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RESEARCH PAPER

Synthesis and Characterization of Silver Nanoparticles from Leaf and Its Impact on Multi Drug Resistant Bacteria

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ABSTRACT

Scientists and chemists have recently become quite interested in the green production of silver nanoparticles. In this regard, Indian flora is still holding back a plethora of resources for inexpensive, non-hazardous lowering and stabilizing substances used in the production of silver nanoparticles. The current research highpoints the biological synthesis of silver nanoparticles by employing *Azadirachta indica* extract and antibacterial use of synthesized silver nanoparticles towards multi-drug resistant super bugs by well-diffusion method. UV-visible spectroscopy study depicted a stronger peak. Silver ions are reduced utilizing the extracts of *Azadirachta indica* and silver nitrate as the reducing agent. Ultra Violet-Visible Spectrophotometer, X-ray diffraction, Scanning- and Transmission electron microscope are used to analyze the biosynthesized silver nanoparticles. These underwent *in-vitro* testing for antibacterial effectiveness against microorganisms that are resistant to many medications. Mostly, silver nanoparticles are found to be spherical, crystal shape, and ranged in size from 2 to 50 nm, according to scanning and transmission electron microscope study. Silver nanoparticles may show to be a viable alternative in the production of pharmaceutical goods and medical equipment that could aid in preventing the spread of infections that are multi-drug resistant. Overall, the synthesized silver nanoparticles are benign to release to the ecosystem and may be used in procedures for pollution cleanup.

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INTRODUCTION

Owing to their distinctive qualities, including minimal cytotoxicity and improved permeability, as well as electrical, optical, biological, catalytic, and magnetic propertiess, different metal nanoparticles (NPs) are created. Silver nanoparticles (Ag NPs) have received the most interest amongst the numerous metal NPs because of their numerous uses in a variety of fields, including cancer therapies, biosensors, antibiotics, anti-inflammatory agents, and drug delivery [1]. Biogenic Ag NPs were found to have potent antibacterial action against a variety of pathogenic microbes, according to numerous recent investigations. Additionally, research indicates that biosynthesized Ag NPs exhibit outstanding anti-inflammatory and anti-cancer properties. Biogenic Ag NPs demonstrated considerable action in the degradation of a variety of hazardous compounds. Emerging applications of biosynthesized Ag NPs include cosmetics, freshwater filtration, nano-insecticides, -pesticides, food preservation, sanitization, and wastewater sanitization [2]. The production of metal NPs has been carried out by employing a change of physico-chemical techniques. It's understood that biological synthesis techniques are safer, simpler, and less affordable than pricy, poisonous, and risky chemical and physical procedures. Different negative effects of chemical

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and physical approaches can be mitigated by their distinctive qualities, including minimal cytotoxicity and improved permeability, as well as electrical, optical, biological, catalytic, followed by magnetic features, different metal NPs are created [3]. Ag NPs have received the most interest amongst the different metal NPs because of their numerous uses in a variety of fields, including cancer therapies, biosensors, antibiotics, anti-inflammatory agents, and drug delivery [4].

Fig. 1 depicts the mode of action of anti-microbial resistance. There are various mechanisms of resistance to common antimicrobials. Additionally, research indicates that biosynthesized Ag NPs exhibit outstanding anti-inflammatory and anti-cancer properties. Globally, however, it was understood that biological techniques are safer, simpler, and inexpensive than pricy, poisonous, and risky chemical and physical procedures [5]. The biogenesis of NPs using several biological components can address several destructive impacts of chemical and physical methods. It is crucial to create a straightforward, affordable, non-toxic, and ecologically benign method for the easy and bulk manufacturing of metal NPs using biological systems. It is probable to achieve biological biosynthesis utilising microorganisms, fungus, floral extracts. Owing to its widespread production, fast rate of development, and ease of handling, microbes are usually favoured among different biological organisms to produce NPs [6]. Numerous research have shown that bacteria such as *Microvirga rosea, Terrabacter humi, Pseudomonas sp*., etc. are used in the production of Ag NPs. Rhizosphere bacteria are thought to be beneficial in several fields, including phytoremediation, biotransformation, and the manufacture of valuable chemicals, as well as to boost plant development and production [7].

A severe hazard to the global community is the growth of multidrug-resistant (MDR) germs as a result of unrestrained, excessive, and repeated use of antibiotics and chemotherapeutics [8]. The bacteria *Klebsiella pneumoniae* is a rod-shaped, Gram-negative, non-motile, facultative anaerobic, summarized illness that result in pneumonia, wounds or medical site contagions, bloodstream, and meningitis. When infected food is consumed, *Salmonella Enteritidis*, a food-borne bacterium, infect human and produce several ailments, including gastroenteritis [9]. A frequent food-borne illness called salmonellosis is brought on by the Salmonella infection. Young children and the elderly are particularly at risk for salmonella epidemics because they have impaired immune systems. Both *K. pneumoniae* and *S. enteritidis* has lately demonstrated resistance to many medications. The definitive answer to this problem is the creation of a novel antibacterial agent. Consequently, a promising drug to inhibit these MDR bacteria might be biosynthesized Ag NPs [10].

MATERIALS AND METHODS

Preparation of Nano-silver Solution

The therapeutic plant *Azadirachta indica* was

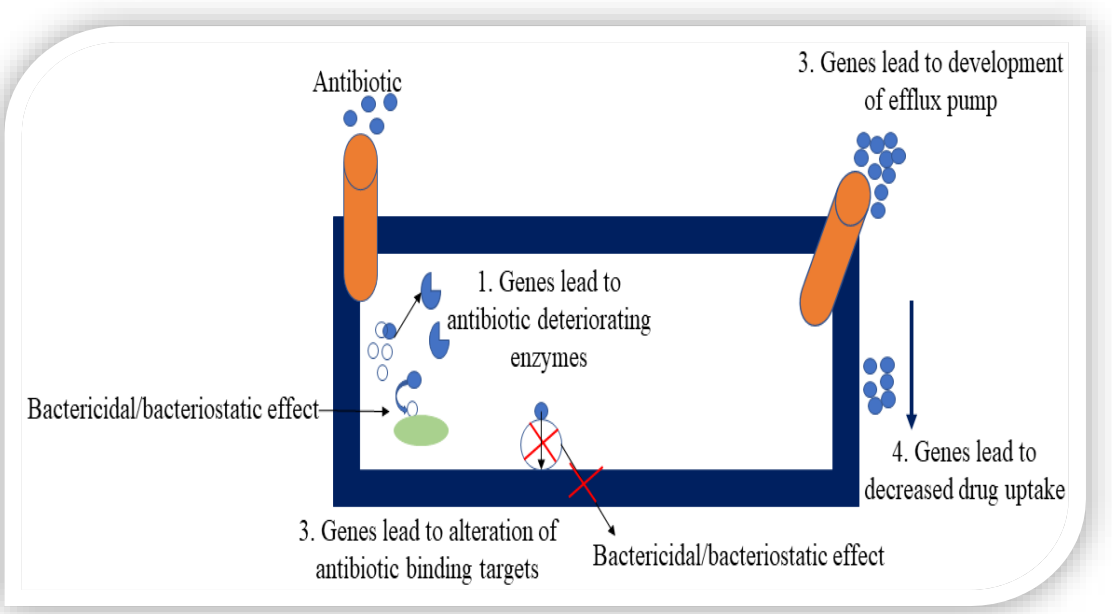

Fig. 1. Mechanism of Anti-microbial resistance.

employed, and the conventional soxlet extraction procedure was used, in which newly dried leaves are first rinsed with water to eliminate dust, then sun dry to eliminate any leftover moisture, and then crushed to produce powder [11]. These leaves have been gathered. Then, 20 g of powder was joint with 300 ml of deionized water to make the plant extract, then bubbled at 800 ^{o}C for 30 minutes. The solution was centrifugation at 7000 rpm for 15 minutes after cooling. The supernatant was taken out and sieved through Whatman filter paper no. 1. The extract was stowed at -200 ^{o}C to protect it for usage in the future. Utilizing a reducing agent such as silver nitrate, this solution was utilized to convert silver ions (Ag^{+}) into Ag^{0} silver nanoparticles. A 99-ml mixture of 1mM Ag NO_3 aqueous solution and leaf extract (1ml) was maintained in a water bath at room temperature. The solution's hue changed from colourless to yellow after 5 minutes, showing the creation of Ag NPs [12].

Characterization of Silver Nanoparticles

We used the Shimadzu UV-1601 spectrophotometer's UV-VIS absorption spectrum to measure the color change over time and confirm the fabrication of the Ag NPs. The X-ray diffraction study showed that Ag NPs are crystalline. By submerging a glass sheet in the solution, a thin coating of the Ag NPs was created, and it was employed for the X-ray experiments. Cu K1 radiation with a wavelength of 1.78 Å was used to capture the diffract pattern. The time constant was 2 s, but the scanning ranged from 20^{o} to 90^{o} for 2θ at 0.02^{o}/min [13].

The morphology and shape of the produced Ag NPs are examined using scanning electron microscopy (SEM), thinner films of the Ag NPs were placed on a metallic stub, and an ultrathin layer of gold was coated using lower vacuum sputtering. This was performed to increase contrast and reduce the buildup of constant electrical fields at specimen caused by the electron irradiation during imaging. High quality pictures of the specimen surface were created using the SEM technique [14].

The size and shape of Ag NPs are visible by employing the transmission electron microscopy (TEM) technique. It has been carried out using the Ultra High-Resolution TEM. The best tool for figuring out the inner microstructure of substances at the nanoscale scale is called TEM. With resolution in the range of a few tenths to a few nanometers, this permitted us to acquire real-space photographs of our material. A droplet of the particle solution was executed to a copper grid that had been coated with carbon to create the TEM grids. The samples on the grids were observed under a TEM, permitted to dry beneath a lamp [15].

Studies on the biological features of the processed Ag NPs

Antibacterial Action

Bacterial strains

Escherichia coli, *Klebsiella pneumoniae*, and MRSA pathogenic clinical isolates were obtained from the certified microbiology laboratories. Medically significant organisms like *E. coli*, *K. pneumoniae*, and *Staphylococcus aureus* are frequently identified from clinical samples.

Bacterial susceptibility testing

The well diffusion study was employed to determine the Ag-NPs' antibacterial activity [16]. The cleansed glassware, swabs, and well cutter are autoclaved at 121°C for 15 minutes to sterilize them. A sterile Petri plate was filled with sterilized Mueller-Hinton agar material, which was then left to set up at room temperature. Using a sterile cotton swab, an inoculant of test bacteria equal to 0.5 McFarland standard was applied equally across the whole surface. The agar media was punctured with holes using a 6 mm cork borer. Twenty µl of the produced Ag-NPs were poured into each hole. The control well was filled with sterile, distilled water. For optimal diffusion, the medium was placed in the freezer for one hour before being maintained at 37°C for 24–48 hours. The zone of suppression within the well was measured to gauge the antimicrobial activity. The zone of suppression was assessed after 24 and 48 hours, respectively. Three duplicates of each experiment were carried out.

Determination of MIC and MBC

By employing the conventional broth dilution technique, the effectiveness of Ag NPs as an antibacterial was evaluated. Utilizing serial two-fold dilutions of 1 mM produced Ag-NPs in volumes ranges from 3.12 to 200 µl and attuned microbial concentrations, the MIC was calculated in Mueller Hinton broth and maintained at 37 ^{o}C for 24 hours. Moreover, 2 controls were kept as antibacterial controls and organism controls. The

MIC is the least amount of antibacterial agent that can be seen to visibly block 99% of microbiological growth. It was done in triplicates, and the MIC was determined through visual turbidity of the tubes both before and after incubation [17].

Aliquots of 50 µl from all tubes that had no discernible microbial growth after the Ag-NPs' MIC was determined were planted in MH agar plates that weren't enriched with Ag-NPs, and they are nurtured for 24 hours at 37°C. Before and after incubation, the MBC was examined to see whether bacterial growth was present or absent on agar plates. The lowest antibacterial agent concentration required to eradicate 99.9% of the starting microbial population is known as the MBC endpoint.

RESULTS AND DISCUSSION

Using $AgNO_3$ as a reducing agent, Ag NPs were created from the neem leaf extract. Using a UV-Visible Absorption Spectrophotometer, we monitored the reaction. With growing reaction period from 5 to 30 mins, it was noticed that the peak in the absorption spectrum shifted from 425 to 413 nm, with the peak centering at 413 nm for 10-, 15-, and 30-min specimens as shown in Fig. 2. One of the quickest biological reducing techniques to create Ag nanostructures, it results in the reduction of Ag ions and the growth of stable nanoparticle within half an hour of the reaction. With the passage of time, the absorption peak's strength grew. More than 90% of the reduction of Ag^+ ions was finished in less than 30 minutes after the metal ions were added to the plant extract, indicating that the metal ion reduction process was rather quick. Even four weeks after their manufacture, the metal particles in solution were shown to be stable. Over time, there was no change in the nanoparticle solution's optical characteristics.

X-Ray Diffraction Studies

As seen by the peaks, the silver particles that developed during our trials were nanocrystals. To confirm the crystal structure of the particles, X-ray diffraction (XRD) was used (Fig. 3). The XRD results showed numbers of Braggs reflections that can be indexed using the face-centered cubic assembly of Ag at 2 values of 27.84, 32.19, 38.48, and 46.23, respectively. These values correspond to (300), (111), and (222) facades of face-centered cubic crystalline shapes. The (111) of cubic face-centered Ag is indexed by the diffraction peaks that were clearly visible in the XRD spectrum at 32 degrees [18].

Scanning Electron Microscope

Synthesized NPs were in the 100 µm size range, according to Scanning Electron Microscope (SEM) analysis (Fig. 4). Most of the time, Ag NPs were ellipsoidal in shape rather than spherical. Owing to the SEM measurements, the larger silver particles

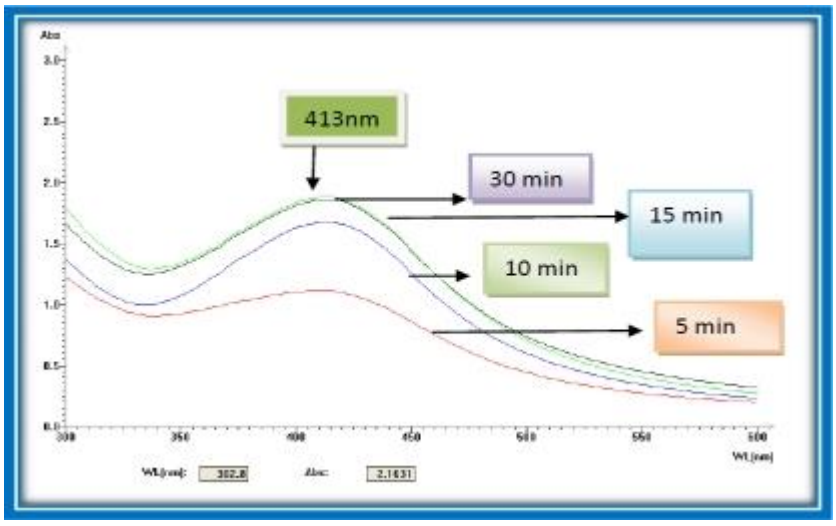

Fig. 2. UV-Vis absorption Spectra noted as a time of aqueous solution of 1mM $AgNO_3$ by *Azadirachta indica* 5 (red color), 10 (blue), 15(black) and30 (green) min.

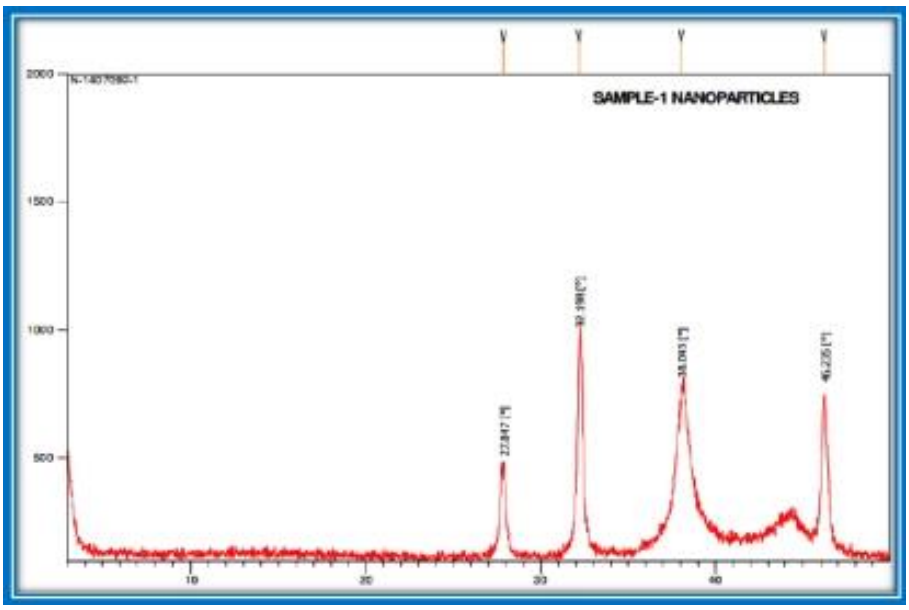

Fig. 3. XRD image of Ag NPs from *Azadirachta indica* extracts.

may have aggregated from the smaller ones.

Transmission Electron Microscope

We discovered through Transmission Electron Microscope (TEM) investigation that the majority of the Ag-NPs had a spherical form and crystalline structure. In Fig. 5, a few sample TEM micrographs of created Ag NPs are displayed at various intensifications. In other locations, a couple agglomerated Ag-NPs were also seen, suggesting potential later sedimentation. A high resolution lattice image of one of these particles can be found in Fig. 6 (on the left). The Ag (111) planes are

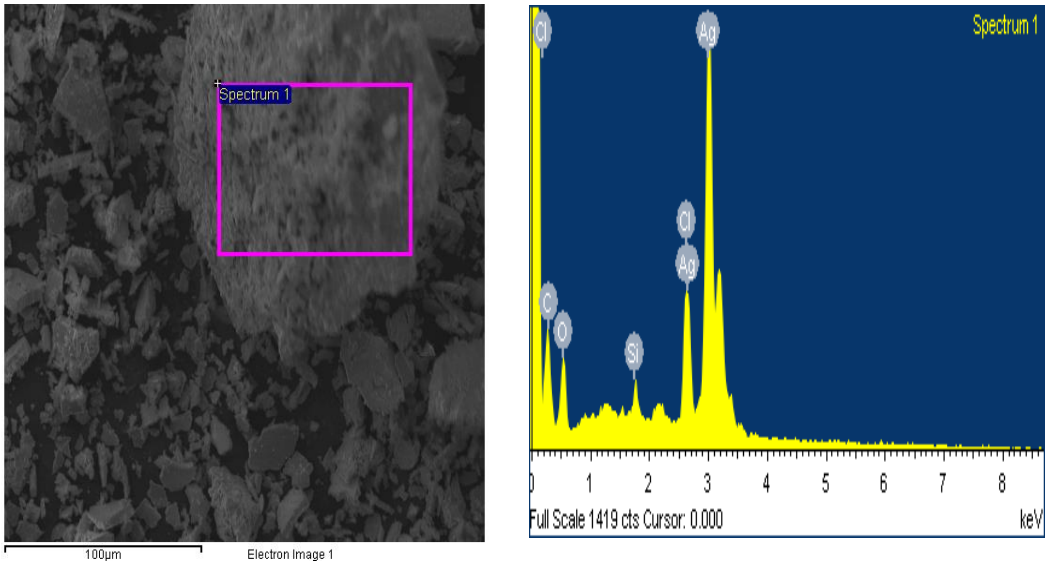

Fig. 4. SEM-EDAX pictures of Ag NPs.

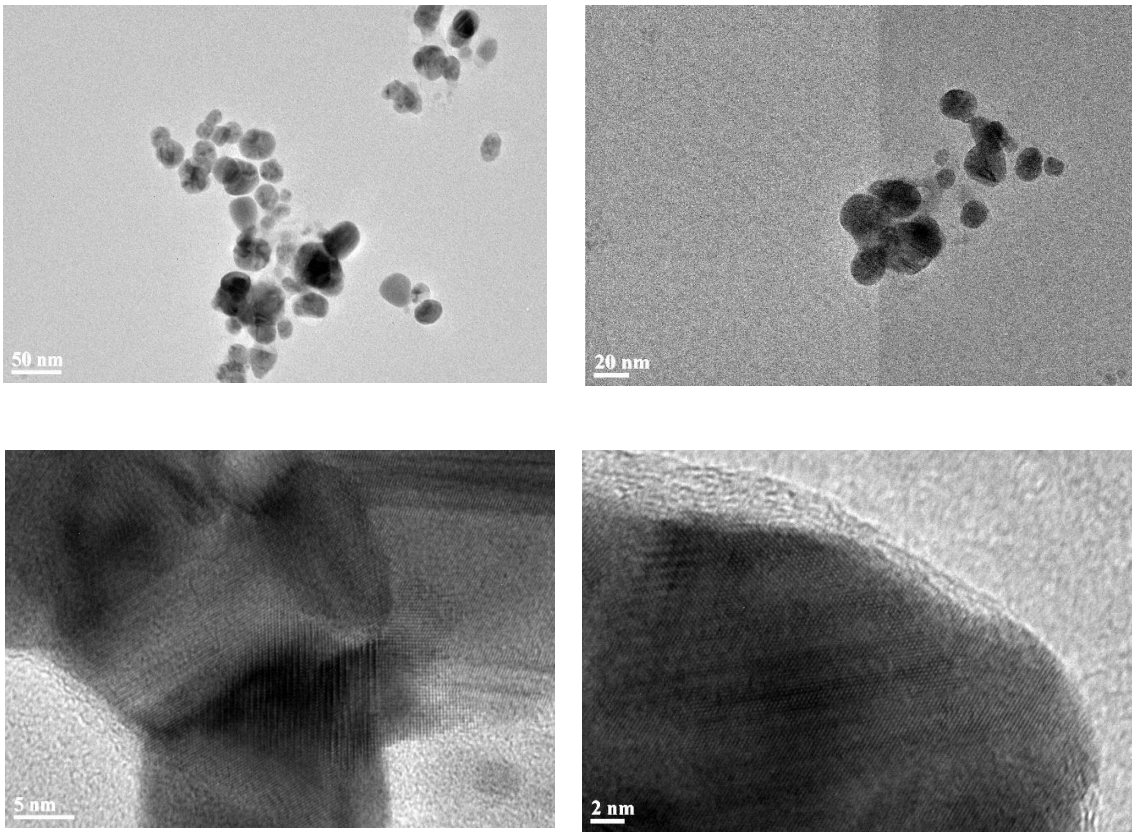

Fig. 5. An image of bright field TEM biologically reduced Ag NPs.

confirmed by the lattice spacing. The histogram obtained from a higher number of micrographs are displayed in Fig. 6 (right). It is clear that particle sizes vary; the estimated average size was 9 nm, and the shape of particles ranging from 2 to 50 nm.

Anti-bacterial action

Fig. 7 shows the antibacterial action of Ag-NPs via well diffusion approach against multi-drug resistance super bugs. In Fig. 8, the MIC and MBC values are shown. By using the well diffusion method, it was discovered that MBL producers were more resilient than ESBL producers, while the broth dilution approach revealed that Klebsiella pneumoniae strains were more resistant than E. coli strains. The 2 species had inhibition zones that differed by 1 mm, although their MICs (6.25) were comparable. There may be a small variation in their MIC, which may range from >3.125 to 6.25 µl. The zone of inhibition differed by 1mm between the two Klebsiella pneumoniae bacteria, although their MIC was the same. MBC was discovered to be 12.5 µl of a 1 mM solution of Ag-NPs for all the investigated species. Even MBC may vary among >6.25 and 12.5 µl depending on the tested strain of the organism.

To create silver nanoparticles, we employed a biological process. The color shift was noticed which results in the reduction of Ag ions to Ag NPs upon contact to neem plant extracts. The Surface Plasmon Resonance phenomena was the cause of the hue change. The decrease of the silver ion and the creation of Ag-NPs having a plasma resonance peaking at 413 nm are both confirmed by the UV-Vis spectroscopic investigation, which also reveals the Plasmon resonance property.

The capping chemical stabilizing the nanoparticle may have caused the sharp Braggs peaks to appear. The centrifugation and redispersing of the pellet in millipore water after nanoparticle production as a part of the purification method ruled out independently crystallization of the capping agents. The Ag NPs created via reduction of Ag^{+}

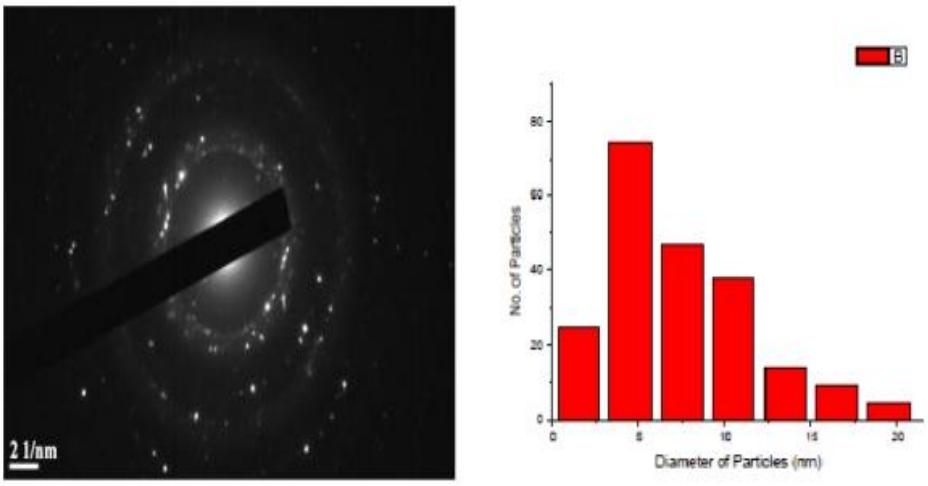

Fig. 6. High Resolution diffraction picture and histogram.

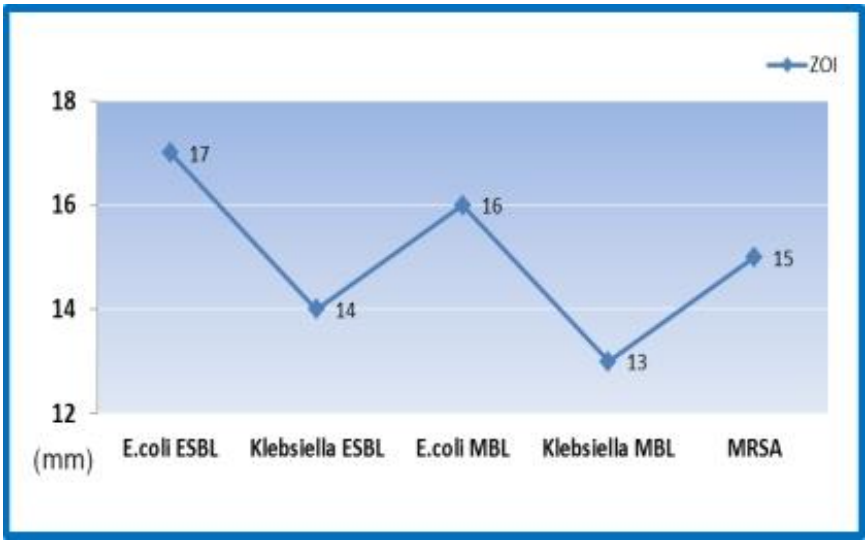

Fig. 7. Antimicrobial action of Ag NPs towards MDR microbes (X-axis) through well diffusion technique. Sizes of zone of inhibitions (ZOI) showed in Y axis.

ions through neem leaf extract are clearly crystal in character, according to the X-ray diffraction measurements. In many cases, the widening of peaks in solids' XRD patterns are due to particle size effects. Broader peaks, which denote lower particle size, show how the testing conditions affected the nucleation and growth of the crystal nuclei.

The size, structure, and composition of the Ag-NPs are identified by employing TEM. Since the therapeutic characteristics of silver nanoparticles are size dependant, it was recognized that spherical as much as non-spherical nanoparticles demonstrate greater physical qualities if they are generated small in size.

The majority of the Ag-NPs are identified as spherical in shape, have a crystal structure, and range in size from 2 to 50 nm, according to all characterization investigations conducted utilizing XRD, SEM, and TEM analysis.

Due to their nano size, surface area, and surface fictionalization, silver nanoparticles have a cytotoxic

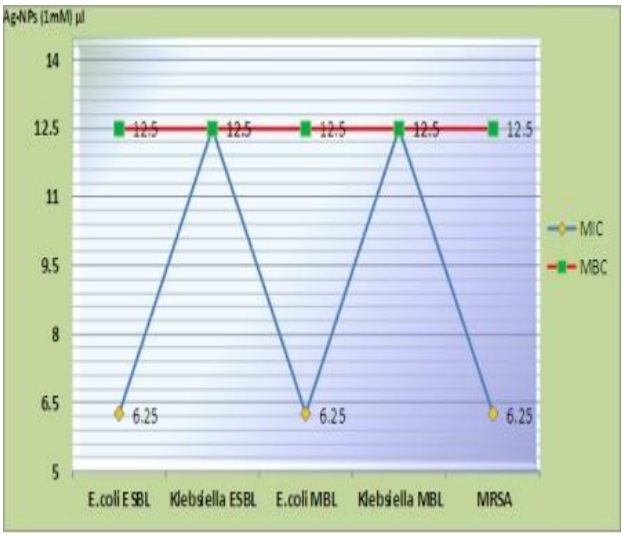

Fig. 8. MIC & MBC of 1 mM Ag NPs (Y-axis) towards MDR microbes (X-axis).

effect that reduces cell viability through controlling bio-kinetics. According to several researchers, Ag-NPs caused cytotoxicity in leukemic cells like THP-1, and K562 cells. The potential mode of action of toxic was hypothesized to involve disturbance of mitochondrial respiratory chain through Ag-NPs, which would disrupt ATP synthesis and result in the production of Reactive Oxygen Species and DNA damage. According to estimates, deposition causes more DNA damage, which is then followed through interactions between Ag-NPs and the DNA, causing cell cycle arrest in the G2/M phase. Additionally, these nanoparticles may work in concert with chemotherapy medicines like cyclophosphamide or busulfan to inhibit leukemic cell growth. To maximize physicochemical qualities and comprehend the medicinal properties will take more effort in the future development of silver nanoparticles based therapeutic medicines. The study's gathered data might offer some fundamental knowledge for subsequent research.

Silver ions are thought to be closely related to the physiological effects of silver. Ag-NPs continually discharge silver ions into an aquatic microenvironment. Since they have a wider surface area for interaction, tiny silver nanoparticles are reported to exhibit stronger and better bactericidal effects than bigger particles. interacting with the stability of the microbial cell by linking to important cell-based structural components such enzymes and proteins, especially to its SH-groups. These are the primary causes of silver ions' bactericidal effects. The effectiveness of Ag NPs towards clinical and non-clinical bacterial species has been investigated in a number of research. However, this study is unique in that it has only evaluated superbugs, or MDR pathogens. Finding new antibacterial compounds from alternative sources, such as plants, has become crucial in the current situation of the growth of MDR in human infective bacteria. India is the top producer of therapeutic plants. In order to use colloidal silver to destroy superbugs (MDR), the development of super medicines will be essential.

CONCLUSION

There is little question that this research area will continue to draw a lot of interest in light of the numerous advantages of green production of AgNPs utilizing plant extracts and their superior antibacterial activity alone or in combination with antibiotic medicines. The use of *Azadirachta indica* leaf extract in this study's demonstration of an easy, straightforward, and ecologically acceptable technique for the manufacture of Ag NPs. In addition to reducing $AgNO_3$, the phyto-molecules in the extract also serve as capping agents for the surfaces of Ag-NPs. Our results are important because they demonstrated the effectiveness of bactericidal substances towards superbugs incluidng ESBL and MBL producers and MRSA, irrespective of resistant mechanisms that

give these bacteria significant as an emerging pathogen. In this work, efficient chemicals and potential functional groups intricate in the reduction of silver ions were identified. Additionally, Ag nanoparticles could be produced economically and employed in pharmaceutical goods to potentially stop the spread of infections that are multi-drug resistant. The discovery of novel biological resources for the manufacture of Ag NPs is preferable to modern physico-chemical processes since these resources are widely accessible, reasonably priced, and easily used. AgNPs can be used to clean up pollution because their antibacterial properties and toxicity testing results show that they are safe to release into the environment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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