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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING AQUEOUS EXTRACT OF SYTIUM AROMATICUM AND ITS CYTOTOXIC AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

The huge potential of *Syzygium aromaticum* (clove) as rich source of polyphenols makes the plant a good choice for the biological synthesis silver nanoparticles. Hence the present investigation was focused on green synthesis of silver nanoparticles (AgNPs) using aqueous extract of clove. For synthesis, aqueous extract of clove was mixed with 0.2mM silver nitrate and incubated at room temperature to observe the color change from colorless to golden brown. To confirm the formation of AgNPs, UV-Vis spectrum was recorded in the range of 200-800 nm which showed the peak of Plasmon resonance at 420nm. Transmission Electron Microscope (TEM) analysis revealed the particles were spherical, monodispersed and size of nanoparticle was around 15nm. Vibrational characteristics studied by Fourier-Transform Infrared spectroscopy (FTIR) showed absorbance bands at 618 cm^{-1}, 1024 cm^{-1}, 1635^{-1} and 3434^{-1} corresponds to out of plane bending of the aromatic –C-H bond, C–O stretching, bending vibration modes of aromatic ring C=C / C=N and O-H or N-H stretching modes respectively, widely present in *S. aromaticum* extract. The zeta potential was found to be -37.63 mV, which indicates that the nanoparticles are quite stable due to charge accumulation on its surface. Reactive oxygen species (ROS) generation of silver nanoparticles was determined in monocytes using Nitroblue tetrazolium (NBT) assay. Biosynthesized AgNPs were incubated with the monocytes for 30 minutes and observed for oxidative burst. The increase in percentage of blue cells indicates the cytotoxic potential of clove silver nanoparticles. This property makes them useful also in the treatment of cancer. The bio-synthesized silver nanoparticles revealed potent antibacterial activity against Gram negative bacteria *Escherichia coli* in Agar disc diffusion method. The Minimum Inhibitory Concentration (MIC) of silver nanoparticles against *Escherichia coli* was found to be 50µg/ml and Minimum Bactericidal Concentration (MBC) was found to be 75µg/ml. Biosynthesized nanoparticles exhibited significant antimicrobial and cytotoxic activity. Based on the above findings it can be concluded that the biosynthesized nanoparticles can be used for various biomedical applications.

KEYWORDS: green synthesis, silver nanoparticles, *Syzygium aromaticum*, cytotoxic activity, antibacterial activity.

INTRODUCTION

Nanotechnology is currently the upcoming successful field in science. It is the field that deals with particles that are less than 100nm in size. There are several reports on nanoparticles made of noble metals over the last few years, as they can be used in medicine, biology, material science, physics, and chemistry (Yokohama and Welchons, 2007). Among the several noble metal nanoparticles, silver nanoparticles (AgNPs) have attracted special attention due to their distinct properties, which include favorable electrical conductivity, chemical stability, catalytic and antibacterial activity (Sharma *et al*., 2009).

The ability of silver nanoparticles to deliver drugs has made it quite useful in the field of medicine. Silver nanoparticles that are chemically synthesized are highly toxic when exposed for a longer duration. Therefore, it is necessary to opt for green synthesis of silver nanoparticles. Products made with plant-based nanoparticles are environment-friendly and hence are an advantage. In addition, the synthesis of nanoparticles using plants offers other advantages, such as the utilization of safer solvents, decreased use of dangerous reagents, milder response conditions, feasibility, and their adaptability in use for medicinal, surgical, and pharmaceutical applications (Mina Sorbiun *et al*., 2018). The use of plants for synthesis of nanoparticles is rapid, low cost, eco-friendly, and a single-step method for biosynthesis process (Huang J *et al*., 2007). Plants contain different biomolecules having different functional groups which helps in the reduction of silver ions and helps in the formation of silver nanoparticles. *Syzygium aromaticum* (Clove) represents one of the richest sources of phenolic compounds such as eugenol, eugenol acetate and gallic acid and posses great potential for pharmaceutical, cosmetic, food and agricultural applications (Cortés-Rojas *et al*., 2014). Hence it proves to be a good biological reducer for synthesis of silver nanoparticles. The antioxidant and antimicrobial activity of clove is higher than many fruits, vegetables and other spices and increases the scope of utilizing it in nanomedicines. The main objective of this research is to synthesize silver nanoparticles using *Syzygium aromaticum* and to check its antibacterial and cytototoxic effect.

MATERIALS & METHODS

Materials

Syzigium aromaticum (Clove) was obtained from Ayurvedic medicinal garden in Kerala, India. Silver nitrate (99.99%), Nitro blue tetrazolium (NBT) were obtained from Himedia and Ficoll- Hypaque from Merck (Mumbai, India). All the reagents and chemicals used were of analytical grade.

Methods

Biological synthesis of silver nanoparticles from S. aromaticum

The *S. aromaticum* (clove) was surface sterilized by 0.1% mercuric chloride and then washed repeatedly in sterile distilled water and kept for drying. 5g of clove was added to 100 ml of distilled water (Satish Kumar *et al*., 2009) in an Erlenmeyer flask and heated at 100°C for 5 minutes. Filtered the spice extract using Whatmann No.1 filter paper. 100 ml of 0.2 mM $AgNO_3$ was mixed with 10 ml of spice extract in a conical flask. Incubated at room temperature for 24 hours and observed for change in colour.

Characterisation of the synthesized silver nanoparticles

UV–Vis spectrophotometry:

The formation of silver nanoparticles (AgNPs) was primarily observed by monitoring the change in colour of the extract after treatment with silver nitrate ($AgNO_3$, 1 millimolar [mM]). The bio-reduction of silver (Ag) ions in aqueous extract was monitored with the UV–visible double beam spectrophotometer (Model no: UV 1800, Shimadzu, Japan) from 300 to 700 nm.

Transmission electron microscopy (TEM)

The particle size and microstructure were studied by transmission electron microscopy PHILIPS CM 200 operating at 200KV. AgNPs were suspended in deionized water followed by sonication to obtain a homogenous suspension of nanoparticles. A drop of the aqueous AgNPs suspension was placed on to carbon-coated copper grid, dried under IR light and loaded in to the TEM microscope to obtain image.

Zeta Potential

Zeta Potential is used as an indicator of stability of nanoparticles. The analysis of the biologically synthesized silver nanoparticles from clove was performed using Horiba Scientific Nanopartica Nanoparticle Analyser SZ-100 (Japan). About 100 microliter (µl) of sample was diluted with 1 ml of sterile deionzed water and loaded in to the folded capillary cells. The measurements were made at 30.5ºC with electrode voltage of 3.3 V.

Fourier Transformed – Infrared (FTIR) spectroscopy

Synthesized nanoparticles were subjected to FTIR spectroscopy, Bruker 3000 Hyperion Microscope with vertex 80 FTIR in the range of 400–4000 cm^{-1} at Spectral resolution of 0.2 cm^{-1}. The AgNPs were grinded with Potassium Bromide (KBr) and pressed to form pellets of around 0.5 to 1.0 mm thickness. The pellets were later placed in the IR path and the spectrum was analyzed.

Anti-bacterial assay for the synthesized nanoparticles

Disc diffusion method for the synthesized silver nanoparticles

The antibacterial activity of AgNPs synthesized was examined against pathogenic Gram negative bacteria *Escherichia coli* using Agar disc diffusion method. A loopful of bacterial culture of *E. coli* was inoculated in sterile Mueller Hinton agar slant (pH 7.4) and incubated at 37°C for 18 hours. After incubation period, the culture was prepared in sterile saline the surface of sterile Mueller Hinton agar plate was swabbed using sterile cotton swab. Sterile standard whatmann filter paper discs of 5 mm diameter were loaded with sterile distilled water, *S. aromaticum* extract, silver nitrate solutions (0.2 mM) were kept as control, Amikacin (30 microgram, µg) and *S. aromaticum* AgNPs (50 microgram, µg). The plates were then incubated at 37°C for 24 hours in upright position. Post incubation, the zones of inhibition was measured.

Minimum Inhibitory concentration (MIC) for AgNPs

The MIC of the biologically synthesizes silver nanoparticles was determined using sterile Mueller Hinton broth with silver nanoparticle concentration ranging from 0.5 microgram/ milliliter (µg/ml) to 500 microgram/ milliliter (µg/ml). 18 hour old bacterial culture of *E. coli* was re-suspended in sterile saline such that the culture density reached that of McFarland`s standard no. 5, having approximate cell density of $1.5x10^8$ CFU/ml. The prepared bacterial culture was inoculated in the standard tubes of silver nanoparticles as shown in Table 1. A positive control was maintained by inoculating the culture in the sterile Mueller Hinton (MH) broth; whereas for negative control, the sterile MH broth was kept uninoculated. All the tubes were then incubated at 37°C for 24 hours. The MIC was noted by observing the tubes for turbidity. The lowest concentration of antimicrobial agent that inhibits the visual growth of the organism is noted as MIC and the concentration of antimicrobial agent that kills the organism is notes as minimum bactericidal concentration (MBC).

TABLE 1: Minimum inhibitory assay for clove AgNPs

Tube no.	Concentration in tube of AgNPs (µg/ ml)	Volume of AgNP stock (µl)	Volume of media stock (ml)	Volume of culture (ml)
1.	0.5	2.5	4.997	0.1
2.	2.5	12.5	4.987	0.1
3.	5.0	25	4.975	0.1
4.	10.0	50	4.950	0.1
5.	25.0	125	4.875	0.1
6.	50.0	250	4.750	0.1
7.	75.0	375	4.625	0.1
8.	100.0	500	4.500	0.1
9.	200.0	1000	4.000	0.1
10.	500.0	2500	2.500	0.1
		Incubate the tubes at 37°C for 24 hours		

Study of Reactive Oxygen Species (ROS) generation:

Monocytes were isolated via Ficoll Hypaque technique. Isolated monocytes were incubated with nitro blue tetrazolium (NBT – 0.3%) for 30 minutes to load them with the NBT. Silver nanoparticles were then incubated with the monocytes for 30 minutes. NBT is reduced if oxidative burst takes place due to generation of ROS and the cells appear blue. The cells were observed and counted under phase contrast microscope at 10X magnification. Three different fields were focused and mean number of blue cells with respect to the total number of cells in the fields were expressed as percentage.

RESULTS

In biological synthesis of AgNPs (Fig.1) after addition of clove extract to the solution of silver nitrate, the solution changed from colorless to dark reddish-brown. The appearance of color in reaction flasks was the indication of AgNPs formation due to bio-reduction process. The color intensity increased as a function of time due to the reduction of Ag+. It is already established that color change of solutions is due to excitation of surface plasmon vibrations with the Ag NPs.

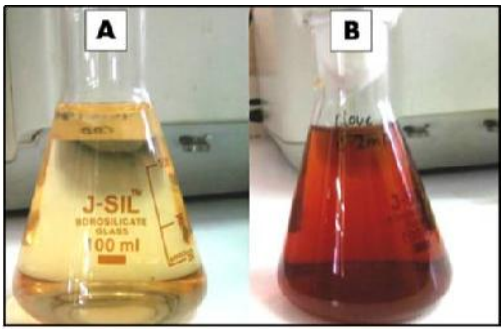


FIGURE 1: Biological synthesis of silver nanoparticles. A: Initial colour of *S. aromaticum* extract with $AgNO_3$ (0 minutes). B: After 24hrs incubation

UV–Vis spectrophotometry

The absorption spectra of the AgNPs are shown in Fig. 2. The sample showed the characteristic surface plasmon of AgNPs. AgNPs had a narrow band with a maximum at 420 nm.

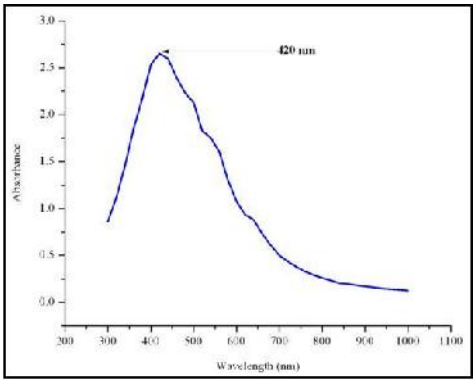
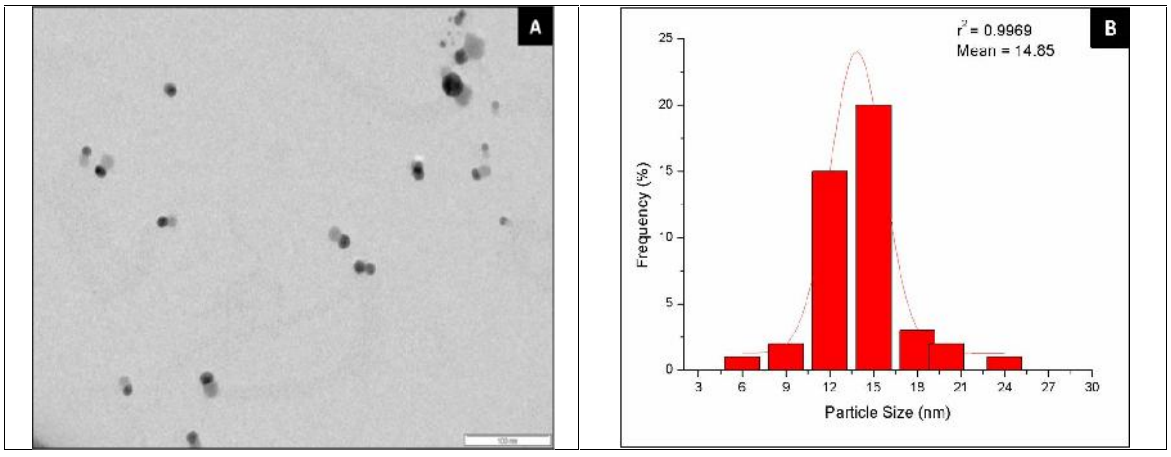


FIGURE 2: UV-Visible Spectrum of Clove AgNPs



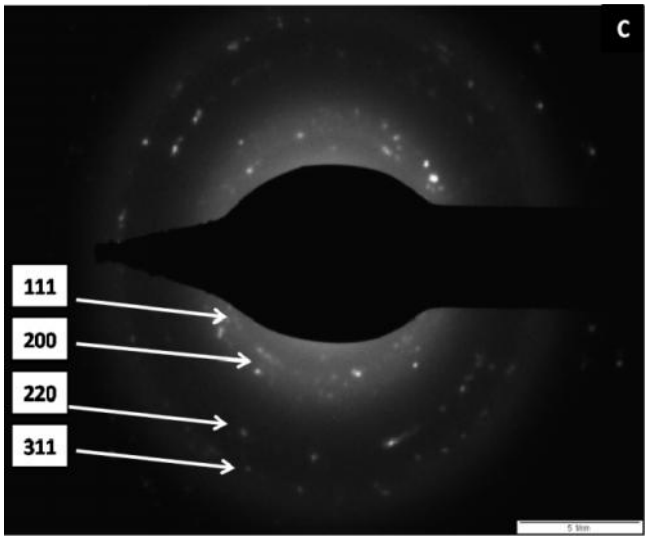


FIGURE 3A. Transmission electron microscope of silver nanoparticles (Scale bar is 100 nm), B. Particle size distribution of biologically synthesized nanoparticles and C. Selected area electron diffraction (SAED) of silver nanoparticles (Scale bar is 5 1/nm).

Transmission electron microscopy

The shape and size of the AgNPs were elucidated with the help of TEM. The particles were of spherical shape & monodispersed as seen in Fig. 3A. The TEM micrograph & the particle size distribution (Fig. 3B) suggest that the sizes of the particles were around 15 nm. The SAED pattern of AgNPs show circular rings as seen in Fig. 3C, indexed corresponding to the reflections from the (111), (200), (220) and (311) planes (JCPDS 4 - 783). These planes correspond to face centered cubic (fcc) Ag & reveal the highly crystalline nature of the synthesized AgNPs.

Zeta Potential

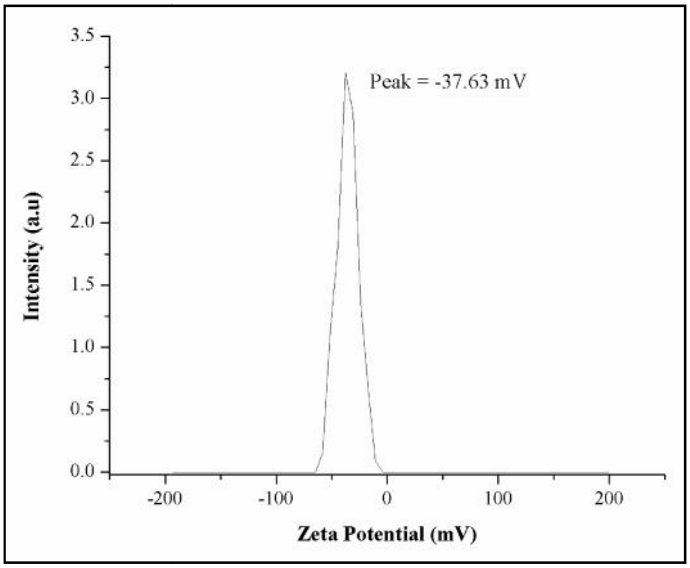


FIGURE 4: Zeta Potential of clove silver nanoparticles

The nanoparticles are most stable if the zeta potential values are higher than +30 mV or lower than -30 mV [5]. The zeta potential of the biologically synthesized silver nanoparticles as synthesized from clove was found to be -37.63 mV, which indicates that the nanoparticles are quite stable due to charge accumulation on its surface. This columbic repulsion helps in keeping the nanoparticles dispersed, prevents agglomeration and thus remains stable for months together.

Fourier transform-Infrared (FT-IR) spectroscopic analysis

To characterize and identify the biomolecules that were bound specifically on the synthesized Ag NPs, FT-IR spectroscopic analysis was utilized. The spectrum so obtained, as shown in Fig. 5, displayed a number of peak FTIR spectrum of processed AgNPs. Absorbance bands at 618 cm^{-1}, 1024 cm^{-1}, 1635^{-1} and 3434^{-1} corresponds to out of plane bending of the aromatic –C-H bond, C–O stretching, bending vibration modes of aromatic ring C=C /C=N and O-H or N-H stretching modes respectively, widely present in *S. aromaticum* extract.

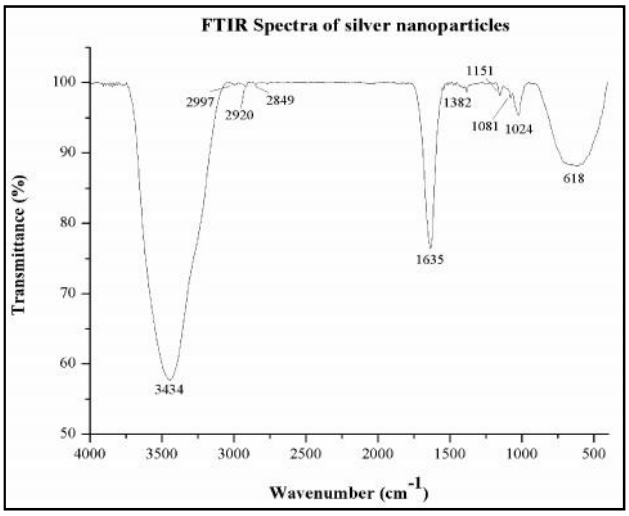


FIGURE 5: FT-IR Spectroscopy

Antibacterial activity

Disc Diffusion method

After incubation, the zone of inhibition was measured in terms of millimeter as shown in table 2.

TABLE 2: Antibacterial assay

Sr. No.	Contents	Zone of Inhibition (mm)
1.	Distilled water	0
2.	$AgNO_3$ (0.2 mM)	9
3.	Clove extract	11
4.	Clove AgNPs	17
5.	Amikacin	16

Minimum Inhibitory Concentration

After 24 hours of incubation of the inoculated tubes, turbidity was observed in all the plates ranging from 0.5 to 25 µg/ml concentration, indicating bacterial growth; whereas from 50 to 500 µg/ ml concentration, no visible growth of bacteria was observed. Thus the MIC of the silver nanoparticles synthesized using clove was found to be 50 µg/ ml for *E. coli* bacteria as indicated in Table 3. Upon inoculation of silver nanoparticles from 50 - 500 µg/ ml on St. Muller Hinton agar plates, no growth was seen from 75 -500 µg/ ml, suggesting the MBC for silver nanoparticles was 75µg/ ml.

TABLE 3: Minimum inhibitory assay for clove AgNPs

Tube no.	Concentration in tube of AgNPs (µg/ ml)	Result
1.	0.5	+
2.	2.5	+
3.	5.0	+
4.	10.0	+
5.	25.0	+
6.	50.0	-
7.	75.0	-
8.	100.0	-
9.	200.0	-
10.	500.0	-
Key: (+) – Visible growth, (-) – No growth		

Reactive oxygen species (ROS) generation

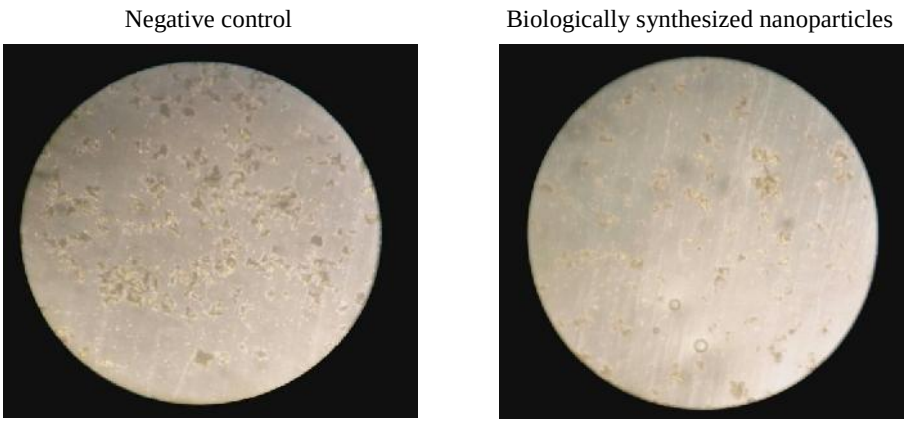


FIGURE 6. ROS generation by bio-reduced AgNPs synthesized from *Syzygium aromaticum*: *In vitro* ROS generation by synthesized silver nanoparticles in monocytes. 48.88% of treated monocytes showed ROS generation as compared to negative control (14.37%) ($P<0.05$). Microbicidal activity of silver NPs maybe due to ROS generation.

DISCUSSION

Silver nanoparticles were synthesized using aqueous extract of *Syzygium aromaticum* and Silver nitrate. Reduction of silver ion into silver nanoparticles might be due to soluble phytoconstituents present in clove extract. Silver nanoparticles exhibit dark yellowish-brown color in aqueous solution due to the surface plasmon resonance phenomenon (Ponarulselvam *et al*., 2012). Characterization using UV-Vis Spectrophotometry showed maximum absorbance of the synthesized particles is at 415nm which falls in the absorbance range of nanoparticles. The TEM results clearly show the structural morphology and size of biosynthesized AgNPs. It was found that the synthesized particles were of spherical shape, monodispersed and approximately 15nm in size thus confirming that the particles are nanoparticles as well as the selected area electron diffraction (SAED) pattern reveals its crystalline nature. The magnitude of zeta potential which indicates surface charge of nanoparticles in solution was found to be -37.63 mV. The zeta potential result indicates that the synthesized nanoparticles are stable in nature. This columbic repulsion helps the nanoparticle to be dispersed and prevents agglomeration, aiding colloidal stability.

The FT-IR graph shows stretching at different wavenumbers due to the different functional groups present on it. The nanoparticles show a -CO- stretch which is obtained due to bioreduction from eugenol, a compound present in *S. aromaticum.* Anti-bacterial activity against *Escherichia coli* was determined by disc diffusion method. The test was performed along with different controls. Silver nitrate showed an inhibition of 9mm zone white silver nanoparticles showed a zone of inhibition of 17mm thus indicating that silver nanoparticles are anti- bacterial in nature and can be used in nanomedicine. The mechanisms of antibacterial activity of silver nanoparticles are by binding on the membrane of microorganisms through electrostatic interactions, cell wall disruption and affecting the intracellular processes such as DNA, RNA and protein synthesis (Wang *et al*., 2007, Wang *et al*., 2009, Wijenhoven *et al*., 2009, Yudha *et al*., 2013). To determine the concentration that can be used, Minimum inhibitory concentration of these silver nanoparticles against *Escherichia coli* was determined. The concentrations from 0.5-25 µg/ml showed growth of culture while concentrations from 50-500 µg/ml inhibited the growth. Therefore the MIC of silver nanoparticles against *Escherichia coli* was found to be 50 µg/ml.These results suggest that growths were inhibited due to the penetration of Ag NPs into the bacterial cell that inhibits the bacterial growth and acts as a bactericidal agent followed by bacteriostatic activity Balaram Das *et al*., 2017). After determining the MIC, the MBC was determined by spot inoculating the tubes that did not show any growth and the MBC results showed growth in 50µg/ml concentration whereas concentrations from 75-500 µg did not show any growth. Therefore, the MBC was found to be 75µg/ml.

ROS generation of silver nanoparticles was determined using NBT assay. The ROS generated by the silver nanoparticles in monocytes was found to be 48.88%. The cytotoxicity of silver nanoparticles may be in part due to the ROS generation that increases their scope and application perspective. It has been suggested that AgNPs produce reactive oxygen species and free radicals which cause apoptosis leading to cell death preventing their replication (Khwaja Salahuddin Siddiqi *et al*., 2018). This property makes them useful also in the treatment of cancer. The present silver nanoparticles synthesised through green synthesis are eco-friendly and pose comparatively lesser threat. They can be used in topical ointments to prevent contamination and sepsis in wounds, *etc*.

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