Characterization of biogenically synthesized silver nanoparticles for therapeutic applications and enzyme nanocomplex generation

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Abstract

The present study describes green synthesis of silver nanoparticles (AgNPs) and inulin hydrolyzing enzyme nanocomplexes (ENC) using *Azadirachta indica* (Ai) and *Punica granatum* (Pg) leaf extracts. Surface topology and physico-chemical characteristics of AgNPs were studied using surface plasmon resonance (SPR), FTIR, SEM, AFM and EDX analyses. Particle size analysis using dynamic light scattering and AFM studies revealed that Ai-AgNPs (76.4 nm) were spherical in shape having central bigger nano-regime with smaller surroundings while Pg-AgNPs (72.1 nm) and ENCs (Inulinase-Pg-AgNPs ~ 145 nm) were spherical particles having smooth surfaces. Pg-AgNPs exhibited significant photocatalysis of a thiazine dye, methylene blue. Both Ai- and Pg-AgNPs showed selective antibacterial action by inhibiting pathogenic *Bacillus cereus*, while the probiotic *Lactobacillus* strains remained unaffected. Ai-AgNPs showed potential anti-biofilm effect (30% viability) on *B. cereus* biofilms. Pg-AgNPs showed anti-cancer effect against human colon cancer cell lines (Caco-2) resulting in 40% cell death in 48 h. Enzymes (inulinase, L-asparaginase and glucose oxidase) were successfully immobilized onto nanoparticles together with the biogenic synthesis of AgNPs and recyclability of the Inulinase-Pg-AgNPs complex was demonstrated. The study elaborates characteristics of green synthesized nanoparticles and their potential applications as anti-cancer, antibacterial and antioxidant nano drugs that could be used in food and nutraceutical industries. Enzyme immobilization on AgNPs without any toxic cross-linker opens up newer possibilities in enzyme-nanocomplex research.

Keywords Biogenic silver nanoparticles · Anticancer · Antimicrobial · Antioxidant · Enzyme immobilization

Introduction

Over the past few years, the fabrication of green synthesized nanoparticles (NPs) with controlled size, shape and plasmon properties has received increasing attention in the field of nanotechnology. NPs can be fabricated by physical, chemical and biological methods which are responsible for

their characteristics and usefulness in various applications. Physically derived NPs have limitations due to their slow formation and requirement of costly equipments for attaining high temperature, pressure etc. Chemical synthesis is usually fast but demands separation of toxic chemicals from NPs. On the other hand, biological synthesis usually occurs at room temperature and is devoid of toxic reactants as well as by-products and utilizes plant flavonoids and antioxidants or microbial metabolites. Microbial and phyto-metabolites derived NPs are produced by an environmental friendly approach and have several biomedical applications including antimicrobial and cytotoxic effects (Garibo et al. 2020). The facile synthesis of nanoparticles with desirable properties has led to increase in the applications of biosynthesized metal nanoparticles including in vivo sensing, bio-imaging, targeted therapy, diagnosis, enzyme immobilization, antibacterial and anti-cancerous applications (Ding et al. 2015; Sathiyaraj et al. 2020). Green synthesis or the plant extract driven fabrication and stabilization of metal nanoparticles is renewable, eco-friendly, economically viable, non-toxic

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and leads to biologically compatible nano-products with relatively high reproducibility. Green synthesis of metal nanoparticles is cost-effective and eco-friendly and hence preferable over chemical and physical methods. Among the noble metal nanoparticles, silver nanoparticles (AgNPs) are the most promising nanomaterials for biomedical applications because of their biomimicking properties and unique surface chemistry (Roy et al. 2019). AgNPs fabricated using aqueous leaf extract of *Pongamia glabra* showed fungicidal effects against phyto pathogen, *Rhizopus nigricans* (Sahayaraj et al. 2020). The generation of AgNPs using plant extract containing medicinal ingredients is a green, non-toxic approach. *Azadirachta indica* (common name: Margo) and *Punica granatum* (common name: Pomegranate) are two such medicinal trees harbouring numerous bioactive compounds (Sreekumar et al. 2014). The medicinal potential of these plants is because of the presence of a number of bioactive compounds such as salannin, nimbolide, nimbin, gedunin, phenolics, terpenes and plant phytochemicals stored in their leaves as secondary metabolites (Roy et al. 2019).

To overcome problem of multidrug resistance due to indiscriminate use of antibiotics, use of novel nano-sized drugs in place of conventional antibiotics is gaining considerable interest (Siddiqi et al. 2018). Nano-sized particles are also being used as enzyme carriers or enzyme immobilization matrix due to their high surface area and small size (Agrawal and Kango 2019). The enzyme-nanocomplex (ENC) carrier formulations are small, less toxic and have high potential to treat a target due to their high surface area: size ratio. Biological synthesis of AgNPs was carried out using from *Bacillus* sp. MB353 strain those have potential antibacterial and antifungal activities (Khan et al. 2020). Yu et al. (2019a) demonstrated the synthesis of cellulose nanofibril/silver nanoparticle (CNF/AgNP) nanocomposite displaying antimicrobial activity against *Escherichia coli* O157:H7 and *Staphylococcus aureus*. A different strategy to fight with multiple drug resistance (MDR) against bacterial pathogens, AgNPs fabricated with antimicrobial peptide isonicotinyl hydrazide and polyoxometalates phosphotungstic acid and phosphomolybdic acid were synthesized (Umapathi et al. 2019). Antimicrobial peptide, silk fibroin was used to synthesize AgNPs–silk fibroin scaffold exhibiting enhanced antimicrobial potential. These films were compatible to fibroblast and osteoblast cells and showed no detrimental effect on human mesenchymal stem cells (Patil and Singh 2019). Biological synthesis of AgNPs have been achieved using different plant extracts including *Bergenia ciliata*, *Bergenia stracheyi*, *Rumex dentatus* and *Rumex hastatus* and their application as antimicrobial agents has been demonstrated (Rashid et al. 2019). Extracts derived from grape seeds and cacao beans are rich in flavonoids and polyphenols which are responsible for the reduction of silver nitrate to metallic silver and subsequent formation

of AgNPs (Ranoszek-Soliwoda et al. 2019). AgNPs generated using *Garcinia indica* (popularly known as Kokum) extract exhibited good antimicrobial, antioxidant and free-radical scavenging activities (Sangaonkar and Pawar 2018). AgNPs fabricated using *Artemisia vulgaris* leaf extract had antimicrobial, antioxidant, and anti-proliferative properties (Rasheed et al. 2017). Motivated from the above studies, we have demonstrated the green synthesis of AgNPs and their multifarious applications including catalytic degradation of azo dyes. Azo dyestuffs such as methylene blue, Congo red, methyl orange etc. represent a major pollutant in textile and wastewater released from printing industries. Some azo dyes generate aromatic amines upon reduction which are highly carcinogenic and therefore these dyes should be completely removed from water bodies (Sarkar et al. 2017). The present study was aimed to synthesize AgNPs using aqueous leaf extracts of *Azadirachta indica* (Ai: Margo) and *Punica granatum* (Pg: Pomegranate) and their characterization using multi-scale spectroscopic and microscopic techniques. Synthesized nanoparticles were tested for antimicrobial, anticancer, antioxidant, photocatalytic dye degradation and enzyme nanocomplex (ENC) development.

Experimental

Materials

Silver nitrate (AgNO_3), dinitro salicylic acid (DNS), inulin from Chicory, ammonium sulphate, trichloroacetic acid (TCA), glucose oxidase (GOD), inulinase from *Aspergillus niger* and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagents were purchased from Sigma Aldrich®, USA. Commercial L-asparaginase (Bionase®) was procured from Zydus oncoscience, India. Media components for cell culture (DMEM, FBS, Trypsin–EDTA, PBS etc.) were procured from Gibco™ and Invitrogen™, USA. Bacteriological media components were obtained from HiMedia®, India. All other reagents were of analytical grade.

Green synthesis of silver nanoparticles (AgNPs) and enzyme-nanocomplex (ENC)

Preparation of plant extracts

Fresh leaves of *A. indica* (Ai) and *P. granatum* (Pg) were collected from local areas of Sagar, India (23.8388° N, 78.7378° E) and washed twice to remove the dust particles. Washed leaves were dried under the sunlight and ground to fine powder. Plant extracts were prepared as described by Khorrami et al. (2019) with some modifications. Plant extracts were prepared by mixing minimum quantities (2.5 g Pg and 0.4 g Ai) of leaf powders for AgNPs synthesis in

100 mL of distilled water. The mix was stirred for 30 min and centrifuged at 5000g for 15 min. The clear supernatant was obtained by filtering through Whatman No. 1 filter paper and was used for the green synthesis of silver nanoparticles (AgNPs).

Green synthesis of AgNPs

AgNPs were synthesized as described by Roy et al. (2017) with some modifications. Briefly, AgNPs were synthesized by adding 1 mL plant extract to 10 mL AgNO₃ solution (1 mM) followed by incubation at 30 °C for 24 h (*Pg*-AgNPs) and 72 h (*Ai*-AgNPs). AgNPs were separated by centrifuging the suspension at 12,000g for 20 min, washed twice with distilled water and used in further studies.

Enzyme-nanocomplex (ENC) formulation

Enzyme nanocomplexes were synthesized by adding appropriate amount of enzyme in reaction mixture as described by Sheikh et al. (2018) for lactoperoxidase immobilization. ENC were prepared by addition of 1 mg enzyme (Inulinase or L-Asparaginase or 0.1 mL GOD) to 10 mL AgNO₃ and 0.5 mL of *Pg*-extract. The reaction was incubated at 30 °C for 45 min followed by incubation at 4 °C overnight. ENC formulation was confirmed by measuring characteristic SPR peak by visible spectrophotometry. ENC were recovered by centrifugation for 20 min at 12,000g, washed once with distilled water and stored at 4 °C for further studies. Immobilization yield was calculated as the following formula:

$$\text{Immobilization Yield (\%)} = A_f/A_i \times 100,$$

where A_f is the final activity of immobilized enzyme and A_i is the initial activity of free enzyme.

The ENC were produced after immobilization were characterized using surface Plasmon resonance (SPR) measurement, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM) and dynamic light scattering (DLS).

Enzyme assay

Inulinase assay Inulinase activity was determined by estimating reducing sugars liberated from inulin using dinitrosalicylic acid (DNS) method (Rawat et al. 2015). Briefly, 100 µL of free inulinase or inulinase-*Pg*-AgNPs was mixed with 900 µL of 0.5% chicory inulin (pH 5.0) and incubated at 50 °C for 10 min. After incubation, the reaction was stopped by adding 1.5 mL DNS reagent and subsequent boiling for 5 min. After cooling, the absorbance was recorded at 540 nm. One inulinase unit (U) was defined as the amount

of enzyme that released 1 µmol fructose per min under the assay conditions.

L-asparaginase assay L-asparaginase activity of asparaginase-*Pg*-AgNPs and free enzyme was determined by Nesslerization method described previously (Agrawal and Kango 2019). In brief, 100 µL of free asparaginase or enzyme nanocomplex was mixed with 900 µL L-asparagine (100 mM w/v, pH 7.0) and incubated for 20 min at 37 °C. After incubation, the reaction was stopped by addition of 100 µL of 1.5 M trichloroacetic acid (TCA). Ammonia liberated in the mixture was measured by diluting 200 µL of above reaction mixture in 800 µL distilled water after adding 100 µL Nessler's reagent. Absorbance of the reaction mixture was recorded at 450 nm against appropriate enzyme blank. One unit (U) of L-asparaginase was defined as the amount of enzyme responsible for release of 1 µmol ammonia per minute under assay conditions.

Characterization of nanoparticles

Localized SPR of AgNPs was recorded with Multiskan GO (ThermoFisher) spectrophotometer in the spectral range of 350–600 nm using distilled water and AgNO₃ as blank and reference, respectively.

Fabricating and stabilizing capping agents responsible for AgNPs formation were examined by FTIR using Bruker Alpha II ECO-ATR system in the spectral range of 4000–500 cm⁻¹. Spectral analysis of each sample was performed for 15 scans with spectral resolution of 2 cm⁻¹ using OPUS software at transmission mode.

Topographical characteristics of AgNPs were recorded using FEI Nova NanoSEM™ 450 scanning electron microscope (SEM) operating at an accelerating voltage of 10–20 keV. Silver detection in nanoparticles was confirmed using energy-dispersive X-ray spectroscopy (EDX) system (Bruker, USA) integrated with SEM. Surface texture of AgNPs was observed under atomic force microscope (AFM; Innova, Bruker, USA).

Polydispersity index (PDI) and hydrodynamic particle size of AgNPs were analyzed by DLS using NanoPlus Zetasizer (Particulate Systems, USA). Suspended samples were sonicated at 250 W by applying 10 pulses of 10 s on, 5 s off and filtered through 0.2 micron filter prior to analysis to remove impurities.

Applications of AgNPs

Anti-bacterial properties

Anti-bacterial efficacy of AgNPs was evaluated by well-diffusion assay as described by Rautela et al. (2019) with some modifications. The activity of AgNPs against pathogenic

Bacillus cereus MTCC 1272 and probiotic strains, *Lactobacillus acidophilus* NCIM 5306 and *Lactobacillus delbrueckii* NCIM 2025, was evaluated on nutrient agar or MRS agar (for *Lactobacilli*) plates. Plates were inoculated with 1×10^6 bacteria and colloidal suspension of AgNPs (20–100 $\mu\text{g/mL}$) was poured in 6 mm wells to assess antibacterial activity. Plates were incubated at 37 °C for 24 h and zone of antibacterial activity was measured.

Cellular damage due to AgNPs treatment was assayed as described by Rautela et al. (2019) with some modifications. To assess the cellular damage upon treatment of nanoparticles, mid-log phase culture (4.8×10^7 cells/mL) of *B. cereus* was treated with 100 $\mu\text{g/mL}$ of AgNPs for 8 h at 37 °C. Cells were centrifuged after incubation and the supernatant was examined for protein and DNA leaching. Quantitative estimation of DNA and protein was done using Nanodrop spectrophotometer (Thermo Scientific, USA). Untreated bacterial culture was taken as control.

Biofilm combating potential of AgNPs was evaluated as described by Bhatwalkar et al. (2019) with some modifications. In brief, 200 μL of mid-log phase culture of *B. cereus* was grown in LB medium in 96 well plates and incubated at 37 °C for 24 h for biofilm formation. After incubation, bacterial film was treated with different concentrations (20–100 $\mu\text{g/mL}$) of AgNPs and incubated at 37 °C for 24 h. After treatment, films were washed twice with phosphate buffered saline (PBS) and finally with distilled water. Films were stained with 0.1% (w/v) crystal violet and incubated at 30 °C for 10 min. Films were then rinsed with distilled water and solubilized using 95% (v/v) ethanol. The optical density was recorded at 570 nm in Multiskan GO spectrophotometer.

Antioxidant activity (DPPH free radical scavenging)

The antioxidant activity of AgNPs was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging assay (Veenashri and Muralikrishna 2011). Briefly, 100 μL of AgNPs at different concentrations (20–100 $\mu\text{g/mL}$) was mixed with 100 μL DPPH reagent (80 mg/L in ethanol) in a 96-well plate. The mixture was incubated at 30 °C for 1 h in dark. After incubation, absorbance was measured at 517 nm. The DPPH scavenging % was calculated using the following equation:

$$\% \text{DPPH radical scavenging} = (A_c - A_s) / A_c \times 100,$$

where A_c is the absorbance of control and A_s is the absorbance of sample.

Anti-cancer properties

Cell culture Caco-2 cells were procured from National Center for Cell Sciences (NCCS), India and maintained on

high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin (Gibco). Cells were grown in 96-well plates at 37 °C and 5% CO_2 in a humidified incubator.

WST-1 cell viability assay About 10,000 cells per well were seeded on 96-well plates and incubated for 24 h for attachment. To determine the cytotoxic effect of nanoparticles against human colon cancer cell lines, Caco-2 cells were treated with 100 μL of AgNPs (20–80 $\mu\text{g/mL}$) and incubated for 24–48 h at 37 °C in a humidified CO_2 incubator. After treatment, cell viability assay was performed using WST-1 reagent (Roche). For this, 10 μL WST-1 reagent was added per well and the plate was incubated for 4 h at 37 °C in CO_2 incubator and the absorbance was measured at 440 nm. Untreated cells were taken as control for the viability assay and the % cell viability was obtained using the following equation (Ahmad et al. 2015):

$$\% \text{Viability} = \text{OD}_{440} \text{ of treated cells} / \text{OD}_{440} \text{ of control cells} \times 100.$$

Dye scavenging

About 500 μL (80 mg/mL) of biosynthesized *Pg*-AgNPs was added to 10 mL of methylene blue solution (1% w/v in DW). The reaction was stirred for 30 min and incubated under the sunlight for activation of AgNPs. The dye at same concentration as reaction without *Pg*-AgNPs was also incubated under sunlight and considered as the control. The reaction was observed for color change and the absorbance was recorded in 400–700 nm spectral range. Scavenging (%) was calculated by measuring absorbance at 660 nm using the following equation (Vanaja et al. 2014):

$$\% \text{Scavenging} = (C_0 - C) / C_0 \times 100,$$

where C_0 is the initial concentration of dye solution and C is the concentration of dye solution after photochemical degradation.

Enzyme-nanocomplex recycling

Enzyme nanocomplex recycling was done as Torabizadeh and Mahmoudi (2018) with some modifications. Inulinase-*Pg*-AgNPs (100 μL) were incubated with 900 μL chicory inulin (0.5% w/v, pH 5.0) and incubated for 10 min at 50 °C. After incubation, ENC were centrifuged and washed with citrate buffer (50 mM, pH 5.0). The procedure was repeated for 4 cycles. The activity was measured as given in Section **Enzyme Assay** and the activity of first cycle was considered as 100%.

Results and discussion

Green synthesis of AgNPs

To obtain stable nanoparticles, parameters such as incubation time, temperature and ratio of reactants were optimized. AgNPs generated from both *A. indica* and *P. granatum* extracts were formed at 30 °C. The reduction of monovalent silver (Ag^+) from AgNO_3 to elemental silver (Ag^0) in AgNPs resulted in the color change from pale yellow to reddish brown because of the characteristic phenomenon i.e. localized surface plasmon resonance (LSPR). LSPR phenomenon is the result of resonating oscillations of conduction band of the reactant metal ions while reacting with light of specific wavelength (Ahmad et al. 2015).

Ai-AgNPs were synthesized and stabilized in 72 h of incubation, whereas, *Pg*-AgNPs were synthesized within 9 min of reaction and stabilized in 39 min only (Fig. 1a, e). The stabilizing time is the measure of completion of the silver ion reduction by the constituent reducing agents present in the plant extract (Ahmed et al. 2017). High concentration (10% w/v) of ethanolic leaf extract of *A. indica* resulted in AgNPs synthesis in 24 h from 100 mM AgNO_3 (Ulaeto et al. 2020). Aqueous extract of *Malus domestica*

resulted in the formation of AgNPs within 20 min at 80 °C (Anand Mariadoss et al. 2019). Aqueous extract of pomegranate (*P. granatum*) leads to AgNP synthesis in 2 h (Khorrami et al. 2019). Methanolic extracts of *A. indica* L. leaves fabricated AgNPs in 2 h while *Punica granatum* L. fruit aqueous extract showed AgNPs synthesis in 10 min under sunlight (Priyadarshini et al. 2019; Mohseni et al. 2020). *Ai*-AgNPs and *Pg*-AgNPs displayed characteristic LSPR peaks at 434 nm and 416 nm, respectively (Fig. 1a, e). AgNPs synthesized by *Enicostemma axillare* exhibited SPR peak at 417 nm (Raj et al. 2018). Comparative account of LSPR peaks of AgNPs are shown in Table 1. Glucose oxidase-*Pg*-AgNPs showed a displacement in LSPR peak from 416 to 409 nm while, inulinase-*Pg*-AgNPs and asparaginase-*Pg*-AgNPs showed SPR at 417 and 420 nm, respectively (Fig. 1i).

Characterization of AgNPs

Surface topographic analysis by SEM and AFM

The size, shape and topography of the biosynthesized AgNPs was recorded using scanning electron microscopy and *Ai*-AgNPs, *Pg*-AgNPs and inulinase-*Pg*-AgNPs were found stabilized in spherical shapes (Fig. 1b, f, j). AFM

Fig. 1 Fabrication kinetics of green synthesized silver nanoparticles using *Azadirachta indica* (*Ai*) and *Punica granatum* (*Pg*) leaf extracts and their characteristics: Surface Plasmon Resonance (SPR) of **a** *Ai*-AgNPs synthesis in 72 h, **e** *Pg*-AgNPs formation in 39 min and **i**

Enzyme nano-complexes (**GA** Glucose oxidase-*Pg*-AgNPs, **IA** Inulinase-*Pg*-AgNPs and **AA** Asparaginase-*Pg*-AgNPs). SEM, AFM and EDX analysis of *Ai*-AgNPs (**b–d**), *Pg*-AgNPs (**f–h**) and Inulinase-*Pg*-AgNPs (**j–l**)

Table 1 Comparative profile of green synthesized AgNPs, properties and applications

Plant	Part	Size (nm)	SPR ^d peak (nm)	Responsible Functional Group	Applications	Reference
<i>Alternanthera sessilis</i>	Leaf extract	< 100 ^c	420	–C≡C, –NH, –C≡N	Anticancer against prostate cancer cells PC-3	Firdhouse and Lalitha (2013b)
<i>Sargentodoxa cuneata</i>	Plant material extract	3–8 ^a	–	–CH, –CC, –CO Methyl or methylene, polyphenolic tannin or tannic acid	Antibacterial against <i>E.coli</i> , <i>P.aeruginosa</i> , <i>S. aureus</i> , <i>B. subtilis</i> , antileishmanial activity cytotoxicity against murine macrophages J774	Ahmad et al. (2015)
<i>Oscinum sanctum</i>	Leaf	16–40 ^a	–	–C=C, –C≡C, –NH, –OH Quercetin, flavonoid, terpenoids, phenolic compounds	Antibacterial against <i>E. coli</i>	Jain and Mahata (2017)
<i>Cynara scolymus</i>	Leaf extract	98.47 ^b 200–223 ^c	400–450	–C–C, –CH ₂ , CN, C–O Aromatics, aliphatics and ether compounds	Anticancer activity against MCF-7 cells	Erdogan et al. (2019)
<i>Dyosma pleiantha</i>	Rhizome extract	76 ^a	460	–C–C, –CH, CO Methyl and carbonyl compounds	Anticancer activity against breast cancer cells MDA-MB-231 and gastric cancer cell lines	Karupaiya et al. (2019)
<i>Phyllanthus emblica</i>	Fruit extract	19.8–92.8 ^c	430	–CC, C–H Amide and proteins	Antimicrobial activity against <i>A. oryzae</i> strain RS-2	Masum et al. (2019)
<i>Berberis vulgaris</i>	Leaf and root extract	90–100 ^b	450	–	Antibacterial against <i>E. coli</i> , <i>S. aureus</i>	Behravan et al. (2019)
<i>Malus domestica</i>	Fruit extract	50–107.3 ^a	425	–OH, –NH, –CH, –CO, –CC Alkaloid, saponins, triterpenoids, steroids, sterols and flavonoids	Antimicrobial against MDR <i>E. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , anti-cancer against breast cancer cells MCF-7	Anand Mariadoss et al. (2019)
<i>Azadirachta indica</i>	Leaf extract	76.4 ^b	434	–CO, –OH	Antibacterial activity against <i>B. cereus</i> , anticancer activity against colon cancer cells Caco-2, free radical scavenging effects	This study
<i>Punica granatum</i>	Leaf extract	72.1 ^b	416	–CO, –OH	Antibacterial activity against <i>B. cereus</i> , selective inhibition of antibacterial properties against probiotic microbes, anticancer activity against colon cancer cells Caco-2, free radical scavenging effects, methylene blue dye scavenging, immobilization of inulinase, glucose oxidase and L-asparaginase enzymes	This study

^aDry nanoparticle size measurement using TEM^bHydrodynamic size measurement using dynamic light scattering by zeta sizer^cSize measurement using SEM^dSPR- surface plasmon resonance

images showed that *Pg*-AgNPs and inulinase-*Pg*-AgNPs had smooth surface, while *Ai*-AgNPs contained central AgNP core with smaller surrounding particles (Fig. 1c, g, k). Elemental analysis of synthesized AgNPs is shown in Fig. 1d, h and l. Energy dispersive X-ray (EDX) graphs showed peaks at 3 keV and 0.25 keV, clearly indicating the presence of Ag.

Functional group analysis by FTIR

Functional groups present on nanoparticles were analyzed using FTIR (Fig. 2). A broad IR stretch at 3271 cm^{-1} confirmed the presence of -OH bond indicating some polyhydroxy compounds, such as reducing sugar or phenolics etc. Similarly, hydroxyl stretch at 3432 cm^{-1} suggested the presence of phenolic compounds in *Sargentodoxa cuneata* extract (Ahmad et al. 2015). Peak at 1635 cm^{-1} showed $>\text{C}=\text{O}$, $>\text{C}=\text{C}$ stretch of plant triterpenoids and flavones or amide group. *Amaranthus dubius* whole plant extract has also been used for the synthesis of AgNPs because of the presence of proteins which are natural capping and reducing agents (Firdhouse and Lalitha 2013a).

Particle size analysis

Particle size analysis was performed to determine the average hydrodynamic size of green synthesized AgNPs and their distribution in the solution. The average hydrodynamic size of *Ai*-AgNPs was 76.4 nm with polydispersity index (PDI) of 0.324, while *Pg*-AgNPs were 72.1 nm in size with PDI 0.300. Inulinase-*Pg*-AgNPs and asparaginase-*Pg*-AgNPs had large particle size of 145 nm and 102 nm with a PDI of 0.341 and 0.295, respectively, possibly due to agglomeration of enzyme nanoparticle complex. Glucose

oxidase-*Pg*-AgNPs showed an average size of 150 nm with PDI of 0.314. A comparative analysis of properties of AgNPs shows that the green synthesized AgNP size ranges between 416 and 450 nm (Table 1).

Applications of green synthesized AgNPs

Antibacterial properties

Both *Ai*-AgNPs and *Pg*-AgNPs showed potential antibacterial activity against *B. cereus* (Fig. 3a, b). Nanoparticle concentration of $100\text{ }\mu\text{g/mL}$ produced 24 mm zone of antibacterial activity in both the cases. Contrastingly, AgNPs synthesized using corn leaf extract showed 9–11 mm zone of inhibition with $50\text{ }\mu\text{g}$ AgNPs (Patra and Baek 2017). AgNPs synthesized using *P. granatum* seed extract produced 16 mm and 17 mm inhibition zone against *Salmonella typhi* and *E. coli*, while polyherb-derived AgNPs showed 23 mm and 12 mm zone of inhibition against *E. coli* and *Bacillus subtilis* (Mohseni et al. 2020; Sathiyaraj et al. 2020). Interestingly, we found no inhibitory action against any of probiotic *Lactobacillus* spp. (Fig. 3c, d). These results suggest the possible use of these AgNPs as food antimicrobials. Moreover, the *B. cereus* biofilm degradation result suggested that *Ai*-AgNPs effectively degraded the biofilm in 24 h (30% viability), whereas *Pg*-AgNPs showed less effect on *B. cereus* biofilm (Fig. 3e). dsDNA and protein leaching profiles of *B. cereus* upon AgNPs treatment showed an increase in DNA and protein concentrations in supernatant after 8 h treatment as compared to untreated control (Fig. 3f). Treatment of *B. cereus*, *E. coli* and *S. aureus* with AgNPs synthesized using *Tectona grandis* enhanced its membrane

Fig. 2 FTIR spectra showing functional group analysis of AgNPs and enzyme-nanocomplex functionalized and stabilized by *A. indica* (*Ai*) and *P. granatum* (*Pg*) leaf extracts. AgNO_3 Silver nitrate solution (1 mM), *Ai*-extract Leaf extract of *A. indica*, *Pg*-extract Leaf extract of *P. granatum*, *Ai*-AgNPs *A. indica* induced silver nanoparticles, *Pg*-AgNPs *P. granatum* induced silver nanoparticles and enzyme nanocomplexes: GA Glucose oxidase-*Pg*-AgNPs, IA Inulinase-*Pg*-AgNPs, AA Asparaginase-*Pg*-AgNPs

Fig. 3 Anti-bacterial action of **a** *Ai*-AgNPs and **b** *Pg*-AgNPs against *Bacillus cereus*. Effect of *Pg*-AgNPs on probiotic strains **c** *Lactobacillus acidophilus* and **d** *Lactobacillus delbrueckii* at different concentrations (20–100 μg/mL). *Ai* *A. indica* (Margo) extract, *Pg* *P.*

granatum (Pomegranate) extract, **c** Control; **e** Anti-biofilm activity of AgNPs against *B. cereus* **f** Leaching of DNA and proteins from *B. cereus* in the presence of *Ai*-AgNPs (filled square) and *Pg*-AgNPs (inverse triangle) in comparison to untreated control (filled circle)

permeability leading to protein leakage (Rautela et al. 2019). Selective bactericidal effects against food borne pathogen of AgNPs suggested their prospective applications as food antimicrobials.

Cattò et al. (2019) have deciphered a unique chemo-preventive mechanism in probiotic gut microbiota to overcome bactericidal effects of AgNPs. They studied the effect of AgNPs on 25 metabolic genes out of which, 21 gene families responsible for essential functions such as membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair, energy metabolism and translation were observed unaltered. While, on the other hand they observed 55% increase in xenobiotics degrading gene's expression in the fecal microbiota with AgNP treatment. AgNPs generate free radicals such as superoxide anion, hydroxyl free radical, singlet oxygen, hypochlorous acid and hydrogen peroxide. Hsueh et al. (2015) also reported the silver ion based antimicrobial mechanism against *B. subtilis*. Cellulose nanofibril /AgNPs (CNF/AgNP) conjugate (2.5–10 mg/mL) inhibited the growth of *E. coli* O157: H7 in 24 h (Yu et al. 2019b). Our results and other reports (Hsueh et al. 2015; Gomaa 2017; Qing et al. 2018; Rautela et al. 2019; Yu et al. 2019b) suggested that the bacterial membrane damage accompanied

with DNA and protein leakage may be the probable mode of antibacterial action of AgNPs (Fig. 4).

DPPH free radical scavenging potential

The antioxidant activity of green synthesized AgNPs in terms of DPPH free radical scavenging is shown in Fig. 5a. High concentration (100 μg/mL) of *Ai*-AgNPs and *Pg*-AgNPs showed significant radical scavenging (46%) in 1 h (Fig. 5a). *Ai*-AgNPs showed greater radical scavenging efficacy (EC₅₀ 1.09 μg/mL) than *Pg*-AgNPs (EC₅₀ 1.14 μg/mL). AgNPs synthesized using *Stychnis lavandulifolia* flower extract showed 50% inhibition in DPPH radical at a concentration of 371 μg/mL (Zangeneh et al. 2019).

Anti-cancer properties

The results of cytotoxicity studies on colon cancer cell lines (Caco-2) suggested no significant inhibition in cancer cells upon treatment with *Ai*-AgNPs, while *Pg*-AgNPs showed potential anti-cancer effects (40% cell death) in 48 h (Fig. 5b). The surface chemistry, size and shape of silver nanoparticles are shown to be responsible for different

Fig. 4 Proposed mechanism of anti-bacterial action of silver nanoparticles: (1) Binding of AgNPs to the membrane proteins results in the increased membrane permeability leading to protein and DNA leaching from bacterial cell; (2) AgNPs penetrate the membrane and release Ag^+ which results in reactive oxygen species (ROS) generation and ultimately bacterial cell death; (3) Released AgNPs or Ag^+ can bind to DNA and proteins and lead to distortion in cellular structure; (4) DNA damage leads to cell death

Fig. 5 Evaluation of anti-oxidant and anti-cancer properties of biogenic AgNPs. Values represent average of three independent replicates $\pm$ SD. **a** DPPH free radical scavenging efficacy of *Ai*-AgNPs and

Pg-AgNPs. **b** Anticancer effect of AgNPs evaluated for 48 h against adenocarcinoma cell lines Caco-2

cytotoxic effect on different cell lines (Sivakumar et al. 2020). Induction of caspase mediated programmed cell death pathway by silver nanoparticles generated using galactomannan from *P. granatum* fruit results in their anticancer property (Padinjarathil et al. 2018). AgNPs generated

using a common weed, *Parthenium hysterophorus* showed inhibition in liver cell lines HepG2. The anticancer activity was concentration dependent and increased from 50% with 53 µg/mL AgNPs to 100% inhibition with 100 µg/mL AgNPs (Sivakumar et al. 2020).

Dye scavenging

Photocatalytic degradation of hazardous dyes using AgNPs can be exploited in bleaching applications. As shown in Fig. 6, the characteristic peak of MB at 660 nm vanished in 48 h after incubation with *Pg*-AgNPs. When incubated under sunlight, 27% scavenging of MB was achieved with *Pg*-AgNPs within 1 h and the degradation reached upto 90% in 48 h (18 h under sunlight) of incubation (Fig. 6).

Singh and Dhaliwal (2018) reported 82.8% MB degradation with AgNPs after 180 min exposure of mercury light. The mechanism of MB degradation into leuco or a colorless product is based on the photocatalyzed generation of free radicals. Silver nanoparticles are reported to cause charge separation of photo generated electron-hole pairs resulting in the overall enhancement of photocatalytic activity (Chen et al. 2010). Several metal and metal oxide nanoparticles such as Ag, Au, Cu, ZnO, MnO₂ are incorporated in biopolymeric materials and are being used for waste water treatment (Sarkar et al. 2020).

Fig. 6 Photocatalytic reduction of Methylene Blue (MB) by *Pg*-AgNPs: *Pg*-AgNPs were added to MB, incubated under the sunlight for activation of AgNPs and scavenging (%) was observed at 660 nm. Methylene blue without *Pg*-AgNPs incubated under sunlight was considered as control

Enzyme-nanocomplex recycling

Inulinase-*Pg*-AgNPs and asparaginase-*Pg*-AgNPs were successfully produced, with immobilization yields of 55.4% and 58.72%, respectively, without employing any toxic cross-linker chemical. The binding resulted probably due to surface adsorption interactions among enzyme and nanoparticles. The inulinase-*Pg*-AgNPs were recycled upto 3 cycles with 90% residual activity, while 63% activity was retained till fourth cycle (Fig. 7).

Misson et al. (2015) documented reports of enzyme immobilization on Zinc oxide and poly acrylonitrile nanofibers due to physical adsorption. Beta-galactosidase was immobilized on nano-carriers for lactose hydrolysis and biodiesel production. Similarly, Lactoperoxidase immobilized AgNPs were synthesized immediately upon addition of a chemical reductant NaBH₄ in the reaction mixture of AgNO₃ and lactoperoxidase, where the enzyme acted as the capping reagent (Sheikh et al. 2018). Syed et al. (2016) reported green synthesis of AgNPs via *Fagonia indica* plant material and used them as immobilization support to immobilize glucoamylase from *Neurospora sitophila* using covalent immobilization linker EDC (1-(3 (dimethylamino) propyl) 3-ethylcarbodiimidehydrochloride) coupling reaction.

Fig. 7 a Schematic representation of Inulinase-*Pg*-AgNP (enzyme nanocomplex formation) in single step. 1 mg enzyme (Inulinase) was added to 10 mL AgNO_3 solution (1 mM) and 0.5 mL of *Pg*-extract. The mix was stirred and incubated at 30 °C for 45 min followed by incubation at 4 °C overnight. **b** Recycling of Inulinase-*Pg*-AgNPs nanocomplex for inulin hydrolysis for fructose generation from Chicory inulin (0.5% w/v) at 50 °C. Values represent mean $\pm$ SD of three independent replicates

Conclusions

Green synthesis of silver nanoparticles (AgNPs) was carried out using readily available leaf extracts from *Azadirachta indica* (Margo) and *Punica granatum* (Pomegranate). A detailed analysis of AgNPs revealed their topographical, compositional and bioactive (anti-bacterial anti-cancer, dye scavenging and anti-oxidant) properties. Immobilization of some biocatalysts on AgNPs without using any chemical cross-linker was demonstrated. The ease of synthesis, downstream processing and variety of applications suggested the immense potential of biogenically synthesized silver nanoparticles as food antimicrobials, nano-sensors and anti-cancer agents. Multifarious bioactive properties of the biogenically synthesized silver nanoparticles suggest their immense potential in diverse fields.

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Author contributions RC and AJ performed characterization experiments. Photocatalytic degradation of MB has been performed by AJ. Rest of all application experiments were performed and data analyzed by RC. NK conceptualized and supervised the work. Writing, review and editing of the manuscript has been done by NK and RC.

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Compliance with ethical standards

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