SYNTHESIS AND ANTIBACTERIAL ACTIVITIES OF SILVER NANOPARTICLE

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled "Synthesis and Antibacterial Activities of Silver Nanoparticle" has been written and submitted by me, Lutful Alam and has been carried out under the supervision of Dr. M Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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Abstract

Antibiotic resistance is on the rise for the last few decades. As commercial antibiotics are failing to combat microbes, alternative methods are being investigated. The properties of silver as disinfectant have been known for years. Synthesizing and using silver nanoparticles to inhibit the growth of drug-resistant bacteria can be the solution to combat this alarming problem. Here, silver nanoparticles were synthesized using 5 different methods. The synthesized particles were analyzed by UV spectrometry, Scanning Electron Microscopy and DLS Spectroscopy. AgNPs obtained from the first and fifth method of synthesis were most stable and uniform in size. Silver nanoparticle was found to have antimicrobial activities but that was not so significant.

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Introduction

1. Introduction

1.1 Nanoparticles

Nanosized inorganic particles, of either simple or composite nature, display unique physical and chemical properties and represent an increasingly important material in the development of novel nano-devices which can be used in numerous physical, biological, biomedical, and pharmaceutical applications (Chan *et al.,* 2002). Any particle of the size from 1 to 100 nm is considered as a nanoparticle. They also contain an interfacial layer which affects its properties. In the current world of science, nanoparticles hold a great potential. These particles have a very high surface area to volume ratio. This property attributes to the function of nanoparticles. A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface area and structural properties (Jouget *et al.,* 2002).

1.2 Uses of silver nanoparticle

Silver nanoparticles (AgNPs) are increasingly being used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties. These include optical, electrical, and thermal, high electrical conductivity, and biological properties (Li *et al.,* 2016). Due to their unique properties, they have been used for several applications, including as antibacterial agents, in industrial, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopedics, drug delivery, as anticancer agents, and have ultimately enhanced the tumor-killing effects of anticancer drugs (Chernosouva, 2013). Recently, AgNPs have been frequently used in many textiles, wound dressings, and biomedical devices. Nanosized metallic particles are unique and can considerably change physical, chemical, and biological properties due to their surface-to-volume ratio; therefore, these nanoparticles have been exploited for various purposes. In order to fulfill the requirement of AgNPs, various methods have been adopted for AgNP synthesis. Generally, conventional physical and chemical methods seem to be very expensive and hazardous. Interestingly, biologically-prepared AgNPs show high yield, solubility, and high stability (Gurunathon *et al.,* 2009). Among several synthetic methods for AgNPs, biological methods seem to be simple, rapid, non-toxic,

dependable, and green approaches that can produce well-defined size and morphology under optimized conditions for translational research.

1.3 Biological activity of silver nanoparticle

The biological activity of AgNP depends on few factors including surface chemistry, size, size distribution, shape, particle morphology, particle composition, coating/capping, agglomeration, and dissolution rate, particle reactivity in solution, efficiency of ion release, and cell type, and the type of reducing agents used for the synthesis of AgNPs are crucial factors for the determination of their cytotoxicity (Carlson *et al.,* 2008). The physicochemical properties of nanoparticles enhance the bioavailability of therapeutic agents after both systemic and local administration (Jo *et al.,* 2015) and on the other hand, it can affect cellular uptake, biological distribution, penetration into biological barriers, and resultant therapeutic effects (Albanese *et al.,* 2012). Therefore, the development of AgNPs with controlled structures that are uniform in size, morphology, and functionality are essential for various biomedical applications (Pancek *et al.,* 2006).

1.4 Preparation of silver nanoparticle

The preparation of uniform nanosized drug particles with specific requirements in terms of size, shape, physical and chemical properties is of great interest in the formulation of new pharmaceutical products (Richards *et al.,* 2000). Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains (Courvier *et al.,* 1987).

Generally, the synthesis of nanoparticles has been carried out using three different approaches, including physical, chemical, and biological methods. In physical methods, nanoparticles are prepared by evaporation-condensation using a tube furnace at atmospheric pressure (Gurav *et al.,* 1994). Conventional physical methods including spark discharging and pyrolysis were also used for the synthesis of AgNPs (Tien *et al.,* 2008). The advantages of physical methods are speed, radiation used as reducing agents, and no hazardous chemicals involved, but the downsides are low yield and high energy consumption, solvent contamination, and lack of uniform distribution (Shameli *et al.,* 2010).

Chemical methods use water or organic solvents to prepare the silver nanoparticles (Tao *et al.,* 2006). This process usually employs three main components, such as metal precursors,

reducing agents, and stabilizing/capping agents. Basically, the reduction of silver salts involves two stages (1) nucleation; and (2) subsequent growth phase. In general, silver nanomaterials can be obtained by two methods, classified as "top-down" and "bottom-up" method (Deepak *et al.*, 2011). The "top-down" method is the mechanical grinding of bulk metals with subsequent stabilization using colloidal protecting agents (Malick *et al.*, 2004). The "bottom-up" methods include chemical reduction, electrochemical methods, and sono-decomposition. The major advantage of chemical methods is high yield, contrary to physical methods, which have low yield. The above-mentioned methods are extremely expensive. Additionally, the materials used for AgNPs synthesis, such as citrate, borohydride, thio-glycerol, and 2-mercaptoethanol are toxic and hazardous. Apart from these disadvantages, the manufactured particles are not of expected purity, as their surfaces were found to be sedimented with chemicals. It is also very difficult to prepare AgNPs with a well-defined size, requiring a further step for the prevention of particle aggregation (Malik *et al.*, 2002). In addition, during the synthesis process, too many toxic and hazardous byproducts are excised out. Chemical methods make use of techniques such as cryochemical synthesis, laser ablation, lithography, electrochemical reduction, laser irradiation, sono-decomposition, thermal decomposition, and chemical reduction. The advantage of the chemical synthesis of nanoparticles is the ease of production, and high yield; however, the use of chemical reducing agents are harmful to living organisms. Recently, a study explained a detailed account of synthesis methods, properties, and bio-application of AgNPs (Ganaie *et al.*, 2015).

Biological methods have emerged as viable options to overcome the limitations of chemical methods. In recent times, biologically-mediated synthesis of nanoparticles was proven to be simple, cost effective, dependable, and environmentally friendly approaches. As an alternative method to chemical methods, much attention has been given to the high yield production of AgNPs of defined size using various biological systems including bacteria, fungi, plant extracts, and small biomolecules. Biological methods are suitable not only for silver nanoparticles, but also for the synthesis of several other nanoparticles like gold and graphene.

1.5 Characterization of silver nanoparticle

The physicochemical properties of nanoparticles have a significant impact on their biological properties. Thus, precise particle characterization should be done upon synthesis. To ensure the safety issue of using the full potential of any nanoparticle in the purpose of human

welfare, like in nanomedicines, or in the health care industry, prepared nanoparticles should be characterized before application. The characteristic features of nanoparticles such as size, shape, size distribution, surface area, solubility, aggregation are needed to be evaluated before assessing toxicity or biocompatibility. Many analytical techniques have been used to evaluate the synthesized nanoparticles. These include ultraviolet visible spectroscopy (UV-vis spectroscopy), Fourier transform infrared spectroscopy (FTIR), X-ray diffractometry (XRD), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and many other techniques.

UV-vis spectroscopy is a useful and reliable technique for the primary characterization of synthesized nanoparticles. This is also used to monitor the synthesis and stability of AgNPs. Since AgNPs have unique optical properties, they can strongly interact with specific wavelengths of light. Besides that, UV-vis spectroscopy is fast, easy, simple, sensitive, and selective for different types of nanoparticles. This needs only a short period of time for measurement. Moreover, a calibration is not required for particle characterization of colloidal suspensions. In case of silver nanoparticles, the conduction band and valence band lie very close to each other in which electrons move freely. These free electrons give rise to a surface plasmon resonance (SPR) absorption band which occurs due to the collective oscillation of electrons of AgNPs in resonance with the light wave. The absorption of silver nanopaticles depends on the particle size, dielectric medium, and chemical surroundings. Observation of this peak, assigned to a surface plasmon, is well documented for various metal NPs with sizes ranging from 2 to 100 nm.

X-ray diffraction, shortly known as XRD, is a popular analytical technique which has been used for the analysis of both molecular & crystal structures, quantitative resolution of chemical species, measuring the degree of crystallinity, qualitative identification of various compounds, particle sizes, etc. As X-ray light reflects on any crystal, it leads to the formation of many diffraction patterns. The physico-chemical characteristics of the crystal structure are reflected by these patterns. In case of a powder sample, diffracted beams typically come from the sample and reflect its structural physico-chemical features. For this reason, XRD can analyze the structural features of a wide range of materials, such as inorganic catalysts, superconductors, biomolecules, glasses, polymers, and so on (Robin, 2009). Analysis of these particles largely depends on the formation of diffraction patterns. Each material has a unique

diffraction beam which can define and identify it by comparing the diffracted beams with the reference database in the Joint Committee on Powder Diffraction Standards (JCPDS) library. In addition, the diffracted patterns also explain whether the sample materials are wholesome or contain impurities. Thus, XRD has long been used to define and identify bulk and nanomaterials, forensic specimens, industrial and geochemical samples.

In recent times, the field of nanoscience and nanotechnology has provided a driving force in the development of various high-resolution microscopy techniques for learning more about nanomaterials using a beam of highly energetic electrons to probe objects on a very fine scale. Among various electron microscopy techniques, Scanning Electron Microscope, shortly known as SEM, is a surface imaging method, fully capable of determining different particle sizes, size distributions, nanomaterial shapes, and the surface morphology of the synthesized particles. By using SEM, we can probe the morphology of nanoparticles and derive a histogram from the images by either by measuring and counting the particles manually, or this can be done by using specific software. The combination of SEM and energy-dispersive X-ray spectroscopy (EDX) can be used to study silver nanoparticle morphology and also to conduct chemical composition analysis. There are few limitations of SEM. SEM is not able to resolve the internal structure, however, it can provide valuable information regarding the purity and the degree of particle aggregation and the modern high-resolution SEM can identify the morphology of nanoparticles under the size of 10 nm.

For the characterization of nanomaterials, TEM is a valuable, frequently used, and important technique. It is used to obtain quantitative measures of particle and/or grain size, size distribution, and morphology. The magnification of TEM is determined by the ratio of the distance between the objective lens and the specimen; and the distance between objective lens and its image plane. TEM has two advantages over SEM: it can provide better spatial resolution and it has the capability for additional analytical measurements. The disadvantages include a required high vacuum, thin sample section, and the vital aspect of TEM is that sample preparation is time consuming. Therefore, sample preparation is extremely important in order to obtain the highest-quality images possible.

1.6 Uses of silver nanoparticle to combat antibiotic resistance

It is well known that silver ions and silver-based compounds are highly toxic to microorganisms (Slawson, 1997) showing strong biocidal effects on as many as 16 species of

bacteria including *E. coli* (Herrera *et al.,* 2001). Thus, silver ions, as an antibacterial component, have been used in the formulation of dental resin composites (Yoshida, 1999) and ion exchange fibers (Nonanka *et al.,* 2000) and in coatings of medical devices. Recently, Tiller and co-workers showed that hybrids of silver nanoparticles with amphiphilic hyper branched macromolecules exhibit effective antimicrobial surface coatings (Aymonier *et al.,* 2001).

Silver nanoparticles seem to be alternative antibacterial agents to antibiotics or it can be conjugated with other antibiotics to overcome the bacterial resistance. Thus, it is necessary to develop silver nanoparticle or nanosilver conjugated antibiotics as antibacterial agents. Silver nanoparticle is a promising nanomaterial. The large surface-to-volume ratios and crystallographic surface structure make AgNPs a potential antibacterial agent. The seminal paper reported by Sondi and Salopek-Sondi demonstrated the antimicrobial activity of AgNPs against *E. coli.* In that study, *E. coli* cells were treated with AgNPs and it showed the accumulation of AgNPs in the cell wall and the formation of "pits" in the bacterial cell walls. Eventually, it leads the cell to death. In addition, smaller nanoparticles with a larger surface-to-volume ratio showed a more efficient antibacterial activity than larger particles when they were treated against same *E. coli*.

Biofilms can lead to antimicrobial resistance. Those are also involved in the development of ocular-related infectious diseases like microbial keratitis. Kalishwaralal and co-workers demonstrated the potential anti-biofilm activity against *P. aeruginosa* and *S. epidermidis*. Likewise, guava leaf extract reduced silver nanoparticle (Gr-Ag-NPs) showed significant antibacterial activity & stability against *E. coli* compared to chemically synthesized AgNPs. The reason for this higher activity could be the adsorption of biomolecules on the surface of the Gr-Ag-NP. Silver nanoparticles synthesized by *Cryphonectria* sp. showed antibacterial activity against various human pathogenic bacteria like *S. aureus, E. coli, S. typhi,* and *C. albicans*. These particular silver nanoparticles exhibited higher antibacterial activity against both *S. aureus* and *E. coli* than against *S. typhi* and *C. albicans.*

Gram negative organisms such as *E coli* and *Klebsiella* species can cause many major diseases. Normally a part of the gut flora, *Escherichia coli* can be found in the oral cavity as well. *E. coli* is gram negative, rod shaped and motile with no capsule (Tortora, 1982). Especially by way of the fecal-oral pathway, *E. coli* can inhabit inside of the human mouth. While not all the strains of *E. coli* are pathogenic, the ones that can cause major infections as

well as severe diarrhea, *E. coli* is capable of causing septicemia, gastrointestinal infection and unitary tract infection. In addition to that, *E. coli* often prolongs the infection healing period. Likewise to many other gram negative bacteria, these are also showing antibiotic resistance.

Another gram negative opportunistic pathogenic bacterium is *Proteus* species. The swarming colony it produces is a distinguishing feature of its major species *P. mirabilis*. It is rod shaped with peritrichous flagella that gives it swarming motility. Additionally, it also exhibit urease activity (Tortora, 1982). Ubiquitous in soil and water, these bacteria can reside in human infection sites. These are capable of causing nosocomial infections that include septicemia and pneumonia.

Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. Previous studies have shown that antimicrobial formulations in the form of nanoparticles could be used as effective bactericidal materials (Fresta *et al.*, 1995). Recently, Klabunde and co-workers demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria (Stoeimenov *et al.*, 2000). Thus, the preparation, characterization, surface modification, and functionalization of nanosized inorganic particles open the possibility of formulation of a new generation of bactericidal materials.

This study deals with the synthesis of silver nanoparticles by 5 chemical methods and analysis of their efficiency. It also checks the effectiveness of the nanoparticles to kill Gram Negative *Escherichia coli.*

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Materials and Methods

2. Materials and Methods

2.1 Materials and instruments

Silver nitrate ($AgNO_3$) was collected from Honeywell Fluka, Polyvinylpyrrolidone (PVP 40000) from Alfa Aesar. Polyethylene glycol and sodium borohydride were collected from Sigma-Aldrich. Hydrogen peroxide and trisodium citrate were purchased from Roth, ethanol and ammonium hydroxide from Merck. All chemicals and reagents were used as received without further purification. Water used in all procedures was obtained from a water purification system.

Experiments were conducted using various instruments UV–vis double beam spectrophotometer (PG Instrument, UK T60 U), Scanning Electron Microscope (SEM) (Model Joel JSM-6490LA, Japan), Micro Centrifuge Machine (Tomy, MX-305), Laboratory incubator (Incucell), Particle Size Analyzer (Malvern).

The other apparatus used are as follows:

- Laminar airflow cabinet (Model SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digi system Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Centrifuge machine
- Glasswares, Laboratory distillation apparatus- fractional distillatory set up, Microscope,
- Petri-dishes, Test-tubes, Micro-pipettes, Bunsen burner, Electric balance, etc.

2.2 Synthesis of Silver Nanoparticle:

2.2.1 Formula One: Using trisodium citrate as reducing agent

In a beaker, 100 mL trisodium citrate (7.0 mM) solution was prepared which was later boiled and then 1 mL of 0.1 M aqueous silver nitrate was added in the TSC solution for initiating nanoparticle synthesis (Dong *et al.*, 2009).

Trisodium citrate (TSC) was dissolved into 100 ml distilled water

↓

TSC solution was boiled at 100 °C

↓

Silver nitrate was added into the solution under stirring

↓

Color started changing gradually from colorless to yellow

↓

Stirring was stopped as color turned into turbid yellow.

2.2.2 Formula Two: Using sodium borohydride as reducing agent

2.0 mM sodium borohydride ($NaBH_4$) solution was prepared in a beaker and chilled at 4°C for 20 minutes followed by dropwise addition of 2.5 ml amount of 0.01 mM Silver Nitrate under magnetic stirrer. (Solomon *et al.*, 2007).

Sodium borohydride was dissolved into 57.5 ml distilled water

↓

$NaBH_4$ solution was chilled at 4°C for 20 minutes

↓

Silver nitrate was added dropwise to $NaBH_4$ solution under stirring

↓

Color of the solution was changed gradually to brighter yellow

↓

Stirring was stopped

2.2.3 Formula Three: Using both TSC and sodium borohydride as reducing agent

A mixture of 0.01 M trisodium citrate and 0.01 M silver nitrate were stirred for 3 minutes at 10 °C followed by addition of 0.002 M sodium borohydride to the mixture which changed the color of the solution to golden yellow indicating the formation of nanoparticles (Brown *et al*, 2012).

Trisodium citrate (TSC) was dissolved in 40ml distilled water and

silver nitrate was dissolved in 10ml distilled water

↓

Combined solution was stirred for 3 minutes at 10 °C

↓

Sodium borohydride solution was added dropwise under stirring

↓

Color of the solution was changed gradually from colorless to golden yellow

↓

Stirring was stopped.

2.2.4 Formula Four: Using TSC, sodium borohydride as reducing agent & H_2O_2 as stabilizer.

Aqueous solution of 0.05 M silver nitrate, 0.075 M trisodium citrate was prepared separately and mixed together with 100 µL H_2O_2 followed by addition of 250 µL of 100 mM sodium borohydride into the solution mixture to initiate nanoparticle synthesis. (Zhang *et al.*, 2011).

10 ml silver nitrate and 30ml TSC solution was prepared

and mixed together under stirring

↓

100 micro liter H_2O_2 was added into the solution

↓

Sodium borohydride was added into the mixture

↓

Color of the solution turned to deep reddish yellow

↓

Stirring was stopped.

2.2.5 Formula Five: Using PEG as reducing agent

Polyethylene glycol (0.05 g) was dissolved in 60 mL of distilled water under magnetic stirring by heating at 120 °C followed by addition of 1.5 g of PVP and 0.05 g AgNO3 to the solution and stirred for 1 hour under continuous heat. (de Oliveira *et al.*, 2017).

Polyethylene glycol (PEG) was dissolved in 60 mL distilled water.

↓

PEG solution was stirred and heated at 120 °C.

↓

PVP & $AgNO_3$ were added to the solution under continuous heat and stirring

↓

Heating stopped, stirring continued until the temperature cooled down to room temperature

↓

Stirring was stopped.

Upon synthesis, initial confirmation of nanoparticle synthesis was done by UV–visible absorption spectra analysis. UV-visible spectroscopy typically shows a signature peak in absorbance between 350 nm and 450 nm confirming the formation of nanoparticle.

Size, shape, size distribution and morphological analysis were carried out by Scanning Electron Microscope and Malvern DLS measurement Instrument. In case of Scanning Electron Microscope, powder nanoparticles were used. On the other hand, colloid suspension was required for DLS observation.

Anti-bacterial activity of silver nanoparticle synthesized by five methods was observed and compared through well diffusion method. Efficiency of same concentration of silver nitrate was also measured as silver nitrate was basis of silver nanoparticle synthesis in all five methods. Apart from that, Minimum Bactericidal Concentration (MBC) of five nanoparticles was determined against an *E. coli* isolate.

2.3 Well Diffusion Test

Anti-bacterial activities of nanoparticles synthesized by five formulas along with silver nitrate of same concentration were observed by well diffusion method.

Mueller Hinton Agar (MHA) media was prepared

and 20 ml of MHA media per conical flask was autoclaved

↓

Autoclaved media was cooled down to a minimum temperature

↓

Organism was inoculated into the media with a loop followed by gentle shaking of the media

↓

Transferring the inoculated media in a Petri dish to solidify

↓

Six wells were made for five type of nanoparticle and silver nitrate of same concentration

↓

Zone of inhibition after overnight incubation was recorded.

2.4 Determination of MBC of five nanoparticles against *E. coli*

i) *E. coli* was inoculated into 5 ml LB broth with sterile tooth pick

Inoculated broth was kept in incubator (37 °C) for overnight incubation

One milliliter of the cultured organism was subcultured into 5 ml LB broth

Subculture was incubated for 2/3 hours

OD of the subculture was adjusted between 0.4-0.6

Figure 2.1: Optical density (OD) measurement

ii) One milliliter of the subcultured media was taken into a test tube

Nine milliliter fresh LB broth was added to the test tube to make diluted cell suspension

iii) Test tube 1: 50 µl cell suspension was added in 5 ml LB broth

Test tube 2: 50 µl cell suspension was added in 5ml LB broth+5mg/L AgNP

Test tube 3: 50 µl cell suspension was added in 5ml LB broth+10mg/L AgNP

Test tube 4: 50 µl cell suspension was added in 5ml LB broth+20mg/L AgNP

Test tube 5: 50 µl cell suspension was added in 5ml LB broth+40mg/L AgNP

Test tube 6: 5ml LB broth only

Six test tubes were incubated at 37 °C for 2-2.5 hours into shaker incubator.

Figure 2.2: Shaker incubator

Figure 2.3: Text tubes containing different concentration of AgNPs

iv) Petri dish 1: Spread 5 µl inoculated media from test tube 1

Petri dish 2: Spread 5 µl inoculated media from test tube 2

Petri dish 3: Spread 5 µl inoculated media from test tube 3

Petri dish 4: Spread 5 µl inoculated media from test tube 4

Petri dish 5: Spread 5 µl media from test tube 5

Petri dish 6: Spread 5 µl media from test tube 6

Petri dishes were kept in incubator for overnight incubation

Number of colonies was counted on each Petri dish.

v) MBC was determined by observing the results.

Results

3. Results

3.1 Confirmation of AgNP synthesis by UV–visible absorption spectra analysis

Upon synthesis, prior confirmation of nanoparticle synthesis was observed by UV–visible absorption spectra analysis using UV-Spectroscopy. For each of the methods, UV-vis absorption spectrum of AgNP was found at desired range which gave an initial assurance of silver nanoparticle formation.

Formula 1: AgNP synthesis using trisodium citrate (TSC) as reducing agent

Figure 3.1: AgNP synthesized by TSC

For the first method, the color of the solution turns to turbid yellow indicating the synthesis of silver nanoparticle.

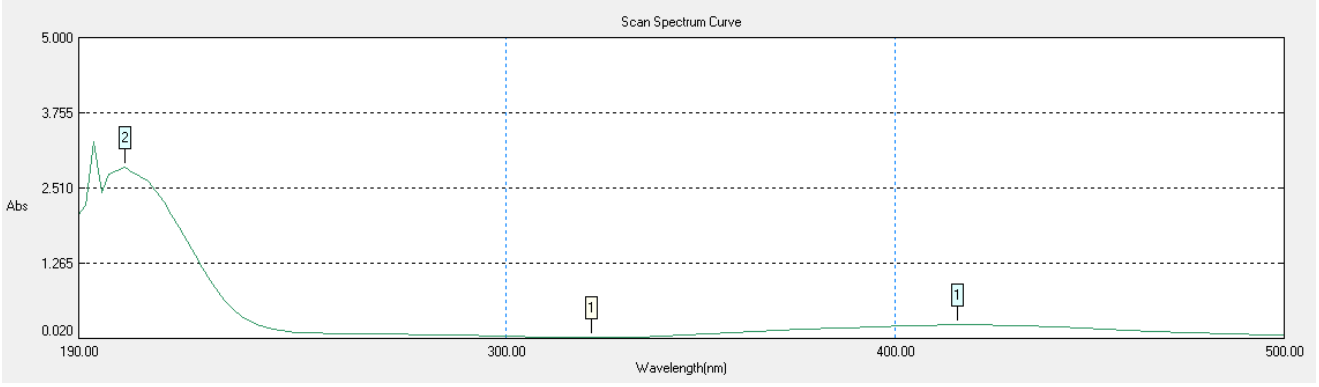

Figure 3.2: UV absorbance spectrum of synthesized AgNP at 416 nm

For UV–visible absorption spectra analysis, the solution was diluted four times. The absorption peak was found at 416 nm confirming the synthesis of AgNP. The above graph shows absorbance-wavelength correlation for AgNP synthesized by this method.

Formula 2: AgNP synthesis using sodium borohydride as reducing agent

Figure 3.3: AgNP synthesized by $NaBH_4$

After the addition of silver nitrate, the color of the solution turned to brighter yellow indicating the synthesis of silver nanoparticle.

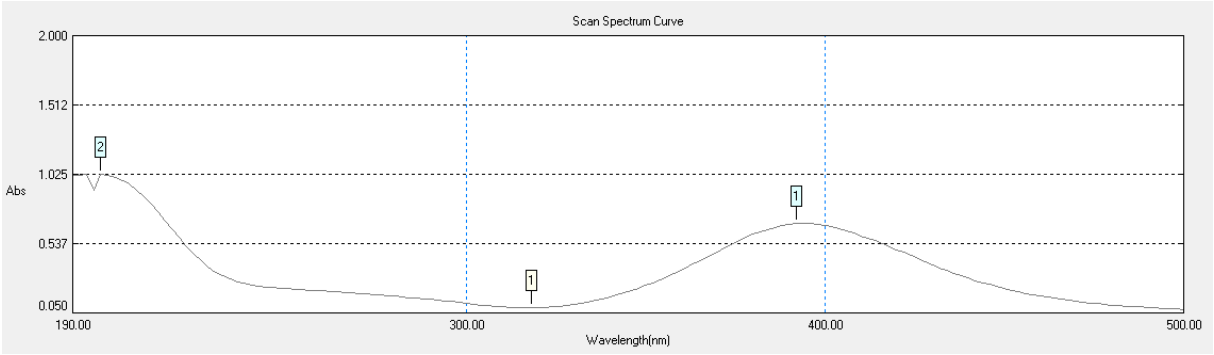

Figure 3.4: UV absorbance spectrum of synthesized AgNP at 392 nm

Like the first method, the solution was diluted four times for UV–visible absorption spectra analysis. The absorption peak was found at 392 nm confirming the synthesis of AgNP. The above graph shows absorbance-wavelength correlation for AgNP synthesized by this method.

Formula 3: AgNP synthesis using sodium borohydride & trisodium citrate (TSC) as reducing agent

Figure 3.5: AgNP synthesized by $NaBH_4$ and TSC

For the third method, the color of the solution turned to golden yellow indicating the formation of silver nanoparticle.

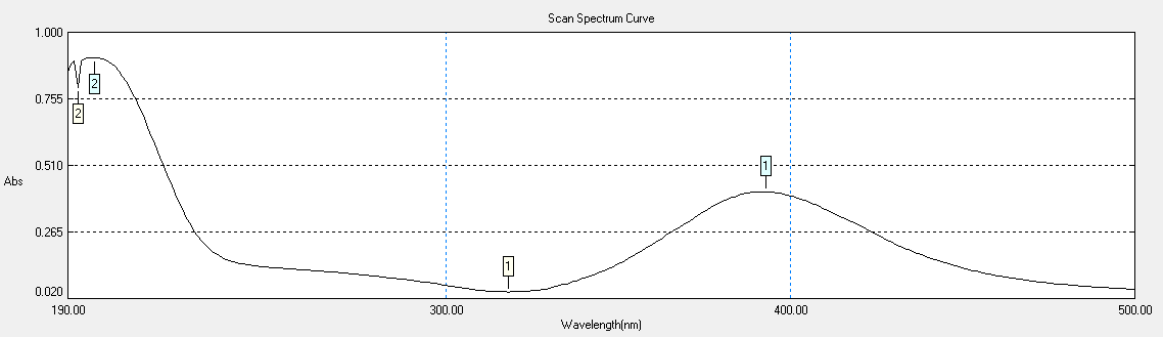

Figure 3.6: UV absorbance spectrum of synthesized AgNP at 393 nm

The solution was diluted four times for UV–visible absorption spectra analysis. The absorption peak was found at 393 nm confirming the synthesis of AgNP.

Formula 4: AgNP synthesis using $NaBH_4$ & TSC as reducing agent & H_2O_2 as stabilizer

Figure 3.7: AgNP synthesized by $NaBH_4$, TSC and H_2O_2

For the fourth method, the color of the solution turned to reddish yellow indicating the formation of silver nanoparticle.

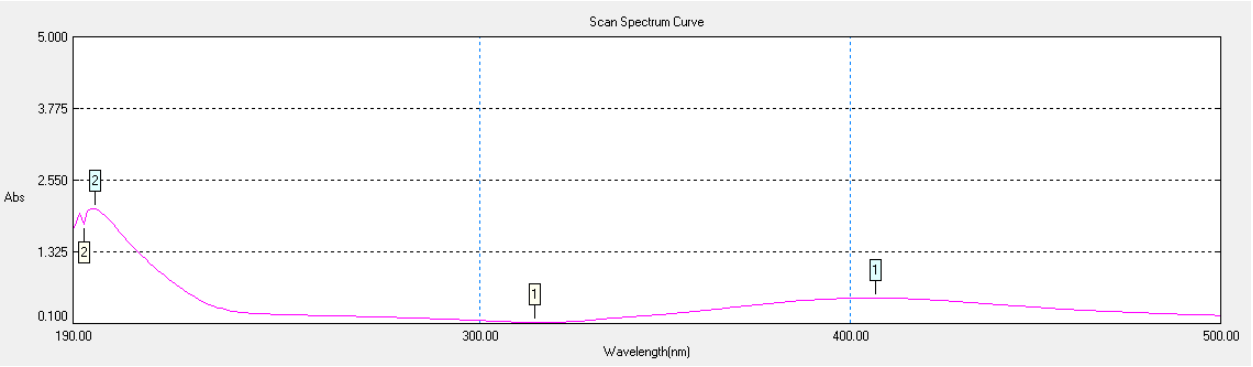

Figure 3.8: UV absorbance spectrum of synthesized AgNP at 403 nm

For this method, the solution was also diluted four times for UV–visible absorption spectra analysis. The absorption peak was found at 403 nm confirming the synthesis of AgNP.

Formula 5: AgNP synthesis using PEG as reducing agent

Figure 3.9: AgNP synthesized by PEG

For the fifth method, the color of the solution turned to light yellow indicating the formation of silver nanoparticle.

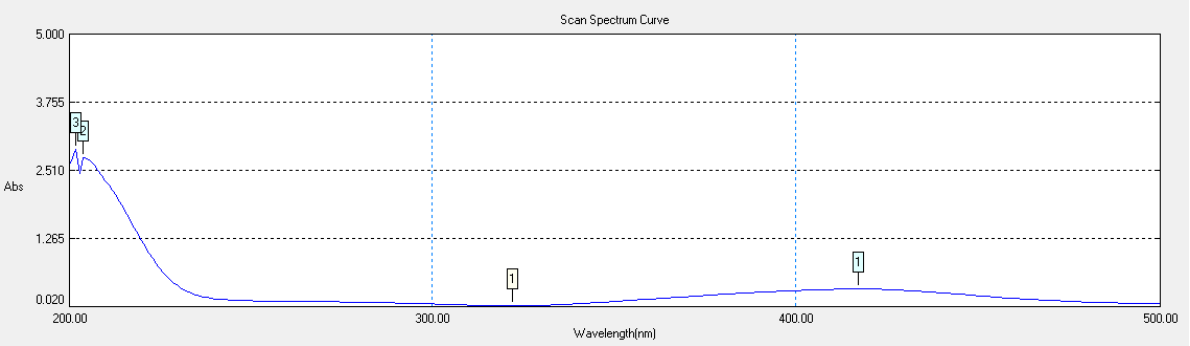

Figure 3.10: UV absorbance spectrum of synthesized AgNP at 418 nm

In case of fifth method, the solution was diluted twice for UV–visible absorption spectra analysis. The absorption peak was found at 418 nm which confirmed the synthesis of AgNP. The above graph shows absorbance-wavelength correlation for AgNP synthesized by this method.

3.2 Size, shape distribution and morphological analysis:

Morphological analysis of AgNp by Scanning Electron Microscope

Powder nanoparticles were spread into carbon coated aluminum stubs to determine morphological characteristics of nanoparticles using Scanning Electron Microscope. SEM analysis showed shape, approximate size and the levelof aggregation of AgNP.

AgNP synthesized by formula one:

Image of AgNP synthesized by first method was observed at 10,000 to 50,000 times magnification. SEM analysis showed that spherical and rod shaped AgNP was formed in case of citrate reduction method. Besides that, silver nanoparticles seemed to be less aggregated which also ensured a good stability of the particles.

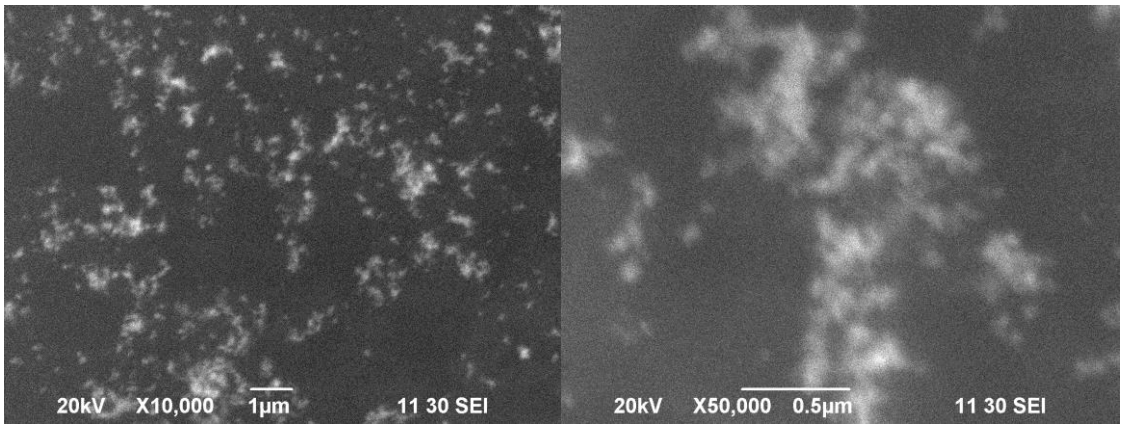

Figure 3.11: SEM image of AgNP synthesized using TSC as reducing agent

AgNP synthesized by formula two:

In case of this method, synthesized AgNP was analyzed by SEM with 25,000 to 50,000 times magnification. The analysis could not show exact shape of nanoparticle since those were mostly aggregated with one another. The shapes seemed to be uniform throughout the mixture but those remained cloudy. The sizes of the particles were found to be around 100 nm. Nanoparticle concentration was much higher than the AgNP synthesized by citrate reduction method.

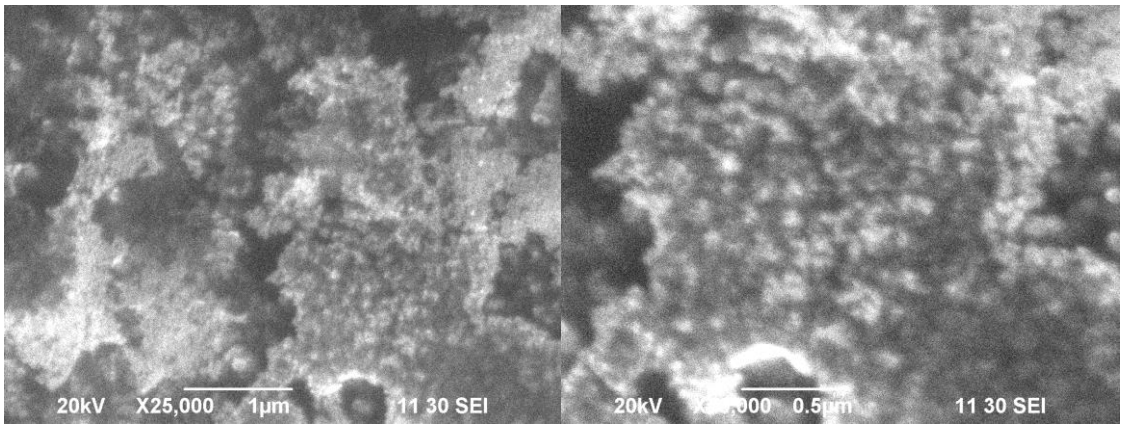

Figure 3.12: SEM image of AgNP synthesized using $NaBH_4$ as reducing agent

AgNP synthesized by formula three:

For this method, synthesized AgNP was analyzed by SEM with 25,000 to 50,000 times of magnification. The shape of nanoparticles was not clear and the sizes of the particles were found to be less than 100 nm.

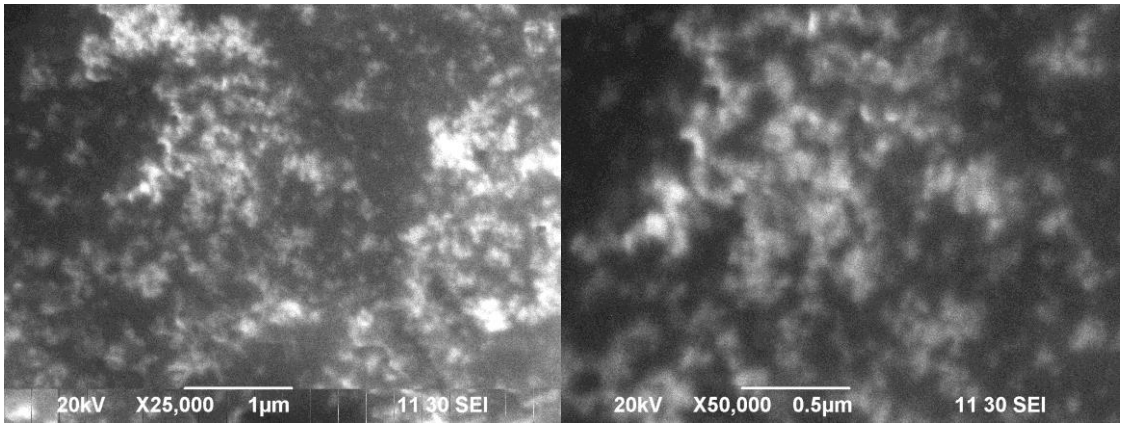

Figure 3.13: SEM image of AgNP synthesized using $NaBH_4$ and TSC as reducing agent

AgNP synthesized by formula four:

Hydrogen peroxide was used in this method for shape control and stability assurance. Synthesized AgNP was analyzed by SEM with 10,000 to 50,000 times of magnification. The shape of nanoparticles was mostly spherical and the sizes of the particles were found to be 100 nm-200 nm.

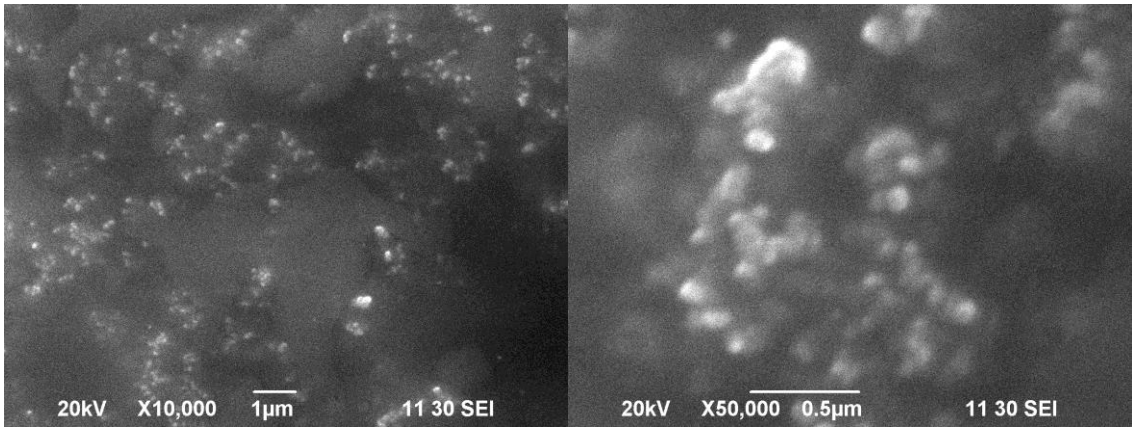

Figure 3.14: SEM image of AgNP synthesized using $NaBH_4$ & TSC as reducing agent & H_2O_2 as stabilizer

AgNP synthesized by formula five: At the time of this SEM analysis, nanoparticle of fifth method was not available. Thus, their morphological properties could not be observed.

3.3 Size distribution of AgNP was observed by DLS Measurement Instrument

Size Distribution Report by Intensity

Malvern DLS Measurement Instrument was used to determine the size distribution of the nanoparticles synthesized in colloidal solution.

Dispersant Name: Water

Temperature (°C): 25.0

Sample one: AgNP synthesized by using trisodium citrate (TSC) as reducing agent

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	107.8	**Peak 1:**	91.71	77.1	58.09
PdI:	0.418	**Peak 2:**	408.9	15.0	129.2
Intercept:	0.863	**Peak 3:**	5.543	4.3	1.711

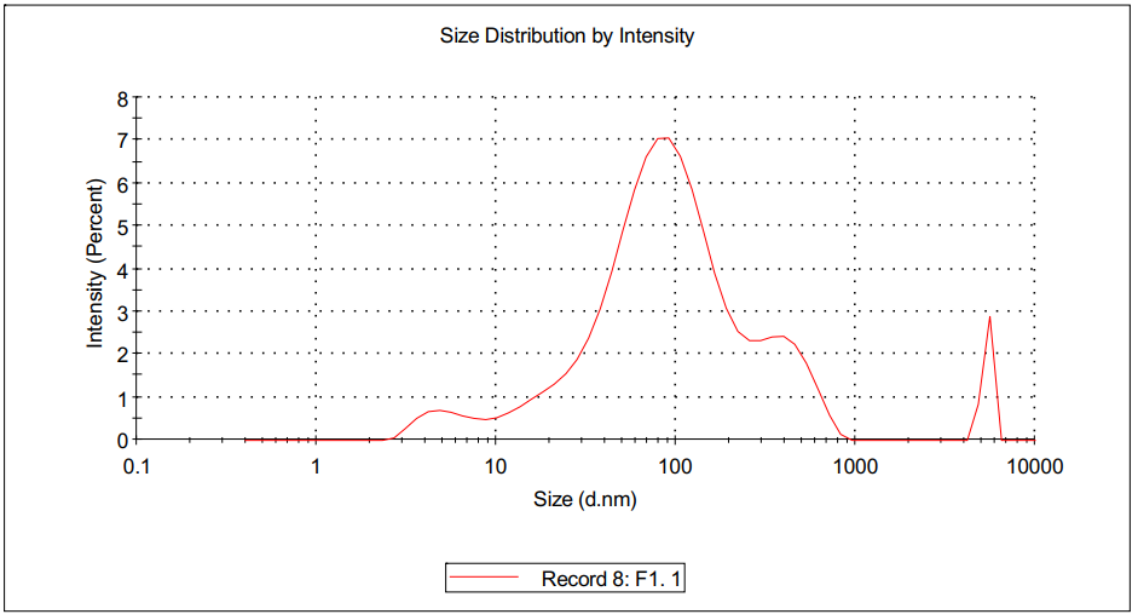

Figure 3.15: Size distribution of AgNP synthesized by TSC

Colloidal silver was synthesized by this method using TSC as reducing agent. The nanosilver suspension was dispersed in water. The suspension was subjected to DLS analysis to determine size and potential stability. In case of this method, the average size of AgNP was determined to be 107.8 nm.

Sample two: AgNP synthesized by using sodium borohydride as reducing agent

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	34.14	Peak 1:	87.15	76.4	56.28
PdI:	0.519	Peak 2:	13.05	20.4	4.687
Intercept:	0.785	Peak 3:	3.533	3.2	0.7677

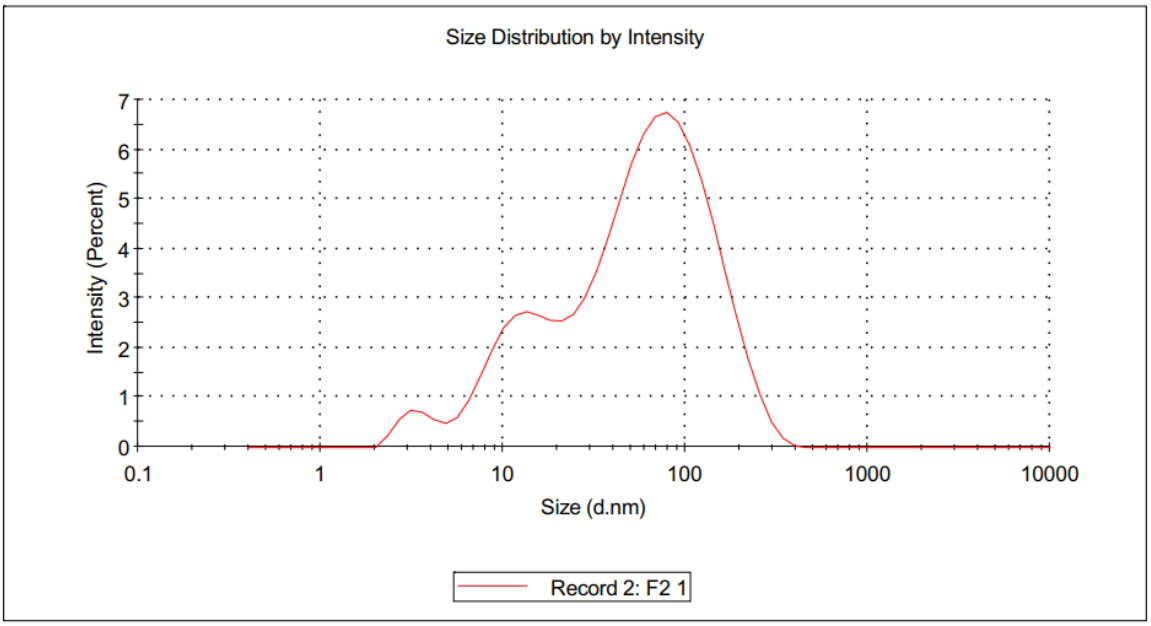

Figure 3.16: Size distribution of AgNP synthesized by $NaBH_4$

DLS spectroscopy showed that the average size of nanoparticle synthesized in this method is 34.14 nm. This is much smaller in size than the nanoparticles produced in the first method. The above graph shows the size distribution in relation with the intensity of the solution.

Sample three: AgNP synthesized by using sodium borohydride & TSC as reducing agent

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	37.22	Peak 1:	65.07	58.7	38.64
PdI:	0.711	Peak 2:	289.1	18.7	113.5
Intercept:	0.821	Peak 3:	2.240	11.6	0.7040

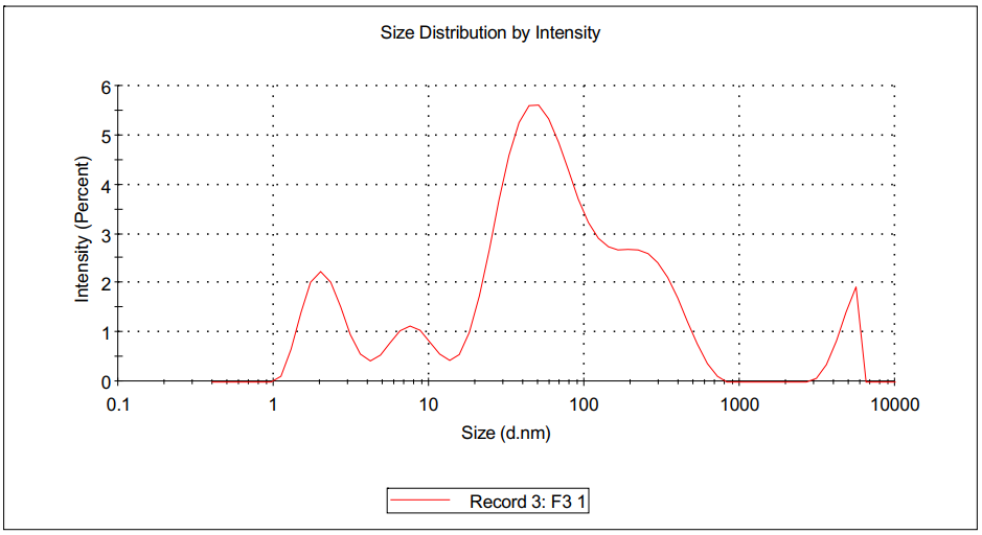

Figure 3.17: Size distribution of AgNP synthesized by TSC and $NaBH_4$

In this method, combined use of TSC and sodium borohydride produced colloidal nanosilver with an average size of 37.22 nm.

Sample 4: AgNP synthesized by using $NaBH_4$ & TSC as reducing agent and H_2O_2 as stabilizer

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	67.34	Peak 1:	30.91	56.0	17.53
PdI:	0.316	Peak 2:	202.1	34.0	89.31
Intercept:	0.874	Peak 3:	4.757	6.6	1.297

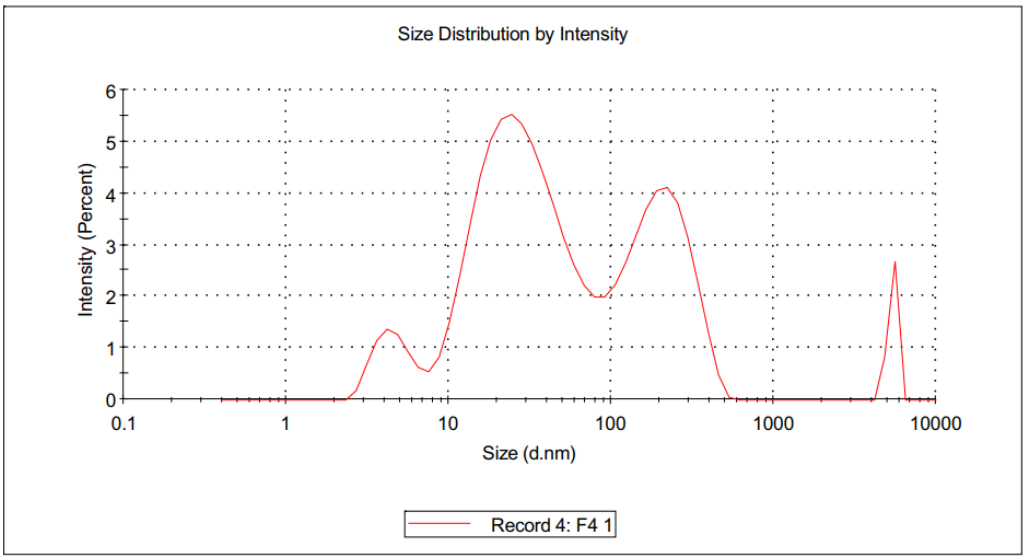

Figure 3.18: Size distribution of AgNP synthesized by $NaBH_4$, TSC & H_2O_2

In this method, NaBH4 & TSC was used as reducing agent & H_2O_2 as stabilizer for the synthesis of silver nanoparticle. DLS spectroscopy showed an average size of 67.34 nm for nanoparticles synthesized in this method.

Sample five: AgNP synthesized by using polyethylene glycol (PEG) as reducing agent

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	47.77	Peak 1:	93.85	81.6	52.90
PdI:	0.554	Peak 2:	11.71	11.7	4.225
Intercept:	0.862	Peak 3:	4069	3.7	1057

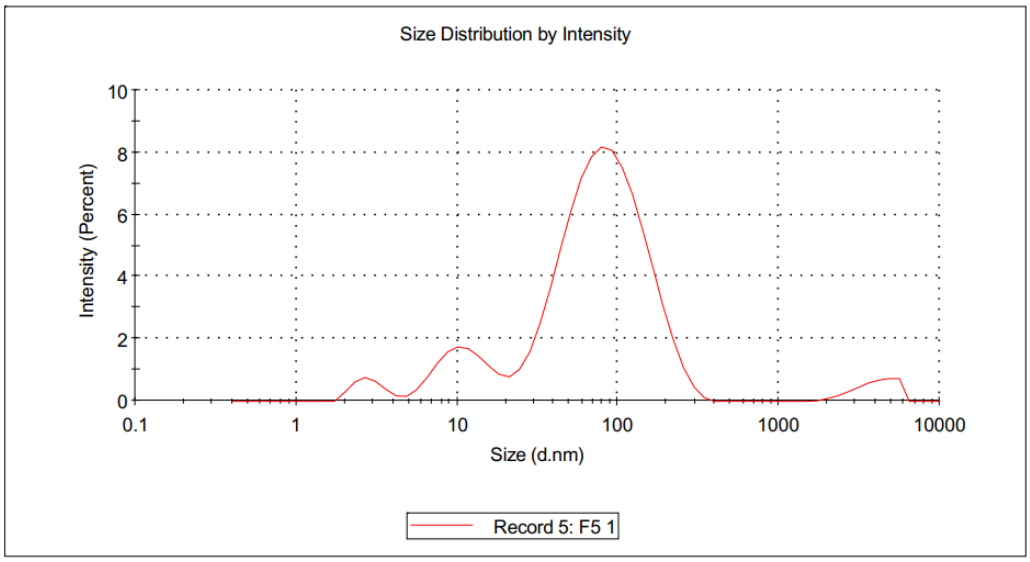

Figure 3.19: Size distribution of AgNP synthesized by PEG

The above graph shows size distribution in relation to intensity of the solution. The average particle size produced in this method was assumed to be 47.77 nm which falls under the desired size range of AgNP.

Determining surface charge and stability of AgNPs by analyzing Zeta Potential Report

Malvern DLS measurement Instrument was used to determine zeta potential (ζ) for a surface charge of Ag nanoparticles.

Dispersant Name: Water

Temperature (°C): 25.0

Sample one: AgNP synthesized by using trisodium citrate (TSC) as reducing agent

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-29.1	**Peak 1:**	-30.1	97.6	8.20
Zeta Deviation (mV):	10.2	**Peak 2:**	9.91	2.4	2.13
Conductivity (mS/cm):	0.603	**Peak 3:**	0.00	0.0	0.00

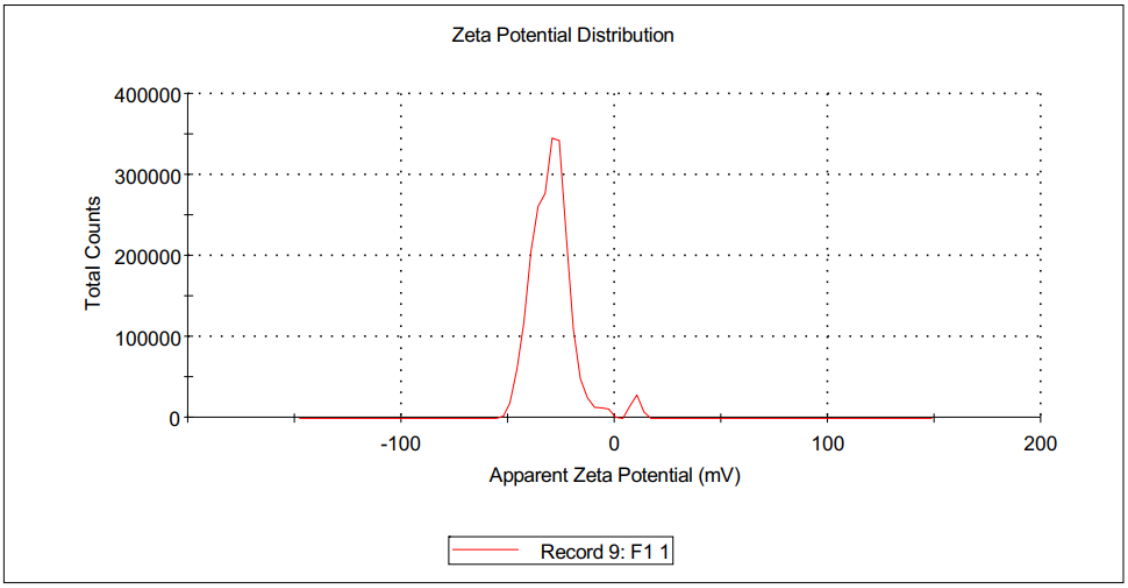

Figure 3.20: Zeta Potential distribution of AgNP synthesized by TSC

Zeta potential represents the stability of the colloidal solution. The above graph shows zeta potential distribution of sample one which is AgNP synthesized by using TSC as reducing agent. For this sample, zeta potential value is -29.1 mV. Thus, stability of the solution is much strong and the particles are less likely to get aggregated. Zeta potential was measured with the same instrument used to determine size distribution.

Sample two: AgNP synthesized by using sodium borohydride as reducing agent

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-39.4	**Peak 1:**	-66.4	45.2	9.30
Zeta Deviation (mV):	28.2	**Peak 2:**	-6.74	28.1	5.68
Conductivity (mS/cm):	0.265	**Peak 3:**	-23.1	21.1	5.73

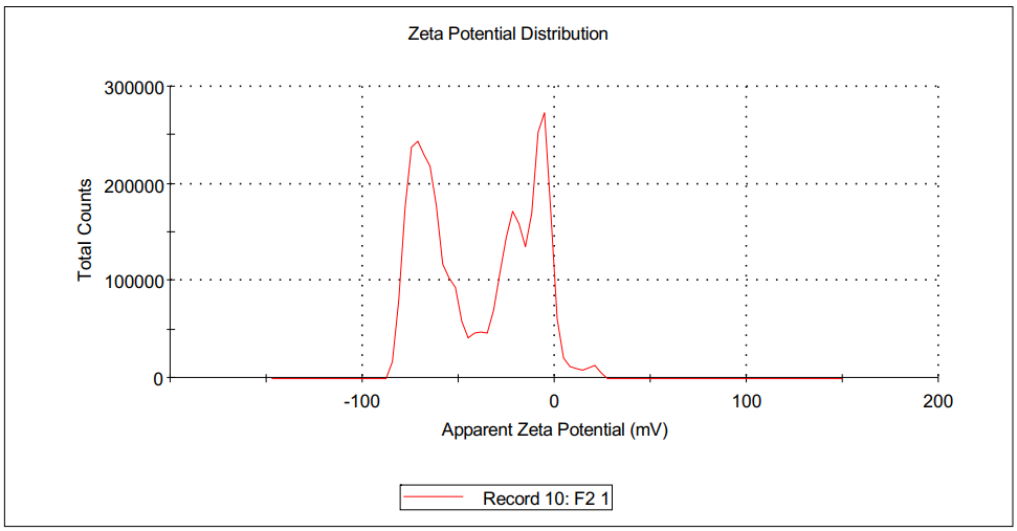

Figure 3.21: Zeta Potential distribution of AgNP synthesized by $NaBH_4$

DLS spectroscopy was used to determine zeta potential for this AgNP sample which showed a value of -39.4 mV. The charge among the colloidal silver is much higher which indicates a good stability of the nanoparticle.

Sample three: AgNP synthesized by using sodium borohydride & TSC as reducing agent

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-41.1	**Peak 1:**	-32.7	61.3	6.85
Zeta Deviation (mV):	13.9	**Peak 2:**	-56.1	37.5	6.43
Conductivity (mS/cm):	0.0491	**Peak 3:**	-1.69	1.1	4.35

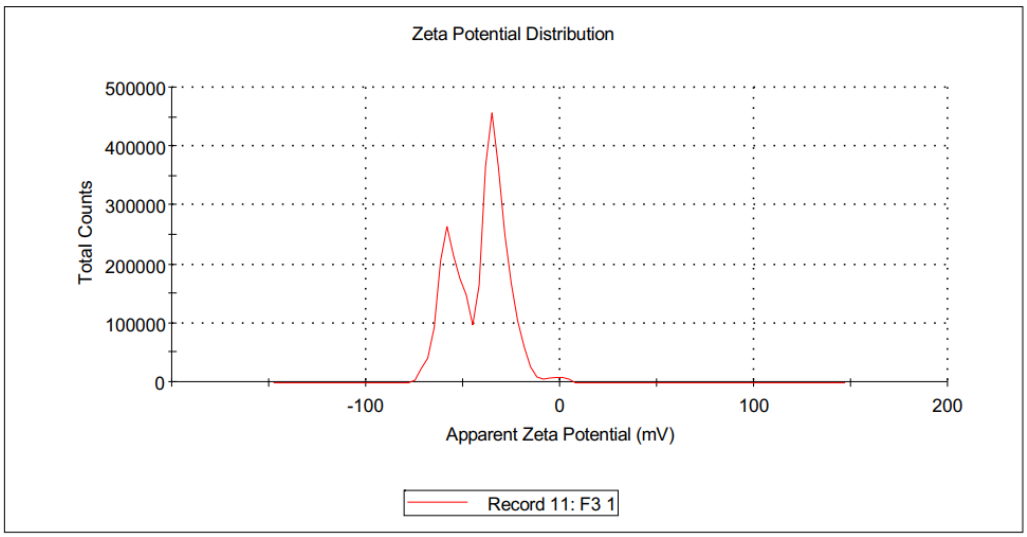

Figure 3.22: Zeta Potential distribution of AgNP synthesized by TSC and $NaBH_4$

Zeta potential of silver nanoparticle produced in combination of TSC and $NaBH_4$ showed a value of -41.1 mV. Thus, the potential stability of the colloidal solution seems quite satisfactory.

Sample four: AgNP synthesized by using $NaBH_4$ & TSC as reducing agent and H_2O_2 as stabilizer

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-36.7	**Peak 1:**	-21.3	47.3	10.8
Zeta Deviation (mV):	21.5	**Peak 2:**	-58.3	36.7	6.62
Conductivity (mS/cm):	0.719	**Peak 3:**	-41.9	14.6	3.59

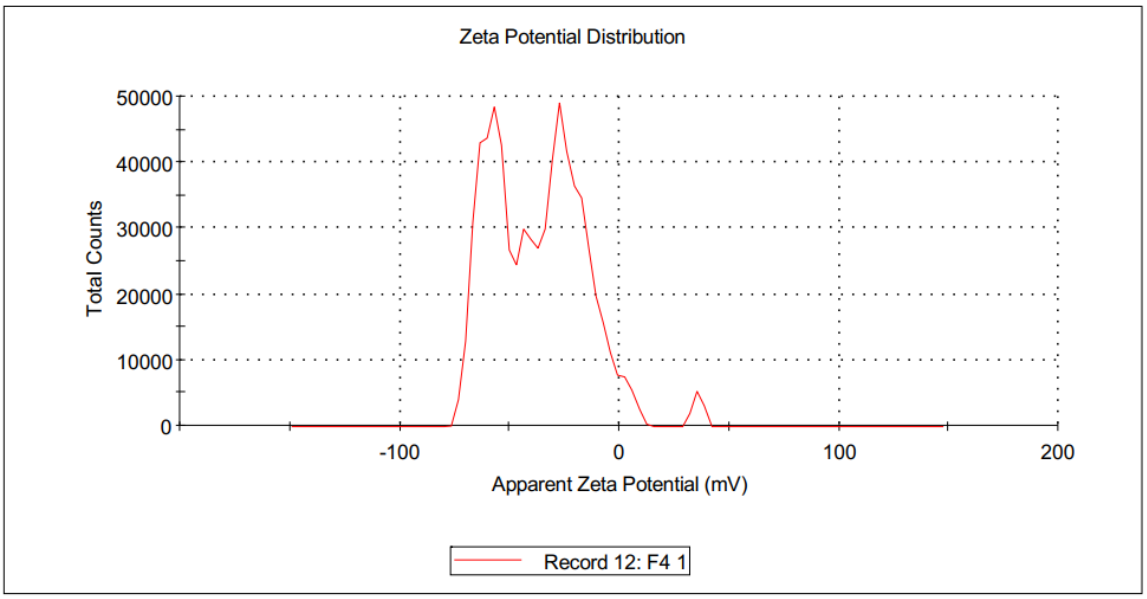

Figure 3.23: Zeta Potential distribution of AgNP synthesized by $NaBH_4$, TSC & H_2O_2

Average zeta potential for this solution is -36.7 mV. All three peaks for zeta potential for this sample is negative which refers to a good stability of the colloidal solution. Zeta potential value for this sample seemed better than any other sample.

Sample five: AgNP synthesized by using polyethylene glycol (PEG) as reducing agent

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-8.00	**Peak 1:**	-7.93	99.7	3.66
Zeta Deviation (mV):	3.90	**Peak 2:**	-32.1	0.3	0.00
Conductivity (mS/cm):	0.194	**Peak 3:**	0.00	0.0	0.00

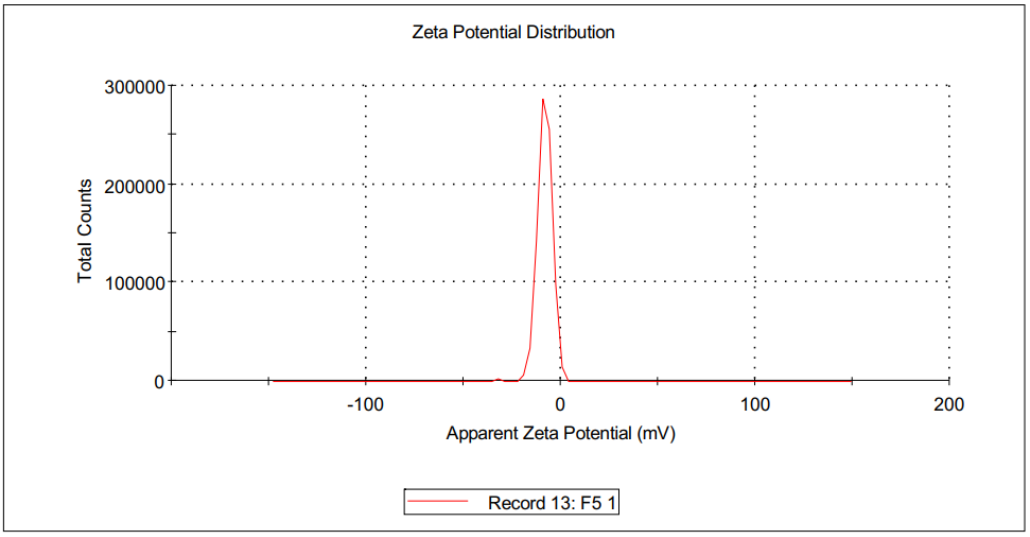

Figure 3.24: Zeta Potential distribution of AgNP synthesized by PEG

The above graph shows zeta potential distribution for AgNP synthesized by fifth method. The zeta potential value is -8.00 mV which is much less than the other AgNPs synthesized by other methods. This sample seemed to be least stable among the five types of nanoparticles.

3.4 Determination of antimicrobial efficiency of synthesized AgNPs by well diffusion test

Well diffusion test was done to compare the anti-bacterial efficiency of nanoparticles synthesized by five formulas along with silver nitrate of same concentration.

Clinical Isolate	Zone of Inhibition (mm)					
	40 µl of 80 PPM $AgNO_3$	40 µl of 80 PPM F1	40 µl of 80 PPM F2	40 µl of 80 PPM F3	40 µl of 80 PPM F4	40 µl of 80 PPM F5
Klebsiella (1)	9.5	9.5	0	9	0	9.5
Klebsiella (2)	11	11	0	11	8	11
E.coli (1)	12	12.5	0	12	9	12.5
E. coli (2)	13	13	0	13	11	13
Salmonella (1)	11.5	11.5	0	11	8	11.5
Bacillus (1)	14	13	0	20	11	15
Staphylococcus (1)	13	13	0	12.5	9	12
Staphylococcus (2)	11	11	0	10	0	10.5

Table 3.1: Zone of inhibition of AgNPs and $AgNO_3$

F1= AgNP synthesized by using trisodium citrate (TSC) as reducing agent

F2= AgNP synthesized by using sodium borohydride as reducing agent

F3= AgNP synthesized by using $NaBH_4$ & TSC as reducing agent

F4= AgNP synthesized by using $NaBH_4$ & TSC as reducing agent and H_2O_2 as stabilizer

F5= AgNP synthesized by using polyethylene glycol (PEG) as reducing agent

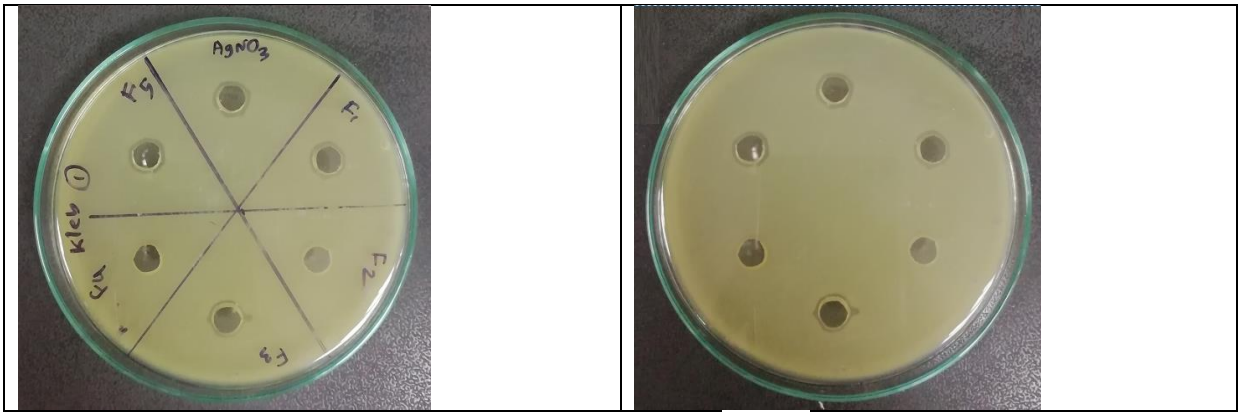

Figure 3.25: Zone of inhibition of AgNPs and $AgNO_3$ against *Klebsiella* (1)

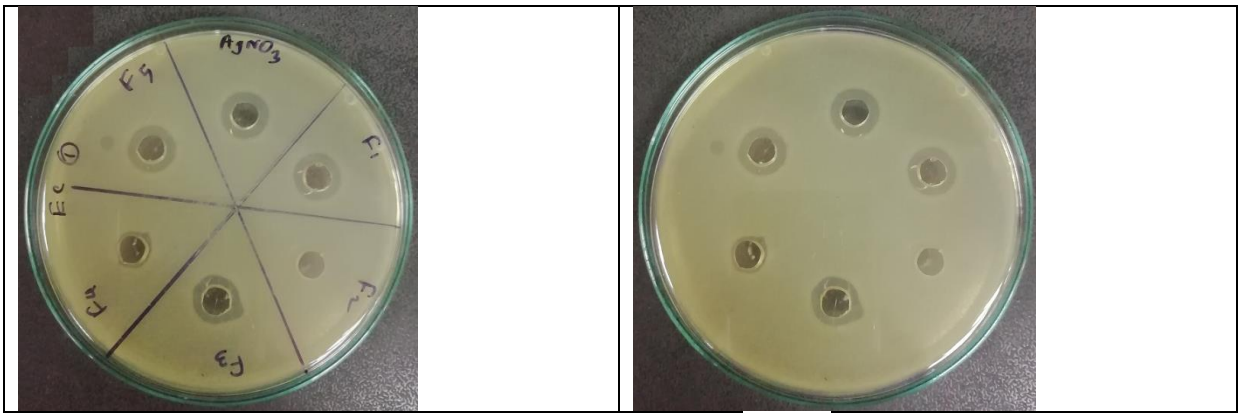

Figure 3.26: Zone of inhibition of AgNPs and $AgNO_3$ against *E. coli* (1)

<u>Determination of MBC of five nanoparticles against *E.coli*</u>

Organism: *E. coli* (1)

OD of *E.coli* after overnight culture: 0.440

Number of colonies was counted after overnight incubation on each plate of 0 ppm to 40 ppm AgNP for each type of nanoparticle.

Petri dish 6, which was inoculated with blank LB broth, didn't show any visible microbial growth. Thus, it was assumed that the LB broth was prior contamination free.

Nanoparticle	Number of Colonies (5 micro liters from each test tube was inoculated in Petri dishes containing nutrient agar.)				
	0 PPM	5 PPM	10 PPM	20 PPM	40 PPM
F1	402	7	2	1	0
F2	402	39	10	8	0
F3	402	106	8	6	4
F4	402	395	176	18	4
F5	402	71	4	2	0

Table 3.2: Number of colonies of *E. coli* on each plate of 0 ppm to 40 ppm

a) Survival (%) of *E. coli* on different concentration of AgNP

Nanoparticle	Survival percentage				
	0 PPM	5 PPM	10 PPM	20 PPM	40 PPM
F1	100% (402)	1.7%	0.5%	0.25%	0
F2	100% (402)	9.7%	2.5%	1.99%	0
F3	100% (402)	26.4%	1.99%	1.5%	1%
F4	100% (402)	98.3	43.8	4.48	1%
F5	100% (402)	17.7	1%	0.5%	0

Table 3.3: Survival percentage of *E. coli* in different concentration of AgNP

b) Determining approximate MBC from the survival rate of *E. coli*

MBC of F1 AgNP: 40 PPM

MBC of F2 AgNP: 40 PPM

MBC of F3 AgNP: >40 PPM

MBC of F4 AgNP: >40 PPM

MBC of F5 AgNP: 40 PPM

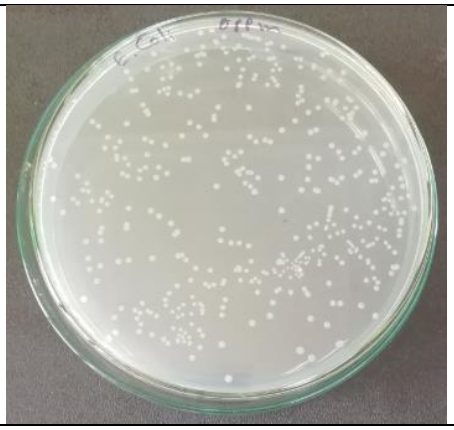

Figure 3.27: Number of colonies of *E. coli* grown on 0 PPM AgNP

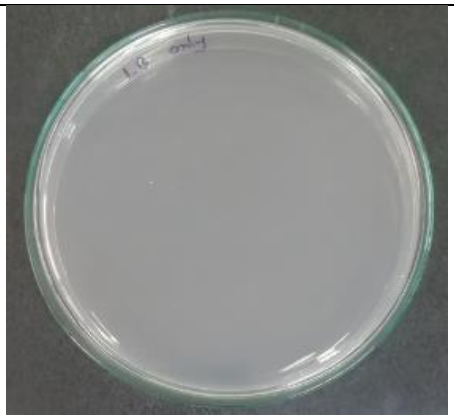

Figure 3.28: No growth of microorganism for LB broth

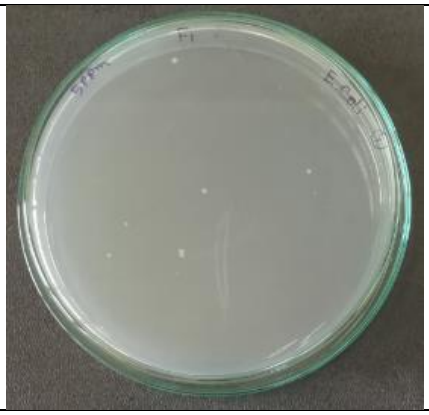

Figure 3.29: Number of colonies of *E. coli* grown on 5 PPM F1 AgNP

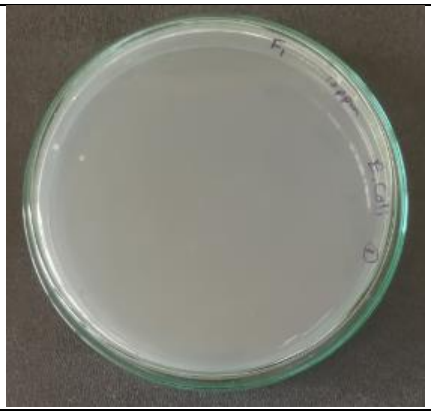

Figure 3.30: Number of colonies of *E. coli* grown on 10 PPM F1 AgNP

Figure 3.31: Number of colonies of *E. coli* grown on 20 PPM F1 AgNP

Figure 3.32: No colony of *E. coli* was found on 40 PPM F1 AgNP

Discussion

4. Discussion

Silver nanoparticles generally show UV absorbance peak around 400 nm (Solomon *et al.,* 2007). Silver nanoparticles synthesized by five methods showed a strong absorption peak between 390 nm to 420 nm which is an initial confirmation of the production of the silver nanoparticle. The absorbance peak never showed any value below 390 nm or above 420 nm. This highest peak is called the Surface Plasmon Resonance (SPR) for the nanoparticles synthesized by different chemical methods (Pradhan *et al.,* 2002).

Size distribution and morphological properties of synthesized nanoparticles were carried out by Scanning Electron Microscope at the initial stage of the study. The shape of the nanoparticle synthesized by formula one was spherical and rod. A comparable study of synthesizing AgNP using trisodium citrate as reducing agent also produced spherical and rod shaped silver nanoparticles (Dong *et al.,* 2011). The nanoparticles of first method were more stable compared to the AgNPs of other methods. In case of formula two, shape of the nanoparticles were uniform with an estimated size of 100 nm but the particles seemed to be aggregated with one another. For formula three, the shape of AgNPs were not very clear but the size of nanoparticles seemed to be less than 100 nm. In formula four, spherical nanoparticles were formed with an estimated size of 100-200 nm. This value is close to the size of nanoparticles synthesized in another study (Zhang *et al.,* 2011). Sample nanoparticles of formula five were not available at the time of SEM analysis so it could not be analyzed. It must be mentioned that the procedures of nanoparticle synthesis were updated after the initial SEM report. Thus, the yield and stability of nanoparticles seemed to be better in later phases.

Size of the nanoparticles in later phases was confirmed by using DLS spectroscopy. In all five methods, nanoparticle size was below or around 100 nm. This value is close to nanoparticle synthesized in another study (de Oliviera *et al.,* 2017). The smallest AgNP was formed by the fourth method and it increased as follows –

F4 > F3 > F1 > F2 > F5

A study showed that zeta potential ranging from -25.5 to -38.3 mV is ideal for ensuring the stability of the nanoparticle (Haider *et al.,* 2014). For the nanoparticles formed by the first four methods, zeta potentials were less than or around -30 mV. This confirms a good stability of the colloidal preparations for these four types of nanoparticles. However, it was -8 mV for the fifth method which proves that it was not as stable or colloid as the others.

Anti-bacterial activities of silver nanoparticle synthesized by the five methods were observed and compared through well diffusion method which is the official method used in clinical microbiology laboratories for routine antimicrobial susceptibility testing (Balouiriet *et al.,* 2016). The efficiency of the same concentration of silver nitrate was also measured as silver nitrate was the basis of silver nanoparticle synthesis in all five methods. Apart from that, Minimum Bactericidal Concentration (MBC) of five nanoparticles was determined against an *E. coli* isolate.

For well diffusion method, two isolates of *Klebsiella* species, two isolates of *E. coli*, one isolate of *Bacillus* species, one isolate of *Salmonella* species, and two isolates of *Staphylococcus* species were used. Muller Hilton agar was used as culture media in case of well diffusion procedure. Due to having multiple resistance mechanisms, these microbes can become very hard to treat (Imming *et al.,* 2000). Addition of silver nanoparticle with antibiotics such as ampicillin increases the activity of the antibiotic in terms of killing resistant microorganism (Fayaz *et al.,* 2009). Each isolate was cultured on separate Petri dishes. In each Petri dish, six wells were made to observe antimicrobial efficiency of five nanoparticles along with the silver nitrate of same concentration. The approximate concentration of silver nanoparticle for each method was adjusted to 80 PPM and a silver nitrate solution of the same concentration was also prepared. Forty micro liter of nanoparticle solution was applied per well from each type of nanoparticle alongside the same amount of silver nitrate in the last well.

After overnight incubation, the zone of inhibition was measured for each nanoparticle and silver nitrate. In a similar study, AgNP also showed antimicrobial efficiency against organisms like *P. aeruginosa*, *S. aureus* and *E. coli* (Franci *et al., 2015*). Likewise, nanoparticles synthesized in this study showed antimicrobial efficiency against different microorganisms. Nanoparticle synthesized by formula one was found to be most effective against the second isolate of *E. coli, Bacillus* species and the first isolate of *Staphylococcus* species. It was found to be least effective against the first isolate of *Klebsiella* species. It should be mentioned that silver nitrate showed almost same efficiency as formula one AgNP except for the isolate of *Bacillus* species. On the contrary, AgNP synthesized by formula one seemed to be least effective against the first isolate of *Klebsiella* species. However, nanoparticle synthesized by formula two seemed ineffective against any of the isolates. Apart from that, nanoparticle synthesized by formula three was observed to be most effective against the *Bacillus* isolate and it was least effective against the first isolate of *Klebsiella*

species. Besides, nanoparticles of formula four were most effective against the *Bacillus* species and the second isolate of *E. coli* whereas it was least effective against the first isolate of *Klebsiella* species and the second isolate of *Staphylococcus* species. Lastly, AgNPs of formula four seemed to be most effective against the *Bacillus* isolate and it was least effective against the first isolate of *Klebsiella* species.

In conclusion, silver nanoparticle synthesized by formula one seemed to be most effective followed by formula five, formula three and formula four consecutively. On the other hand, nanoparticle of formula two was observed to be least effective as it didn't actually show any measureable zone of inhibition. It can be assumed that nanoparticle synthesized by formula two was the least stable. However, it showed promising result while determining the MBC of all types of nanoparticles against *E. coli* as the method of AgNP synthesis in formula two was updated accordingly to obtain a stable form of AgNPs in later phases. On the other hand, nanoparticles of formula three and four seemed to be lesser effective than the same concentration of silver nitrate. This clearly indicates that the silver nitrate used in formula three and four gave a lesser yield of AgNP. Lastly, formula five had a better yield of silver nanoparticle.

The MBC test was done to determine the lowest concentration at which an antimicrobial agent will kill a particular microorganism (Balouiri *et al.*, 2016). In case of determining Minimum Bactericidal Concentration of silver nanoparticles of each formula, the first isolate of *E. coli* was used. *E. coli* culture was grown on Luria Bertani broth till reaching the desired range of Optical Density. One milliliter of culture was diluted ten times before using the organism into a series of test tubes containing different concentration of silver nanoparticle. For each type of silver nanoparticle, a serious of test tubes containing Luria Bertani broth was prepared using serial dilution method. Five test tubes contained 0 PPM, 5 PPM, 10 PPM, 20 PPM, 40 PPM of silver nanoparticle and there was an additional test tube which contained only LB broth. Fifty micro liter of cell from the diluted culture was applied into each test tube except for the last one which contained only LB broth. After 2 hours of incubation, 5 micro liter media from each test tube was spread into separate Petri dishes containing nutrient agar. This step was followed by an overnight incubation before counting the number of colonies per Petri dishes. The LB broth was cultured to get an assurance of contamination-free procedure in determining MBC for each type of AgNP.

Prior inoculation, the OD of *E. coli* culture was set to 0.440. After overnight incubation, numbers of colonies were counted on each Petri dish. The Petri dish containing only LB broth showed no visible growth of any microbial colony. Thus, it was assured that the broth was prior contamination free. Apart from that, 402 colonies were found for 0 PPM concentration of silver nanoparticle which basically contained no nanoparticle. Number of colonies of *E. coli* on other PPM concentration of AgNP was also counted to determine the survival rate of *E. coli*. The number of colonies significantly declined even on a lower concentration of silver nitrate (5 PPM) for formula one AgNP. Besides, the number of colonies moderately declined on the 5 PPM concentration of AgNPs synthesized by formula two, three and five. Lastly, the number of colonies very slowly declined for formula four.

The survival percentage of *E. coli* for silver nanoparticles synthesized by formula one, two and five was least at their 20 PPM concentration whereas it was declined to zero at 40 PPM concentration. Thus it can be concluded that 40 PPM or a lower PPM concentration of silver nanoparticle was the MBC of AgNPs synthesized by formula one, two and five. On the other hand, nanoparticles of formula three and four had least survival rate on 40 PPM concentration. Similarly, MBC for the third and fourth AgNP would be more than 40 PPM. However, MBC was determined to be 4 PPM against *E. coli* in a similar study where AgNP was also synthesized by combination of $NaBH_4$ and TSC (Brown *et al.*, 2012). After analyzing the MBC, it can be said that silver nanoparticle of formula one is the most effective, followed by formula five and formula two. Lastly, formula three and four was least effective in case of determining MBC against *E. coli*.

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