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Evaluation Of Antimicrobial Activity And Characterization Of Silver Nanoparticles By Bacterial Isolate

MS.RESMI NAIR[1] , DR. M.REJADHEESH[2]

[1]RESEARCH SCHOLA, LNCT UNIVERSITY, BHOPAL, INDIA.

[2]RESEARCH SUPERVISOR, DEPARTMENT OF MEDICAL MICROBIOLOGY, LNCT UNIVERSITY, BHOPAL, INDIA.

ABSTRACT

Silver nanoparticles revealead to be more powerful tool against Gram-positive and Gram-negative bacteria with bactericidal activity together with multi resistant strains. The cell supernatant of E.coli species was used in this study for the silver nanoparticles synthesis. The synthesized Ag NPs optical density was measured in different cell supernatant. The multi drug resistant pathogen E.coli was chosen due to its highest Ag NP synthesis. The spectrophotometric analysis of Ag NPs at the band ƛ 431 nm for characteristic Surface Plasmon Resonance indicated that Ag nanoparticles are in spherical or roughly spherical shaped. FT-IR spectrum of Ag NPs observed at 1359.35 cm–1, 1350.3 cm-1 and 1398.40cm-1. The Ag NPs synthesis showed the antimicrobial activity for the E.coli measured was 18.67±2.082 diameter in zone on inhibition. The Ag NPs synthesized values of MIC against the tested clinical pathogen showed the antimicrobial activity at 200 ppm for E.coli respectively.

Keywords: Microbial production of Silver Nanoparticles; E. coli bacteria; UV-Vis spectroscopy; Transmission Electron Microscopy; Antibacterial activity; Bacterial growth curve.

INTRODUCTION

In nanotechnology industry the silver nanoparticles are very promising products. Synthesis of silver nanomaterials is an important feature of present nanotechnology field. Several physical, chemical and biological methods are involved in the synthesis of Silver nanoparticles. From the past years the chemical method is rapid method, avoiding toxicity and increased quality so it is mostly used in the synthesis.

Synthesized silver nanoparticles biologically involved in the coatings for absorption of solar energy and electrical batteries intercalation material, as optical receptors, as chemical reactions catalysts, for biolabelling and antimicrobials. Silver nanoparticles are considered to be cytotoxic, so it applied in bimolecular detection, diagnostics, antimicrobials and therapeutics, catalysis and micro-electronics (Rai et al., 2009). The antibacterial properties of silver nanoparticles utilize household items includes nano silver lined refrigerators, air conditioners and washing machines.

Ag NP has high antimicrobial property to bacteria like common kitchen microbe, E. coli and it depends with the outer membrane of bacteria and obstructs the respiration and some other metabolic pathways that cause the bacterial death (Panacek et al., 2006).The nanoscale sized particles of reducing silver integrate with the antimicrobial into larger materials such as plastics, coatings, foams and natural and synthetic fibers. The nano size silver gives a more antimicrobial protection for the life (Jha et al., 2009).

Inorganic nanomaterials have good antimicrobial properties that used in the current research of industries of pharmaceutical and medical. Silver metal is used to kill microorganisms and they act as an antimicrobial agent for both extracellular and intracellular targets. And also it show heavy bactericidal activity against Gram positive and Gram negative multi drug resistant bacteria (Shrivastava et al., 2007), and described in new research studies.

In recent years, the antimicrobial compounds have the growth and changes with improved uptake. The metallic nanoparticles have the applications in biomedical research that concerned with Ag, Au, Cu, Pt, etc. Between these Ag NPs has the major attraction of unique antimicrobial properties. In recent research, the small sized Ag NPs can simply perforated in to the cell wall of microbes and illustrated the reactive oxygen species (ROS) and free radicals, to apoptosis (Mukherjee et al., 2001).

In this present study, synthesis of silver nanoparticles by microbes was determined using the bacterial strain Escherichia coli as a reducing agent, this research study hinted the synthesis of silver nano particles and characterization of particles spectro metrically and to understand its inhibitory activity against bacterial species.

MATERIALS AND METHODS

Materials

Silver nitrate was bought from Merck (Mumbai), India. The products used for bacterial study were EMB media, Nutrient agar, LB broth, PDA of Hi Media, India. The Cultures were acquired from the Drug Radiation Research Department at National Center, Pune. The slope cultures were preserved at 4˚C in nutrient agar. The microbes were preserved in glycerol at -70˚C for long term storage. The antimicrobial activity of Silver nanoparticles was tested against Gram negative infectious multidrug resistant bacteria obtained from specimen at the National Cancer Institute, Pune.

Glassware and Apparatus

All glass wares such as conical flasks, measuring cylinders, beakers, petri plates and test tubes etc. were obtained from borosil, India.

Ready Made Media

According to the manufacturer's instruction the following ready made media were prepared and included.

Nutrient agar

4 gm/l of Yeast extract, 5 gm/l of tryptone , 50 gm/l of glucose, 0.55 gm/l of potassium dihydrogen phosphate, 0.425 gm/l of potassium chloride, 0.125 gm/l of calcium chloride, 0.125 gm/l of magnesium sulphate, 0.0025 gm/l of ferric chloride, 0.0025 gm/l of manganese sulphate, 0.022 gm/l of bromocresol green and agar at 15 gm/l (oxoid).

Muller–Hinton Agar

300gm/l of Beef, 17.5gm/l of casein hydrolysate, 1.5gm/l of starch and 17gm/l of agar.

Luria bertani broth

10gm/l of Tryptone, 5gm/l of yeast extract and gm/l of sodium chloride.

Prepared Media

Nitrate media

1.5 gm/l of Glucose, 15 gm/l of peptone, 3.5 g m/l of yeast and 3.5 gm/l of KNO_3, after fusing and autoclaving of these components at 121°c for 15 min then cool and inoculate the pathogen.

Optimized media

The mixing of the components such asglucose 1 gm/l, peptone 10 gm/l, yeast 4 gm/l and KNO3 4 gm/l for the production of the Ag NPs from E.coli then mixed the components and autoclaved at 121°c for 15 min then cooled and inoculated the pathogen.

Production of cell supernatant from E.coli

For the synthesis of silver nanoparticles, the cell supernatants from bacterial isolates were grown aerobically in nitrate containing medium. The bacterial cultured flasks were inoculated using shaker and agitated at 120 rpm. After 24 hours the collected cell supernatants were centrifuged by using centrifugation at 6,000 rpm for 10 minutes at 6˚C.

Synthesis of silver nanoparticles

The method of **s**ynthesis of silver nanoparticles was described by (Kalishwaralal et al., 2008) with little changes. For the synthesis of silver nanoparticles the cell supernatant was prepared by the above method, was placed in the Erlenmeyer flasks containing AgNO3 at a concentration of 1 mM and incubated for 5 min. By using UV–visible spectrophotometer the absorption spectrum of the sample was recorded at a resolution of 1 nm. The cell supernatant was irradiated one time at before and after mix with the 1 Mm of silver nitrate and it was exposed to room temperature at 0.25 to 3kGy Gamma-rays. After mixing the silver nitrate filtrate was irradiated then we use different ranges of silver nitrate.

Characterization of silver nanoparticles

Before and after mixing with AgNO3 solution to gamma irradiation of cell supernatant at room temperature by the dose of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 and 3.00 k Gy. The produced Ag NPs after irradiation were specified by the UV–vis spectroscopy, DLS, TEM and the FT-IR spectroscopy.

UV/vis Spectral Analysis

The UV/v is spectral analysis was measured from200-900 nm operated at a resolution of 1 nm as a function of wavelength.**(didn't understand)**

Dynamic Light Scattering (DLS)

Size distribution and average particle size were determined by particle sizing system.

Fourier Transform Infra-Red Spectroscopy (FT-IR)

For infra-red spectrometer the measurements were carried out using by employing KBr pellet technique.

Transmission Electron Microscopy (TEM)

The synthesized nanoparticles size and morphology were measured by TEM. To drop coating silver nanoparticles onto carboncoated film on the TEM grids and allow drying, the unwanted solution was detached by a blotting paper.

Atomic Absorbtion Spectrophotometry

Silver nanoparticles aggregation was estimated by Atomic Absorbtion Spectrophotometry, provided with denterium background correction. Each solutions were constructed with ultra pure water with certain resistance.

Antimicrobial study of the synthesized silver nanoparticles

Determination of zone of inhibition

The antimicrobial activity of Ag NPs synthesized was tested by the method of agar well diffusion (Bauer et al., 1966) against pathogenic multidrug resistant bacteria. The tested pathogenic bacterium (108cfu/ml for bacteria) was swabbed onto sterile Muller-Hinton Agar plates (MHA) uniformly by sterile cotton swabs. The agar medium was poured in to the well containing 10mm diameter prepared using gel puncture. The silver nanoparticles solution (200ppm) was added into each well using micropipette, incubated at 37˚C for 24hr and the inhibition zones were measured. For antimicrobial activity, tetracycline was used as positive control and the mean values ± SD were measured.

Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

Gram negative bacteria E.coli strain was selected and their MICs were determined by using LB broth under two-fold dilutions of Ag NPs in the levels of 1600-0.049 ppm, with tubes of positive and negative control (CLSI, 2009). After 24 hours of incubation at 37˚C with initial inoculums of 0.1 OD at 600 nm the MIC was measured. The MIC concentration of Ag NPs inhibit the growth of 50% and 90% of microorganism and completely visually inhibited 99% growth.

The aliquots of invisible growth tubes were seeded without supplement of Ag NPs in MHA plates and incubated for 24 h at 37˚C. The invisible growth of lowest concentration of Ag NPs on MHA medium is called as MLC.

The synthesized Ag NPs antimicrobial activity was measured by the zone of inhibition and MIC, MIC50, MIC90 and MLC ranges were recorded in vitro exposure to a dose level of 24.41 Gy gamma radiations. 24.41Gy is a single dose biologically equivalent to the fractionated multiple therapeutic doses of 70 Gy/35 fractions given to treated patients and was calculated using the linear quadratic (LQ) formula (Barton, 1995).

The bacteristatic effect of the Ag NPs against the tested bacterial strain was represented by the MIC. The antimicrobial study of the Ag NPs synthesized including zone of inhibition, MIC, MIC50, MIC90 and MLC ranges were carried out for all the tested strains before and after their in vitro exposure to a dose level of 24.41 Gy gamma radiations. This was done to study the antimicrobial activity of the Ag NPs against microorganisms causing infections in radiotherapy treated patients. 24.41Gy is a single dose naturally identical to the fractionated more remedial doses of 70 Gy/35 levels given to cure patients and was recorded by linear quadratic (LQ) formula (Barton, 1995).The irradiation origin wosrned was 137Cs Gamma cell and its dose rate at the time of experiments was 0.774 rad /sec.

Statistical Analysis

For nitrate reductase production and their results of optimization were calculated by standard analysis of variance (ANOVA) statistical version 6.0 Statsoft inc. USA. The significance of antimicrobial activity of Ag Nps was measured and calculated by standard analysis of variance (ANOVA) Version 9 by SAS Institute Inc. Cary, NC, USA. The calculation were done in triplicates and the mean values were represented as ± SD.

RESULT AND DISCUSSION

Silver nanoparticles revealed more powerful bactercidial activity against Gram-positive and Gram-negative bacteria activity together with multi resistant strains, as well as possible anti fungal activity (Shrivastava et al., 2007).

Synthesis of silver nanoparticles

The cell free supernatant of E.coli was added to the aqueous Ag+ within 10 hours after incubation the Ag NPs were reduced before optimization of media. At the time of incubation, a color change was observed from whitish yellow to brown observed and the control with no colour change. The SPR band for Ag NPs is obtained at ƛ 431 nm which was measured by spectrophotometric analysis and denotes the appearance of spherical or roughly spherical Ag nanoparticles.

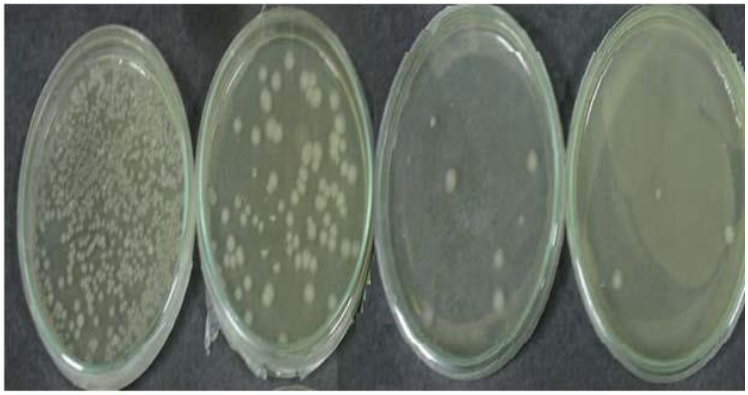
The concentration of 1 mM of silver nitrate and 5 ml cell free supernatant in UV-Vis spectra absorbance denoted the completed reaction within 24 hours and the formation of Ag NPs does not influence the increasing time. The enzyme production of the nanomaterials synthesis is based on the growth over a level of chemical compositions, shapes and separation. The datas of silver nanoparticle solution were obtained in a fast way by absorbance spectroscopy. The data and the correlation with an optical model of silver nanoparticles must be done in this absorbance spectroscopy (Pedersen, 2005).

Characterization of silver nanoparticles

The distribution of silver nanoparticles exhibited vigorous colors due to the Plasmon resonance absorption. So, the characteristic optical absorption spectrums in the UV–visible region have metallic nanoparticles. The synthesized Ag NPs have strong, broad peak UV– visible spectrum located UV– visible spectrum of Ag NPs was strong, with broad peak and located in the middle of 420 and 440 nm. The cell free supernatant of bacteria with silver nitrate solution treated with various doses of gamma radiation after mixing with AgNO3 showed more effective than which noticed before mixing. The formation of silver nanoparticles measured with the peak at 430nm. The fig-5 showed the peaks of irradiation dose of 0.75 kGy appeared as high absorbance, decreased Surface Plasmon Resonance band intensity and increased irradiation of above 0.75 kGy.

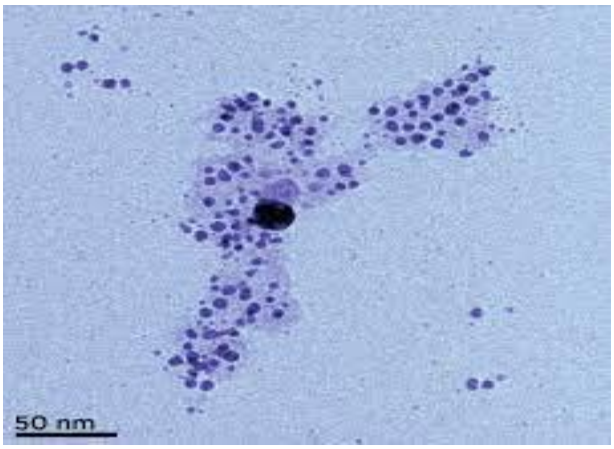
The electrons specific vibrations were based on the size and shape of the particles. The collective oscillations of valence electrons in the electromagnetic field have the peak at 431 nm of characteristic of Ag nanoparticles (Majeed et al., 2011). The surface Plasmon mode is 'restricted' because of little dimensions of electrons confined in the metal nanoparticles. The metal nanoparticle's surface plasmon oscillation is varied from the bulk metal plasma frequency. The optical properties and spectral profile influence were scattered by the SPR of the metal nanoparticles. Among the metal nanoparticles and silver nano particles are known to exhibit strong SPR. The size of the particle may be observed by the Mie theory which solves Maxwell's equations that described the extinction spectra of arbitrary size particles (Power et al, 2010).

Fig-5: Digital photograph of E. coli colonies grown on nutrient agar plate as a function of silver nanoparticle concentration. A) MIC B) MIC50 C) MIC90 D) MLC silver NP concentration



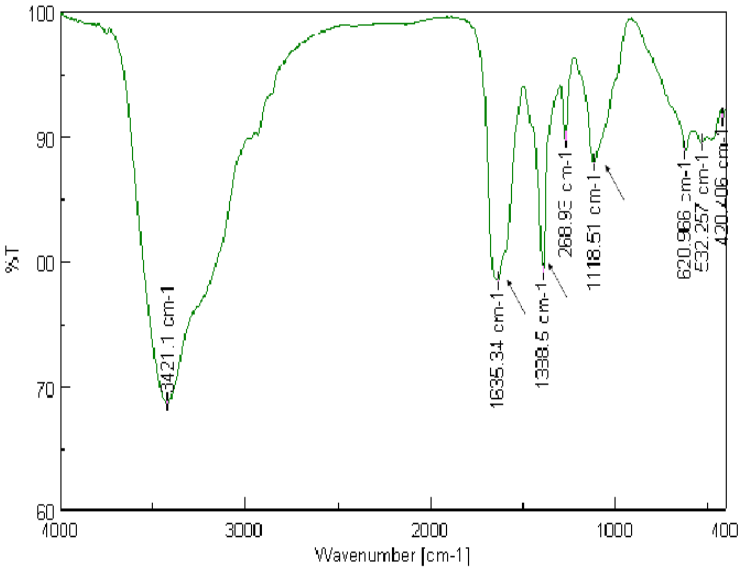
The silver nitrate concentration affects strongly and increases (what) the 5ml of cell free supernatant with various ppm such as 200, 300, 400, 800 and 1600. TEM micrographs (Fig-3) showed the mono dispersed and spherical particles besides significant aggregation. After vigorous shaking the very tiny particles have also been observed ranges from 8.16 to 20.5 nm and 15 ± 2.5 nm. These observations were similar with the results of DLS measurements in mean diameter 17.8 nm.

Fig-3: TEM image for silver nanoparticles



From the FT-IR spectrum of Ag NPs the bands observed at 1359.35 cm–1 (correspond to a primary amine NH band), 1350.3 cm-1 and 1398.40cm-1 (correlate to a secondary amine NH band (Fig-4) and primary amine CN stretch vibrations of the proteins, respectively). These observations denote the secondary structures of proteins and do not affect the consequence of reaction with Ag+ ions or binding with Ag NPs. The methylene scissoring vibration from the protein band was observed at 1312.3cm–1. This indicated the releasing of extracellular protein molecules from bacterial pathogen. Binding of silver ions or silver nanoparticles have not been affected by the secondary structure of proteins by the FTIR results. Here the protein molecules play a vital part in the size and shape of the protein molecules (Jain et al., 2010).

Fig-4: FT-IR spectrum of fermented extract with silver Nanoparticles



The methylene scissoring vibration of protein solution showed the band at 1388.5 cm-1(Sheikh et al.,2009). It implies the releasing of extracellular protein molecules from bacteria and carry out the formation and stabilization of Ag NPs in aqueous medium.

The significance factors were used to confirm both the t-value and p-value statistical parameters. The large coefficient measured in t-value is related to the standard error. The p-value is the chance of getting a larger t value by chance. The larger t-value and smaller the p-value have the more significance than the corresponding coefficients (Myers and Montgomery, 2007).

Antimicrobial activity

The antimicrobial activity of Ag NPs synthesis against bacterial pathogen and it was compared with antibacterial drugs like tetracycline. The average particle size used was 14 ± 4 nm and the antibacterial activity of synthesized Ag NPs against tested multi drug resistant pathogen was proved (Table-1). The zone of inhibition was measured against E.coli was 18.67±2.082. From the standard broth macro dilution method, the MIC, MIC50, MIC90 and MLC values of the Ag NPs for the bacterial pathogen was showed in table-2 & fig-5. The Ag NPs synthesized values of MIC against the tested clinical pathogen showed the antimicrobial activity at 200 ppm for E.coli. The inhibitory effect of the synthesized Ag NPs on bacterial pathogen was specific and differed from each other before and after in vitro application of gamma irradiation.

Table-1: The antimicrobial activity (inhibition zone in mm) of the Ag NPs synthesized against different strains before and after in –vitro gamma irradiation

Tested strains	Tetracycline (Standard antibacterial agent)	*Diameter of inhibition zone. (mm) produced by Ag NPs		P-value
		B Mean ± SD	A Mean ± SD	
E.coli	26	27.67± 0.577	22.67±1.155	0.0131●

* Three repeats were performed for each tested strain

B: Before in-vitro gamma-irradiation. A : After in-vitro gamma-irradiation

●P- value significant < 0.05

P- value non-significant > 0.05 Ag NPs (200ppm), concentration of antibiotics:20 (µg/ ml)

Table-2: Minimum inhibitory and minimum lethal concentration values of the Ag NPs synthesized for the selected strains

Tested strains	MIC (µg/ml)	MIC50 (µg/ ml)	MIC90 (µg/ ml)	MLC(µg/ml)

	B	A	B	A	B	A	B	A
E.coli	200	200	50	50	100	100	400	400

B: Before in-vitro gamma-irradiation
A: After in-vitro gamma irradiation

The antibacterial properties of nano-sized particles synthesised are called as "nano antibiotics", and it is used in the growth of new pharmaceutical products (Huh and Kwon, 2011). The Ag NPs is used as a significant antibacterial agent in modern era (Haung et al., 2011).

The zone of inhibition was measured for Ag NPs against MRSA and E.coli has good antibacterial activity and similar study was reported by (Guzman et al., 2012). The antibacterial activity against MRSA before and after in vitro gamma irradiation were greater than compared to various studies (Ayala-Nunez et al. ,2009) which observed MIC and MBC ranges of Ag NPs all over 1800 ppm and 2700 ppm, respectively.

The before and after in vitro treated gamma irradiation of each microorganism was specific and differs in the inhibitory impact of the Ag NPs (Fig-1 & 2). Treatment of Ag NPs on microorganisms implies the loss of DNA replication ability of ribosomal subunit protein expression and enzyme and cellular protein inactivation by ATP production (Yamanaka et al.,
2005).

Fig-1: UV-VIS Spectrum of cell free supernatant irradiated after mixing with Ag NO3 solution

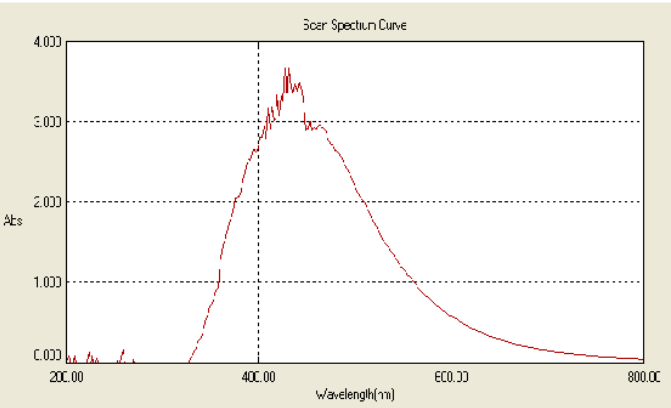
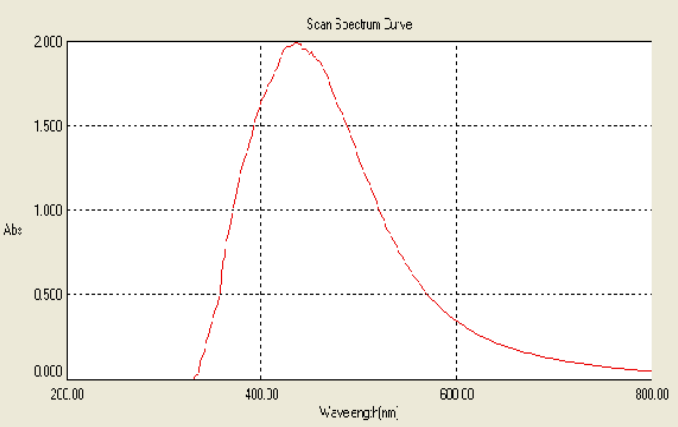


Fig-2: UV VIS-Spectrum of cell free supernatant irradiated before mixing with silver nitrate solution



Many research studies showed that Ag+ ion positive charge is crucial for its antimicrobial activity by electrostatic attractions among positive and negative charged cell membrane of microorganisms Tiwari et al., (is this needed) 2008.And also the Ag NPs interact with the surface of membrane and penetrate inside of the bacteria. It has intensive property to react with the sulfur and phosphorus groups of the cell membrane such as DNA Ag NPs (Dehkordi et al., 2011).

The antibiotic therapy of bacterial infections of Ag NPs does not cause the bacterial resistance due to the fact that antibiotics do not exert the bacterial wall, protein synthesis and DNA which means the pathogens have to adapt a lot of changes to protect themselves (Morones et al., 2005).

CONCLUSION

This study concluded that the silver nitrate was added to supernatant of E.coli synthesized the Silver nanoparticles in the range of 14±4 nm. The Response surface methodology estimated the main factors and explored the interaction among different factors. These factors exhibited antimicrobial activity against both Gram-positive and Gram negative bacterial strains, considered as a drug-resistant mechanisms(needs rewording)and also could be considered as a potential antifungal agent. The antibactericidal activity have proved that Ag NPs kill microorganisms at such low levels (units of ppm), does not disclose acute dangerous effects on human health, in addition to that resistance, and low cost compared to conventional antibiotics. It was found to be a cost effective and eco-friendly method.s

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