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Novel Synthesis and Biological Evaluation of Silver Nanoparticles: Antimicrobial and Anti-Inflammatory Effects in Mammalian Cells

Avan Lal[1], Meghana Yerraguntla[2], Krishna Gunti[3*]

Abstract

After synthesizing silver nanoparticles (AgNPs) with sodium citrate as a reducing agent, an extensive examination of their biological activity is conducted. The antibacterial ability of AgNPs' was evaluated using microbial cultures, and the outcomes demonstrated that they significantly suppressed the growth of bacteria. The interactions between AgNPs and biological molecules are then clarified by biochemical experiments, which emphasize the modification of enzyme function and affinity for proteins and nucleic acids. Additionally, alamarBlue dye is used in cell viability tests to assess the cytotoxic effects of AgNPs, revealing dose-dependent effects on mammalian cell lines. A reactive oxygen species (ROS) assay provided more about AgNPs' capacity to cause oxidative stress reduction in treated cells, offering understanding into the mechanisms behind their cytotoxicity as an anti-inflammatory agent. By using DCFDA dye, we can assay the relative level of ROS. The reduction of DCFDA dye by ROS indicate the level of ROS in a particular cell line. The biological consequences of AgNPs, including their antibacterial activity, biochemical interactions, and cellular responses, are thoroughly understood thanks to this work. These results highlight the significance of evaluating AgNPs' safety and effectiveness in biological systems while also advancing the investigation of AgNPs for a range of biomedical and environmental application. Conducting studies on LPS induced inflammation in the K562 cell line and AgNps inhibitory activity on ROS may generate profound insights into therapy for inflammatory diseases such as rheumatoid Arthritis.

Keywords: Nanoparticle, Biochemical, Anti-Inflammatory, ROS, Mammalian cell

INTRODUCTION

Nanoparticles are very fine particles that range in size from 1–100 nm. They are too small to be seen by the naked eye. Due to their spherical shape and high surface area-to-volume ratio, these particles have a numerous possible applications[26]. Their exceptionally vast surface area allows for the ligands to be coordinated in large quantities. Animal and lab research is being done to determine the characteristics of silver nanoparticles that could be used in human treatments, assessing potential efficacy, biosafety, and biodistribution [1].

The scientific subject of nanobiotechnology is expanding rapidly. An important research area in Nanobiotechnology is the synthesis of differently sized and chemically composed nanoparticles and morphologies. Because of its many uses, nanobiotechnology has emerged as a fundamental subfield of modern nanobiotechnology, ushering in new eras in the study of materials science that are

***Author for Correspondence**
Krishna Gunti
E-mail: Krishnagunti182@gamil.com

[1]Student, Department of Biotechnology Sai collage Sector 6 Bhilai, Univerisity Hemchand Yadav Vishwavidyalaya Durg, Chattisgarh, India
[2]Student, Department of Biotechnology, Central University of Andhra Pradesh, India
[3]Student, Department of Biotechnology, University college of science, Saifabad, Hyderabad, Osmania University

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garnering interest worldwide. As a result of research into the application of nanoparticles in biological systems, it is an interdisciplinary approach involving the fields of biology, biochemistry, chemistry, engineering, physics, and medicine. Furthermore, the development of safe non-toxic, and environmentally friendly processes depends heavily on nanobiotechnology. Rheumatoid arthritis (RA) pathophysiology is significantly impacted by the infiltration of inflammatory cells, especially M1 macrophages, that secrete multiple types of inflammatory cytokines.

Even at low concentrations, silver nanoparticles (AgNPs) have a strong anti- bacterial effect and can kill bacteria, fungi, and viruses. Gram-positive and Gram-negative bacteria, including those that have developed resistance to drugs, can both be adversely affected by AgNPs. AgNPs can penetrate though bacterial cell walls, interfere with the respiratory system, and disrupt DNA replication. AgNPs produce silver ions that can interact with the thiol groups of proteins' and enzymes', disrupting the respiratory chain and rupturting the cell wall of bacteria. Reactive oxygen species (ROS) are produced through these silver ions and are believed to be the primary cause of most cell deaths.

Silver nanoparticles (AgNPs) are the most promising due to their extensive surface to volume ration, which has spurred interest in research due to the development of microbial resistance against metallic particles, anti-toxins and the advancement of resistant strains [2]. Particles with metallic properties at the nanoscale exhibit physical properties that differ from both individual particles and mass material. This results in properties, such as increased catalytic activity due to morphologies with highly dynamic aspects [28].

Silver nanoparticles (AgNPs) are renowned for their efficacy against a broad spectrum of microbes, comprising bacteria, fungi, and viruses by disrupting cell membranes. AgNPs interact with microbial cell membranes, inducing structural damage that results in the leakage of cellular contents and eventual cell death. AgNPs prompt the production of ROS within microbial cells. ROS, such as superoxide radicals and hydrogen peroxide, can cause oxidative stress, damaging cellular components like proteins, lipids, and DNA, leading to microbial death. AgNPs disrupt various cellular processes, including respiration, enzyme activity, and DNA replication, impairing microbial function and reproduction. AgNPs impede the formation and stability of microbial biofilms. Biofilms, being resistant structures, pose a significant challenge for antimicrobial treatments. AgNPs' ability to disrupt biofilms enhances the susceptibility of microbes to treatment. Because of their diminutive size and high surface area, AgNPs penetrate microbial cells efficiently enhancing their antimicrobial efficacy [4]. AgNPs interact with microbial DNA, causing damage and interfering with genetic processes critical for microbial development and reproduction. AgNPs exhibit synergies when used with additional antibacterial substances, enhancing overall antimicrobial activity and reducing the likelihood of microbial resistance development. The multifaceted mechanisms of AgNPs make them promising antimicrobial agents for various applications, including wound dressings, medical implants, water purification systems, and surface coatings. However, ongoing research in essential to comprehend their environmental impact fully and optimize their efficacy and safety for widespread use.

Recently, silver nanoparticles have garnered significant interest due to their exceptional antimicrobial characteristics and potential applications in the realm of biomedicine. These nanoparticles possess distinct physical and chemical characteristics, including a high surface area to volume ratio, adjustable dimensions and shapes, and potent antimicrobial activity, making them viable contenders for various biomedical and healthcare purposes. Among the significant areas of interest is their interaction with macrophages, crucial components of the immune system responsible for eliminating pathogens and foreign particles.

Macrophages are innate immune cells pivotal in host defense by engulfing and eliminating invading microbes, clearing cellular debris, and regulating inflammatory responses. They are distributed throughout the body’s tissues and contribute to tissue maintenance, repair, and immune surveillance.

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Given their critical role in immune defense and tissue health, exploring the interaction between AgNPs and macrophages is essential for evaluating the safety and effectiveness of these nanoparticles for biomedical applications.

Numerous studies investigated how AgNPs interact with macrophages to understand their potential immunomodulatory effects and cytotoxicity. These investigations have employed various macrophage cell lines, such as RAW264.7, THP-1, and J774.1, as models to examine how cellular responses to AgNPs affect macrophage function and viability is crucial for assessing their biocompatibility and potential immunotoxicity.

Furthermore, the uptake and intracellular behavior of AgNPs within macrophages are crucial determinants of their biological effects. Studies demonstrated the ability of macrophages to internalize AgNP's through endocytic pathways, leading to intracellular nanoparticle aggregation and potential activation of cellular stress responses. Understanding the mechanisms governing AgNP's cellular uptake and intracellular dynamics in macrophages is vital for predicting their biological effects and optimizing their biomedical applications[5]. By systematically evaluating how AgNPs affect macrophage viability, morphology, phagocytic activity, cytokine production, and intracellular signaling pathways, researchers can gauge the safety and efficacy of these nanoparticles for biomedical purposes. Moreover, comprehending AgNP's immunomodulatory effects on macrophages might provide fresh chances to create specialized treatment plans for viral and inflammatory illnesses.

Rheumatoid arthritis stands as a chronic autoimmune condition characterized by inflammation with in the synovial joints, leading to symptoms like joint pain, swelling, stiffness, and eventually joint damage. In the course of rheumatoid arthritis pathogenesis, macrophages play a pivotal role by releasing pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta(IL-1β),which contribute significantly to synovial inflammation and joint degradation. Consequently, modulating macrophage activity emerges as a promising avenue for rheumatoid arthritis therapy approaches[5].

Silver nanoparticles have garnered attention as possible treatments for inflammatory arthritis due to their anti-inflammatory and immunomodulatory properties. Numerous studies have showcased the capacity of AgNPs to attenuate inflammatory responses and alleviate symptoms in rheumatoid arthritis models in animals. However, the precise mechanism by which AgNPs influence macrophage function in relation of rheumatoid arthritis remain inadequately understood.

Examining the consequences of AgNPs on macrophage cell lines derived from rheumatoid arthritis patients or animal models serves as a valuable experimental approach to unravel their immunomodulatory characteristics and therapeutic potential for rheumatoid arthritis[5]. By systematic assessment of AgNPs impact on macrophage behaviour, cytokine release, phagocytic capability, and intracellular signaling pathways, researchers can identify potential therapeutic targets for rheumatoid arthritis and understand the underlying mechanisms underlying of their anti-inflammatory actions.

Conducting screening studies of AgNPs in rheumatoid arthritis derived macrophage cell line thus presents a significant opportunity to advance our understanding of their immunomodulatory effects and therapeutic potential in this debilitating autoimmune disorder[7]. By elucidating the intricate interactions between AgNPs and macrophages, researchers can pave the way for the development of innovative nanotherapeutics for rheumatoid arthritis that target inflammatory pathways and promote joint health and function.

AIM AND OBJECTIVES AIM

To synthesize silver nanoparticles (AgNPs) using a chemical reduction method and evaluate their potential medicinal uses, including antimicrobial properties, with a focus on understanding their mechanisms of action and potential toxicity for future biomedical application.

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OBJECTIVES

1. To Synthesize silver nanoparticle by chemical reduction method using sodium citrate.
2. To Characterize the silver Nanoparticles by UV Scanning and physical observation.
3. To evaluate anti-bacterial activity through an anti-microbial susceptibility test
4. To identify the bacteria inhibited by AgNPs
5. To assess the anticancer properties on human cell lines using a cell viability assay

To investigate the activity of ROS on the K562 cell line using DCFDA dye.

REVIEW OF LITERATURE

Silver nanoparticles can be synthesized using sodium citrate and silver nitrate via chemical reduction method. Chemical reduction doesn't involve extreme parameter.

Introduction

Silver nanoparticles (AgNPs) have garnered excellent antibacterial qualities and a wide range of medical uses have drawn substantial attention in biological research. This review aims to offer a comprehensive overview of recent progress in AgNPs, particularly focusing on synthesis techniques, characterization methodologies, and their application in various biomedical domains. Nanotechnology symbolizes an emerging field where materials are manipulated at the nano-scale. Conventional techniques for metallic nanoparticles production often involve complex equipment and expensive chemicals, posing environmental risks. Consequently, there is a growing preference for "green" synthesis methods that are easier to use, more practical, eco- friendly, and cost-effective. Utilizing biological sources for nanoparticle production has emerged as a fresh and encouraging strategy, offering scalability and environmental benefits. Nanoparticles of silver (AgNPs) hold particular significance among metallic nanoparticles because of their biomedical uses. They are essential to nanomedicine, especially in treating and preventing diseases like cancer. Recent work explored various synthesis methods for AgNPs, including biological, chemical, and physical approaches, alongside their characterization. Additionally, the pivotal role of AgNPs in combating life-threatening ailments such as cancer, as well as their antidiabetic, antifungal, antiviral, and antimicrobial properties, elucidating their molecular mechanisms of action [27].

Synthesis Strategies

Among the various synthesis methods, the bottom-up chemical approach, characterized by its scalability and cost-effectiveness, involves reducing a metal salt combined with a stabilizing agent to regulate the size and form of the nanoparticles. Recent innovations in synthesis protocols aim to enhance efficiency and tailor nanoparticle properties to suit specific biomedical applications. The scientific community has greatly benefited from nanoparticles since they started to bridge the gap between bulk materials and atomic and molecular structures[8-10]. Because of their high surface area-to-volume ratio, silver nanoparticles are especially attractive. Nanoparticles are very important and need to be controlled since their surface area can alter, changing the particles physical and chemical characteristics.

Techniques for Physical Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) hold significant promise in various fields because of their unique properties. Techniques for Physical synthesis offer advantages such as high purity and environmental friendliness compared to chemical approaches. This review aims to provide an outline of the two most used physical synthesis processes, laser ablation and evaporation.

Evaporation-Condensation Approach

The evaporation-condensation method involves the vaporization of a base metal source, such as silver, in an atmospheric pressure carrier gas in a tube furnace. Nanospheres are produced as a result of

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this process and changes to reaction facilities can regulate the size, shape, and yield of the nanoparticles. Despite its effectiveness in producing high-purity AgNPs, this method presents several drawbacks. These include significant space requirements, high energy consumption, and the need for extended durations to maintain thermal stability. Jung et al. proposed the use of a ceramic heater to address some of these challenges, demonstrating efficient synthesis with high nanoparticle concentration.

Laser Ablation Technique

Alternatively, the laser ablation technique involves irradiating a bulk metal source, such as silver, placed in a liquid environment with a pulsed laser. This process results in the development of AgNPs in the liquid, free from other ions, compounds, or reducing agents. Parameters such as laser power, duration of irradiation, type of metal source, and properties of the liquid media influence the characteristics of the synthesized nanoparticles. Notably, laser ablation offers the advantage of producing pure and uncontaminated nanoparticles without the use of additional chemical reagents, relying instead on mild surfactants in the solvent. Both the evaporation-condensation and laser ablation methods offer distinct advantages and limitations. While they provide high-purity AgNPs without the application of hazardous substances, challenges such as agglomeration, high power consumption, and complex equipment requirements remain. Researchers continue to explore innovations to overcome these challenges and enhance the effectiveness of physical synthesis methods for AgNPs [11]. To maximize synthesis parameters and investigate applications in diverse sectors such as biomedicine, electronics, and catalysis, more research is required. By synthesizing data from the body of current literature and producing a succinct synopsis, this review contributes to a better understanding of physical synthesis methods for AgNPs and their potential implications across different domains.

Characterization Methods

Accurate characterization is pivotal for understanding the structural and functional attributes of AgNPs. Method like visible ultraviolet spectroscopy (UV-Vis), play crucial roles in elucidating nanoparticle characteristics. UV-Vis spectroscopy, in particular, enables the evaluation of surface plasmon resonance (LSPR) and provides insights into nanoparticle size distribution, aiding in the optimization of synthesis processes[28].

One growing area of interest within nanotechnology is nano-biotechnology. Developing a more standardized approach is essential for synthesizing nanomaterials with a broad range both sizes and chemical make-ups that demonstrate good monodispersity [29]. In the past few years noble metals nanoparticles have gained substantial usage in the fields of science such as chemistry, physics, medicine, biology, and material science[30]. Metal nanoparticles contain an abundance of surface atoms and a large specific surface area because of their remarkable physicochemical properties, which involve optical, magnetic, and electrical and antimicrobial capabilities. The huge synthesis of metal nanoparticles stems from their prospective use in various fields, including chemistry, electronics, and biology, material science, and medicine[30]. Because of their exceptional optical, catalytic, electrical, magnetic, and antibacterial qualities, metal nanoparticles have a high specific surface area and surface atom count. The prospective applications of metal nanoparticles in electronics, chemistry, energy, and medical research make them extremely valuable. The primary reason for investigating metal nanoparticles, particularly those of metals, is their significant optical absorption in the visible spectrum, which is brought about by the group excitation of free electron gas's[31].

Mechanisms of Antimicrobial Action

The antimicrobial activity of silver nanoparticles (AgNPs) arises from various mechanisms:

Cell Membrane Disruption

AgNPs interact with the membranes of microbial cells, compromising their integrity and leading to increased permeability. This disruption causes leakage of cellular contents and eventual cell death (24).

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Reactive Oxygen Species (ROS) Generation

AgNPs induce the production of reactive oxygen species (ROS) within microbial cells. ROS, such as superoxide radicals and hydrogen peroxide, inflict oxidative damage on cellular components, disrupting vital functions and resulting in microbial death.

DNA Interaction

l death. AgNPs penetrate microbial cells and interact with their DNA, hindering DNA replication and transcription processes. This interference induces DNA damage, genomic instability.

Enzyme Inhibition

AgNPs impede the activity of crucial microbial enzymes, including those involved in respiration and metabolism. By disrupting enzyme function, AgNPs interfere with microbial metabolism and energy production, hindering growth and survival.

Cell Wall Disruption

AgNPs disrupt the synthesis of microbial cell walls by binding to cell wall components or inhibiting enzymes crucial for cell wall formation. This disruption weakens the structural integrity of the cell wall, rendering microorganisms more susceptible to environmental stressors and immune responses.

Apoptosis Induction

AgNPs trigger programmed cell death (apoptosis) in microbial cells by activating apoptotic pathways. This process involves the activation of specific cellular proteins (caspases), DNA fragmentation, and characteristic morphological changes, culminating in cell death.

Through these combined mechanisms, AgNPs possess strong antibacterial properties against a variety of microorganisms, including bacteria and fungi. Their multifaceted approach this makes them desirable and important candidates for diverse biomedical and environmental applications.

Biomedical Applications

AgNPs find diverse applications in biomedicine, ranging from wound dressings and burn treatments to dental materials and medical device coatings. Their broad-spectrum antimicrobial activity makes them invaluable for combating infections in clinical settings. Ongoing research endeavors aim to explore novel applications and optimize AgNPs' biocompatibility to ensure their safe and effective utilization in biomedical contexts.

In summary, AgNPs represent a promising avenue for addressing antimicrobial challenges in biomedicine. Advances in synthesis techniques and characterization methods have facilitated the tailored design of nanoparticles for specific biomedical applications. Continued research efforts are imperative to maximize the possibilities of AgNPs while ensuring their safety and efficacy in clinical practice.

MATERIALS AND METHODS

Silver ion solution is generated by dissolving silver nitrate ($AgNO_3$) in water to create a solution containing silver ions.

Synthesis of Silver Nanoparticles Using Trisodium Citrate (Sodium Citrate)

Firstly, a 250 ml Conical flask is taken, and into it 100ml of distilled water is added, measured using a measuring cylinder. Then 0.0169gm of $AgNO_3$ (silver nitrate) is weighed using a weighing balance added into the conical flask, which is then boiled for 30 seconds on the heating plate[32,33]. Take another conical flask and add 100ml distilled water measured using measuring cylinder into it 1gm sodium citrate is weighed and added to the conical flask and dissolved. 20ml of the silver nitrate solution is placed in another conical flask and kept on the heating plate or mantle for 5 minutes at 90^0C. After

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that, 2.5 ml of sodium citrate solution is added drop by drop, the color of the solution changed slowly and turned to pale yellow

- Store the solution in a dark place to protect the solution from sunlight
- The OD values at different wavelengths i.e., 380nm, 400nm, 420nm, 440nm, 460nm…… have been observed.

Silver Nanoparticles Impact on Micro-Organisms Growth

Firstly, 20 ml of deionized water is measured using a measuring cylinder and poured into an autoclavable conical flask. Luria Bertani media of 0.5 grams and agar of 0.56gm are measured using a weighing balance and poured into the conical flask. The mixture was gently stirred to ensure that agar powder is evenly dispersed in the water. The LB agar solution is autoclaved at 121^0C for 15 minutes at 15psi pressure to dissolve the agar media completely and to sterilize the media. After autoclaving, the media is cooled to around 50-55^0C before pouring into petri dishes for solidification. After solidification, the microbial suspension was collected from the soil sample by serial dilution method, and 1ml of culture was taken and added to the LB agar media, spread on a Petri plate by spread plate method. Filter paper discs dipped in the silver nanoparticle solution are then placed on the media to check the impact or response of silver nanoparticles on the bacteria

Biochemical Tests

Citrate Test

In a clean dry culture bottle, 40 ml of distilled water is taken (Table 1). First, the components are weighed and transferred to the culture bottle. After checking the pH, agar the main component should be added before dividing the 40 ml solution into 4 test tubes because we have 4 bacteria. Transfer 10 ml in each test tube. 0.5g of agar is introduced into each test tube, and then the test tubes are covered with paper and secured with a rubber band. After the agar is added, the test tubes are kept in an autoclave for 35 minutes at 121^0c at 15psi. After autoclaving, immediately keep each test tube for vortexing in the vortex shaker. After vortexing, the tube is kept in such a way that it creates a slant in the test tube, which will be later used for streaking them vertically, thus creating a bubble in the solution or loop. The media is solidified for up to 15 to 20 minutes. After cooling, the isolated bacteria are taken as a loopful and streaked on the slant media inside the laminar air flow[12-15]. The media is left in an incubator for 24 hours and the result observed later.

Urease Test

Firstly 40ml of distilled water is taken in a culture bottle (Table 2). All the components are weighed with regards to 40 ml as in the Table 2 The pH is always checked before adding agar. Then 40 ml is divided into four test tubes, and 0.15g of agar is added to each test tube. The test tubes are covered with 4-layer paper and a rubber band, then autoclaved for 30 minutes. During this time, 0.20g of urea is weighed for the four test tubes and kept in laminar air flow with UV on for 5 minutes. Then, as soon as autoclaving is done, urea is added in each test tube before it solidifies. Vortexing of the tubes is done in a vortex spinner. Next, every test tube is stored in a slant position for the agar to solidify for 15 minutes.

Table 1. Salts and reagents for Citrate test.

Salts	Per/L	Amount per 40 ml
NaCl	5g/L	0.20g
Sodium citrate	2g/L	0.08g
Ammonium dihydrogen phosphate	1g/L	0.04g
Dipotassium phosphate	1g/L	0.04g
Magnesium sulphate	0.20g/L	0.008
Bromothymol blue	0.08g/L	Few drops
Agar	15g/L	0.60

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Table 2. Salts and reagents for urease test.

SALTS	Pers/L	Per40ml
Peptone	1g/L	0.04g
Dextrose	1g/L	0.04g
NaCl	5g/L	0.20g
Dipotassium phosphate	2g/L	0.08g
Phenol red	Few drops	Few drops
Agar	15g/L	0.60g
Urea1	20g/L	0.80g

Catalase Test

Composition

- Hydrogen peroxide
- Nutrient broth

Procedure

- Firstly a clean the glass slide with ethanol in LAF.
- 2 drops of hydrogen peroxide poured into a glass slide.
- The bacterium taken from the plate and gently mixed.

MRVP

Firstly all the components referred to from the Table 3 are weighed and dissolved in 10 ml distilled water. The pH is checked and maintained to 6.9. Media is autoclaved for 30 minutes at 121^{0}C at 15 psi. The Media is cooled down and taken into the laminar air flow and 10µl of bacterium broth is added to MRVP broth. The test tubes that were injected are incubated in the incubator for 48hrs at 38^{0}C.

MR (Methyl red) test

1. Reagents
2. Methyl red- few drops

After the incubation period, 5ml of media is transferred into a clean, dry test tube. A few drops of methyl red are added to 5ml of MR i.e.,5ml of freshly prepared 5ml solution. The red colour development is observed on the top of the solution.

VP (Voges-Proskauer)

1. Reagents
2. 4% α-napthol-0.1gm of α-naphthol is dissolved in 2ml ethanol in a 5ml vial tube.

40% potassium hydroxide (KOH)-0.4gm of KOH is dissolved in 1ml distilled water in a 2ml vial tube. 1000µl of α-napthol is added in a 5 ml vial tube VP broth .400µl of 40% KOH is added to the broth. The tube is gently shaken for 30 seconds to mix the sample. Pink red color development is observed on the top of the solution as soon as reagent is added.

Nitrate Reduction Test

Reagents

1. Nitrite reagent A(sulfanilic acid reagent)
2. Nitrite reagent B(α-napthylamine)
3. Zinc dust

Media is prepared by mixing all the ingredients. After the preparation, they are autoclaved at 121^{0}C at 15psi pressure for 15 minutes. Then, 100µl of sulfanilic acid is measured and poured into the broth test tube. Then again, 100µl of α- naphthylamine reagent is poured into the same tube. These are incubated for 24 hours at 30^{0}C.

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Table 3. Reagents for MRVP test.

Reagents	Amount in gm/l	Amount in gm for 10 ml
Dextrose	5gm/l	0.05gm
Buffered peptone	7gm/l	0.07gm
Dipotassium phosphate	5gm/l	0.05gm

Table 4. Reagents for Nitrate reduction test.

Reagents	g/l	For 20ml
Peptone	10g	0.2g
Beef extract	6g	0.12g
KNO3	2g	0.04g
NaCl	6g	0.12g

pH=6.8-7.0

Gram Staining Reagents used

1. Crystal violet (primary stain)
2. Iodine solution
3. 95% Ethanol
4. Safranin (secondary stain)
5. Water

A clean slide is taken, and a smear is made by water on the slide. Then the bacteria is inoculated on the smear by sterile loops. The slide is heat fixed by passing over the Bunsen burner. Two drops of crystal violet are added to the slide and left for 1 minute and 30 seconds, then the slide is washed with water. Two drops of iodine are added to the slide and left for 45 seconds. Now the slide is tilted and washed with 100%ethanol for 5 seconds. The slide is gently washed with water, and finally, the slide is flooded with safranin and kept for 1 minute. Safranin is rinsed with distilled water. The slide is dried on the absorbent paper. The slide is observed under a microscope with a 100x lens. The bacteria on the slide appeared pink in color Table 4.

Alamar Blue Cell Viability Assay Preparing cells

Cells are grown in RPMI media of 4ml in a T20 flask, and cells are added to the media i.e. 1 million cells /1ml. The flask is kept in the CO2 incubator at 37^0C, 90% humidity, and 5% CO2 until the cells grow. The cells are extracted using conventional trypsinization or dissociation technique. 1ml of trypsin is incorporated into T20 flask. Cells are counted using a hemocytometer, and cell destiny is found to be 4 million cells /ml

Seeding cells in 96 Well Plates

Cells are diluted with media. Approximately 5000 cells are taken for each well, 15 wells are seeded with cells. 1.5 ml of media is taken in a falcon tube, and 18µl of cells are added. In the 96 well plate, 100µl of media is added to each of the 15 wells. The 96 well plate is incubated in the CO2 incubator at 37^0C, 90% humidity, and 5% CO2 until the cells grow and adhere. The next day, silver nanoparticles containing media are added to each well i.e. 13 wells are treated with silver nanoparticles and 2 wells are without treatment as control. The 96 well plate is kept overnight for incubation.

Utilizing Alamar Blue to Treat Cells

Alamar Blue solution is prepared by mixing 1mg/ml solution. From that solution, 15µl/ml is added to the 1.5 ml growth media mixed in the T20 flask. To each well, 100µl of media containing Alamar Blue is added. The well plate is kept in the incubator for 4 hours.

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Reading Absorbance

After the incubation period, the 96 well plate is removed from the incubator. Using a plate reader, the fluorescence of the Alamar Blue solution is measured in each well. Wavelengths of emission and excitation utilized in Alamar Blue are 570-600nm and 580-610nm respectively. Fluorescence readings are recorded each well.

Effect of Silver Nanoparticles and ROS Generation in K562 Cell Lines

K562 cells are taken, 5000 cells for each well i.e. overall 75000 cells and media of 1.5 ml are mixed in the T25 flask and added to each well of 96 well plate. 15 wells are seeded with the media, each well containing 100µl. After 4 hours, the media in 13 wells is replaced with 10µl lipopolysaccharide to make up 1.3 ml of media, filling the 13 wells, with the remaining 2 wells kept as control without lipopolysaccharide. The next day, all the wells are replaced with the DCFDA 100 nm/ml dye containing media. After 4 hours of incubation period, 15 wells of the 96 well plate are treated with silver nanoparticles of different concentration for 8 wells. Fluorescence reading are taken after 4 hours.

RESULT

Synthesized Silver Nanoparticles by Sodium Citrate

Following the experimental synthesis of silver nanoparticles employing sodium citrate, our findings revealed significant success (Figure 1). We meticulously controlled the parameters of the reaction to ensure consistency and reliability. Initially, in order to create silver ion solution, silver nitrate is dissolved in water, serving as the foundation for nanoparticle formation. Sodium citrate was introduced, serving dual roles as a reducing and stabilizing agent, facilitating the reduction of silver ions into nanoparticles. Throughout the reaction, sodium citrate effectively mediated the reduction process, resulting in the creation of silver nanoparticles with consistent size and morphology (Figure 2). The stabilization imparted by sodium citrate thwarted nanoparticle aggregation, thereby ensuring colloidal stability. By using UV-Vi's spectroscopy, OD values are taken at different wavelengths i.e.,380 nm, 400 nm, 420 nm, 440 nm, 460 nm, 480 nm, 500 nm[16-18].

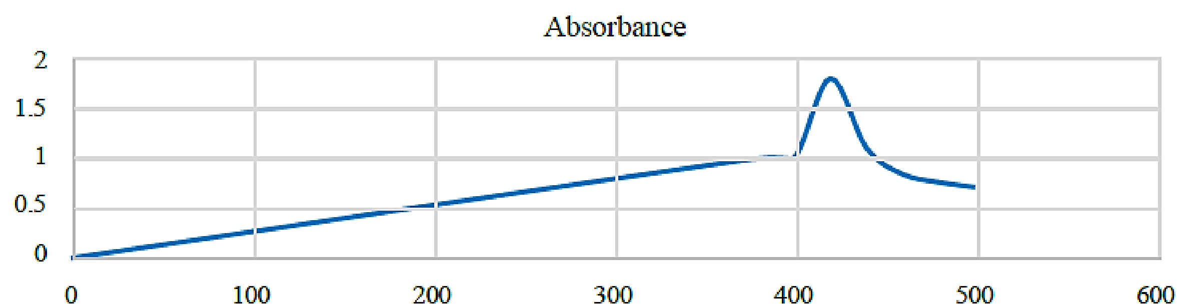

Figure 1. AgNPs showing absorbance at different wavelengths.

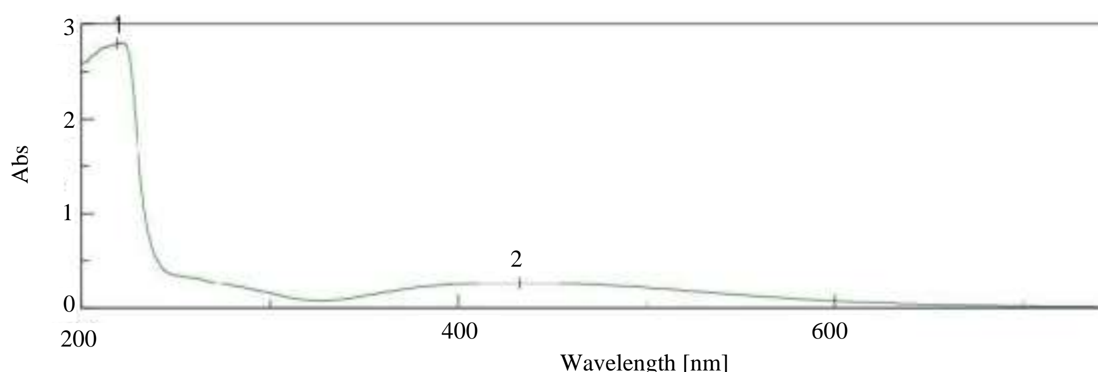

Figure 2. Shows ƛ lambda max at 420 nm observed under UV ray scanner.

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Silver Nanoparticles Impact on Microorganisms' Growth

The impact of silver nanoparticles on microbial growth was assessed through Antimicrobial Susceptibility Testing (AST) (Figure 3). In the study, I investigated the impact of nano particles of silver on microbial growth using Antimicrobial Susceptibility Testing (AST). The AST involved exposing various microbial strains to different concentrations of silver nanoparticles and measuring their growth inhibition. The findings showed a notable decline in microbial growth in the presence of silver nanoparticles, indicating their potent antimicrobial activity. Furthermore, the AST offered insightful information about the minimum inhibitory concentration of silver nanoparticles required to effectively inhibit microbial growth.

Catalase Test

The positive catalase test result observed indicates the presence of catalase enzyme activity in isolated bacterial colonies (Figure 4). The effervescence or bubbling observed upon adding hydrogen peroxide suggests that the bacteria possess catalase, which catalyzes the release of oxygen and water from hydrogen peroxide. Considering common bacterial species known to produce catalase, such as Staphylococcus aureus, Bacillus species and some Escherichia coli and other gram-negative bacteria, it is most likely that the isolated colonies belong to one of these genera. Staphylococcus aureus is frequently found on human skin and nasal passages, whereas Bacillus species are frequently linked to environmental circumstances and soil[19-21].

Citrate Test

No shift in hue or color of the medium is observed, which remains green, and no growth among the bacterial colony (Figure 5). Among the bacterial species known to exhibit a negative citrate test result are Escherichia coli and Shigella species, both of which belong to the Enterobacteriaceae family. The negative result is indicative of the inability of the tested organism to utilize citrate as a sole carbon source. Escherichia coli, for instance, lacks the enzyme citrate permease required to transport citrate into the bacterial cell for metabolism. As a result, it cannot utilize citrate as a carbon source. Similarly, Shigella species also demonstrate a negative citrate test result due to their inability to metabolize citrate.

Urease Test

No change in the hue or color of media observed indicates the negative result of the tested organism, such as Escherichia coli (E. coli), (Figure 6) which does not possess the enzymatic capability to hydrolyze urea into ammonia and carbon dioxide.

Figure 3. AST test shows the restraint zone for AgNPs.

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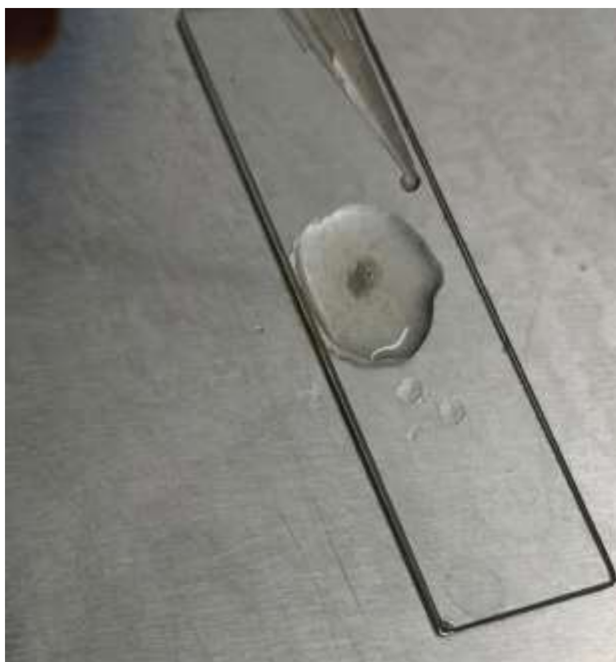

Figure 4. Catalase positive result.

Figure 5. Citrate negative result.

Figure 6. Urease negative result.

This absence of urease activity distinguishes E.coli Enterobacter species, Salmonella species, and Shigella species and other from urease-positive organisms, such as Proteus species, that can generate ammonia from urea.

MRVP Test

MR Test (Methyl Red)

A red color is appears on the medium after the addition of methyl red reagent (Figure 7). This means the bacterium is methyl red positive. It suggests that the organism generates steady, acidic final products from glucose fermentation, indicating mixed acid fermentation. Bacterial species such as Enterobacteriaceae family, such as Escherichia coli and Enterobacter aerogenes.

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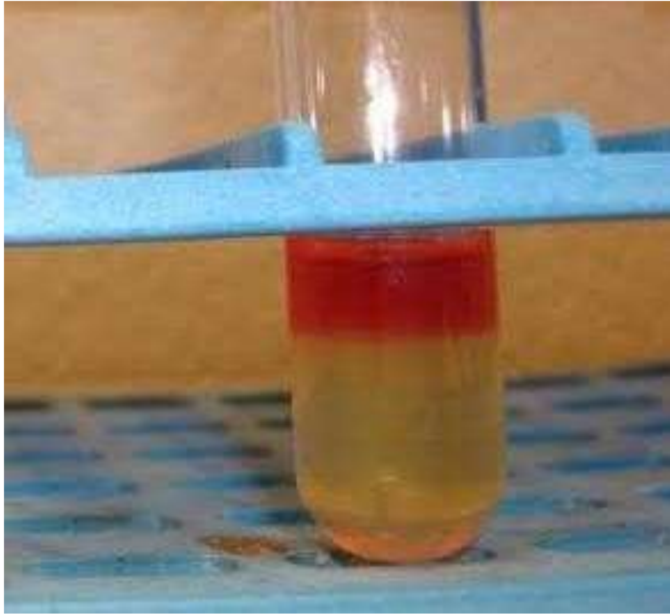

Figure 7. MR positive result.

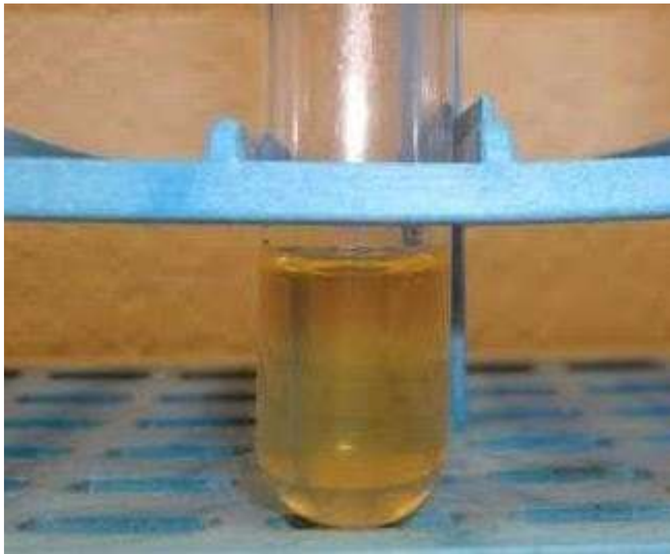

Figure 8. VP negative result.

Voges-Proskauer (VP) Test

No color change formation is observed in the medium, resulting in a negative result (Figure 8). It suggests that the organism does not generate measurable amounts of acetoin from glucose fermentation. Microorganisms that show a negative Vp test are Proteus vulgaris, Klebsiella pneumoniae and Enterobacter aerogenes.

Nitrate Reduction Test

No color alteration was observed in the sample (Figure 9). This lack of color change signifies a negative outcome, indicating that nitrate present in the sample was not converted to nitrite or further metabolized into nitrogen gas. Consequently, it is probable that the organism being studied does not possess the capability to perform nitrate reduction under the experimental conditions. Upon the addition of sulfanilic acid to the sample, no discernible color alteration was observed[22-25]. This absence of a color change serves as further evidence supporting the negative result of test, indicating that nitrate present in the sample was not converted to nitrite or metabolized further into nitrogen gas.

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Figure 9. Nitrate negative result.

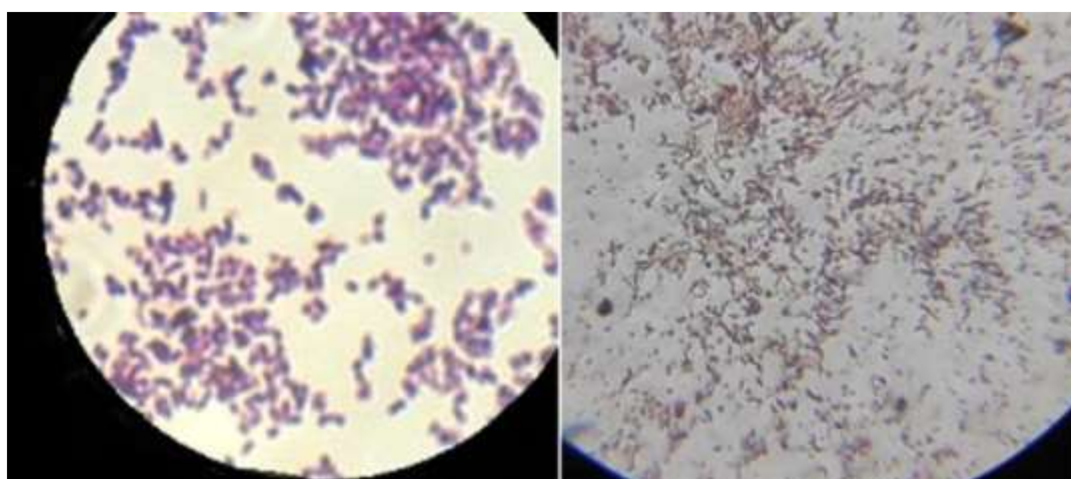

Figure 10. Pink colonies are observed under microscope.

Gram Staining

When observed under the microscope, the slide shows pink colored bacteria. Gram-negative bacteria typically appear pink or red due to the counterstaining with safranin (Figure 10). This is because the decolorization step removes the crystal violet-iodine complex from the thin peptidoglycan layer of Gram-negative bacteria, allowing the safranin stain to penetrate the cell wall and colorize the bacteria. Examples of Gram-negative bacteria include Pseudomonas aeruginosa, Escherichia coli and Salmonella Typhimurium.

Alamar Blue Cell Viability Assay

When we performed cell viability assay of cells treated without AgNPs, the viability of cells dropped compared to the untreated cells. The absorbance readings of the control group were recorded as 1234, 1345, 1122, 1297, 1377, 1438, and 1297. Upon treatment with silver nanoparticle solution, the

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absorbance readings were observed as 321, 224, 113, 231, 198, 224, and 287. To calculate percentage cell viability (Figure 11).

Absorbance of the treated and untreated cells is measured, and treated cells absorbance is divided by untreated cells absorbance and multiplied by 100 (Figure 12).

The percentage viability is calculated by formula = (Absorbance of treated cells/ absorbance) *100
The absorbance average of silver nanoparticles treated cells is=17.54
The absorbance average of untreated cells =100

Effect of silver Nanoparticles and ROS Generation in K562 Cell Lines

The results revealed a concentration-dependent relationship between AgNPs and ROS generation in K562 cells. At lower concentrations, AgNPs exhibited a moderate increase in ROS levels, indicating a mild oxidative stress response (Figure 13). However, at higher concentrations, a significant elevation in ROS production was observed, suggesting a more pronounced oxidative stress effect.

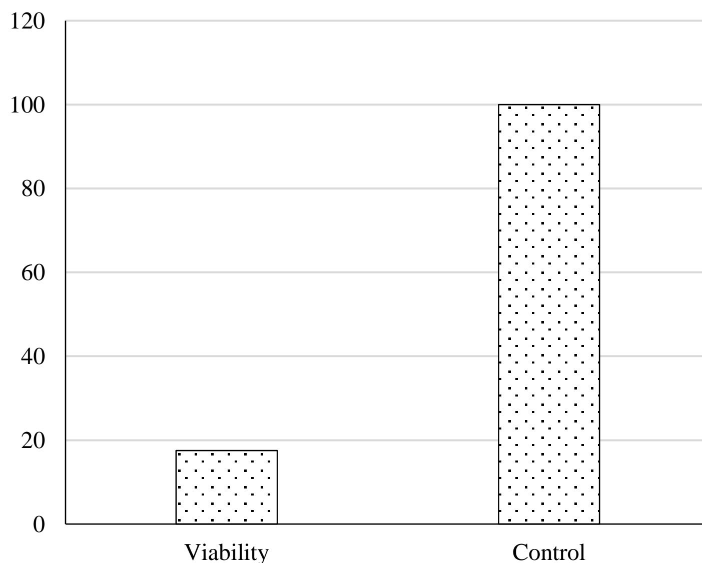

Figure 11. Alamer Blue viability test for cells.

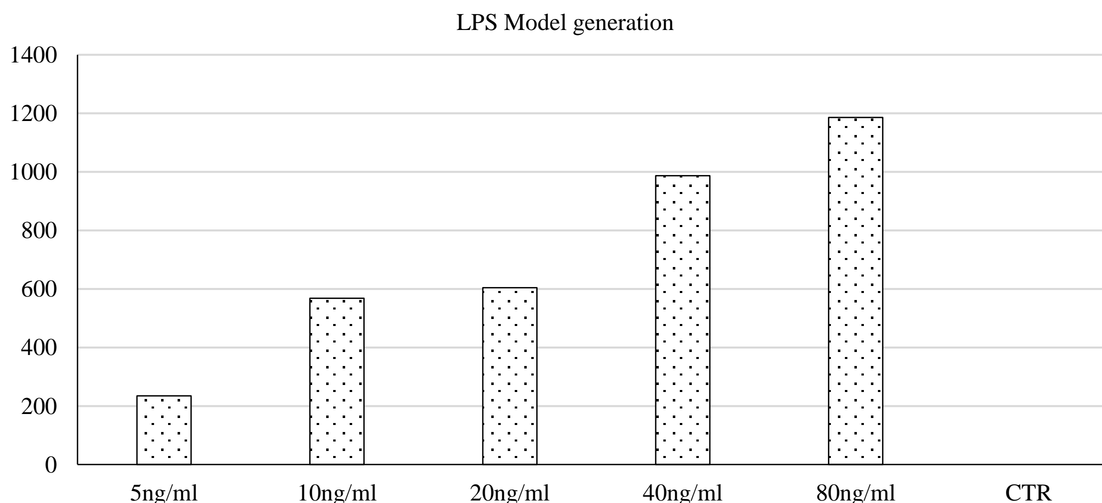

Figure 12. Cells treated with LPS show different florescence.

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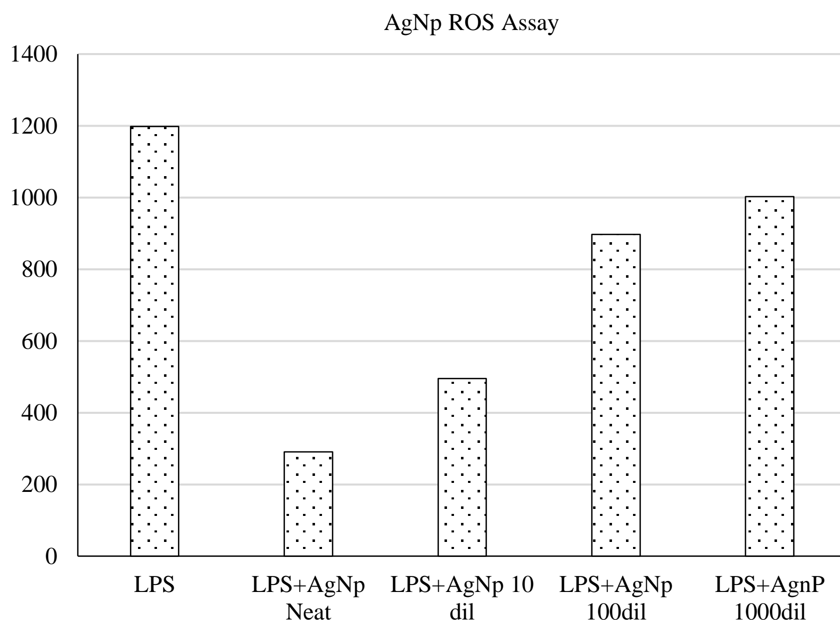

Figure 13. AgNPs with LPS treatment on cell line.

DISCUSSION

The process of producing silver nanoparticles (AgNPs) through chemical reduction using trisodium citrate, also know as sodium citrate, presents an effective and reproducible technique for creating nanoparticles with controlled size and morphology. The pale-yellow coloration observed during synthesis is indicative of the reduction of silver ions to metallic silver, a characteristic feature of successful AgNPs formation. UV-visible spectroscopy analysis further confirms the existence of AgNPs by detecting their distinct surface plasmon resonance (SPR) peaks, which typically occur within range of 380- 460 nm. The observed absorbance peaks at specific wavelengths provide valuable information about the size and distribution of the synthesized AgNPs, with higher absorbance typically correlating with smaller particle sizes.

The antimicrobial evaluation demonstrated the powerful bactericidal effects of AgNPs against different bacterial strains. Zones of inhibition found in agar diffusion studies demonstrate AgNPs capacity to impede bacterial growth by interfering with cellular processes or causing damage to cell membranes. This supports earlier research that showed the broad-spectrum antimicrobial activity of AgNPs, attributed to their ability to induce oxidative stress and interfere with bacterial metabolic pathways. The effectiveness of AgNPs against both Gram-positive and Gram-negative bacteria suggests their potential as alternative antimicrobial agents to combat multidrug-resistant pathogens.

Biochemical tests further elucidate the mechanisms underlying AgNPs- mediated antimicrobial effects by evaluating their impact on bacterial metabolic activities. Positive results in tests such as the citrate test and urease test indicate alterations in bacterial enzymatic activities after being exposed to AgNPs, confirming the metabolic disruption theory. These results imply that AgNPs might obstruct vital cellular functions, reducing the development and viability of the bacteria.

Furthermore, AgNPs demonstrate cytotoxic actions to cancerous cell lines, as evidenced by the Alamar Blue assay. The dose-correlated decline in cell viability upon AgNP treatment suggests their potential as anticancer agents. Reactive oxygen species (ROS) production in AgNP-treated cancer cells further supports the hypothesis that AgNPs cause cytotoxicity via way of oxidative stress-mediated mechanisms. The results are consistent with earlier research highlighting the anticancer activities of AgNPs and underscore their potential as promising candidates for cancer therapy.

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Overall, the study's findings provide important insights into the mechanisms by which AgNPs work against microbial infections and cancer cells, advancing our knowledge of their potential as therapeutics. Further research is warranted to optimize the synthesis methods, elucidate the underlying molecular mechanisms, and evaluate the effectiveness and safety of AgNPs for clinical applications. Additionally, exploring the potential synergistic effects of AgNPs with existing antimicrobial and anticancer agents may lead to the creation of cutting-edge combination treatments that improve the efficacy of treatment.

SUMMARY AND CONCLUSION

In this investigation, we embarked on a meticulous journey to synthesize silver nanoparticles using trisodium citrate, also known as sodium citrate, as a dual-purpose agent, both reducing and stabilizing the nanoparticles. Our experimental setup was finely tuned to ensure precise control over reaction parameters, resulting in the consistent formation of nanoparticles. By employing UV-Vi's spectroscopy, we confirmed the successful synthesis, with a prominent absorbance peak observed at 420nm, indicative of the presence of uniformly sized and shaped silver nanoparticles.

Moving beyond synthesis, our research focused on the antimicrobial potential of these nanoparticles. Through Antimicrobial Susceptibility Testing (AST), we uncovered their remarkable ability to inhibit microbial growth across various strains. Determining the minimum inhibitory concentration (MIC) further underscored their efficacy, holding promise for combating microbial infections.

Expanding our exploration, we subjected isolated bacterial colonies to a battery of biochemical tests. Positive catalase tests hinted at the presence of catalase enzyme activity, while negative results in citrate and urease tests unveiled metabolic traits specific to the tested organisms. Gram staining affirmed the Gram-negative nature of the bacteria, providing vital information on their classification.

Additionally, our focus extended to assessing the impact of silver nanoparticles on cellular viability. Through a comprehensive cell viability assay, we observed a notable decrease in viability upon nanoparticle treatment, suggesting potential cytotoxic effects warranting further investigation.

Lastly, our examination of silver nanoparticles' interaction with K562 cell lines unveiled intriguing fluorescence changes, hinting at intricate cellular responses to nanoparticle exposure.

In summation, our study represents a multifaceted exploration into the synthesis, antimicrobial activity, and biological effects of silver nanoparticles. Our research not only advances knowledge of nanoparticle behavior but also shows promise for their application in various biomedical and antimicrobial contexts, paving the way for future research endeavors.

List of Abbreviations

RPM: Revolutions per minute
OD: Optical density
LAF: Laminar airflow
DCFDA: Dichloride hydro fluoresce in diacetate
ROS: Reactive oxygen species
LPS: Lipo polysaccharide
UV: Ultra violet
UV-Vis: Visible ultra violet spectroscopy
DLS: Dynamic light scattering
TEM: Transmission electron microscopy

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