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Bio-fabrication of silver nanoparticles from Garlic clove extract as a novel antibacterial agent

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Abstract

Recent research on the biosynthesis of nanoparticles is based on the use of biopaths with low-cost and safe production strategies, biocompatible materials to manufacture the structures, and nano-based formulations for specific applications. Current work is aimed at assessing the biological activity of bio-silver nanoparticles *in vitro* using clove extracts from Ginger (*Allium sativum* L). Garlic is a notably studied herb and contains key bioactive compounds. Organosulfur or thiol-based organic compounds are the main bioactive compounds present in garlic. In many research these organic compounds proved for having numerous biological and therapeutic activities and hence marked for formulation and synthesis of various biogenic nanocomponents. Using garlic extract, silver nanoparticles are produced by an environmentally friendly method and their antibacterial activity was evaluated. Size and the structural confirmation of nanoparticles was done by using UV-Visible, Electron Microscopy, X-ray Diffraction and Fourier-infrared spectroscopic studies. Polydisperse type of distribution of silver nanoparticles was confirmed by Transmission electron microscopy study.

Our nature comprises both living and non-living entities. Plants are among the most important living entities in the world of medicine. Therefore, it is important to discover the utilization of plants and its application in the field of nanomedicine. Plants are preferable system for nanoparticle production since they are non-toxic, function as natural capping agents, and minimise the expense of microbe isolation and culture[18].

Nanomaterials refers to the nanoscale particles which are about 1-100 nm and are the one-twentieth the thickness of a human hair and are invisible to the human eye[9]. Fabrication of varied nanoparticles with

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varying chemical compositions, sizes, and forms is a key topic of nanotechnology study. Silver nanoparticles (AgNP) are of current importance in all fields due to their easy fabrication process and unique physico-chemical properties, which enhances optical nature, electroconductivity, absorption, and effective charge distribution[9]. AgNPs have attracted a lot of interest as an effective antibacterial and antitumour drugs, anticoagulant, thrombolytic, anti-inflammatory agents, sensors, detectors and larvicidal agent, they also have reduced toxicity in *in vitro* and *in vivo* studies[3,10]. Phyto fabrication of AgNPs offers several advantages over conventional and physiochemical methods[11] such as eco-friendliness, low cost and avoid of the use of high energy, temperature and toxic chemicals. Medicinal plants with potential therapeutic properties and no side effects, are currently occupying a leading position in the phytopharmacology. Due to limited availability, less in solubility and permeability and high in instability, delivering of plant based therapeutic molecules is bit problematic in nature[9]. These natural medicine obstacles are overcome by using them in the synthesis of powerful nanomaterials that can significantly improve their therapeutic properties and significantly improve their appearance and performance. Therapeutic plants are of particular concern because they control the size and condition of nanoparticles by providing a coating layer. Several medicinal plants, including spices, have been used in the preparation of AgNPs [42].

Garlic (*Allium sativum* L.) belongs to the family Liliaceae and is a perennial herb, which is broadly known for spice and Phyto-medicine[28]. Various studies have previously shown that garlic may be beneficial in protecting against cardiovascular, carcinogenic, and age-related diseases[15,26]. Garlic extract is a rich source various secondary metabolites that plays an essential role in the reduction of metal-to-metal ions[25]. Organosulfur components of garlic extract such as allyl cysteine and allicin, allyl sulfur groups, alliin, ajoene, flavonoids, amino acids and fatty acids that contributes their medical properties in several treatments[1,3]. Garlic extract has been shown to help in preventing many illnesses. In some recent studies, garlic extract has extreme antibiotic activity against a wide range of antibiotic-resistant pathogens, especially against both Gram positive and Gram-negative bacteria and other microbes such as fungi and protozoans[21,31]. According to Shuford[36] report, fresh garlic extract confirmed the inhibition properties against Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. In addition, fresh garlic extract could help improve the antibiotic susceptibility of these strains to some traditional antibiotics. However, the inhibitory activity of this material could gradually decrease due to decomposition of allicin over time[12]. The nanotechnology could then be used to solve this problem and improve the antimicrobial activity of the plant extract[7]. In continuation of other research work on the medicinal, nutraceutical, and economic usage of garlic, our present work aims at the synthesis, characterization, and evaluation of the antibacterial activity of nanoparticles of aqueous silver nitrate ($AgNO_3$) and garlic extract.

Materials collection:

Allium sativum L. (garlic), was used for the green synthesis of silver nanoparticles purchased from the local market of Ballari, Karnataka in the month of January 2021. 1 mM silver nitrate ($AgNO_3$) stock was prepared and stored in a brown bottle, further used for the synthesis of silver nanoparticles.

Preparation of garlic extract :

The garlic was washed with redistilled water and air dried at room temperature for 1 hour. The outer layers were then manually peeled off by sterile hands. The garlic cloves to be separated were washed and extracted as follows. Exactly 200 g of garlic cloves were crushed into small pieces in a mortar and pestle and soaked in 100 ml of redistilled water. It was shaken for 1 day to ensure proper extraction, then filtered through a sterile muslin cloth, then the extract was collected and stored at 4 ° C until needed for AgNP synthesis.

Biological synthesis of silver nanoparticles (AgNPs) :

5 ml of 1 mM silver nitrate aqueous solution was added to 100 ml of fresh garlic extract. Later flask was placed in a shaker at 150 rpm speed. At 28±2°C reaction was carried out for 72 hrs. Garlic extract acts as a reducing agent and stabilizer. AgNP was gradually obtained by erosion and chemical decomposition of the extract. The brown colour formation indicates that AgNP was synthesized from the garlic extract and centrifuged at 5000 rpm for 10 minutes.

Physical characterization for AgNP structural confirmation :

Visual observation :

The preliminary confirmation of the synthesis of AgNPs from fresh garlic extract in the medium was characterized by the observation of changes in colour appearance.

UV-visible spectrophotometer :

AgNPs have unique optical properties which make them firmly cooperate with particular wave length so flight UV-Visible spectroscopy is quick, simple, basic, and specific for various sorts of nanoparticles, needs just a brief period of time for estimation. Reduction of silver nitrate to silver ions in the reaction mixture was confirmed by using UV-visible spectroscopy at scanning wavelength between 200-1100 nm.

Electron microscopy studies :

Scanning Electron Microscopy (SEM) with Energy Dispersive x- ray Spectroscopy (EDS):

The surface morphology of AgNPs was determined by SEM. Samples were prepared by centrifugation of the post-reaction colloidal solution at 10,000 rpm for 5 min. Then, the purified AgNPs pellet were soaked for 10 min to obtain them in suspension. The prepared and dried suspension sample was analysed in the voltage 20 kV. Further elemental composition of AgNPs was examined by using EDS.

Transmission electron microscopy (TEM) :

TEM micrographs of the sample were taken at an accelerating voltage of 200kV. ImageJ software was used to measure the nanoparticle size distribution.

Fourier transform infrared spectroscopy (FTIR) :

FTIR can give exactness, reproducibility, and furthermore an ideal signal-to-noise ratio. This spectroscopy is often used to see if biomolecules are associated with amalgamation of AgNPs, which is more articulated in scholarly and modern research. After 4 hours of incubation the AgNPs were isolated by repeated centrifugation (3-4 times) of the reaction mixtures at 10,000 rpm for 15min. The dried AgNPs powder were subjected to FTIR analysis using the potassium bromide pellet with a scanning range of 4000 - 400cm^{-1} the resolution of 0.2 to 4cm^{-1}.

X-ray crystallography (XRD) :

For XRD analysis, dried AgNPs powder form of samples was run at 400 °C for 4 h in an oven. A thin film of the sample was created on a glass plate and then the diffraction pattern was recorded. Using Scheerer's formula the crystal size was calculated[6].

Antibacterial activity of synthesized AgNPs:

Test Bacterial Pathogens used: Standard MTCC cultures, *Escherichia coli* MTCC 9537, *Klebsiella pneumoniae* MTCC 109, *S. aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 741 (Microbial Type Culture Collection and Gene Bank, Chandigarh, India).

Testing the Minimum Inhibitory Concentration (MIC) of AgNPs and standard antibiotics: against both Gram-positive and Gram-negative bacterial pathogens MIC of synthesized AgNPs and antibiotics were tested by following Clinical and Laboratory Standards Institute (CLSI, M07-A9-2012 guidelines)[6].

Antibacterial activity by Well diffusion method: The antibacterial property of synthesized AgNPs was tested using the standard Well diffusion method (according to CLSI, M02-A11, 2012 guidelines)[7].

In our living system many plants are used as traditional medicine to treat or prevent the highly infectious diseases and this has created the huge scientific interest in this world[28]. Garlic is a medicinally important plant that is used in many home remedies as it has antioxidant, antibacterial, and antihyper-glycemic activities. Allicin contents of garlic act as key antioxidant compound[19]. In the reduction of Ag ions to AgNPs, these compounds are the main contributors.

In the present study AgNPs were successfully synthesized by the green synthesis method (Fig. 1). During exposure to garlic extract filtrate sample, as a result of reduction reaction the yellow colour reaction mixture changes to dark brown colour after certain duration of incubation, is further characterized by various bio analytical techniques. Our results agreed with the findings of Shankar[33] and Andleeb[2] showing the synthesis of AgNP through colour alteration from pale yellow to brown when garlic extracts were mixed with $AgNO_3$.

The synthesized AgNPs were subjected to analysing the size, shape, and surface chemistry by using various physical characterization techniques.

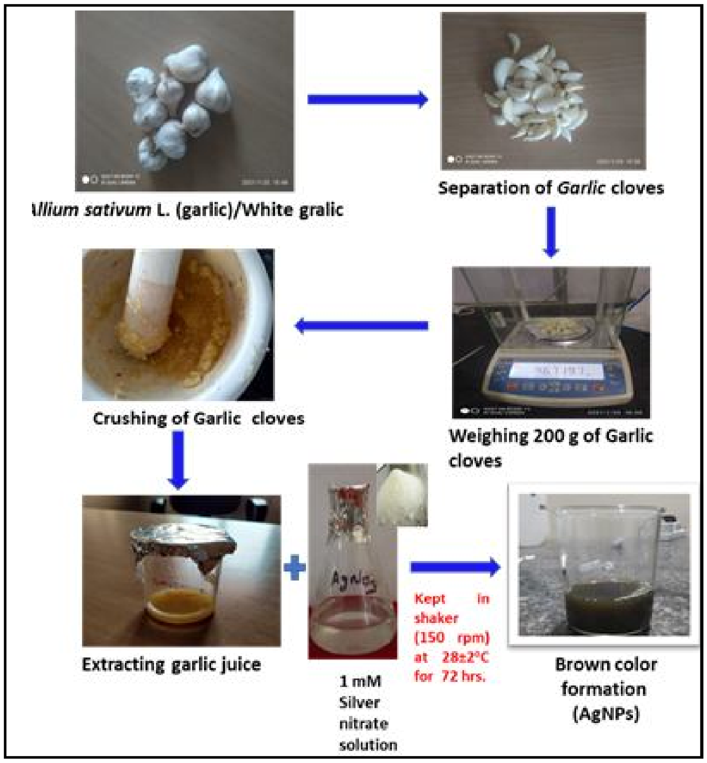

Fig. 1 Green synthesis of Silver nanoparticles from fresh garlic extract at room temperature

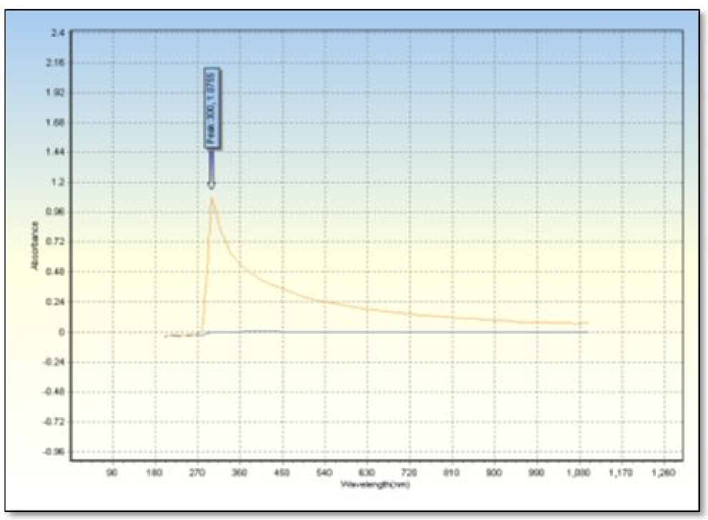

Fig. 2 Graphical representation shows UV-visible spectrum analysis, a sharp peak was observed at 300nm (AgNPs).

UV-vis spectroscopy : When beam of UV-Visible light passes through a sample or after reflection from a sample surface a streaking peak/absorption bands will form. Sometime absorption measurements can be at a single wavelength or over an extended spectral range UV–Visible. Absorbance spectra were recorded in the range of 200-1100 nm. A sharp peak was observed at 300 nm which is the characteristic peak for AgNPs (as shown in Fig. 2) thus giving a partial confirmation that AgNPs are synthesized from fresh garlic extract. Absorption bands are formed according to the size, shape, and chemical constituents of the AgNPs produced[23]. Our findings are in conjunction with El-Refai[9] and Ajayi[1], UV-visible absorption spectrum of AgNPs fabricated from fresh garlic extract, and a clear peak was obtained in ranges from 300-375 nm. Further Ejaz[8] concluded that AgNPs produce the absorption band at around 200–800 nm in the UV-Visible spectra.

Electron Microscopy Studies
SEM with EDS :

SEM was done to know the surface, morphological and elemental information of AgNPs. SEM studies showed irregular-shaped AgNPs, with size 5-100 nm, and they were more aggregated in their arrangements (as shown in Fig. 3). Our results are in agreement with a previous study[22] that reported the formation of individual irregularly shaped AgNPs in the range of 3 keV using *Allium sativum*.

Energy-dispersive X-ray (EDX) :

EDS is performed by measuring the generated energy and X-ray signals distribution through a focused electron beam on a specimen. EDX reports are useful in finding the purity of metal nanoparticles and its elemental contamination. It has revealed that

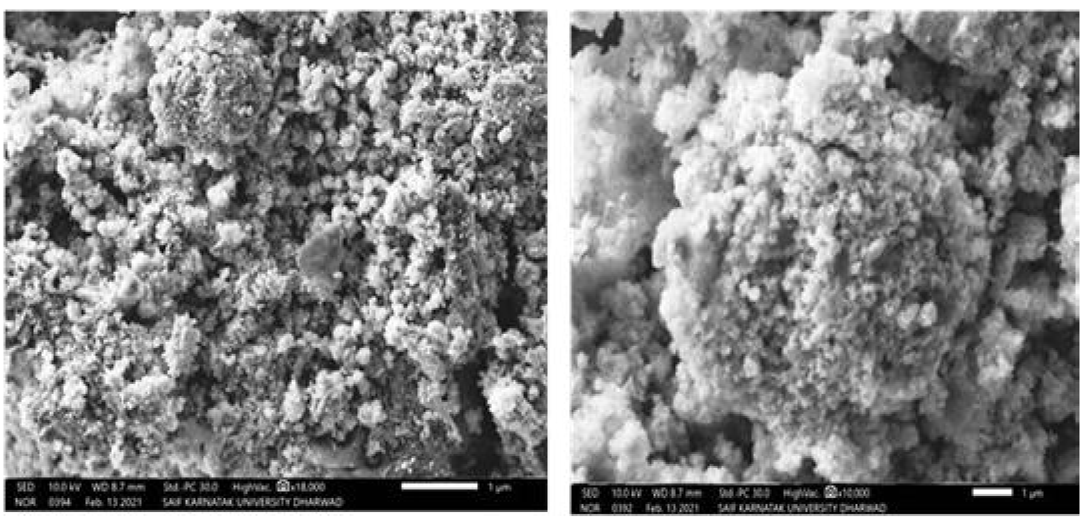

Fig. 3 Scanning electron microscopy image shows irregular shape of AgNPs size ranges between 30-200nm

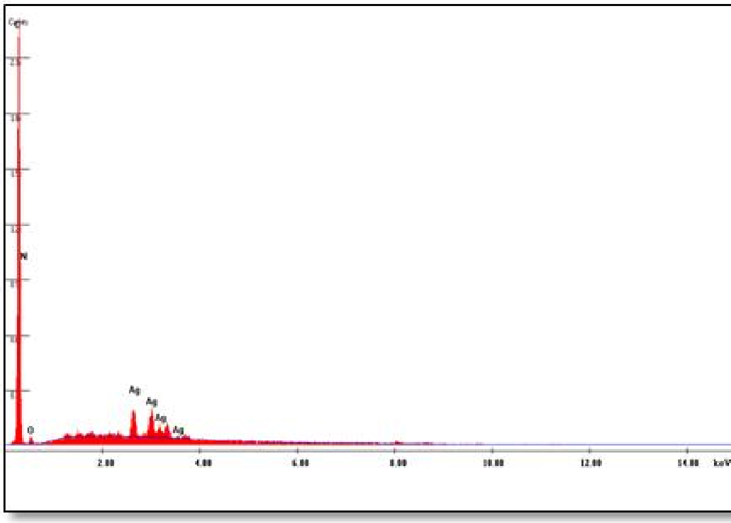

Fig. 4 EDX image shows strong signal peaks of Silver (Ag).

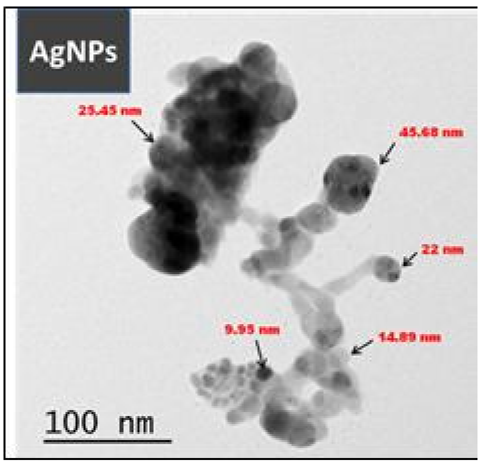

Fig. 5 Transmission electron microscopy image shows roughly spherical AgNPs with size ranges between 9-50nm

strong signal of silver (Ag) at 3Kev which is typical for silver-nano crystallite absorption. Elemental composition and surface morphology depicted in Fig. 4. Earlier reports authorise that, due to surface Plasmon resonance, AgNPs typically absorb in the region of 3 keV[30].

Transmission electron microscopy (TEM) :

TEM is the most suitable microscopy method to analyse detailed and satisfactory information about the very small structure of nanoparticles. In the present investigation, the fresh garlic extract was found to be remarkable in the production of AgNPs with sizes ranging from 5-100 nm. TEM analysis reported the presence of a maximum number of AgNPs with core-shell morphology ranging from 9-100 nm in size (as shown in Figure 5) and almost spherical, and a few in the aggregate form.

Fourier transform infrared (FTIR) spectroscopy :

FTIR spectroscopic analysis was studied to characterize and spot the biomolecules specifically bound to the surface of synthesized AgNP. In this study, the measurements of AgNP FTIR spectrum showed 4 intense bands at ~3427, 2924, 2855, 1640,1029cm^{-1} (depicted in Fig. 6). The spectra exhibited a broad peak located at groups. The less intense peak at 2924cm^{-1}could be assigned the presence of amino acids (aliphatic group), -OH stretching vibrations at 3427cm^{-1}, indicates the presence of hydroxyl secondary amines and the -CH stretch. One more strong peak at 2855 cm^{-1} was attributed as alkenes groups. The band at 1640cm^{-1}assignedas a –CO aloxy groups. The spectrum supports the presence of all the functional groups in the synthesis of AgNPs.

Our infrared spectroscopy reports are slightly correlated with FTIR analysis done by Ajayi[1], his study revealed various stretching vibration at different peaks for each of the spices. The garlic nanoparticles showed peaks of CO (acid and ester stretch), CO (amine) and O–H stretch/bends.

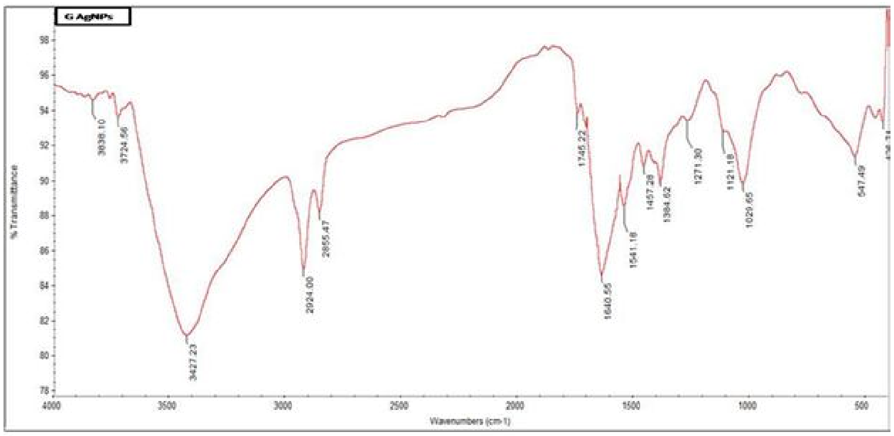

Fig. 6 Fourier transform infrared spectrum of synthesized AgNPs
*(Spectral image delivers the evidence for existence of functional groups and their contribution in conversion of Ag ion to AgNPs. The graph depicts Wavenumber (cm-1) on x-axis and Absorbance Units on y-axis)

X-ray crystallography (XRD) :

The average crystalline nature and size of the biogenic nanoparticles were confirmed by XRD analysis. The X-ray diffraction (XRD) pattern verifies that AgNPs formed from silver nitrate using fresh garlic extract. The four prominent peaks obtained at 2θ angle (38.19 °, 44.22 °, 64.65 ° and 77.7 °) and their corresponding fraction between the intensity of the diffraction peaks (111), (200), (220), and (311), which are known as Bragg reflections peaks (as shown in Fig. 7). Intensity of the facets (111) for the sharp diffraction peak at 38.19° is considered for the face centred cubic structure. The XRD facets of the AgNPs compared and indexed with standard, JCPDS card No. 04-0783. The average crystallite size (D) of AgNPs was calculated and the similar Bragg reflection was found to be around 96.64 nm. Our XRD results are consistent with Huang[16] results, where the silver nanoparticles formed by the reduction of Ag ions by the *Allium cepa* and *Allium sativum* extracts are crystalline with an average size of approximately 30 nm. It shows that our green method is effective in synthesizing silver nanocrystals from fresh garlic extract.

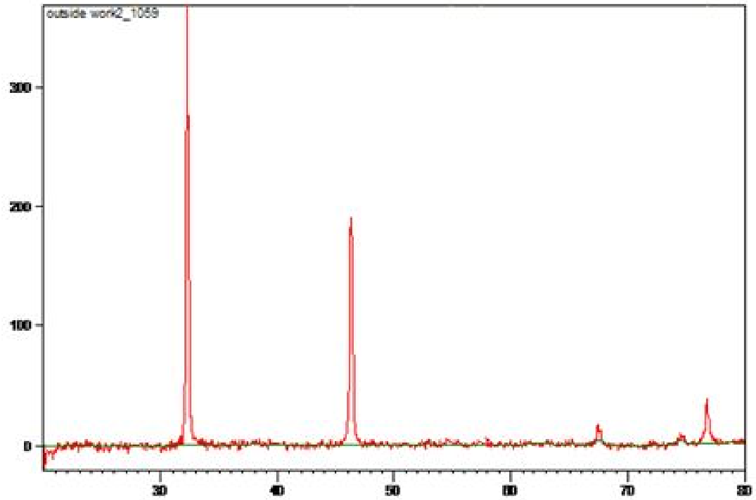

Fig. 7 X-ray diffraction pattern of AgNPs, Crystalline size: 96.64 nm calculated by using Scherrer formula

Antibacterial activity of synthesized AgNPs:

Determination of MIC of biogenic nanoparticles and Antibiotics :

The antimicrobial activities of the synthesized AgNPs against multi-drug resistant (MDR) bacteria were estimated through broth dilution method. The tested concentrations for biosynthesized AgNPs ranged from 2µg/ml to 128µg/ml. The results showed that the effective concentration of synthesized AgNP and standard antibiotics differed between Gram-positive and Gram-negative bacteria. In contrast to antibiotics, biosynthesized AgNP is a potent inhibitor of both Gram-positive and Gram-negative bacteria. Minimum inhibitory concentration of biosynthesized AgNPs against Gram-negative bacteria's, such as *E. coli* MTCC 9537 and *K. pneumonia* MTCC 109 ranging from 3 to 7µg/ml. In addition, 8 to 17µg/ml concentration of biosynthesized AgNPs showed inhibitory kinetics against Gram-positive pathogens including *S. aureus* MTCC 96 and the gram-negative bacteria *P. aeruginosa* MTCC 741 (as shown in Table 1). Inhibition concentration of antibiotics were more similar to those of AgNPs against several Gram-negative bacteria. Dissimilarity of inhibition concentration between Gram-positive and negative bacteria's might be due to differences in the membrane structure and the composition of the cell wall, thereby affecting the access of the biosynthesized AgNPs.

Agar-well diffusion method :

Recently many antibacterial reports demonstrated that phyto-fabricated metal-based nanoparticles have good antibacterial activity. From ancient times, it has proved that their compounds have strong inhibitory and bactericidal effects as well as broad spectrum of antibacterial activities against pathogenic bacteria[40]. The antibacterial performance of

Table-1 Minimum inhibitory concentration (MIC) of biosynthesized AgNPs and Antibiotics against various bacterial pathogens

PathogenicBacteria's	MIC level of Bio-AgNPs in (µg/ml)	MIC level of Antibiotics in (mcg/ml)
E. coli MTCC 9537	7±0.25	11±2.47
K. pneumoniae MTCC 109	3±0.45	13±1.6
S. aureus MTCC 96	8±0.16	09±0.7
P. aeruginosa MTCC 741	17±0.25	12±0.7

various nanoparticles is the most exploited in the medical field[14]. The antibacterial activity of plant mediated synthesised AgNPs by agar well-diffusion method was evaluated, and our findings are matched and agreed with the results of previous studies[22,38]. A significant antimicrobial activity of biosynthesized AgNPs was achieved against multi-drug resistant (MDR) pathogenic bacteria. In well diffusion assay, the zone of inhibition (ZOI) was expressed and calculated the average, mean by taking it in triplicates. After the incubation Gram negative bacteria such as *E. coli* MTCC 9537 and *K. pneumoniae* MTCC 109 showed increased zone of inhibition with increase in concentration. Whereas, the inhibition against *E. coli* MTCC 9537 showed 18 mm with 30 µg/ml concentration and 16 mm with 10 µg/ml of concentration for *K. pneumoniae* MTCC 109. In the case of *P. aeruginosa* MTCC 741, less inhibition (18 mm) was observed with the highest dose (50 µg/ml) of AgNPs. The ZOI acquired around the positive control (respective antibiotics, as shown in Table 2) was less in diameter compared to AgNPs. Gram-positive bacteria include *S. aureus* MTCC 96 and also showed sensitive prototype against AgNP (described in Table-2 & Fig. 8).

Table-2. Zone of Inhibition (nm) of Biosynthesized AgNPs and Antibiotics against bacterial pathogens

Pathogenic bacteria	Zone of Inhibition in mm		
	Antibiotics (10µg/ml)		AgNPs
E. coli MTCC 9537	Ampicillin	-	18±2.65 (30µg/ml)
	Piperacillin	14±2.54	
	Cephalexin	-	
K. pneumoniae MTCC 109	Cephalexin	-	16±2.73 (10µg/ml)
	Ampicillin	-	
	Penicillin	-	
S. aureus MTCC 96	Ampicillin	10±2.89	17±2.97 (10µg/ml)
	Methicillin	09±2.16	
	Penicillin	11±2.64	
P. aeruginosa MTCC 741	Cephalexin	-	18±3.45 (50µg/ml)
	Penicillin	-	
	Ampicillin	-	

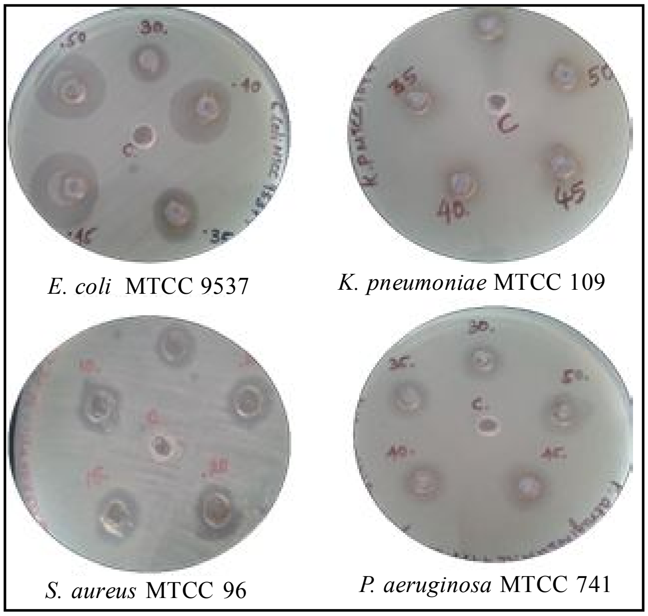

Fig. 8 Antimicrobial activity of AgNPs against pathogenic bacteria by the Agar well diffusion method

Metal-based nanomaterials and their ions were highly toxic to several microbial species and exhibited significant bio-killing activity due to the presence of reactive species with a large surface charge distribution[11,20]. Various reports on antibacterial activity have shown that synthesized nanoparticles are more effective than plant extracts[17,11]. Recently, it has been shown that silver nanoparticles are composed of silver atoms (Ag) and are larger than silver ions (Ag +), which promotes the more reaction with more molecules. As a result, stronger reactions directly enhance their antibacterial activity.

Using fresh *Allium sativum* L clove extract AgNPs was successfully synthesized. The synthesis protocol and later stability of synthesized AgNPs are found to be more efficient. The fabricated AgNPs are found to have a significant antibacterial effect. Because of this property, AgNPs can further efficiently can use in the medical field for the treatment and prevention of infectious diseases.

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Conflict of interest :

There are no conflicts of interests.

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Author's contribution :

Author, Ashajyothi C: Project administration, Investigation, Methodology, Formal analysis, writing original drafting and editing. The author has read and agreed to the published version of the manuscript.

Data availability statement :

The data and materials that support the results or analyses presented in my paper are freely available upon request.

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