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Characterization of chemical mediated synthesis of silver nanoparticles (Ag-NPs) and their antibacterial efficacy against selected bacterial pathogens

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ABSTRACT

Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications. The main objective of the present study is focused on synthesis of silver nanoparticles by two different techniques using tri-sodium citrate as a reducing agent by the reduction of silver nitrate to get different types of silver nanoparticles. Synthesis of silver nanoparticles was carried out using tri-sodium citrate as reducing agent and silver nitrate as an inducer by constant heating and cyclic heating technique. The silver nanoparticle was characterized by UV-visible spectroscopy, SEM and XRD to analyse the size, morphology and chemical composition. The formation of the silver nanoparticles was confirmed by changing colour and monitored using UV-visible spectroscopy. The UV-visible spectrum revealed the formation of silver nanopartícles by exhibiting the typical surface plasmon absorption maxima at 450-460 nm. SEM micrograph demonstrates the spherical shaped and some irregular shaped nanoparticles with the size range of 60- 80nm. XRD pattern at 2θ deg showed the whole spectrum value ranging from 20^o to 80^o by displaying intense peaks at 38.08^o indicating that the particle was made of pure silver. Ag-NPs were subjected to perform antibacterial activity against selected bacterial pathogens. Maximum inhibition activity was noticed against Shigella sp. (25mm) using silver nanoparticle mediated by tri-sodium citrate with constant heating. The outcome of results clearly pin points that the silver nanoparticles produced using tri-sodium citrate as reducing agent and silver nitrate as an inducer by constant heating is a highly efficient and cost effective technique.

Key words: nanopartcles, trisodium citrate, antimicrobial, SEM, XRD

INTRODUCTION

Silver is one of the most significant universal antimicrobial substances. Since roman period silver has been used as antimicrobial substance. But nanotechnogy enables us to expand the vast array of silver particles markedly with numerous applications [1]. Nanotechnology is the engineering of molecular technology at the nanoscale level. It is the collective term for a range of technologies, techniques and process that involve the manipulation of matter at the smallest scale (1-100 nm). The preparation of Nano-sized 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. Among the various metal nanoparticles, silver nanoparticles have been widely investigated because they exhibit unusual optical, electronic, and chemical properties depending on their size and shape with respect to technological applications. Silver nanoparticles are being viewed as fundamental building blocks of nanotechnology. Silver nanoparticles have received considerable attention due to their attractive physical and chemical properties and as known to be an effective antimicrobial agent [2]. Though biosynthesis of Ag-NPs are

considered to be more eco- friendly [3], chemical or physical method of synthesis of preparation of silver particles are normally used because they are readily available and can be used to synthesize in large amounts in relatively less time. Chemical reduction is the most frequently adopted method for the synthesis of Ag-NPs as colloidal silver dispersion in water is stable. The reduction of silver ions in aqueous solution generally yields colloidal silver with size of particles being several nanometers in diameter [4]. It involves reduction of an ionic salt in the presence of surfactant as a reducing agent. Chemical mediated synthesis is cost effective, easily scaled up for bulk synthesis without the use of high pressure, energy and temperature [5]. These nanoparticles can be taken up for further studies to develop other biomedical applications.

A lot of chemical reduction methods have been applied to synthesize stable and various shapes of silver nanoparticles in water by the use of different reducing agents such as ascorbic acid [6], hydrazine hydrate [7], dimethyl formamide [8] and sodium borohydride [9]. The most popular preparation of Ag colloids is through chemical reduction of silver salts by sodium borohydride or sodium citrate. The size induced properties of nanoparticles enable the development of new applications. The size and shape of the nanoparticles depended on the tendency of the organic substrate used to reduce the silver ions. In the present study we have used tri-sodium citrate as a reducing agent to reduce the silver salts of silver nitrate. The main objective of the present investigation is to synthesize the crystals of silver nanoparticles using tri-sodium at moderate temperature by different stirring times under constant heating and cyclic heating techniques. The synthesized nanoparticle was subjected to perform its characterization and antibacterial assay against selected bacterial pathogens.

MATERIALS AND METHODS

Test pathogens

The synthesized silver nanoparticles were subjected to antibacterial assay against selected gram negative human pathogens *viz., Salmonella paratyphi* A, *Shigella* sp., *Enterococci* sp., and gram positive pathogen *Staphylococcus aureus.* The test pathogens were maintained as pure culture in our laboratory.

Chemicals and culture media

All chemicals, media components and Hi media were procured form Hi media Laboratory Private Limited (Mumbai, India) for the present silver nanoparticle synthesis and their characterization investigation.

Chemical mediated synthesis of silver nanoparticles (Ag-NPs)

Two different techniques have been adopted for chemical mediated reductions of silver salt using tri-sodium citrate as a reducing agent in order to obtain nanoparticles with potential biomedical application.

Synthesis of nanoparticles using tri sodium citrate under constant heating

Tri-sodium citrate ($C_6H_5O_7Na_3$) of analytical grade (AG) as a reducing agent and silver nitrate ($AgNO_3$) as starting material were used for the synthesis of nanoparticle under constant heating. The silver colloid was prepared by using chemical reduction in deionised Milli-Q water. 1mM of $AgNO_3$ solution was prepared in 250ml of Erlenmeyer flask by adding 17.0mg of silver nitrate in Milli-Q water by constant heating in a microwave oven till it dissolves absolutely. Then 10ml of 1% tri-sodium citrate was added drop by drop, mixing vigorously. The reaction mixture was heated until colour change was evident from colourless to yellowish brown. Then the flask was removed from microwave oven and the solution was stirred until chilled to room temperature (28-30^0 C). The consequential solution was subjected for centrifugation at 10000 rpm for 15 minutes. The supernatant was discarded and the precipitate was transferred into quartz glass for drying in room temperature for 48-72 hrs to obtain the nanoparticles. The dried nanoparticles were stored in dark bottle for further characterization studies.

Synthesis of nanoparticles using tri-sodium citrate under cyclic heating

In the second method, cyclic heating technique has been adopted to synthesis silver nanoparticles. Tri-sodium citrate of analytical grade (AG) as a reducing agent and silver nitrate as starting material were used for the synthesis of nanoparticle under cyclic heating at regular intervals. The silver colloid was prepared by using chemical reduction method in deionised Milli-Q water. 1mM of $AgNO_3$ solution was prepared in 250ml of Erlenmeyer flask by adding 17.0mg of silver nitrate in Milli-Q water by constant heating in a microwave oven till it dissolves absolutely. Then 10ml of 1% tri-sodium citrate was added drop by drop while mixing vigorously. The reaction mixture was intermittently heated and stirred at 30 sec cyclic interval until the colour change was evident from colourless to yellowish brown. Finally the flask was removed from the oven and the solution was blended continuously until

cooled to room temperature (28-30^0 C). The resultant solution was centrifuged at 10000 rpm for 15 minutes. The clear supernatant was discarded and slurry precipitate was transferred into quartz glass for drying at room temperature for 48-72 hrs to obtain nanoparticles. The dried nanoparticles were stored in a dark bottle for further characterization. Characterization of synthesised silver nanoparticles was performed by the following standard techniques of UV-visible spectroscopy, Scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis.

UV-Visible spectral analysis

The chemical mediated reduction of silver ions was monitored by measuring the UV-Vis spectrum of the reaction mixture by diluting a small aliquot of the sample into double distilled water after 2 hours. UV-Vis spectral analysis was carried by using CARY Conc 100 – EL06023680 model UV-Vis spectrophotometer.

Field Emission scanning electron microscopy (FESEM) analysis

Field emission scanning electron microscopy (FESEM) is a powerful technique for the morphological examination of organic and inorganic materials. This gives information about the structure of a specimen in the micrometre and sub-micrometre range. The structural characterization, size and morphology of the subject under consideration can be performed by FESEM. Thin films of dried nanoparticles were prepared on a carbon coated copper grid by dropping a trace amount of the sample on the grid and the film on the SEM grid was allowed to dry by putting it under a mercury lamp for 5-10 minutes. FESEM analysis was carried out using German made SUPRA-55, CARL ZEISS machine.

X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) has become a very important and powerful tool for the structural characterization in solid state physics and materials science. The silver nanoparticle obtained by constant heating which showed potential antibacterial activity was subjected to XRD analysis to study the composition of nanoparticles. Silver nanoparticles were determined by x-ray diffractometer (SMARTLAB, RIGAKU JAPAN – power model – 9kw) operated at a voltage of 40 kV and a current of 30 mA with Cu K radiation in a theta-2 configuration. The crystallite domain size was calculated from the width of the XRD peaks. It is assuming that they are free from non uniform moieties. The average size of the silver nanoparticles can be calculated using the Debye–Scherrer equation.

Antibacterial assay

The antimicrobial susceptibility assay of silver nanoparticles was evaluated by standard Kirby-Bauer disc diffusion method against selected bacterial pathogens. Different concentrations (5 µl, 10 µl, 15 µl, 20 µl, 25 µl per disc) of silver nanoparticles synthesised by two different methods was impregnated with commercially available sterile empty disc (Hi-media) with the size of 6mm diameter for antibacterial assay. Sterile Muller Hinton agar (MHA) plates was prepared and swabbed with overnight broth cultures of each test pathogens (10^8 cells) separately. Silver nanoparticle impregnated disc was placed at the center of the plate aseptically. Triplicates were maintained for each test pathogens to obtain mean value of zone of inhibition. The disc impregnated with tri-sodium citrate alone (25µl/disc) was used as a negative control to compare the antibacterial efficacy. The zone of inhibition was measured after 24 hrs of incubation at 37^{o}C. The different levels of zone of inhibition around the discs were measured and recorded in mm diameter for each test pathogen.

Statistical analysis

The antibacterial assay results of silver nanoparticle synthesized by two different techniques were calculated as mean diameter of zone of inhibition in mm ± standard deviation (mean ± SD).

RESULTS AND DISCUSSION

Silver Nanoparticles

Nanobiotechnology is the important and prompt novel emerging discipline in the field of both nanotechnology and biological science. Biosynthesis of nanoparticles and their characterization have been well documented by various researchers across the world. Microbes with the ability to produce extracellular primary metabolites have industrial [10] and medical applications [11]. The reduction of silver nitrate into silver ion is due to the extracellular enzymes produced by the microbes. In the current investigation chemical mediated silver nanoparticles were produced by two different methods as described in the previous section for characterization and antibacterial efficacy against selected bacterial pathogens. The reaction mixture containing tri-sodium citrate induced the colloidal solution which turned yellowish brown on both the methods of constant heating and cyclic heating indicating that silver nanoparticles was

formed. The colourless solution containing 1mM silver nitrate turned yellowish brown while adding 1% tri-sodium citrate solution with different heating techniques indicating the formation of silver nanoparticles. Van Dong et al [12] reported that chemical mediated silver nanoparticle produced using sodium citrate showed ultra-small and well controlled size of silver nanoparticles by the reduction of silver nitrate at room temperature.

UV visible spectroscopy

Ultraviolet and visible spectrometry is almost used for quantitative analysis of compound known to be present in the sample. UV-visible spectroscopy is one of the most widely used techniques for structural characterization of silver nanoparticles. The absorption spectrum of yellowish brown silver colloids prepared using tri sodium citrate reduction by constant heating and cyclic heating is portrayed in fig.1&2 respectively. The reaction mixture showed a surface plasmon resonance absorption band with a maximum peak of 450 nm and 460 nm respectively indicating the presence of spherical or roughly spherical shape silver nanoparticles. Aashritha [13] noticed that UV-Vis spectroscopy shows the plasmon band of silver nanoparticle suspensions mediated by sodium citrate exhibiting a typical absorbance peak at 430nm. The symmetrical shape and size distribution of the plasmon band indicate relatively sharp particle. The particles size ranges in 14nm with mean diameter of 10nm. This result is corroborated with our present study of silver nanoparticle synthesized using tri-sodium citrate as a reducing agent by constant heating with mean particle size of 40 nm. The shape and size distribution of silver nanoparticle is highly depended on the tendency of the organic substances that reduces the silver ions.

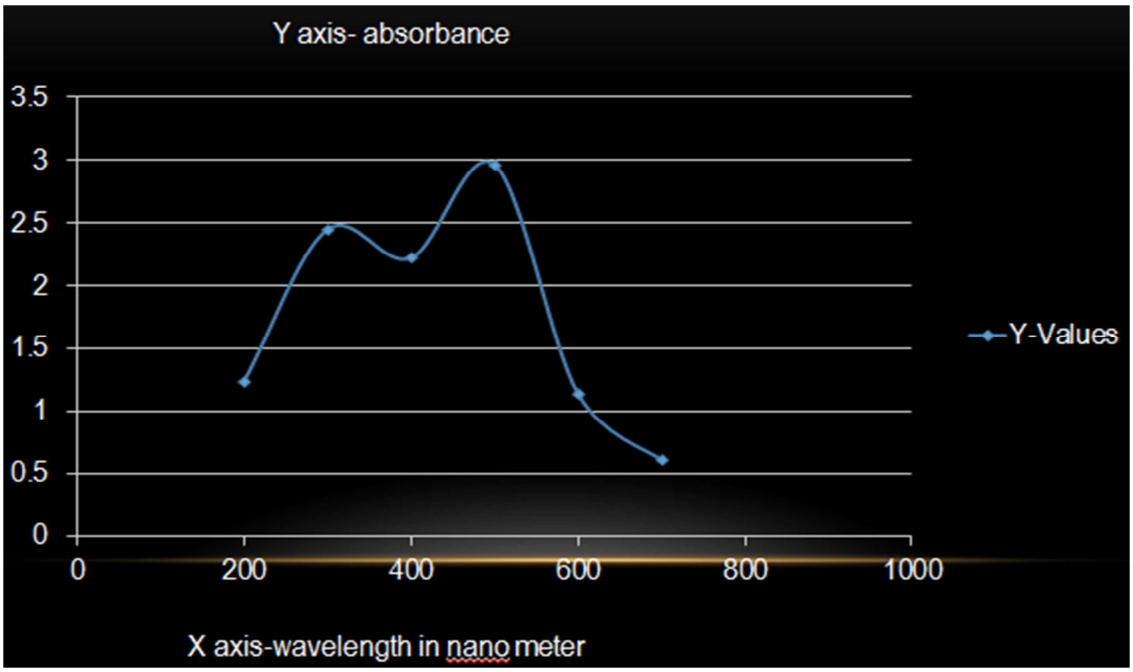

Figure 1. UV-visible spectroscopy of silver nanoparticles synthesized by constant heating technique

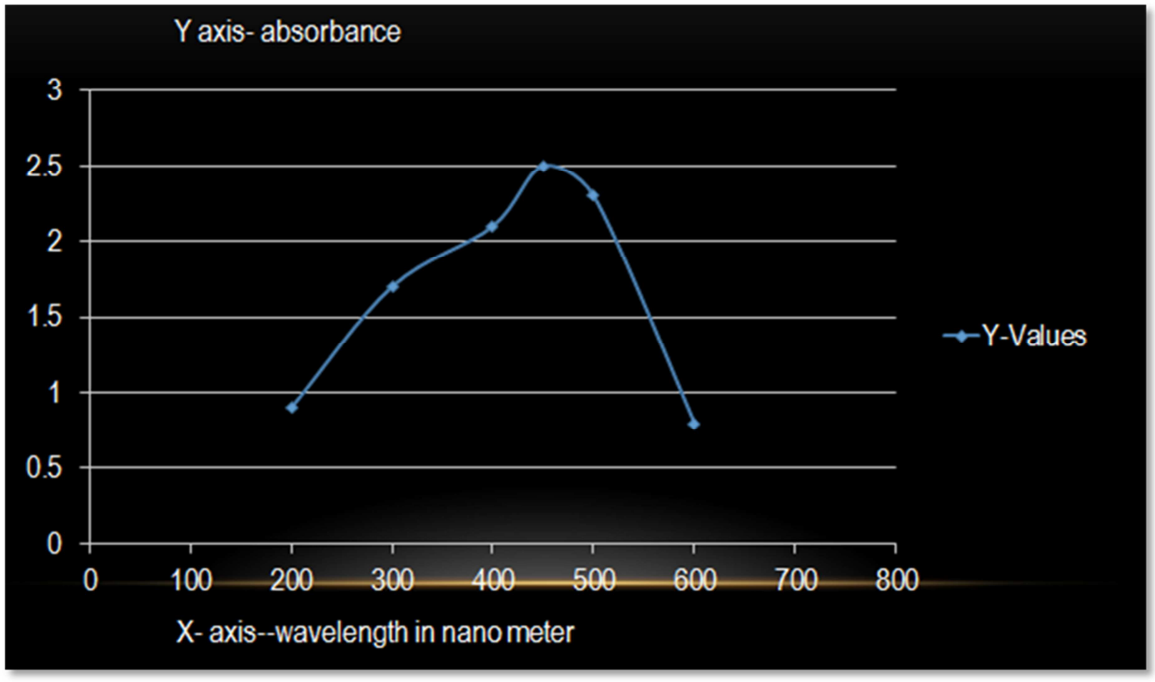

Figure 2. UV-visible spectroscopy of silver nanoparticles synthesized by cyclic heating technique

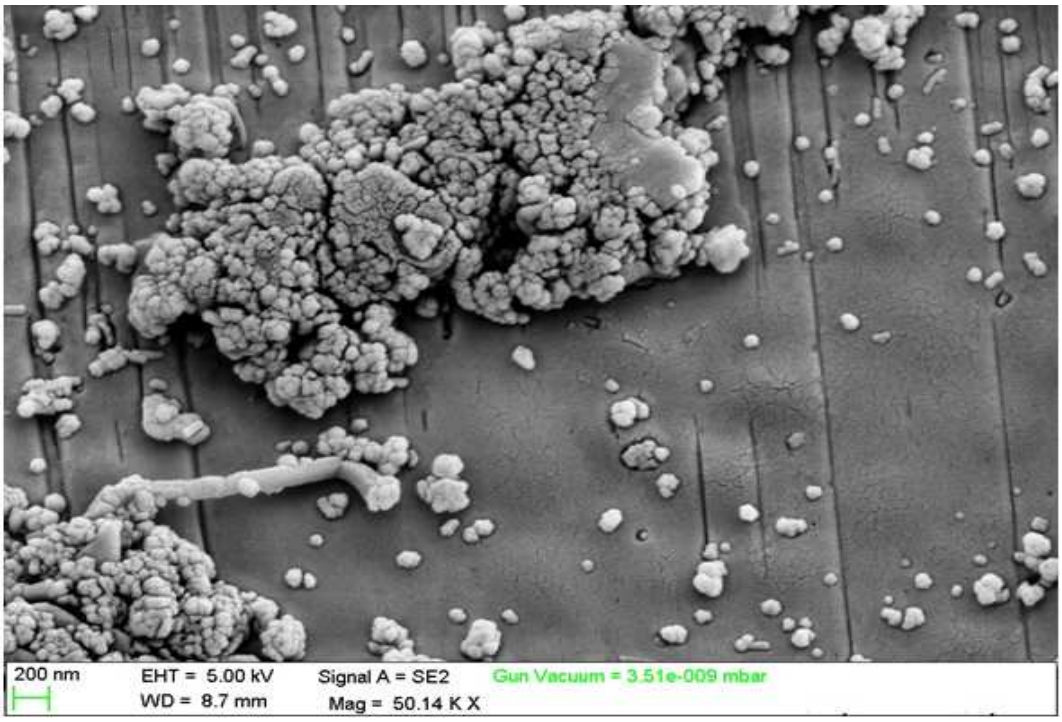

Figure 3. SEM image of silver nanoparticle mediated by tri-sodium citrate synthesized by constant heating technique

SEM

Silver nanoparticles were subjected to SEM micrograph analysis to understand the topology of silver ions. The silver nanoparticles size and morphology were studied by means of scanning electron microscopy (SEM). SEM micrograph of nanoparticle being synthesized using trisodium citate is displayed in fig. 3. The present investigation

of nanoparticle using SEM micrograph clearly illustrates the spherical shaped or roughly spherical shaped and some irregular shaped nanoparticles having the size range of 60- 80nm. The agglomerated and scattered pattern of small grains of nanoparticles was confirmed by SEM imaging. The variation in particle size is possibly due to the fact that the nanoparticles are being formed over periods of time. Tri-sodium mediate synthesized Ag-NPs have strong signals of silver ions at 5keV with magnetic resonance of 50.14Kx.

X-ray diffraction (XRD) analysis

Energy dispersive X-ray diffraction (XRD) analysis was performed to understand the chemical composition of nanoparticles. The chemical mediated synthesized silver nanoparticles using tri-sodium citrate as a reducing agent by constant heating technique was further characterized by XRD analysis. The XRD characterization of nanoparticles was made by taking small amount of samples from the stored dark bottle and drying it on a quartz plate at room temperature. The nanoparticles demonstrated and confirmed by the characteristic peaks observed in the XRD image is depicted in fig. 4. Van Dong et al [14] indicated that X-ray diffraction of chemical mediated silver nanoparticles exhibit remarkably intensive diffraction of the characteristic peak at 2 theta value of 38.1 corresponding to the reflections of crystalline metallic silver particles in the face-centered cubic (fcc) structure. This corresponds to the present XRD pattern of 2 theta deg showing whole spectrum value ranging from 20^o (2θ) to 80^o (2θ) and displaying intense peaks at 38.08^o (2θ) indicating that the particle is made of pure silver. Four more additional broad bands are observed at 38.085^o (2θ), 244.14^o (2θ), 64.37^o (2θ), and 77.38^o (2θ) by the XRD image of silver nanoparticles. These results corroborated with the previous research of Prema [15]. The peak position and their intensities were clearly agreed and compared with the standard literature [JCPDS No 030931]. Average size of the particles was found to be 40^o (2θ) with size range 20-80^o (2θ). The XRD result shows that the nanoparticles are crystalline in nature and the crystals are cubical in shape. The result also confirms that the main component of the particle is silver.

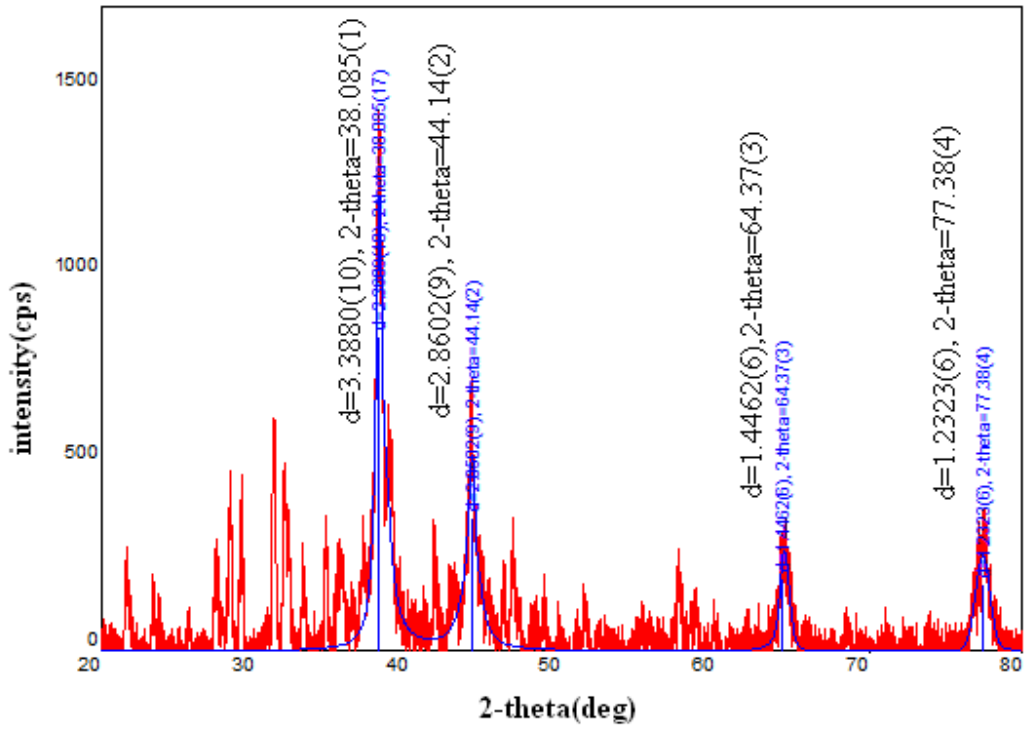

Figure 4. XRD image of silver nanoparticles mediated by tri-sodium citrate synthesized by constant heating technique

ANTIBACTERIAL ACTIVITY

Biosynthesed silver nanoparticle exhibit antimicrobial activity against human pathogens [16] and plant pathogens [17]. In the persent investigation, we approched chemical mediated synthesis of silver nanoparticle. The antibacterial activity of silver nanoparticles synthesized using tri-sodium citrate as reducing agent by the constant heating and

cyclic heating technique was studied against selected bacterial human pathogens. Different volume (µl) of silver nanoparticles was used to optimize the concentration of nanoparticle. Different levels of antibacterial efficacy were measured in terms of mm in diameter by means of inhibition zone. Antibacterial activity of tri-sodium mediated silver nanoparticle synthesized by using constant heating and intermittent heating to asses the efficiency of nanoparticles is portrayed in table 1 &2 respectively. Among the bacterial pathogens tested *Shigella* sp. exhibited maximum inhibition zone of 25mm by using silver nanoparticle mediated by tri-sodium citrate with constant heating technique. Guzman et al [18] reported that chemical mediated synthesis of silver nanoparticle using hydrazine hydrate as a reducing agent exhibit highest antibacterial against gram negative pathogens. This result agrees with the present investigation showing maximum antibacterial activity against gram negative bacteria *Shigella* sp., *Salmonella paratyphi* A and *Enterococci* sp. by silver nanoparticle mediated by tri-sodium citrate with constant heating method.

Table 1 Antibacterial activity of silver nanoparticle mediated by tri-sodium citrate by constant heating

S.No	Bacterial pathogens	Zone of inhibition in different concentration of nanoparticles (mm)				
		5 µl	10 µl	15µl	20 µl	25 µl
1	*Shigella* sp.	15	25	20	20	19
2	*Salmonella paratyphi A*	20	18	15	13	15
3	*Staphylococcus aureus*	12	23	20	20	14
4	*Enterococci* sp.	13	15	15	12	20

Values are the average of three replicates

Table 2 Antibacterial activity of silver nanoparticle mediated by tri-sodium citrate by cyclic heating

S.No	Selected pathogens	Zone of inhibition in different concentration of nanoparticles (mm)				
		5 µl	10 µl	15 µl	20 µl	25 µl
1	*Shigella* sp.	12	23	17	17	18
2	*Salmonella paratyphi* A	17	15	12	10	12
3	*Staphylococcus aureus*	12	20	17	17	13
4	*Enterococci* sp	10	12	13	14	16

Values are the average of three replicates

Morones *et al*. [19] defined chemical mediated synthesis of silver nanoparticles and its potential antibacterial application against Gram negative bacteria. They suggested that silver nanoparticles penetrate through the cell wall of bacteria and attach to the surface of the cell membrane and disturb its function by releasing silver ions. Similar antibacterial activity was determined against gram positive bacteria [20, 21, 22]. Comparatively silver nanoparticles mediated by tri-sodium citrate by constant heating technique exhibit significant broad spectrum of antibacterial activity against all the tested pathogens. The present investigation shows the chemical mediated synthesized silver nanoparticles have almost equally bactericidal effect on both gram positive and gram negative pathogens. The bactericidal mechanism of silver nanoparticles on bacteria is not clearly understood and almost unknown. However, it has been proposed that the effect is caused by the same mechanism of bactericidal effect of silver ions [23].

CONCLUSION

The present study mainly focused on chemical mediated synthesis of silver nanoparticles by the reduction of silver salt. Ag-NPs were successfully synthesized under diverse temperature at different stirring times using tri-sodium citrate as a reducing agent. The formation of Ag-NPs reduced by tri-sodium citrate was determined by UV–visible spectroscopy where surface plasmon absorption maxima can be observed at 450–460 nm from the UV–vis spectrum. The synthesized nanoparticles were characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis. The peaks in the XRD pattern confirmed that the Ag-NPs possessed a face-centered cubic. SEM revealed that Ag-NPs were roughly spherical in shape. The tri-sodium citrate mediated silver nanoparticle synthesized by constant heating exhibited average particle size with mean diameter of 40 nm. Highest antibacterial activity was observed for Ag-NPs with nano-sized particles. This study clearly demonstrated that tri-sodium citrate induced Ag-NPs with constant heating exhibit utmost antibacterial efficiency towards selected bacterial pathogens. The stability, toxicity and bio compatibility of nanoparticle must be analysed using cell line studies in future for biomedical application.

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