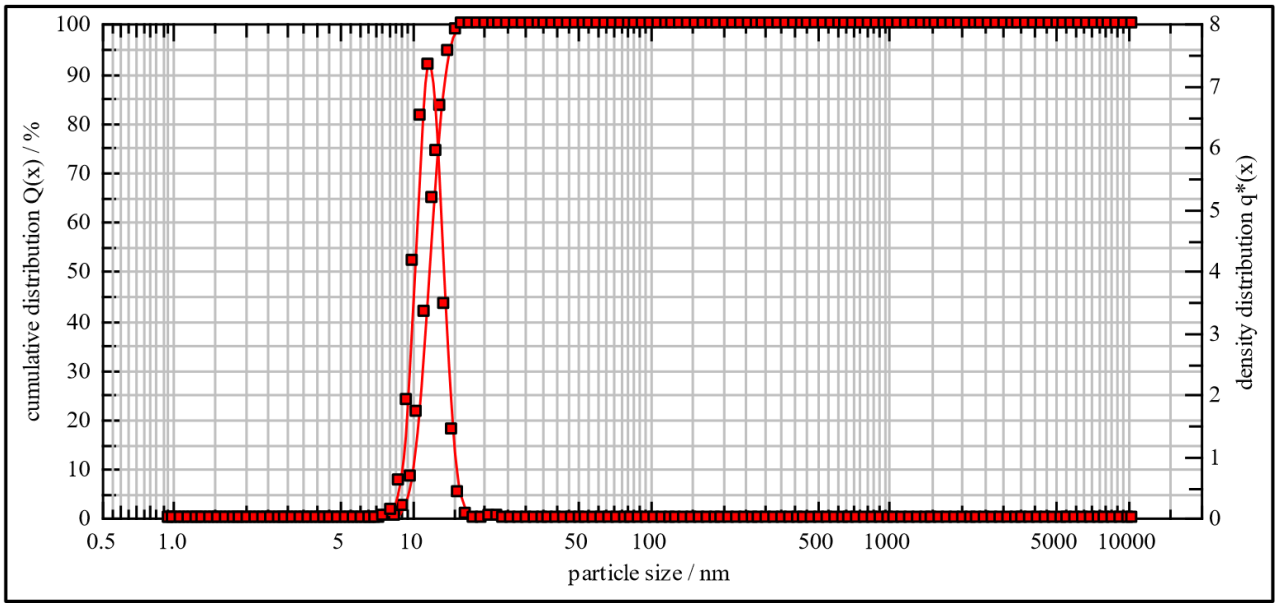

Fig 4: PSA of synthesized Ag NPs using Cotton plant extract.

Antibacterial assay

For assessing the antibacterial effect of synthesized Ag NPs, gram positive (*Staphylococcus aureus*) and gram negative (*E. coli* and *P. aeruginosa*) were used. Different volumes (100 µl, 200 µl, 300 µl, 400 µl and 500 µl) of Ag NPs synthesized were used.

Ampicillin was used as positive control, whereas sterilized water was used as negative control. Results were recorded after 24 hrs of incubation with NP suspension. Zone of inhibition were measured in millimeter (mm) and mean of the triplicate data calculated. Table 2 displays the zone of inhibition of Ag NPs against the representative bacteria. From table 2, it is observed that as the concentration of NPs increases, zone of inhibition increases. There was higher inhibitory activity observed at higher concentrations. Similar results were observed by [33] and [34] that showed density dependent bactericidal activity. Also, synthesized NPs were of small size around 12 nm exhibiting higher surface area due to which it remains in contact with bacteria. These NPs attach to the cell surface of bacteria, release silver ions, disrupts cell membrane and weaken cell metabolism ultimately leading to cell death [20]. Also, these ions may produce reactive oxygen species that may damage cell membrane, leak the cell components and lead to cell lysis [34].

Table 2: Zone of inhibition of synthesized Ag NPs against gram positive and negative bacteria

Concentration of Ag NPs (Volume)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*E. coli* (mm)
Control	0	0	0
100 µl	0	11 ± 0.58	0
200 µl	0	11 ± 1.15	9 ± 0.58
300 µl	9 ± 0.58	13 ± 1.73	13 ± 0.58
400 µl	9 ± 0.0	12 ± 1.53	14 ± 0.58
500 µl	10 ± 0.58	15 ± 0.58	16 ± 1.53
	Data is expressed as mean ± standard deviation		

Conclusion

In the current study, Ag NPs were successfully synthesized from cotton plant extract using biological approach. The method was cost-effective, simple, without use of toxic chemicals and making use of agricultural waste produced from left over crop. The phytochemicals present in plant extract were employed as a bioreductant to reduce silver ions into silver nanoparticles. The synthesized NPs were characterized using UV-Vis spectroscopy, TEM and PSA. Ag NPs synthesized were polydispersed, cuboidal shaped, coated with organic layer, while few NPs found to be agglomerated having size in the range of 11 to 14 nm. To our knowledge, this is the first report on synthesis of small size NPs (11 to 14 nm) by biological approach utilizing cotton plant extract.

Optimizing various parameters (pH, incubation time, temperature and silver nitrate concentration) influencing Ag NPs formation, acidic pH was found to be effective. Further, significant antibacterial activity was shown against gram-positive and gram-negative bacteria in linear correlation. With increase in concentration of NPs there was increase in inhibition of bacterial growth. Thus, this research provides an insight on production of silver NPs using cotton agricultural waste and its use as an antimicrobial agent in medicinal field.

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