Supplementary Information

Bacteria against bacteria: Green silver nanoparticle fabrication, antioxidant, anti-biofilm and antibacterial activities

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In vitro Antioxidant potency of biosynthesized Ag-NPs

Total content of phenolics

The overall phenolics of Ag-NPs were assessed using the Folin-Ciocalteu (F-C) technique[1] with only minor modifications. F-C reagent (0.2 N, 2.5 mL) was thoroughly mixed with water dispersed nanoparticles in a range of concentrations (0.5 mL). These reagents were mixed properly and incubated in dark for 120 min, O.D. was thereafter measured at 760 nm spectro-photometrically of the deep blue colour that resulted from the addition of around 2 mL of Na_2CO_3 to the above blend in comparison to a blank. The overall phenolics present in the biogenic silver nanoparticles were determined using the calibration curve of gallic acid as a reference polyphenol, and quantified as gallic acid equivalents (GAE), µg mg^{-1} of Ag-NP.

Total content of flavonoids

Total flavonoids in green synthesized Ag-NP was calculated by means of an altered form of the aluminum chloride technique spectro-photometrically[2]. In order to get different final NP concentrations in the system, 125 µL of an aqueous extract of Ag-NPs was combined with $NaNO_2$ (5%, 75 µL).After that, the mix was left standing for 6 min. Then 10% $AlCl_3$ (150 µL) was added and mixed well, following further incubation for 5 min. Finally 750 µL of 1M NaOH was combined with the above mixture. The solution was finally made upto 2500 µL using dd water, and finally incubated for 15 min before noting the absorbance 510 nm. The flavonoid amount present in Ag-NP was expressed as standard flavonoid quercetin equivalents (QE) in µg mg^{-1}.

DPPH (2,2-diphenyl-2-picrylhydrazyl) radical scavenging efficacy:

The technique of Rahman et al., was marginally adjusted for investigating Ag-NP's ability to scavenge stable DPPH radical[3]. 1 mL of the green synthesized nanoparticles was diluted to different concentrations and mixed with 2 mL of DPPH (0.1 mM, methanolic solution). The change of colouration (light yellow from deep violet), was noted by measuring absorbance spectrophotometrically after it had been incubated in dark for 30 min, at 517 nm which shows that the radicals are efficiently destroyed. The IC_{50} or concentration (µg mL^{-1}) showing 50% inhibition was calculated from percentage inhibition.

Blank used was double-distilled water. Trolox, BHT, and BHA served as positive control. DPPH scavenging or inhibitory activity was calculated as:

$$\% \text{ inhibition (I\%)} = \{(A_0 - A) / A_0\} \times 100 \qquad \ldots(1)$$

A_0= absorbance of blank; A= absorbance of test material.

Hydrogen peroxide (H_2O_2) scavenging activity

To determine the Ag-NPs' ability to scavenge H_2O_2, Ruch et al.'s methodology was slightly modified[4]. About 0.1 mL of this sample which had various concentrations of Ag-NPs dispersed in water was placed into an Eppendorf tube. Phosphate buffer (400 µL, 50 mM, pH 7.4) and H_2O_2 (600 µL, 2 mM) was added, and mixed properly with subsequent incubation for 10 min. Next, absorbance

was taken at 230 nm. Blank used was water while positive controls included trolox, BHA, and BHT. The outcomes were shown using IC_{50} values and formulae of section 2.5.3 was utilized for calculation.

Ferrous ion chelating potency

The ferrous ion-ferrozine complex approach was applied for evaluating Ag-NP's metal ion chelating capacity for ferrous ions (Fe^{2+}) of the silver nanoparticles[5]. The reaction mixture comprises water (1500 µL), different concs. of Ag-NP (2000 µL), and $FeCl_2$ (2 mM, 25 µl) and incubated for 30 s. Then ferrozine solution (5 mM, 50 µL) was added and the blend was incubated for 600 s at RT. Water served as the blank, and the substances trolox, BHT, and BHA served as positive controls. The solution absorbance was noted at 562 nm. From the chelating rate calculated by following formula IC_{50} values were determined:

% of Chelating activity = $[1-(A/A_0)] \times 100$. Where A= Test absorbance and A_0 =Control absorbance

Ferric reducing antioxidant power (FRAP) assay:

With a few changes, the approach of Biglari et al., was applied[6]. FRAP reagent constitutes a mixture of acetate buffer of pH 3.6 (300 mM), aqueous $FeCl_3.6H_2O$ (20 mM), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (10 mM, dissolved in 40 mM HCl) in 1:10:1. In varied concentrations, 900 µL of the FRAP reagent and 100 µL of aqueous Ag-NP solution were combined well followed by incubation for 15 min at 37 °C and colour measurement at 593 nm using dd water as blank. We employed BHT, Trolox, and BHA as our positive controls. A standard calibration curve was created using a 200-1000 µM solution using 1000 µM L^{-1} $FeSO_4.H_2O$. The Fe(II) (M) equivalents were used to depict the FRAP data.

ABTS radical scavenging assay

Using the $ABTS^+$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) test, the TRAP of the Ag-NP was calculated[7]. $K_2S_2O_8$ (2.45 mM) and ABTS (7 mM) are thoroughly mixed and left overnight at RT in dark to generate $ABTS^{+\bullet}$ radicals. Once the absorbance of 0.700 ± 0.010 was attained by dilution with water, a thorough blending of 100 µL of various Ag-NP concentrations (diluted with water) was performed with 2000 µL of the ABTS reagent following incubation for 6 min in dark and the spectra was measured in visible range at 734 nm. As standard antioxidants BHA, Trolox and BHT were utilized. The results were displayed as IC_{50}, which were computed using the I% determined using the formula previously indicated. Triplicate measurements were taken.

Lipid peroxides radical scavenging capacity:

Empty Liposome preparation: Any lipids that remained after many acetone washes were applied were removed from crude lecithin. Phosphatidylcholine mixed in chloroform (5 mg/mL) was extracted with a rotary evaporator to remove the organic solvent, leaving a thin coating on the round-bottom flask wall. Tris-HCl buffer (pH 7.4, 0.1M) was applied to hydrate the lipid film, which was then shook erratically. Liquid nitrogen freezing and room temperature thawing were used to disseminate the liposome suspension. The complete method was performed five times[8].

Based on the formation of TBARS, the anti-oxidative effects of Ag-NP on oxidation induced by liposome of $FeCl_3/H_2O_2$/ascorbic acid and lipid peroxidation were assessed. 500 µL of liposome solution, 500 µL of tris-HCl, and 100 µl of $FeSO_4.7H_2O$ made up the reaction mixture (4 mM). 2 mL of the liposome solution were put to each tube along with. Liposome peroxidation was started with 100 µL of ascorbic acid (2mM). The test tubes labelled as the sample and standard, respectively, were then filled with 0.5 mL of $CuSO_4$ (100 mM), different AgNP water concentrations, BHA, Trolox, and BHT. After being incubated for 60 min at 37°C with 20% TCA (1000 µL) and 0.67% TBA (2000 µL), the test tubes were then heated for 20 minutes at 100°C. After being added, chloroform was mixed (2000 µL). The supernatant of mixtures were separated by centrifugation for 10 min (3000 x g). The absorbance was calculated at 532 nm. IC_{50} values were presented as final findings derived from I% determined by applying the earlier formula.

Nitric oxide radical scavenging activity:

The procedure of Makhija et al. was followed[9]. In order to produce nitric oxide radicals, sodium nitroprusside solution was mixed with phosphate buffer (10 mM, 1000 µL), and various

concentrations of Ag-NP are mixed with the reagent. 150 min were spent incubating the mixture at 25 °C. Griess' reagent (1000 µL) was combined with the above mixture. Finally, at 546 nm absorbance was noted. The Ag-NP inhibited nitric oxide radicals, and IC_{50} values were determined. Water served as the blank while Trolox, BHA and BHT served as standards. Each measurement was done three times before calculating their mean values.

Hydroxyl radical scavenging assay:

The Fenton reaction was used to evaluate Ag-NP's ability to scavenge hydroxyl radicals[10]. Briefly, different aqueous Ag-NP solutions concs. (1000 µL) were mixed with $FeSO_4$ (1.5 nM, 2000 µL), H_2O_2 (6 mM, 1000 µL) and sodium salicylate (20 mM, 300 µL). The mixture was incubated at 37 °C for 1hfollowed by cooling to RT and absorbance was taken at 510 nm. BHA, trolox, and BHT were used as standards. The approach based on I% that was previously mentioned was used to calculate IC_{50} values.

In-vitro antimicrobial efficacy evaluation of bio-fabricated AgNP

Primary anti-bacterial Activity of AgNP against bacteria

The *in-vitro* anti-bacterial activity of the bio-synthesized AgNP was evaluated upon four bacterial pathogens and spoilage species on a Mueller-Hinton agar (MHA) plate using the Kirby-Bauer disc diffusion technique. The discs were prepared by impregnating each sterile filter disc (Hi-media, Mumbai, India) with 20 µL of Ag-NP solutions having different concentrations (2.5-20 µg mL^{-1}). Each suspension had its turbidity balanced to 0.5 MacFarland standard, or 1.5 x 10^8 CFU mL^{-1} of bacteria. The inoculum for every bacterium was sub-cultured in sterilized MHB. On each MHA plate, the 20 µL of each bacterial culture used in this investigation were added along with the four Ag-NP discs at the aforementioned concentrations. The zones of inhibitory activity (ZOI) on the plates were measured (in cm) through calculating the widths of the clear regions without any growth surrounding the filter discs following 18–20 h of incubation in a BOD incubator at 37°C. In the study, as positive control filter discs impregnated with streptomycin (2.5 and 5 µg mL^{-1}) were used and dd water served as negative control, and compared to that of Ag-NPs against each bacterium. The amount of test sample that can demonstrate lethal effects on bacteria was represented by a disc with various concentrations of NPs that showed visible inhibition of bacterial growth. This provides preliminary screening for the antibacterial effects of the four nanoparticle concentrations above against the four various bacterial infections. Each test was carried out three times. Mean with Standard deviation (SD) are used to express the results in centimeters[11].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The resazurin-based broth-microdilution approach was applied for calculating MIC, and the MBC assay was conducted using the traditional spread plate inoculation technique[12]. According to the CLSI (Clinical and Laboratory Standards Institute) recommendations, each test bacterial organisms were sub-cultured in Tryptic Soy Broth (TSB), resulting in 1x 10^8 CFU/mL (https://clsi.org, accessed on 1st March 2023). The modified inocula were then further diluted in TSB by 1:100, yielding 1x10^6 CFU/mL. Ag-NP's original stock solution was made in double distilled water using various concentrations. There was only 200 µL of the bacterial broth solution put into each well in column 1. For bactericidal investigation, 190 µL of tryptic soy broth (TSB) were present in each well in columns 2 through 11 at the same time. Using a multichannel micropipette, 10 µL was added to each column from 2 to 11 to create different Ag-NP concentrations ranging from 1 to 100 µg ml^{-1} in the plate columns. As the negative control (NC) column 12 included 10 µL of water and 190 µL of standardised inoculum suspensions. In a BOD incubator the plate was incubated overnight at 37 °C. Then, each well of columns 1 through 10 and 12 received 20 µL of sterile solution of resazurin dye (0.015% w/v), and the plates were once more incubated for 1-2 h to look for a colour shift. Columns were scored as MIC if there was little to no colour change following incubation (the colour of the blue resazurin remained constant). The well contents with no colour change (i.e. at and above the MIC value) were subsequently seeded to estimate MBC on sterile tryptic soy agar (TSA) plates. Different sterile tryptic soy agar (TSA) plates was filled with the contents of the wells, which remained blue, and this mixture was allowed to incubate for further 24 h at 37 °C to detect any presence of bacteria. The test bacteria which did not develop distinct colonies at the lowest possible

concentration of Ag-NPs on seeded agar surfaces that were thought to represent the MBC. The outcomes are expressed in μg mL^{-1}.

Ag-NP's tolerance level against various bacterial strains was computed using the corresponding bacteria's MBC/MIC value in order to perceive the nature of their antibacterial impact. The degree of tolerance reflects whether the nanomaterials have a bacteriostatic or bactericidal effect on the pathogen of choice. Ag-NP was regarded as bactericidal when the ratio was less than or equals to 4, and the values greater than 4 was noted as bacteriostatic[13].

Kinetics assay for antibacterial activity

The dynamic variations in Ag-NPs' antibacterial properties were evaluated using a sequential dilution method in microplates utilising Nutrient broth media (HiMedia, Mumbai, India) and a culture suspension of $1x10^7$ CFU/mL. The bacteria were suspended again in 10 mM potassium phosphate buffer. Ag-NPs were used to treat the bacterial suspension at 0.5×MIC, 1×MIC, and 2×MIC. The bacterial cells were incubated at 37°C in a BOD incubator with concomitant shaking at 180 rpm. After regular time intervals of incubation (0, 1, 2, 4, 6, 12 and, 24 h) triple O.D. measurements at 600 nm were used to count the number of bacteria present[14].

Determination of mechanism of action of nanoparticles on microbes

It was reported that proteins, reducing sugars, total polysaccharides, and DNA leaks or gets released through the microbial cell membrane. *E. coli, Bacillus cereus, Staphylococcus aureus*, and *Vibrio cholerae* overnight grown cultures were used for this. 2 mL of sample from each culture was taken and labeled as 0 h sample. Each culture was incubated with 100 μL of Ag-NPs solution (10 μg mL^1) in a BOD incubator at 37°C with shaking condition of 200 rpm. Now, from the each culture sample was taken after 2 h, 4 h, and 6 h. Each sample was spinned for 5 min (10000 rpm) and the bacterial pellet was discarded. The supernatant was promptly frozen at -20°C, and the concentrations of DNA, proteins, reducing sugar, and total polysaccharides were ascertained as soon as possible using the Di-phenylamine (DPA)[15], Folin-Lowry[16], Di-nitro salicylic acid (3,5-DNSA)[17] and anthrone methods [18].

For quantitative estimation of the biomolecules, Briefly, 10 mL of cell cultures in exponential phase (OD_{600} = 0.3–0.5) was collected by centrifugation and immediately frozen in dry ice. Cell pellets were first washed once with 1 mL 0.45 M $HClO_4$ and then washed again with 1 mM $HClO_4$. The cell pellet was then hydrolyzed by 0.5 mL 1.6 M $HClO_4$ at 70°C for 30 min. After cooling to room temperature, 1 mL diphenylamine reagent (0.5 g diphenylamine in 50 mL glacial acetate, 0.5 mL 98% H_2SO_4, and 0.125 mL of 32 mg/mL acetaldehyde water solution) was added for colorimetric reaction. After a 16 h to 18 h overnight reaction, the reaction mixture was centrifuged, and the A_{595} of the supernatant was measured. At the same time, a series of standard calf thymus DNA (10 mg/mL in stock solution) reaction was set up in parallel, in order to obtain the DNA standard curve. Bacterial total DNA content was determined from the calf thymus standard curve[15].

Bovine serum albumin standard solution was prepared using BSA powder dissolved in distilled water, and it is diluted to a concentration of 100 mg/100 mL, which is then stored at 4 °C. A series of dilutions (0, 2, 4, 6, 8, and 10 mg/test tube) were made in duplicates and kept for standard curve generation. 1 mL of hydrolysed samples were taken in each test tube. 1 mL of distilled water was taken before taking the protein sample to prevent the degeneration of the sample protein. To these solutions, 4 mL of alkaline copper solution was added. Then the mixtures were mixed thoroughly. The solutions were incubated at room temperature for 10 min. Then 0.5 mL of Folin-Ciocalteau phenol was added to these solutions and mixed properly by using vortex Shaker. Then these solutions were incubated for 30 minutes at room temperature and in dark condition. Duplicates test tube were prepared for each sample. The reading of the absorbance of unknown samples was taken at 750 nm[16].

The reducing sugar content (RSC) was determined using the 3,5-dinitrosalicylic acid (DNSA) method. The measurement was performed according to the procedure of Krivorotova and Sereikaite with slight modification. DNSA reagent was prepared by dissolving 1 g of DNSA and 30 g of sodium-potassium tartaric acid in 80 mL of 0.5 N NaOH at 45 °C. After dissolution, the solution was cooled

down to room temperature and diluted to 100 mL with the help of distilled water. For the measurement, 2 mL of DNSA reagent was pipetted into a test tube containing 1 mL of hydrolysed microbial solution and kept at 95 °C for 5 min. After cooling, 7 mL of distilled water was added to the solution and the absorbance of the resulting solution was measured at 540 nm using a UV-visible spectrophotometer (Shimadzu UV-1800). The reducing sugar content was calculated from the calibration curve of standard D-glucose (200-1000 mg/L)[17].

Total cellular carbohydrates were estimated using the anthrone method. The sample was then reacted with anthrone reagent and a high concentration of sulphuric acid. An aliquot (0.2 mL) of sample was mixed and vortexed with 0.4 mL of pre-chilled 75% H_2SO_4 solution in a test tube. To this mixture 0.8 mL of the anthrone reagent(2 g L^{-1} in75% H_2SO_4,freshly prepared) was added and subsequently boiled at 100 ℃ for 15 min. After cooling, the absorbance was read at 578 nm by using a spectrophotometer[18].

In vitro biofilm inhibitory activity against bacteria

Microbial biofilm inhibition potential:

By calculating their minimal biofilm inhibitory concentration (MBIC) with modified micro-dilution method, silver nanoparticles that were synthesised in a green manner were inspected for their impact on biofilm development. A range of concentrations of Ag-NPs were tested, with the final concentration falling between 0 and 32 µg mL^{-1}, In brief, bacterial samples were infected into test tubes comprising Luria-Bertani (LB) broth (5 mL), and mixture was then incubated overnight at 37 ℃ in a shaking incubator. In order to make the final volume 250 µL, 2.5 µL of bacterial culture and 2.5 µL of various Ag-NP concentrations were separately mixed with 245 µL of LB broth in 96-well plate. As a substitute for the nanoparticles, PBS was utilised. After that, samples underwent a static incubation period of 18 h at 37 °C. The modified crystal violet assay was followed to measure the production of biofilm[19]. This is how the biofilm inhibition % was calculated and MBIC was determined from that:

$$\text{Inhibition \%} = (OD_{untreated} - OD_{treated})/OD_{untreated} \times 100 \quad \ldots(2)$$

Microbial biofilm eradication potential

By aliquoting 50 µL of standardised *V. cholerae* and *S. aureus* (1 x 10^7 CFU/mL) and incubating the mixture for 4 h at 37°C, biofilms were formed in a 96-well plate. This period of time saw the formation of sufficient biofilm biomass, which was also confirmed by others. 50 µL of bio-synthesised Ag-NPs were added to the tubes after incubation. Further incubation took place for a further 24 h at 37 °C. After the incubation time, the biomass of the biofilm was measured with crystal violet technique[20]. I% was calculated using Eq. (2) and the extracts' minimal biofilm eradication concentration (MBEC) was determined.

Statistical analysis

Every data is presented as mean ± SD. The SAS Institute, located in Cary, North Carolina, USA, provided Origin Pro statistical software for the analysis of the variations in each experiment's results. To compare various groups, we used t-test. One-way ANOVA was used preceding Tukey's honestly significant difference (HSD) analysis for assessments comprising three groups or more. A comparative evaluation based on the t-test was used for the study. An outcome was considered meaningful if its P value (≤ 0.05).

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