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OPTIMIZATION AND CHARACTERIZATION OF GREEN SYNTHESIZED SILVER NANOPARTICLES AND ITS INHIBITORY ACTIVITY AGAINST BIOFILM FORMING BACTERIAL PATHOGENS.

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ABSTRACT

In the present study, antibacterial activity of silver nanoparticles synthesized from entophytic fungi extracts were analysed against biofilm forming bacteria include the *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa* isolated from clinical specimens. Therefore, we tried to establish a combination of medicinal and nanotechnology possibly with the field of medicine for the development of antibacterial agents against these strains. The nanoparticles were characterized by UV–visible spectroscopy, the presented and capped molecules of proteins with nanoparticles was confirmed by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD).

UV–visible studies display an absorption band at 420 nm due to surface Plasmon resonance (SPR) of the silver nanoparticles. The intra cellular synthesized silver nanoparticles (Ag NPs) size 15-35 nm was determination of X-ray diffraction (XRD) crystal calculation, antimicrobial activity higher than the standard antibiotic. The nanoparticle treated with isolated bacteria as result macromolecule of proteins denatures and oozing out the cells and measure the Bradford method. The nanoparticle treated bacterial cells were compared to negative control more amount of protein released. The nano size establishing the silver nanoparticles directly bind with DNA of the pathogenic bacterial strains most important to higher antimicrobial activity.

Key words: Silver nanoparticles, Endophytic fungi, FTIR, XRD, Protein leakage assay and DNA damage activity.

1.INTRODUCTION

In recent years, the number of infections associated with antibiotic-resistant bacteria has increased. Many of these infections are caused by microorganisms growing in biofilms. Both Gram-negative and Gram-positive bacteria can form biofilms on indwelling medical devices such as catheters, mechanical heart valves and prosthetic joints. The most common biofilm-forming bacteria associated with human disease are *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* [1].

Silver ions have long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities [2]. It is generally believed that heavy metals react with proteins by combining the SH groups, which leads to the inactivation of the proteins [3]. The effects of silver ions on bacteria problematical; however, direct observation of the morphological and structural changes may provide useful information for understanding the comprehensive antibacterial effects and the process of inhibition of silver ions. It is generally accepted that free silver ions, present or released from the nanomaterials, are able to bind cell membrane structures, destabilizing the membrane potential and causing proton leakage [4, 5 and 6].

Nanotechnology is an evolving field with wide range of biomedical applications including the use of nanoparticles like silver nanoparticles (AgNPs) as alternative antimicrobial agents. Silver nanoparticle act as an antimicrobial and antibiotic agent when incorporated in proteins, nanofibre, first aid bandages plastics, soap and textiles, in cell cleaning fabrics and as a conductive filler.

There is also an effort to incorporate silver nanoparticles into a wide range of medical devices, including but not limited to bone cement, surgical instruments, surgical masks, Wound dressings. Currently most of the applications of silver nanoparticles are in antibacterial/antifungal agents in textile engineering, bioengineering, water treatment, and silver-based consumer products. There is also an effort to incorporate silver nanoparticles into a wide range of medical devices, including but not limited to surgical instruments, bone cement, surgical masks and Wound dressings.

2.MATERIAL AND METHODS

Screening of Fungi Biomass Aerobically and Mycogenic Synthesis of Silver Nanoparticles

The Potato dextrose broth (PDB) media was using fungi biomass aerobically cultivated then it were incubated at 27°C for 21 days. After incubation, sterile distilled water using the successfully grown fungal mat was complete washed and removes the impurities of medium ingredients components. Weighted 10 g of wet fungal mat was transfer with 100 mL sterile distilled water in conical flask which was kept under shaker condition 120 rpm for 72

h at 27°C. Then, the biomass of fungal mat filtrates with Whatman filter paper No.1. After $AgNo_3$ treated with screened fungi biomass were synthesized silver nanoparticles.

Optimization of Different Concentrations Ratio of Fungal Biomass and Synthesized Silver Nanoparticles

Different concentrations ratio of fungal biomass such as 1:10, 2:10, 5:10, and 10:10mL were optimized for the better synthesis of silver nanoparticles [7]. Different concentrations of silver nitrate (0.25, 0.5, 0.75, 1, 2 and 5 mM) were added to the mycelial mat free extract and optimized pH where the reaction pH was maintained at 3, 5, 7, and 9, respectively. 0.1 N HCl and 0.1 N NaOH using the adjustment of pH and temperatures (25, 35, 45, 55, 65°C) for various time periods up to 12, 24, 36, 48, 72h. The pH was adjusted using 0.1 N HCl or 0.1 N NaOH solutions and the observed absorbance in the presence of reddish brown colour indicate silver nanoparticles formation was measured spectrophotometrically. In addition, the stability of as-synthesized silver nanoparticles was also monitored.

FT-IR Spectra Analysis for Synthesized Silver Nanoparticles

FT-IR measurement was carried out for fungal free filtrate and silver nanoparticles to identify the possible bioactive molecules responsible for the reduction of the Ag+ ions and the capping of the bioreduced silver nanoparticles in the diffuse refluctance mode at a resolution of $4cm^{-}$; using KBr pellets and the spectrum was recorded in the wavelength interval 4000 to 400 cm^{-}.

X-ray Diffraction Studies for Synthesized Silver Nanoparticles

X-ray diffraction (XRD) measurement of the fungal reduced AgNPs was carried out using powder X-ray diffractometer instrument in the angle range of 10°-70° operated at a voltage of 40KVand a current of 30mA with CuKα radiation in a θ-2θ configuration. The crystallite domain size was calculated by using Debye – Scherrer formula.

Antimicrobial Activity of Mycogenic Synthesized Silver Nanoparticles

Well diffusion assay

In this study, seven different isolate human infected pathogenic bacteria *E. coli, Pseudomonas aeruginosa , Klebsiella pneumonia, Proteus mirabilis (Gram-negative) and methicillin-resistant S. aureus (MRSA), (Gram-positive)* were used as test organisms and grown in nutrient broth media overnight culture and stick with Mueller-Hinton agar (MHA) medium. Well diffusion assay was best to determine the antimicrobial activity of mycosynthesized silver nanoparticles. 30 micro litre of silver nanoparticles were loaded separately into each well of the Petri plates. Mycelial free extract was used to compare the negative control antimicrobial activity of synthesized nanoparticles; also standard (1 mg/mL) was used as a positive control for bacterial strains after inoculation, the plates were incubated at 37 ◦C for 24 h and the zone of inhibition was measured in terms of

millimeter. These assays were carried out in triplicate [8].

Nanoparticles treated with bacterial cells and mechanism were measurement by Protein leakage assay

The protein leakage assay was carried out using the method of Kim et al. [9]. The bacterial cells cultures were treated with known concentration of silver nanoparticles a 0 and 5h after centrifuged at 5000 rpm for 20 min. For each sample, 2 ml of the supernatant was mixed with 8 ml of Bradford reagent and then incubated for 15 min. The optical density was measured at 595 nm using spectrophotometer. Bovine serum albumin (BSA) was used as a standard protein and the experiments were done in triplicate.

Mycosynthesized silver nanoparticles used as DNA damage activity

The DNA damage study was established according to the procedure [10]. *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, methicillin-resistant S. aureus* these DNA isolated and silver nanoparticles were treated for 30 min. Silver nanoparticles without bacterial DNA as negative control and DNA without treatment of nanoparticles was served as positive control. Electroporation was carried out using 1% agarose gel at 75 V for 45 min.

3.RESULTS AND DISCUSSION

Different concentration fungal extract treated $Agno_3$ solution gives complete synthesis silver nanoparticles which volume ratio on 1:10, 2:10, 5:10, and 10:10 but 2:10 ratio concentration quickly resulted and changes to yellowish red colour as well as absorbance of the reaction mixture was monitored spectrophotometrically for every 30 sec then an absorption peak at 421nm taken to record. 1mM concentration of silver nitrate produces perfect shape and size of nanoparticle. Optimized pH was maintained at 7 because of alkaline condition reaction more than compared acidic condition. The silver nanoparticles synthesis optimum temperature at 35°c the absorption maximum was measured spectrophotometrically.

UV-VIS Spectroscopy analysis

The UV Vis spectra noted from the fungal extract could be used to study the size and shape of AgNPs in aqueous suspensions (fig 1). The light absorption pattern of fungal cell extract was checked in the range of 200–800nm through a UV–visible spectrophotometer. UV–visible spectrum of the aqueous suspensions was also recorded to study the change in light absorption profile of the medium and change in intensity of the brown color during long term incubation. The appearance of yellow colour indicated the formation of AgNPs in the reaction mixture, as it is well - known that AgNPs exhibits striking colours (light yellow –brown) due to the excitation of surface plasmon vibrations in the particles. The intensity of absorbance in the range of 350–420nm gradually increased significantly resulting in gradual appearance of a peak at 420 nm (fig 2). This observation indicates the bind of fungal extract or

organic macromolecule a possible reduction mechanism for the metal ions present in the $AgNo_3$ solution.

FT-IR analysis

FT-IR spectrum of fungal extract was taken after the synthesis of AgNPs. The spectrum was recorded in the wavelength region between 400cm^{-} to 4000cm^{-}. The spectrum shows peaks at 3448cm^{-}, (strong O-H bonding) which indicates the presence of –O-H stretching of carboxyl group and N-H stretching of secondary amides. These peaks indicate the presence of bonded hydroxyl groups. Further, the peaks observed at 2922cm^{-}, 2852cm^{-}; represents the C - H stretching bonds of alkanes. The peak observed at 1622cm^{-}; and 1340cm^{-}1 represent the N-H deformation, and C=C aromatic conjugates (Fig 3). The sharp peak at 1022cm^{-}; is assigned to C-N stretching vibrations of proteins. The C-S stretching appears as a weak band in the 700-600cm^{-}. The position of these bands is close to that reported for native proteins. The IR spectrum of the AgNPs indicates the absence of many fundamental groups and peaks of lower intensity. This disappearance of the bands and decrease in intensity is attributed to reduction of silver ions.

X-ray diffraction pattern analysis

XRD patterns taken using powder X-ray diffractometer instrument in the angle range 10°- 70° of the AgNPs at 2θ, scan axis 2:1sym. A number of Bragg reflections corresponding to (111), (200) and (220) sets of lattice planes are observed, which can be indexed to face-centred cubic silver (fig 4). The peaks match with the joint Committee on powder Diffraction Standards which further proves the formation of crystal AgNPs. Furthermore, the average diameter of the AgNPs is calculated as 26 nm by Scherrer formula using FWHM obtained from the diffraction peaks: $D = 0.89\lambda/\beta\cos\theta$ Where D is the mean grain size, λ is the wavelength of Cu target, β is the FWHM of the diffraction peaks and θis the diffraction angle. Thus XRD is commonly used to determine the chemical composition and crystal structure of a material.

Anti-microbial activity

Silver nanoparticle has potent and natural antibiotic and antibacterial agents which are exhibited anti-bacterial properties against a gram positive and gram negative bacteria. Anti-bacterial activities of the synthesized silver nanoparticles have been investigated against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa* (table 1). Synthesized AgNPs shows actual strong inhibitory action against 21 mm zone of inhibition while as standard antibiotic.

Effect of silver nanoparticles on protein leakage in bacteria

Absorbency of isolated bacterial pathogens cells initially, protein leakage from the membranes of bacterial cells treated with nanoparticles was almost the same as that from cells in the control group. At 5 h after incubation, protein leakage from cells treated with nanoparticles considerably increased; but there was no change in the

Fig1: Colony morphology of *Aspergillus flavus* in PDA.

Fig2: Mycogenic synthesis of silver nanoparticles

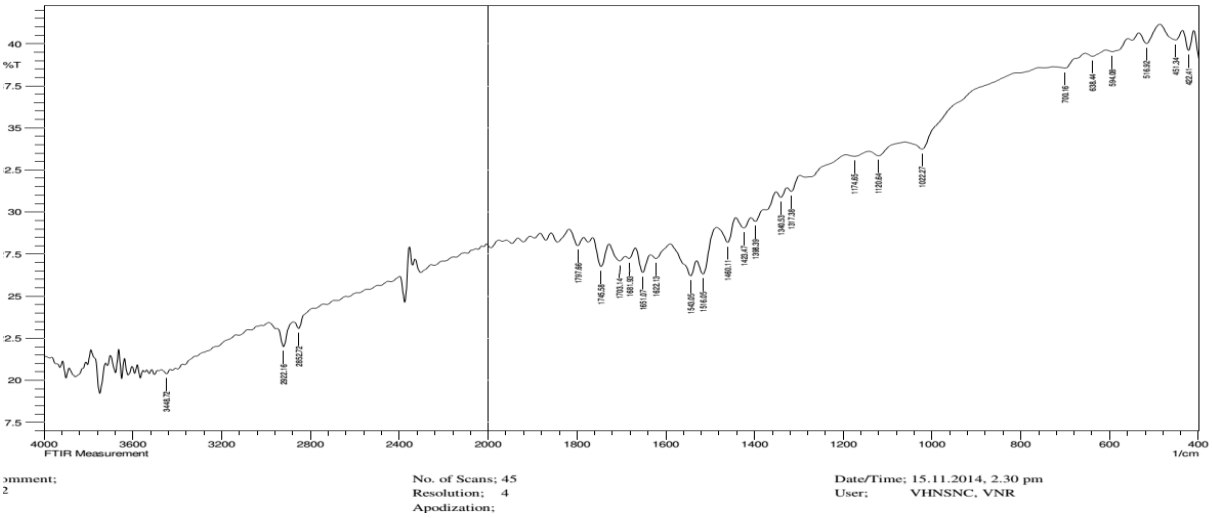

Fig 3. FTIR spectrum recorded from mycogenic synthesised of silver nanoparticles.

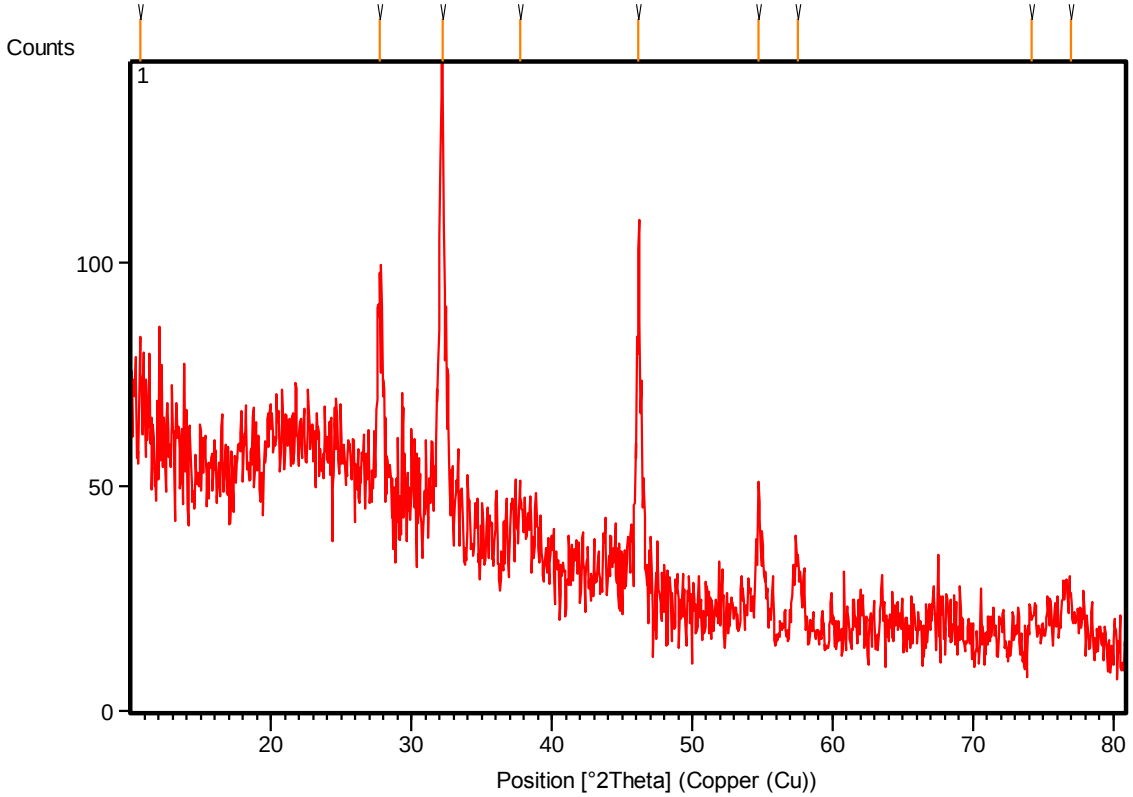

Fig 4: X-ray diffraction patterns of **mycogenic synthesised of silver nanoparticles.**

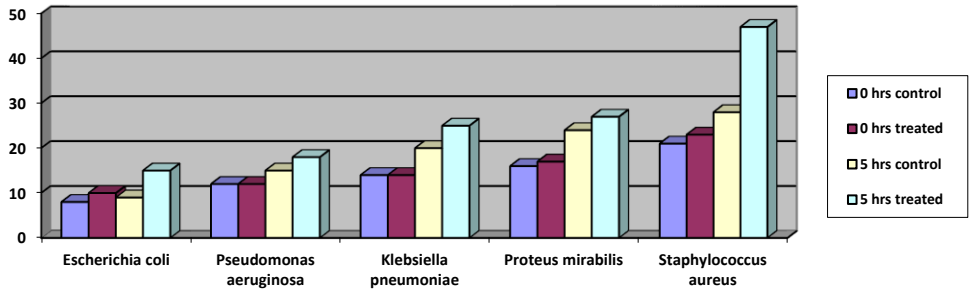

Fig 5: Effect of biosynthesized silver nanoparticles on protein leakage activity in isolated bacteria

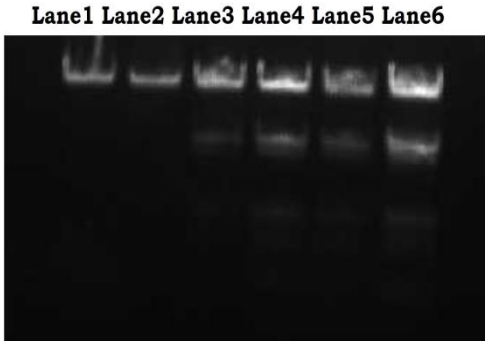

Fig 6: Effect of biosynthesized silver nanoparticles on DNA damage activity in isolated bacteria

Lane1- control, lane 2- *Staphylococcus aureus,* lane 3- *Escherichia coli,* lane 4- *Klebsiella pneumoniae,* lane 5- *Proteus mirabilis,* and lane 6- *Pseudomonas aeruginosa*

Biofilm forming bacteria	**Silver nanoparticle Zone of inhibition (mm)**
Escherichia coli	21
Pseudomonas aeruginosa	16
Klebsiella pneumonia	15
Proteus mirabilis	15
Staphylococcus aureus	13

Table1: Effect of biosynthesized silver nanoparticles on anti-microbial activity of isolated bacteria.

amount of protein leakage from cells in the control group. Leakage from cells treated with Ag-NPs was significantly higher than that from cells in the control group. Furthermore, the initial protein leakage from the membranes of *E. coli* cells treated with Ag-NPs was almost the same as that from cells in the control group. At 5 h after incubation, protein leakage from *E. coli* cells treated with Ag-NPs was significantly increased compared to that from cells in the control group, indicating that Ag-NPs can increase membrane permeability. Particularly, more than amounts of proteins leaked over the *E. coli* membranes compared to other bacterial cell membranes, suggesting that the antibacterial sensitivity of the Gram-positive *S. aureus* was lower than that of the Gram-negative bacteria (fig 5). This difference was maybe assign to the depth of the peptidoglycan layer of *S. aureus* an essential function of the peptidoglycan layer is to shield against antibacterial agents such as toxins, antibiotics, chemicals, and derivative enzymes.

DNA damage activity

The electrophoresis gel showed intact band with the control DNA without silver nanoparticles treated for lane1. The DNA extracted from *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa were* treated with silver nanoparticles were loaded in lane2, lane3 lane4 lane 5 and lane 6 respectively (fig 6). The DNA fragmentation were observed in nanoparticle treated wells.

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