BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2022. Vol. **19**(4), p. 1065-1078

Antibacterial and Anti-Quorum Sensing Studies of Extracellularly Synthesized Silver Nanoparticles from *Azadirachta indica* (Neem) Leaf Extract

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http://dx.doi.org/10.13005/bbra/3056

(Received: 01 May 2022; accepted: 04 October 2022)

Azadirachta indica (Neem) is an important medicinal plant with proven strong antiseptic, antiviral, antifungal and antibacterial properties. The study here presents the antibacterial and QS (Quorum Sensing) inhibitory potential of biogenic Silver nanopaticles (AgNPs) from Azadirachta indica leaf extracts. The nanoparticles were synthesized using an aqueous extract of Azadirachta indica leaves and silver nitrate solution. The size, crystal structure, elemental composition and other physical properties of nanoparticles were determined using different microscopic and spectroscopic techniques. The average diameter of the nanoparticles was found to be between 20-43 nm with crystalline morphology. These extracellularly synthesized AgNPs strongly inhibited Gram negative pathogenic species and exhibited demonstrable anti- Quorum Sensing (QS) activity as evident from pigment inhibition and 75- 80% decrease in biofilm mass on AgNPs treatment in a dose dependent manner.

Keywords: Azadirachta indica; AgNPs; Anti –Quorum Sensing; biofilm; biomedical application; nanotechnology; nanoparticle concentration; Anti –Quorum Sensing.

A serious public issue is emergence of rapidly growing bacterial resistance and refractory biofilm -induced infections[1]. These infections usually do not respond to any existing drugs. The unwarranted and indiscriminate use of antibiotics for prophylactic or remedial purposes has contributed to this global concern.

Serratia marcescens is a Gram negative opportunistic pathogen responsible for many hospital associated infections . It is resistant to multiple antibiotics including cephalosporins and impenemes. Biofilms are consortiums of single or multiple species of bacteria. An extracellular polymeric substance, generally composed of eDNA, proteins and polysaccharides makes the matrix in which these populations are embedded [2, 3]. The impervious nature of matrix structure and host defence systems are the major hurdles in treating the infections[4]. Bacterial biofilms are responsible for majority of infectious diseases in humans including bacterial otitis media, infections of wounds, lungs, urinary tract and most nosocomial infections.

Thus, novel biofilm inhibitors must be searched for. The potential of nanoparticles to control the formation of biofilms, as a function of their biocidal / biostatic, anti-adhesive capabilities can be exploited to treat these infections [5]. Their small size, large specific surface area to volume ratio, with high percentage of atoms/molecules on the surface is well suited for combating microbial infection.

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They can be used in biosensing, bioimaging, bimolecular identification for diagnostic purposes and also for delivery of drugs. Silver nanoparticles also find great usage in several other industries such as cosmetics, food and consumer goods.

Due to increasing demand of the industry and economic feasibility of the products there is need to utilize a cost effective, environment friendly, easy to use and scalable ' Green' pathway for synthesis of metallic nanoparticles.

Varied microbial resources such as yeast, fungi, bacteria, algae have been used for synthesis of nanoparticles consisting of Cu,Agand Au [6]. Plants being non pathogenic in nature, are widely used as biological resource for synthesis of NPs[7] and compared to NPs from other organisms, plant derived nanomaterial are relatively more stable.

AgNPs have been reported /studied for antibacterial activity against different Gram positive and Gram negative genera [8,9,10].There is a need to assess the effect of NPs on biofilm as a tool to combat infections ,with few reports available.

Azadirachta indica (Neem) is an important medicinal plant in the Indian subcontinent with strong antiviral, antifungal and antiseptic properties.

The study aims to synthesize AgNPs from *Azaadirachta indica* (Neem) leaf extract as biological source .The synthesized NPs will be characterized using UV-VIS spectroscopy ,FESEM , XRD , FTIR and evaluated for their antibacterial efficacy.

Anti-Quorum Sensing (QS) potential of the biosynthesized AgNPs will be assessed by pigment inhibition and quantification of biofim/ EPS mass .

MATERIALS AND METHODS

Bacterial Strains and Media

Bacillus subtilis MTCC 441(*B.subtilis*), *Micrococcus luteus* MTCC 1538 (*M.luteus*), *Pseudomonas aeruginosa* MTCC 1688 (*P.aeruginosa*), *Escherichia coli* MTCC 1687(*E. coli)* were obtained from IMTECH, Chandigarh.

S. marcescens was obtained from Vishakha Clinical Microbiology Labs, Nagpur.

Bacterial strains were grown on appropriate agar slants. *P. aeruginosa*, *S. aureus* were first grown on blood agar at 37 °C and stored at 4 C. For each experiment, one colony was inoculated in Try tone Soy Broth, BHI (Brain Heart Infusion) broth respectively and cultured for 16 h.

Neem Leaves Extract (NLE) Preparation

The leaves of *Azadirachta indica* (Neem) were obtained from Botanical Garden in Nagpur, Maharashtra. The plant material was identified by comparing it to reference material (authentication number 11212) at RTMNU Nagpur's Department of Botany. After thoroughly washing and drying the leaves, 10.0 g were ground into a fine paste and boiled in 100.0 ml water for 30 minutes.

The extract was filtered and stored in amber colored bottle. 1.0 ml of the extract was made to 100 ml with 1.0 mM silver nitrate solution and incubated at 37 °C for 24 h with continuous agitation and by regularly monitoring at short intervals. Complete reduction of Ag^{+} ions was confirmed by the change in colour from light or faint to yellowish colloidal brown. The colloidal solution was kept aside for 24 hour for complete bio-reduction and saturation denoted by UV-Vis spectrophotometric scanning. The suspension was centrifuged at 10,000 rpm for 10 minutes; the pellet was retrieved and used for further studies.

The effects of different concentration of $AgNO_3$ solution, percentage of leaf broth and temperature on the synthesis rate and morphology of the synthesized nanoparticles were also investigated.

Characterization of Nanoparticles

UV-Vis Spectra Analysis

The NPs synthesis was measured using an aliquot of reaction mixture diluted with distilled water on an Elico BL 198 Bio Spectro-photometer spanning the spectrum range of 200-900 nm.

Field Emission Scanning Electron Microscopy (FESEM)

FESEM was used to determine the shape and size of silver nanoparticles. The micrographs were taken with a Joel JSM -7610 F at 80 kV. To make the silver nanoparticles more conductive to current, a tiny layer of gold was applied to them to create the film.

Fourier Transform Infra-Red spectroscopy (FTIR)

The FTIR spectra of the NPs were acquired on a Perkin Elmer Spectrum one FTIR spectrometer over the 400-4,000 cm-1 range. The KBr pellet technique was used to perform FTIR

measurements. 12 scans were done in transmission mode at a resolution of 4 cm-1.

X-Ray Diffraction Analysis (XRD)

X-ray diffraction was used to determine the crystalline structure and phase purity of the synthesized Ag NPs. XPert Pro (PANanalytical, Japan) X-ray diffractometer was used to obtain the XRD pattern. The target was Cu (ká) radiation 1.54 A°, the generator operated at 45 kV and 40 mA. The scanning mode was continuous with scanning range 2Ö from 10- 99.

Antibacterial Study

The antibacterial activity of the biosynthesized Ag NPs was evaluated using the agar well diffusion assay method. In sterilized Petri dishes, 20.0 ml of molten agar appropriate for the test organism was poured and checked for sterility by leaving them at room temperature overnight. The bacterial test organisms were cultivated in appropriate conditions for 24 hours before being utilized to generate bacterial lawns (1×10^5 cfu/ml) by pour plate method. A sterilized steel borer was used to prepare 5.0 mm diameter agar wells. Wells were loaded with varying concentrations (20, 40, 60 80, 100 ug/ml) of suspended Ag NPs in water, incubated overnight at 37 °C, and inspected for the presence of inhibition as a clear area surrounding the wells. The diameter of the inhibitory zone was measured, and the mean value was given in millimetres. As controls, Ciprofloxacin (25 ìg/ml), 1.0 mM silver nitrate solution, and inoculated media without nanoparticles were used.

Anti Quorum Sensing Activity studies

Biofilm Inhibition Assay

For biofilm inhibition test, 100 µL bacterial cultures (OD_{600} = 0.132) and 100 µL of NP suspension of varying concentration(20- 100 ug /ml) were transferred into 96-well micro titre plates (polystyrene) and incubated at 37 °C for 48, 72, 96 and 120 hrs without agitation. Adherent cells were rinsed gently twice with distilled water and allowed to air dry. The adherent biofilms were stained with 0.4% (w/v) crystal violet solution for 10 min. Subsequently, the dye was discarded, wells rinsed twice with distilled water and then air dried. Ethanol was used to solubilise the dye and optical density was determined at 595 nm using a microplate reader. BHIB was used as blank and bacterial cultures without NPs were used as control.

ALI biofilm assay

The air-liquid interface (ALI) assay was used as system for microscopic analysis of biofilm formation over a time range of ~4 to 48 hr.

The *test* organism was inoculated in a 3-5-ml broth, grown to stationary phase and diluted in appropriate media. Carefully pipette an aliquot (~200 ìl) of each diluted culture and 100 ìl of NP suspension of test concentrations into separate wells in the angled 24-well plate such that the upper edge of the aliquot just reaches the center of the bottom of the well and entire bottom is not wetted. Covered the plate with lid and incubated at 37 C for 48 hours. Care was taken that the meniscus of the liquid passes through the center of the well (this ensures that the air-liquid interface is appropriately positioned for viewing). Culture was aspirated and the wells were gently washed twice, each time by adding 400 ìl sterile medium and then aspirating. Bacteria were stained by adding 0.4 % crystal violet for 10 min. Rinsed off excess dye and then allowed the plates to air-dry. The bacteria at the air-liquid interface were visualized by microscopy and also quantified by solubilising the dye and measuring OD at 595 nm

Effect of AgNPs on the Production of Extracellular Polymeric Substance (EPS)

In order to investigate the effect of AgNPs on the production of extracellular polymeric substances (EPS), overnight bacterial suspension in LB broth was prepared and diluted to adjust its turbidity according to 0.5 McFarland Standards to achieve final concentration of 5×10^5 cfu/ml. 2 ml of the inoculum was added in 100 ml of LB broth having AgNPs at different testing concentrations. Control was prepared without the addition of AgNPs. Both flasks were incubated at 37° C for 24 hours on a shaking incubator. After incubation, EPS was extracted. For that purpose, bacterial culture was centrifuged at 6000 rpm for 30 minutes at 4° C and the supernatant was collected. Two volumes of acetone were added to the supernatant, and the mixture was refrigerated overnight (at 4° C) for the precipitation of EPS. EPS product was finally collected by centrifugation of the mixture at 6000 rpm for 30 minutes (at 4° C) to collect pellet. Wet weight of the pellet was measured and dry weight was estimated after drying it at 40° C for 24 hours [11].

Pigment inhibition Activity

The anti quorum sensing activity of the

biosynthesized Ag NPs was evaluated using the agar well diffusion assay method. In brief, 1 ml of freshly grown (OD 600nm 0.7) *S.marcescens* culture was introduced to 20 ml of nutrition broth to generate bacterial lawns (1×10^5 cfu/ml) by pour plate method . Cultures without NP suspension served as control . A sterilized steel borer was used to prepare 5.0 mm diameter agar wells. Wells were loaded with varying concentrations of suspended Ag NPs in water, incubated overnight at 37 °C, and inspected for the presence of pigment inhibition as a turbid halo area surrounding the wells. . A positive QSI result was indicated by inhibition of pigmentation around the test bacteria. Negative results were indicated by the presence of pigmentation.

Statistical Analysis

Experiments were performed in triplicates and results represented as mean ± SE. The significance of the results of each experiment was checked by Student's t-test and ANOVAs using Microsoft, Excel. $P < 0.05$ was considered to suggest statistical significance.

RESULTS AND DISCUSSION

The distinct plant parts like roots, latex, stem, seeds and leaves are being used for NPs synthesis. Gardea-Torresdey *et al.,(* 2003) illustrated the first approach of using plants for the synthesis of metallic NPs using Alfalfa sprouts[12].

Synthesis of nanoparticles from *A.indica* (Neem) Leaf Extract elicited great interest due to wide application and well documented medicinal properties of Neem .

Different type of metal oxide NPs; Mn_3O_4, Co_3O_4, MoO_3, CuO have been synthesized from the precursors using *A. indica* as plant resource material by Green route [13,14]. FTIR spectrum revealed the presence of metabolites like alkaloids, saladucin, triterpenoids, valassin, meliacin, nimbidin, flavonoids, and geducin on NPs surfaces making them highly stable without changing their crystallinity

In recent years, there is a renewed interest in silver as a therapeutic option, to investigate the process/techniques which might enhance its antimicrobial properties, thus broadening the possibilities for applications. Silver is known to be biologically active when it is dispersed into its monoatomic ionic state (Ag^+), when it is soluble in aqueous environments; the principal form of metal released from NPs during their action.

Many researchers have reported the use of materials such as fungi, enzymes, bacteria, plant leaf extract,root, stem, bark, leaf, fruit, bud ,shell extract and latex for the synthesis of silver nanoparticles[15-18].

Though, the green synthesis of silver nanoparticle is cost effective, environment friendly; few studies are reported on the use the leaf extract of *A. indica* (Neem) a member of the Meliaceae family for the synthesis of silver nanoparticles and their antimicrobial effects[19].

There was demonstrable occurrence of extracellular synthesis of AgNPs when*A.indica* (Neem) leaf extract is allowed to react with silver nitrate solution (1.0mM).The progression in synthesis is traced by gradual change in colour to yellow brown due to reduction of silver ions present in the solution (Fig.1).

Fig. 1. Colour change observed during synthesis of Ag nanoparticles

The change in colour can be attributed to collective oscillation of free charges present on the surface of a metal by the electric field generated by the light incident on the solution defined as a plasma wave or Plasmon (SPR, Surface Plasmon Resonance).The characteristic wavelength of SPR varies with metal, shape, size and degree of aggregation of NPs. The medium used in the preparation (carrier fluid) and its constituents also affect the vibration peaks. For metals, SPR

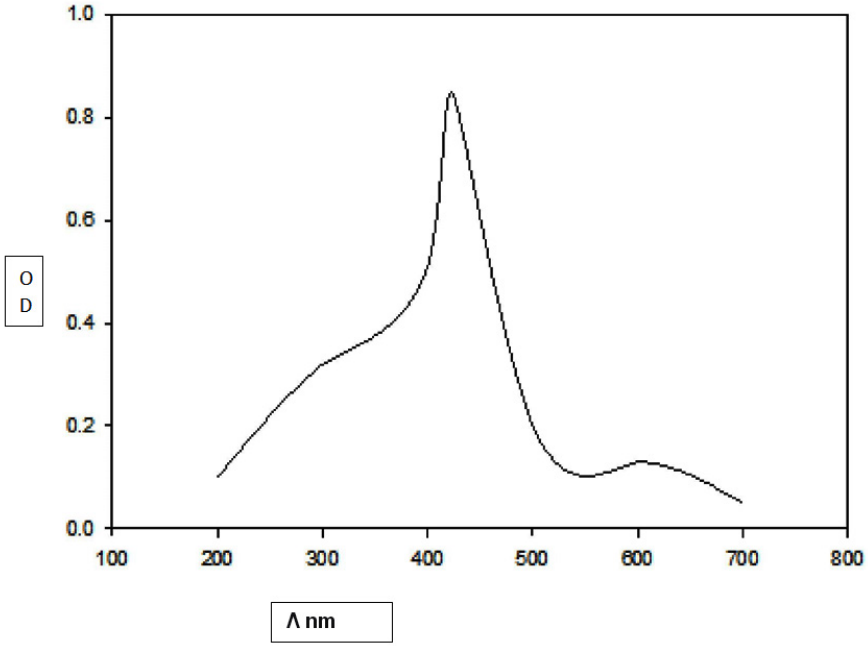

Fig. 2. Absorbance spectra of Ag NPs prepared using Neem Leaf Extract solution

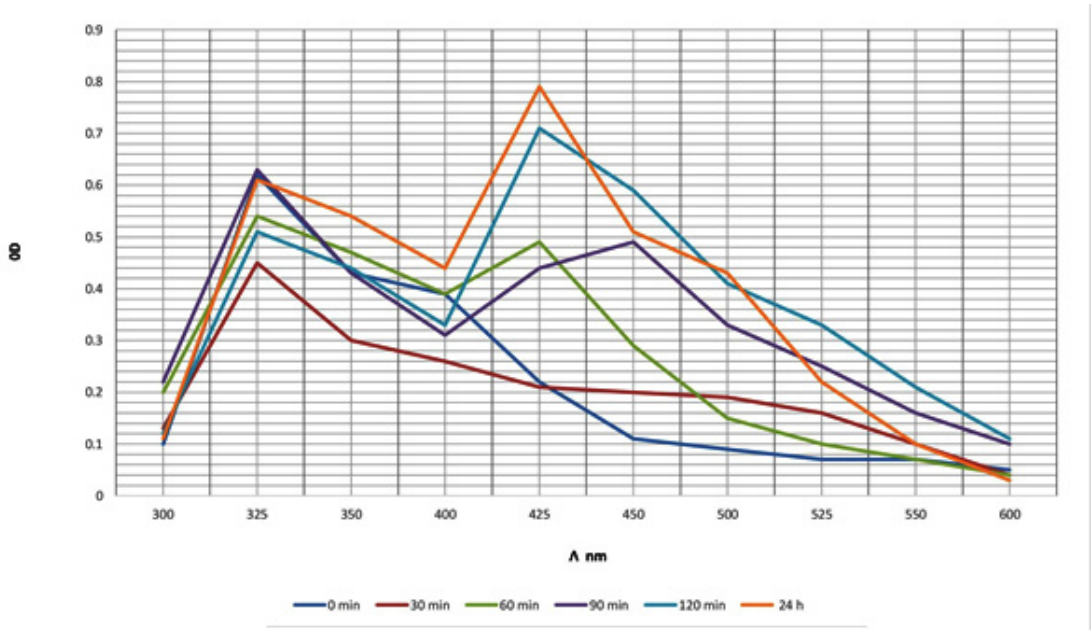

Fig. 3. UV-VIS spectra recorded as a function of reaction at different wavelength versus absorbance during synthesis of silver nanoparticles at different time intervals

bands usually lie in the visible range of spectrum with exception of Cu, Ag and Au. The presence of electrons in the S- atomic orbitals shifts the plasma bands closer to the visible range .

The SPR band for the biosynthesized AgNPs is at 425 nm, where they absorb the blue colour of light and appear yellowish (colour complementary to blue) (Fig. 2).

The formation of NPs was followed and there is evident an increase in peak height with time (Fig.3). The single SPR band of the biogenic NPs suggest 3D spherical configuration [20]. This is corroborated by FESEM images.

AgNPs in spherical architecture with an average size of 40.15 nm are seen in the FESEM pictographs (Fig.4). The magnified images show sphere like morphologies aggregated as clusters where the spheres are in close proximity but still not in direct contact.

The biologically active molecules in different crude extracts from Neem are rich in hydrocarbons, terpenoids, phenolics and alkaloids [21].

Absorption of mid IR region (4000 – 400 cm^{-1}) of Electromagnetic Radiation of the spectrum is quantified in the FTIR spectroscopy. When a sample absorbs IR radiation, the dipole moment is modified and the molecule becomes IR active. Molecular interactions and structure can be derived from bands of IR spectrum suggesting strength and nature of bonds and specific functional groups present in the sample .

The recorded IR spectra helped to identify the active biomolecules present in the leaf extract which reduces the ionic silver to metallic state and stabilize the resultant reduced state.

IR spectrum (Fig. 5) of NLE in KBr pellet show presence of phenolic and alcoholic compounds with -OH functional group; the stretching of this O-H bond specifies the absorption peaks in 3400-3200 cm-1 region. The absorption peaks in 1640-1550cm-1 suggest N-H (bend) of are due to primary (1) and secondary (2) amines. The bands in 1450-1375 cm-1 are due to CH

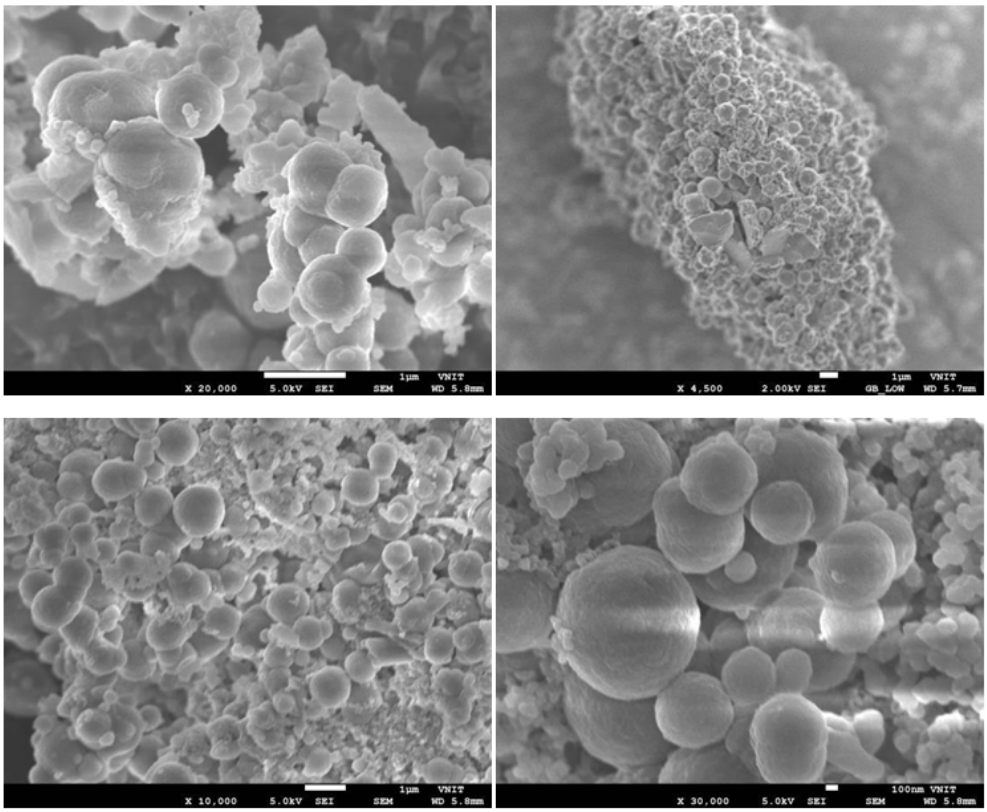

Fig. 4. FESEM images of biogenic AgNPs at different magnifiifcation (4500-30,000X)

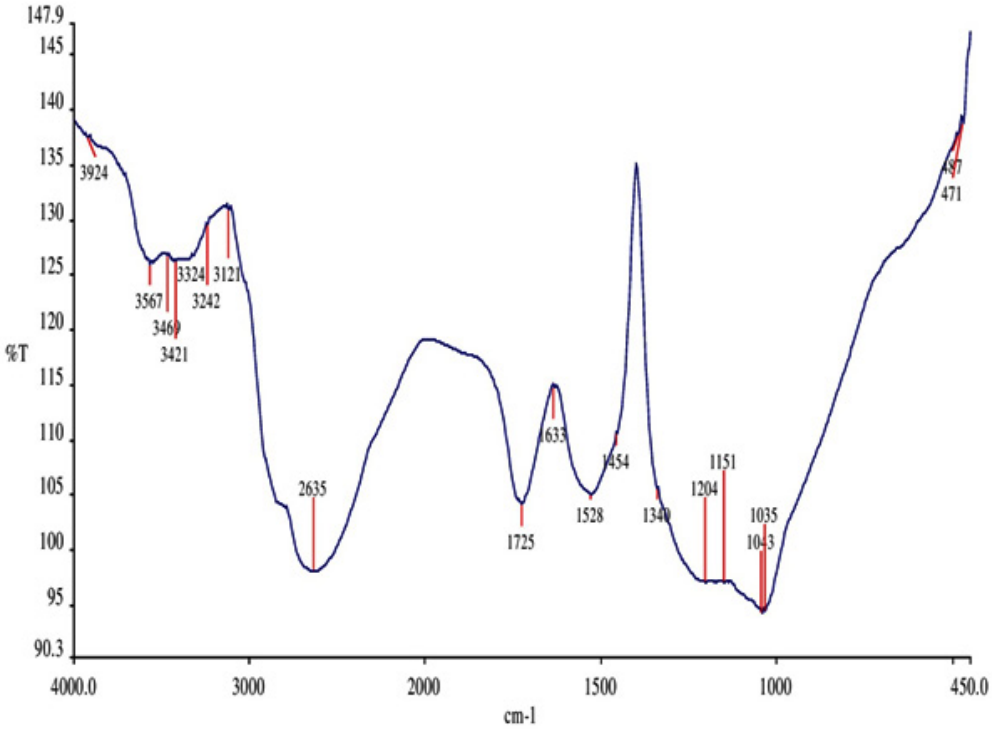

Fig. 5. FTIR Analysis for Ag NPs synthesized using Neem Leaf Extract

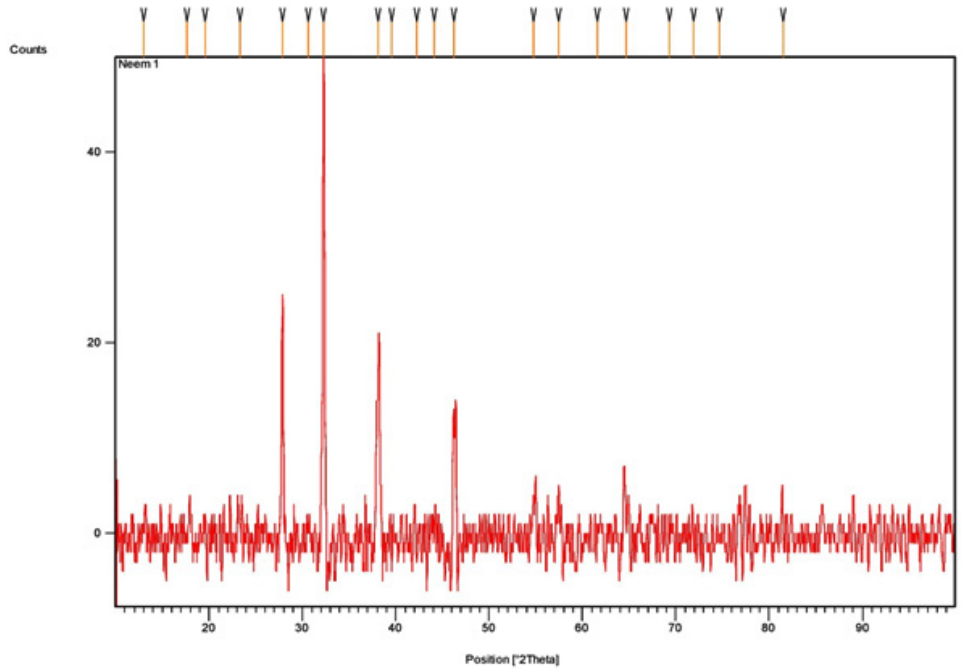

Fig. 6. XRD pattern of Ag NPs synthesized using Neen Leaf Extract after the complete reduction of Ag^{+} to Ag^{0} under the optimized conditions

(-CH3) bend of alkanes. The peaks 1350- 1000cm-1correspond to -C-N- stretching vibration of the amine or -C-O- stretching of alcohols, ethers, carboxylic acids, esters and anhydrides.

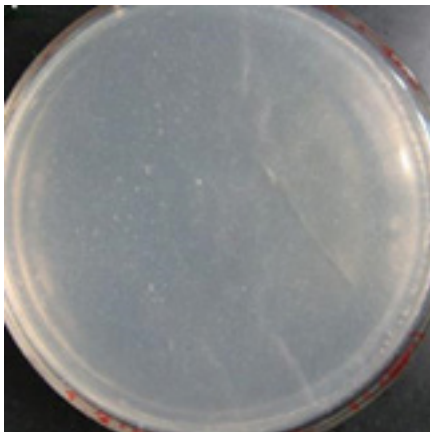

Fig. 7. MBC plate for biosynthesized AgNPs (10 ug/ml)

As was also seen in FESEM images, the NPs were in a cluster but did not impinge upon one another. This evident minimalization of aggregation is partly contributed by strong binding of the metal by carbonyl functional groups of amino acids and proteins present in the plant sample. This 'caps' the metal nanoparticle and forms an envelope around it; thus preventing aggregation. Therefore, biomolecules present in the leaf extract simultaneously acts as reducing and surface –active agent.

As observed in the XRD pattern (Fig. 6), the four characteristic diffraction peaks at 2è values of 27.84°, 32.23°, 38.12°, and 46.27 ° can be indexed to the (111), (200), (211), and (220) reflection planes of simple cubic (sc) structure of silver (JCPDS card no 04.0784).

It is clearly evident from the XRD pattern that the synthesized AgNPs are of explicit , finite dimensions as suggested by the broadening of well

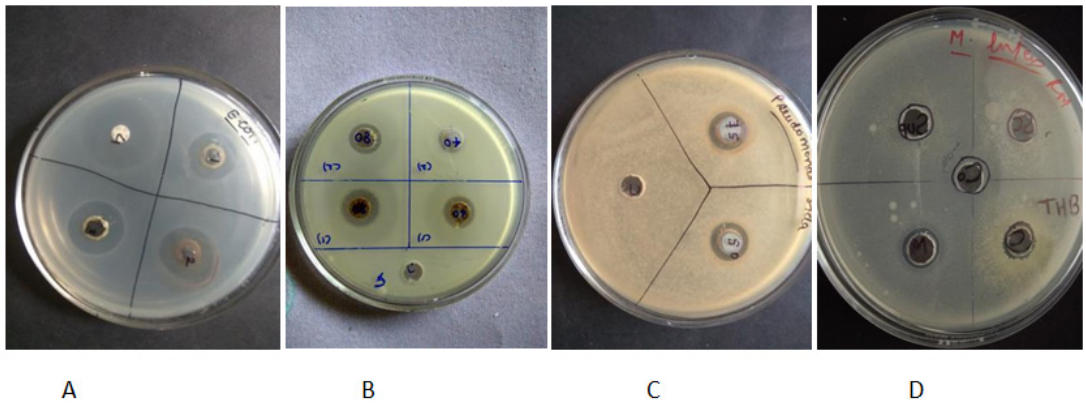

Fig. 8. Antibacterial activity of AgNPs against A. *E. coli* B. *Bacillus substilis* C. *Pseudomonas spp*. D. *Micrococcus luteus*

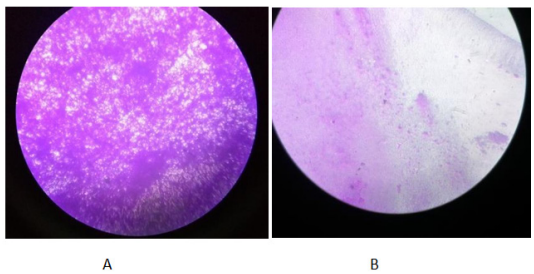

Fig. 9. Effect of AgNPs on cell adherence during biofilm development of S. marcescens
A : untreated control; B: AgNPs treated *S. marcescens*

documented peaks. In the graph, some additional unidentified peaks are also seen.

To evaluate the susceptibility of antibiotic resistant bacteria *S. marcescens* to AgNPs , MIC and MBC were determined by broth dilution method. MIC was found to be 5 ug/ml. As evident from the complete inhibition of colony formation in the plate (Fig .7), MBC was 10 ug/ml ,being two fold higher than MIC.

Disk diffusion method was employed to test the anti bacterial activity of the AgNPs. The study suggests that the AgNPs from Neem

leaf extract can arrest the growth of pathogenic test species under laboratory conditions in a dose dependent manner(Fig.8)

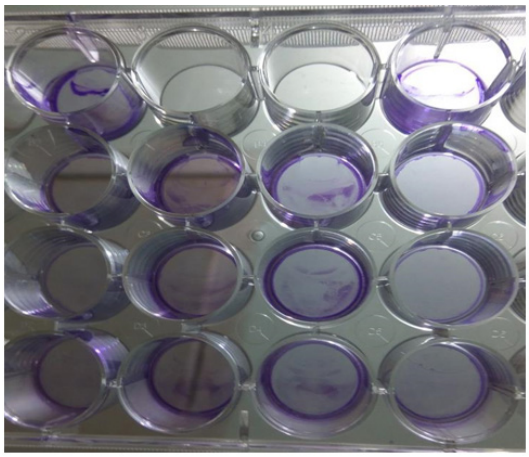

Fig. 10. *S. marcescens* biofilm mass as seen from ALI plates ; A,D : treated with AgNPs B,C : untreated control

The antibacterial activity of AgNPs has been reported by many researchers. However, the MIC values exhibit a large range of variation. Therefore, the comparison of the results is difficult as there is no standard method for determination of antibacterial activity of AgNPs and different methods have been applied by the researchers [22].

They are evidently more potent against Gram negative organisms compared to Gram positive species as inferred from zone of inhibitions.

In an earlier study, the antimicrobial property of AgNPs was investigated by growing *Bacillus* and *E. coli* colonies on nutrient agar plates supplemented with AgNPs [23]. A smaller zone of inhibition (12mm) was seen in *E.coli* as against a larger zone of inhibition (16mm) with *Bacillus* spp. which is in contrast with our study where Gram negative test organisms were found to be more susceptible to inhibition by AgNPs from *A. indica*(Neem) Leaf extract. Roy *et.al.*(2017) also communicated the reduced antimicrobial activity of silver nanoparticle for Gram positive bacteria compared to Gram-negative bacteria[19]. Similar

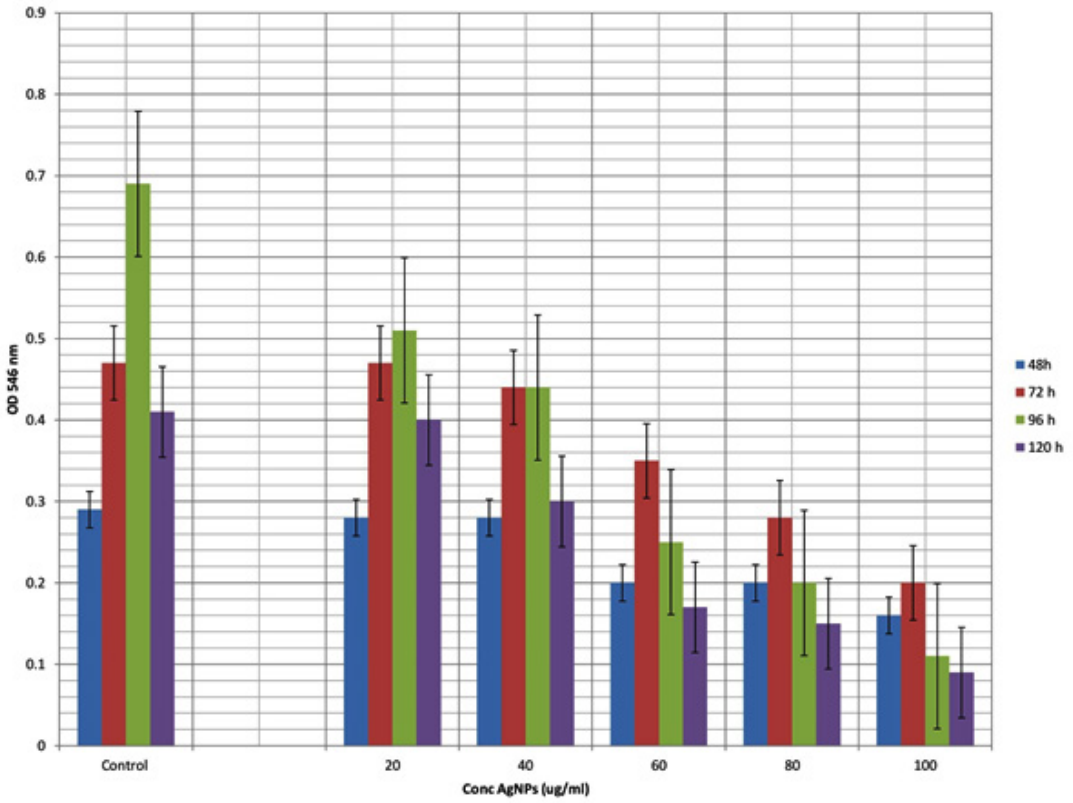

Fig. 11. Percent inhibition of biofilm formation by various concentrations of AgNPs against *S.marcescens*

results have been reported earlier for Neem as well as other plant extracts [24, 25] .

This could be due to the presence of a single layered, smooth thick cell wall with a bulky multilayered peptidoglycan layer in Gram positive bacteria. In contrast , a double layered wavy thin cell wall and a thin peptidoglycan layer is present in Gram negative organisms. Consequently, the thin peptidoglycan layer and an additional envelop of lipopolysaccharides in Gram negative species render them more susceptible to metallic NPs attack. The lipopolysaccharides exhibit strong propensity to the positively charged metal ions released by NPs with concomitant uptake of ions leading to intracellular damage. The adsorbed NPs also cause membrane stretching resulting in mechanical deformation and ultimately cell rupture[26]. Damage to cell membrane from free radicals on attachment of NPs[27], interaction of Ag ions on dissolution of NPs with proteins and other

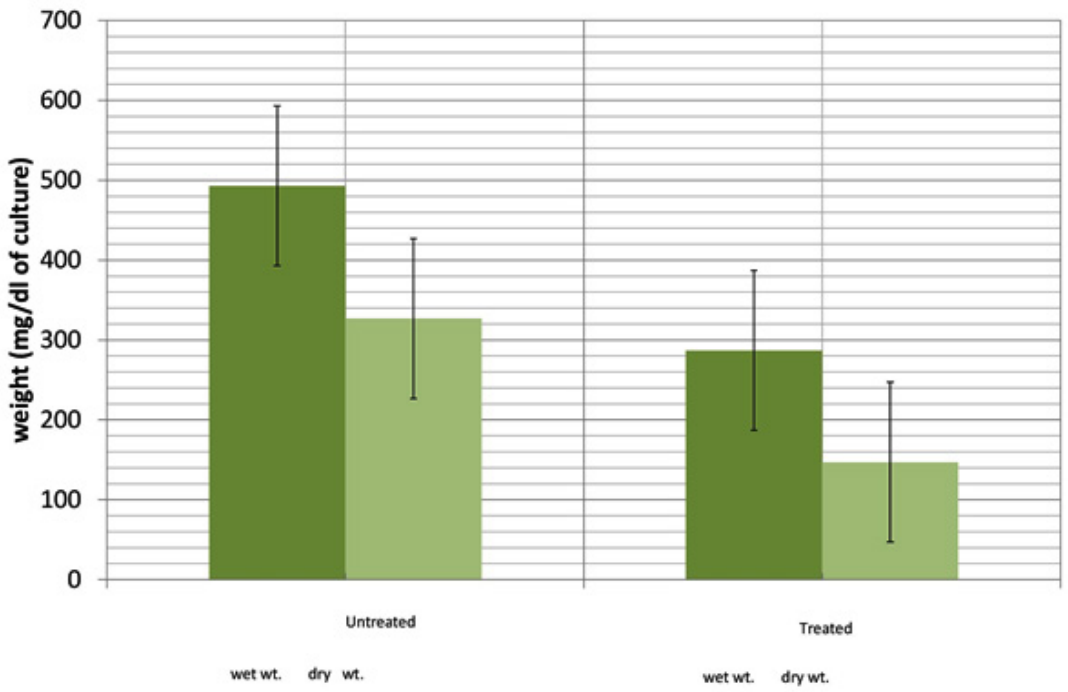

Fig. 12. Quantification of EPS extracted from bacterial cells in the presence and absence of subinhibitory concentrations of AgNPs. The results are expressed in terms of wet and dry weights of EPS extracted

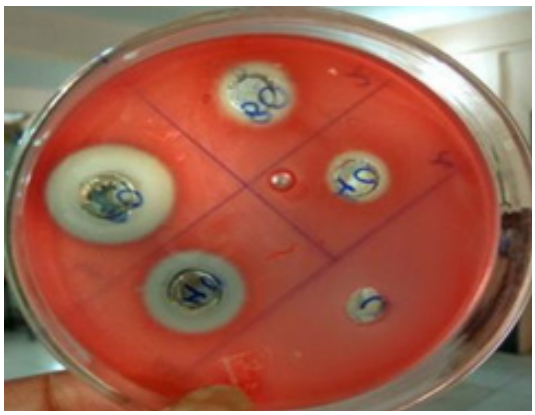

Fig. 13. Pigment inhibition in *S. marcescens* on AgNPs treatment

molecules with O, N.P, S atoms in their structure leads to inactivation of many enzymes[28].

Additionally, NPs alter the trans/cis ratio of unsaturated fatty acids in membrane resulting in its modified fluidity and integrity.

Plant derived NPS demonstrated enhanced antibacterial efficiency due to synergistic or supplementary antibacterial property of plant metabolites in addition to their capping and stabilizing capability during NP biominiralization.

Quorum Sensing (QS) is a cell density dependent communication arrangement involved in virulence, resistance to antibiotics and formation of biofilm by bacterial species

Targeting QS controlled virulence is an attractive anti- infective drug target. The inhibition of QS might attenuate and eradicate the pathogen in synergy with host immune system.

QSI (Quorum Sensing Inhibition) have been identified in all kingdoms but predominantly in plants. Ethanol extracts of 5 plants among 24 Indian medicinal plants, namely *Hemidesmus indicus* (root), *Holarrhena antidysenterica* (bark), *Mangifera indica* (seed), *Punica granatum* (pericarp), and *Psoralea corylifolia* (seed), inhibited violacein production by *C. violaceum* and swarming by *P. aeruginosa* PAO1.[29] Adonizio *et al.,* (2007) reported that among 50 medicinal plants from southern Florida, 6 plants inhibited QS: *Conocarpus erectus, Chamaecyce hypericifolia, Callistemon viminalis, Bucida burceras, Tetrazygia bicolor, and Quercus virginiana*. Furthermore, the extracts of all plants caused inhibition of QS genes and QS controlled factors, with marginal effects on bacterial growth, suggesting that the QQ (Quorum Quenching) mechanisms are unrelated to static or cidal effects [30].

Liimited studies have been conducted on QS from leaves and only a few QSI molecules have been identified [31,32].

QS signal molecules N-acylhomoserine-lactones(AHL ,C4 and C6) regulate different genes responsible for production of various virulence factors, secondary metabolite, prodiogosin and biofilm formation in *Serratia* [33-35]. Eugenol, a plant metabolite could prevent the production of QS-controlled virulence factors like pigment prodigiosin, protease and hemolysin.The QS-mediated biofilm formation stages such as swarming motility, formation of microcolony and extracellular polysaccharide production were inhibited and expression of genes responsible for QS system, adhesion, motility, and biofilm formation were down-regulated[36].

Metal NPs have been shown to possess promising anti QS activity both *in-vivo* and *in- vitro* [37-39]. Though QQ studies on Green NPs are few , reports on synthesis and characterization of Ag NPs from different plant species are available [40-42].

The study rests on assumption that any interference with QS will alter pigment production and encroach the process of biofilm formation.

Inhibition of biofilm formation by AgNPs was tested against *S.marcescens*. There was an inhibition in biofilm in proportion to the concentration of NPs used. From the pictographs of biofilms(Fig.9), treatment with NPs leads to decrease in cohesion of cell on the surface with reference to control.

The biofilm formed was quantified (Fig.10) and percent inhibition of biofilms subjected to AgNPs treatment was also evaluated. It is evident that following AgNPs treatment, there is significant reduction in bioflim biomass($p< 0.05$). At high concentrations of 80, 100 ug/ml AgNPs, a reduction in mass of biofilm was registered(Fig.11).

Formation of biofilm is a gradual process involving adhesion of planktonic cells to the surface which becomes trapped in exopolymeric substances and initiate biofilm formation. Subsequently these entrapped cells secrete more of Extracellular Polymeric substances (EPS) and become firmly bound to the substratum which results in cell clumping and formation of cementing matrix material. The constituents of EPS; polysaccharides, eDNA, lipids and proteins help in attachment of biofilm to the substratum, serve nutrients to the cell population, shield from different bacteriostatic and bactericidal agents and also facilitates cell to cell communication – Quorum Sensing (QS) to efficiently manage density of their population[43].

This study was designed to evaluate the AgNPs as novel bioactives which could destabilize the biofilm architecture, either through prevention or complete suppression of EPS formation.

When challenged with sub inhibitory concentration of AgNPs, there was evident a reduction in biomass of EPS (Fig.12).

The loss in dry mass was less (26% by weight) ,while the wet weight was lowered by half its value (47%). This significant decrease ($p<0.05$) in the EPS biomass implicates AgNPs in the interference with biofilm microsystem through interaction with EPS.

For *S.marcenscens* to initiate and sustain the infection, it will require its entire complement of external virulence factors including production of pigment, prodiogisin. There was observed complete inhibition of pigment synthesis as indicated by a turbid ,halo zone on plates in the treated cultures as compared to control(Fig.13).

This inhibition of pigment production suggests presence of Quorum sensing Inhibition (QSI). Prodigiosin, red tripyrrole pigment is often

accountable for hydrophobic nature of cell surface in *S.*marcenscens[44]. This hydrophobic cell surface enables the *Serratia* cells to attach to the surface and other cells. The loss of this hydrophobic colourant alters the nature of cell surface and cause loosening of consequently biofilm structure complex. This loss in biolim mass was attributed to lowered microcolonization and decrease in EPS production.

It is clearly demonstrable that AgNPs interfere with QS mediated production of secondary metabolite, Prodigiosin resulting in loss of biofilm architecture.

ACKOWLEDGEMENTS

The authors wish to thank management of Hislop College, Nagpur for providing the infrastructure facilities to carry out the lab work. We are also grateful to Department of Material sciences ,VNIT Nagpur for facilitating characterisation of NPs studies.

Conflict of Interest

The authors declare that they have no competing interests.

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