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Feather Protein Hydrolysate mediated synthesis of silver nanoparticles and assessment of anti-microbial potential of silver nanoparticle-treated silk cloth

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In this study, we have performed green synthesis of silver nanoparticles using Feather Protein Hydrolysate (FPH) formed by degradation of chicken feathers by *Stenotrophomonas maltophilia* KB13. The synthesized nanoparticles were characterized by techniques such as, UV-Vis Spectroscopy, Fourier Transformed Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy coupled with Energy Dispersive Spectroscopy (SEM- EDS) respectively. TEM and SEM-EDS confirmed the formation of silver nanoparticles. The size range was found to be 20-40 nm. FTIR analysis suggested the role of amino acids and proteins present in FPH during the synthesis process. XRD analysis of silver nanoparticles confirmed the formation metallic silver nanoparticles with the average size of 29.957 nm. The antimicrobial activity of nanoparticles was also studied on clinically relevant pathogenic bacteria. These nanoparticles exhibited excellent anti-microbial properties. The antimicrobial property of silver nanoparticles was employed for the rescue of silk cloth from microbial deterioration.

Keywords: Anti-Microbial, Feather Protein Hydrolysate (FPH), Silver Nanoparticles

In recent years silver nanoparticles and their derived Nano-materials have become the most sought after in consumer products due to their wide applications in both fundamental and applied research, such as X- ray optics, photography, labelling, photoelectronic, antimicrobial sterilization and surface enhanced Raman scattering[1,2]. They also have been extensively used in nano-chemistry to enhance the immobilization and activity of catalyst, in medical and pharmaceutical nano-engineering for the delivery of therapeutic agents in chronic disease diagnostics and in sensors[3]. Researchers are aspiring to prepare industrially attractive, feasible and commercially viable nanoparticles[4].

Several salts of silver and their derivatives are commercially manufactured as antimicrobial agents against pathogenic microorganisms[4,5]. Compared with organic antibacterial agents, inorganic nanoparticles particularly silver nanoparticles are economical, stable, and effective[6]. Their role as antimicrobial agent have been examined for their ability to reduce microbial infection of skin and burn wounds and to prevent bacterial colonization on various surface devices such as catheters and prostheses[3]. Further, these nanoparticles were found highly toxic against different multidrug resistant human pathogens[7]. Various physio-chemical methods for the preparation of metallic nanoparticles have been reported. Among the physical methods, sputtering, molecular beam epitaxy, microwave assisted and electro-deposition are widely known[8,9]. The most common chemical methods are reduction of metal salt precursors using reducing agents such as citrate, polymer substances like borohydride or other organic reagents[10]. However, in most of the cases chemical route of synthesis causes potential hazards to health and the environment[11]. To minimize the toxic effects of these chemicals, the use of biological compounds and their derivatives as reducing agents and search for new methodologies for production of nanostructures has increased[12]. In this context several green methods for synthesis of nanoparticles including bacteria, fungi, plant leaf extract, seed extract, plant latex and some biopolymers have been reported[11,13,14].

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Chicken feathers are keratin rich waste material generated from poultry industries[15]. Around 8.5 billion tons of chicken feathers are produced annually worldwide and alone, India's contribution is about 350 million tons[16]. Microbial degradation of chicken feathers is considered as an eco-friendly approach for the disposal of this waste material. Degradation of keratin present in chicken feathers leads to generation of a pool of short peptides and free amino acids, which is collectively termed as Feather Protein Hydrolysate (FPH) that can be utilised in multiple ways such as in plant growth promotion as a rich source of nitrogen[15], as ample source of antioxidants[17]. The FPH possesses high reducing power and can be employed for the reduction of hexavalent chromium[18]. It is anticipated that FPH can also be used for the synthesis of metallic nanoparticles owing to its high reducing power. In a similar study, synthesis of silver nanoparticles through casein hydrolysate has been described earlier[19]. Literature survey has suggested that the use of FPH for the synthesis of silver nanoparticles has not been explored. In the present study, we have made an attempt to synthesize silver nanoparticles from FPH obtained after the degradation of chicken feather by a potent keratinolytic bacterium *Stenotrophomonas maltophilia* KB13. The synthesized silver nanoparticles designated as FSNP's were characterized by FTIR, TEM, SEM-EDS, XRD and particle size analysis and its anti-microbial activity was assessed. Further, silk cloth was treated with FSNP's and its anti-microbial activity was determined. Results of this study clearly indicate that FSNP's synthesized from FPH exhibited potent anti-microbial activity against tested strains.

Materials and Methods

Materials

Feathers used in the study were obtained from a regional poultry farm, Bilaspur, Chhattisgarh. They were washed thoroughly with detergent followed by water for 2-3 times to remove dust and blood stains and, were dried in hot air oven for 24 h at 60°C. The chemicals used in this study were of pure analytical grade and was purchased from sigma chemicals and Hi-media, India.

Microorganism, Media, and culture condition

A strain of *Stenotrophomonas maltophilia* KB13 (accession number KC 818432.1) isolated from poultry waste site of Bilaspur, Chhattisgarh, India was employed for hydrolysis of feathers due to its high feather degradation potential[18].

Feather meal broth (FMB) used for culturing of bacterial cells of strain KB13 consisted of (g/L): K_2HPO_4, 0.3; KH_2PO_4, 0.4; NaCl, 0.5 and feather 10, pH 7.2[20]. Peptone water (g/L): peptone, 10; and NaCl, 5, pH 7.2 was used for inoculum preparation. The media described above was autoclaved at 121°C and 15 psi for 15 min. The bacterial culture was maintained at 30°C and 120 rpm.

Preparation of Feather Protein Hydrolysate (FPH)

A single colony of strain KB13 was inoculated in 100 mL Erlenmeyer flask containing 50 mL peptone water and kept for incubation at 30°C and 120 rpm in orbital shaker (24 BL, Remi, India). After 24 h of growth, the culture was centrifuged at 8000 rpm for 10 min in a Sorvall RC-5B super speed refrigerated centrifuge (Du Pont Instruments, USA). The cell pellet was washed twice and suspended in 5 mL FMB (without feathers) and 5% (*v/v*) of the suspension was transferred to 100 mL of fresh FMB and incubated at the same conditions. After 3d of incubation the culture was centrifuged at 12000 rpm for 10 min and supernatant or clear hydrolysate of digested feathers was separated, it was autoclaved and further used for the synthesis silver nanoparticles. The amount of protein present in the lysate was estimated by Bradford method using Bovine serum albumin as standard[21]. The total amino acid in the lysate was estimated using Ninhydrin method using leucine as standard[22].

Synthesis of silver nanoparticles using FPH

A highly reproducible method for the synthesis of silver nanoparticles using FPH was carried out by reducing the salt of silver nitrate ($AgNO_3$). Freshly prepared FPH was used for all the experiments. Silver nitrate (1 mM) was used for all analytical experiments unless otherwise specified. Typical reaction mixture was prepared by addition of 1 mM $AgNO_3$ in 10 mL of FPH with its native pH 8.0 and incubated at 65°C in dark in water bath for 10 min. The effect of pH on FSNP synthesis was determined by adjusting the pH of the reaction mixture (10 mL hydrolysate, 1.0 mM $AgNO_3$) to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, respectively. Unless otherwise stated, all the experiments were performed in triplicate.

Characterization of FSNP's

The reduction of Ag^+ ion in presence of FPH was monitored by measuring the Surface Plasmon Resonance (SPR) properties and spectral analysis of FSNP's (Shimadzu 1800). X-ray diffraction(XRD)

analysis was performed to understand the particle shape and size of FSNP's. X-ray diffraction pattern of air-dried FSNP's were recorded using Bruker D8 Advance eco, X-ray diffractometer (USA). Zeta potentials of FSNP's were determined by the Dynamic Light Scattering (DLS) technique with a Zeta-sizer (Zetasizer Ver. 7.11 Malvern, UK). The structure of FSNP's was studied by TEM. TEM was carried out using Morgagni 268D (Fei Electron Optics). Air dried FSNP's were subjected for Scanning Electron Microscopy (SEM) analysis. SEM was performed using an analytical Scanning Electron Microscope (JEOL JSM-6390) equipped with energy dispersive spectrometer (JEOL JED-2300).

FTIR analysis

FTIR analysis was performed to determine the functional group on FPH, involved in the synthesis of FSNP's. Control sample (FPH without silver nitrate) and test sample (nanoparticles synthesized after the treatment of FPH) were independently dried and blended with KBr to obtain a pellet. The FTIR spectra were recorded using Shimadzu 8400 FTIR spectrometer in the transmission mode 4000-500 cm^{-1} at a resolution of 4 cm^{-1}.

Antimicrobial activity of FSNP's coated silk cloth

Four bacterial strains *Staphylococcus aureus* MTCC740*, E. coli* DH5α*, Pseudomonas aeruginosa* MTCC 647 and *Brucella anthropi* (Accession number-ON510296) were used for the analysis of anti-microbial activity of FSNP's by using well diffusion and disc diffusion method. Before performing experiments air dried powder of FSNP's was suspended in sterilized deionized water. Each bacterial strain was grown over night in Luria Bertani broth at 35°C. After incubation bacterial strain was spread onto nutrient agar media using sterilized swab. For well diffusion assay, sterile borer was used to make well on agar plate and 10 µL (1 mg/mL) of FSNP's was introduced into the well, while FPH taken as control was introduced into the separate well of bacterial inoculum spread over the solid nutrient agar plate. While, disc diffusion method was performed. For this, circular discs of 10 mM diameter made up of silk cloth were employed[23]. Prior to anti-microbial activity, the silk cloth was immersed in different concentrations of FSNP's solution prepared in dH_2O (1, 2.5, and 5 mg/mL, respectively), for 24 h. After incubation, the silk cloth was washed twice with dH_2O and air dried. Silk cloth immersed in FPH/autoclaved double distilled water taken as control. For assessment of anti-microbial activity, silk cloth was placed on the surface of nutrient agar plate previously spread with overnight grown culture of selected bacterial strains as stated above[23,24]. After overnight incubation at 35°C, zone of inhibition was measured and pictures of the agar plates were acquired.

Results and Discussion

Silver Nanoparticles, owing to their smaller size and potential anti-microbial activity are widely used in medicine for the control of pathogenic strains[25]. In a number of studies, nano particles and their conjugates have been employed for the control of bacterial infections[26]. Thus, there is a constant thrust for novel methods for facile synthesis of nano particles. In the present study, we have made an attempt to synthesize silver nano particles from Feather Protein Hydrolysate (FPH) produced after degradation of chicken feathers by a keratinolytic strain *Stenotrophomonas maltophilia* KB13. The strain *S. maltophilia* KB13 was recovered from feather dumping site of Bilaspur, Chhattisgarh using culture enrichment technique. The feather degradation potential of strain KB13 has already been reported[18]. For the preparation of FSNP's, the FPH containing soluble protein(153 µg/mL±4.9), amino acid (88 µg/mL±5.3) was incubated with 1 mM $AgNO_3$. The reaction was instantaneous and the colour change from colourless to dark brown started after 5 min of incubation and after 10 min the colour intensified and no further colour change was observed with further increment of incubation time. The UV-Vis spectra of reaction mixture containing FPH and silver nitrate was analysed in the range of 380-780 nm. It was observed that silver nitrate treated with FPH for 5 min and 10 min exhibited the characteristic surface plasmon absorbance/resonance band of silver nano particle between 470-484 nm (Fig. 1A). Similar results have been obtained by researchers with chemical agents[10] and non-chemical agents[4]. Green synthesis of silver nanoparticles is widely researched by scientific community because it is less polluting and more efficient to synthesise as compared to their chemical counterparts. It was pertinent to study the effect of pH during the synthesis of FSNP's, as it influences the ionization state of amino acids. The effect of pH on the synthesis of nanoparticles is depicted in Fig. 1B. The results indicated that the optimum surface plasmon peak of FPH mediated

silver nanoparticles was observed at pH 7, as at this pH, the amino acids were at their optimal ionization states. Variation of pH in both acidic and basic range resulted in the loss of the plasmon peak was observed at pH 2-6 and 10, which suggested that at this pH, the ionization of amino acids were affected leading to a significant decrease in the reduction potential of FPH.

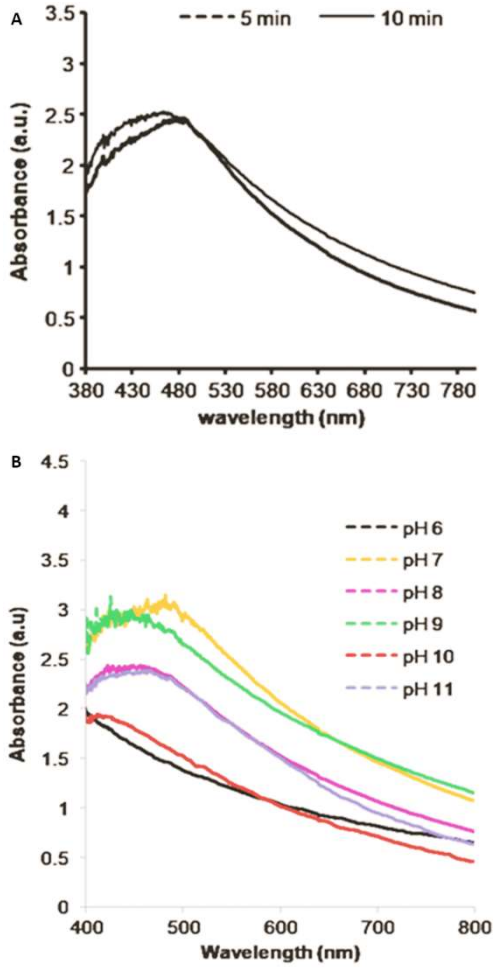

Fig.1 — (A) UV- Vis Absorption spectrum of FSNP's after 5 and 10 min incubation; and (B) Effect of varying pH on the synthesis of FSNP's

During the synthesis of FSNP's, it is anticipated that amino acids, which act as strong electron donor such as glutamic acid, aspartic acid, proline and alanine, weak electron donors such as isoleucine, leucine and valine, will act as reducing agent and reduce silver ions to elemental silver[27]. It is known that aspartic acid and glutamic acids exist in ionized state at pH 7, so it is anticipated that these amino acids play a prominent role in synthesis of FSNP's. This result is contradictory to the reports which claimed that alkaline pH is more suitable for silver nanoparticle preparation as alkaline pH provide large number of functional groups for the association of silver ion[28]. However, similar results have been reported, in which pH 7 favoured the synthesis of silver nanoparticles[29]. Our result suggested that acidic and basic pH does not facilitate the FSNP production.

The morphology of FSNP's was studied by TEM. It was observed that the size of the nanoparticles synthesized by FPH mediated reduction were in the range of 20-40 nm (Fig. 2A). Similar to this study, the size of AgNPs, in the range of 30-50 nm, prepared using *Chenopodium murale* leaf extract[13]. The results clearly demonstrate that the size range of FSNP's were similar to previous reports[13,30,31]. The nanoparticles were spherical, uniformly distributed and no clumping was observed[25]. To further validate the results, SEM EDS was performedin order to understand the structure of FSNP's (Fig. 2B & C). The results clearly indicate the crystalline nature of FSNP's[14]. The EDX spectrum confirmed the presence of elemental silver validating the synthesis of FSNP's. The strongest peak of silver at ~3 keV confirmed the elemental property of FSNP's. Silver nanoparticles typically exhibiting optical absorption signals near at~3 keV could be due to the strong SPR. The result is similar to that of previous reports[32].

XRD was also performed for the evaluation of size distribution of metallic nanoparticles. The diffraction pattern at 2θ = 37.21° (111), 46.27° (200), and 77.11° (311) indicated the Face Centered Cubic (FCC)

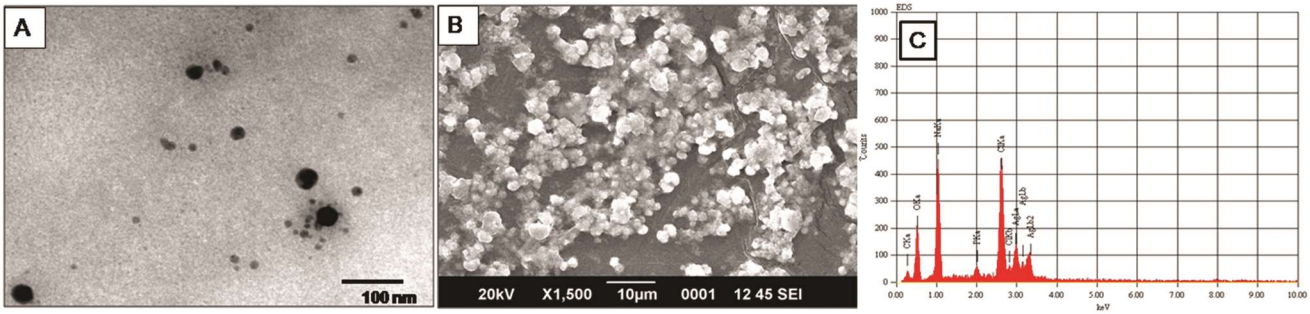

Fig. 2 — (A) TEM image of FSNP's; and (B & C) SEM and EDX analysis of FSNP's

structure of silver nanoparticles are the characteristic peaks of FSNP's (JCPDS 03-0921). Particle size of FSNP's was calculated from the XRD peaks by the Debye-Scherrer's equation; $D = 0.9\lambda/\beta \cos\theta$. Here, D is the crystalline domain diameter of silver nanoparticles, λ is the wavelength of the X-Ray source used (1.541 A°), β is the full width at half maximum of the diffraction peak and θ is used as a diffraction angle in radians. The average size was 29.957 nm confirmed the nano-sized FSNP's and corroborated with size range of 20-40 nm, as determined by TEM analysis. The diffraction pattern of FSNP's represented in (Fig. 3A), exhibits the peaks at 2θ 37.75° (111), 45.49° (200) and 77.1 (311)° reflection planes respectively represents the face centered cubic structure of metallic silver. Similar results have been observed in silver nanoparticles prepared from hydrogen reduction method and from banana peel extract[1,14]. Zeta potential value describes the hydrodynamic stability and quality of nanoparticles present in solution. Zeta potential -13.8 mV of FSNP's (Fig. 3B) represents that synthesized nanoparticles were stable, good in quality and this will prevent their cluster formation due to repulsive interactions between nanoparticles[33,34].

In order to identify the functional groups involved in reduction process, FTIR was performed. Figure 4 shows the FTIR spectrum of feather protein hydrolysate and FPH derived silver nanoparticles. The peak in FPH at 2927 cm^{-1} signifies the side chain vibrations consisting of C-H stretching. The presence of peak at 2357 cm^{-1} was attributed to the N–H stretching or the C- O stretching vibrations. Peak at 1658 cm^{-1} belongs to C-H bending. A shift in this peak (from 1658 to 1664 cm^{-1}) shown the possible participation of carboxyl of the FPH in nanoparticle synthesis. The vibration shift around 1469-1438 cm^{-1} was suggestive of the connection of aliphatic and aromatic (C–H) plane deformation vibrations of methyl, methylene groups in the reductive process. The result suggested that the amino acids with carboxylic group such as aspartic acid and glutamic acid may be involved in reducing the silver salt to silver nanoparticles[14].

Silver nanoparticles possesses excellent anti-microbial properties against clinically relevant pathogenic bacteria[25,35,36]. Anti-microbial potential of FSNP's was assessed on four clinically relevant pathogenic bacteria namely *S. aureus* MTCC740, *P. aeruginosa* MTCC647 and *B.anthropi* (Accession number-ON510296) and a non-pathogenic strain *E. coli* strain DH5α was also employed (Fig. 5). The results clearly demonstrated that FSNP's exhibited potent anti-microbial activity against all the tested strains. The zone of inhibition found in well diffusion as well as silk cloth immersed in FSNP's of different concentration (Fig. 6 and Table 1).

Similar result was also found against *S. aureus* and *P. aeruginosa* bacterial strain in well diffusion assay[37]. In this study, an attempt was also made to study the antimicrobial activity of FSNP coated silk cloth. The silk cloth was immersed for 24 h in different concentration of FSNP's (1, 2.5, and 5 mg/mL, respectively). The silk was washed twice to remove loosely bound FSNP's and their anti-

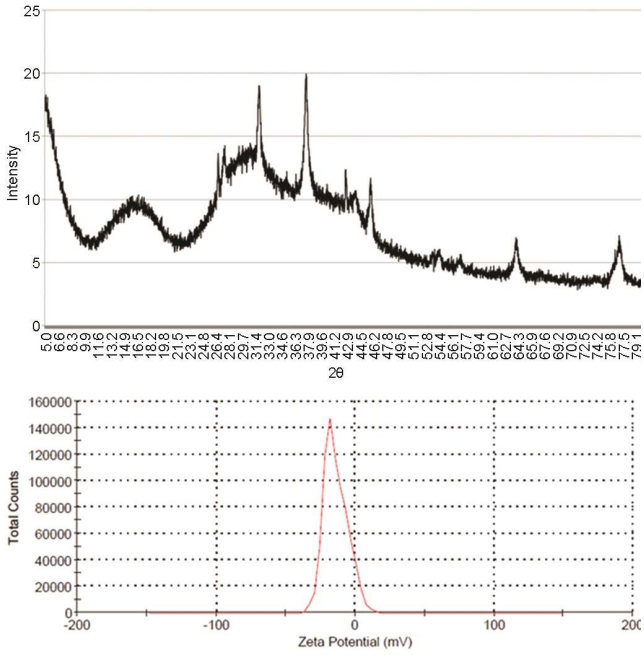

Fig. 3 — (A) XRD spectrum of FSNP's; and (B) Zeta Potential of FSNP's

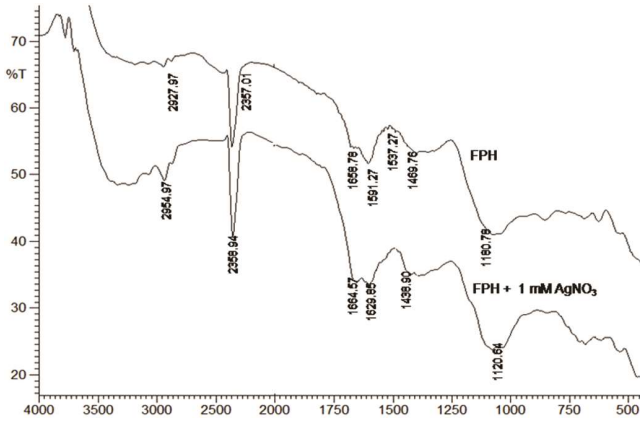

Fig. 4 — FTIR spectrum of FPH and FPH supplemented with 1 mM $AgNO_3$

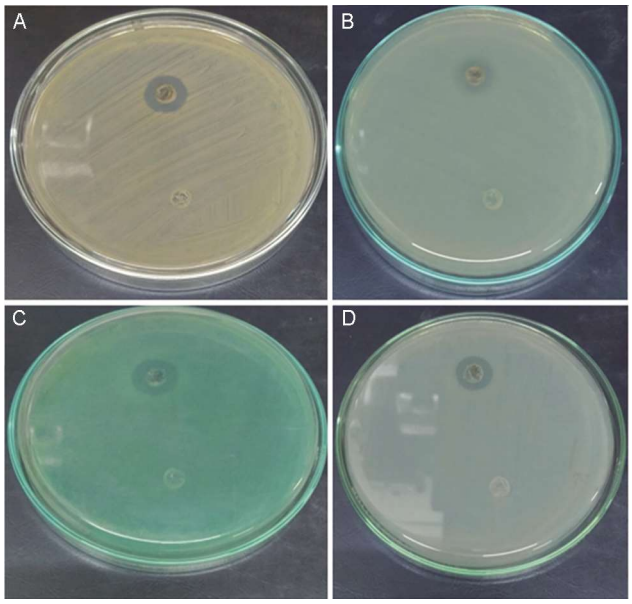

Fig. 5 — Anti-bacterial activity of FSNP's (1 mg/mL) on clinically relevant bacterial strains namely (A) *B. anthropi*; (B) *E. coli* strain DH5α; (C) *P. aeruginosa*; and (D) *S. aureus* respectively. In each well FSNP at a concentration of 10 µg (10 µL of 1 mg/mL) was added in each well and FPH was used as control

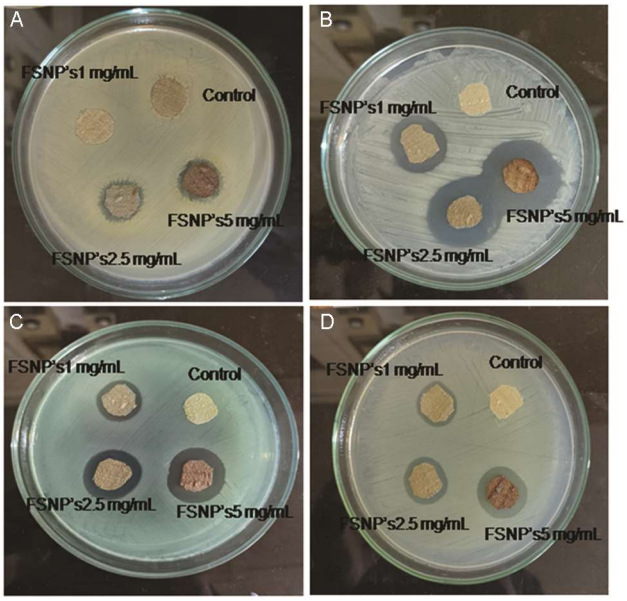

Fig. 6 — Anti-bacterial activity of silk cloth immersed in different concentration of FSNP's on clinically relevant bacterial strains namely (A) *B. anthropi*; (B) *E. coli* strain DH5α; (C) *P. aeruginosa*; and (D) *S. aureus,* respectively

microbial activity was tested by disk diffusion assay. The results demonstrated that a dose dependent antimicrobial activity was observed (Fig. 6). Silver nanoparticles owing to their high affinity with sulphur moieties, tend to bind strongly with keratin, which is

Table 1 — Anti-microbial activity of FSNP's

	Zone of inhibition					
	Well diffusion		silk disc loaded with AgNPs			
	Control	1 mg/mL	Control	1 mg/mL	2.5 mg/mL	5 mg/mL
S. aureus	-	15 mM	-	16 mM	18 mM	20 mM
E. coli	-	10 mM	-	20 mM	26 mM	30 mM
P. aeruginosa	-	14 mM	-	16 mM	20 mM	22 mM
B anthropi	-	12 mM	-	10 mM	12 mM	14 mM

main component of silk. Therefore, after two washes with distilled water, antimicrobial activity was observed. Hygroscopic nature of silk cloths makes its prone to damage and degradation facilitated by microorganism. In order to enhance the shelf-life, nanoparticles impregnated of silk cloths are in trend[38]. Silver nanoparticles are also incorporated in wound dressing for increase anti-microbial activity[39]. Nanoparticles are gaining importance as antimicrobial agents due to its broad-spectrum antimicrobial potential against bacteria and fungi[2]. Silver nanoparticles acts as prominent antimicrobial agent because they easily penetrate into the bacterial cell wall due to their small size and larger surface area. Large surface area facilitates better contact with cell wall of microorganism and while smaller size range provides good diffusion within the bacterial cell[38,40,41]. Silver nano particles have affinity for sulphur and phosphorus moieties present on cell membrane and cell machinery of bacteria[26]. Affinity with phosphorus moieties attract, accumulate the nanoparticles over the surface of bacterial cells and DNA which further get enter in bacterial cell as well as disrupt the replication frame work[42,43].While affinity with sulphur moieties provides interaction with bacterial proteins which accelerates the disintegration of respiratory and replication framework of bacterial cells resulting in cell death[6, 44].

Conclusion

We have reported a facile method for the green synthesis of silver nanoparticles using feather protein hydrolysate. The nanoparticles thus obtained exhibited typical characteristics of silver nanoparticles and also showed excellent anti-microbial properties. The silver nanoparticle synthesis through FPH is chemical free and nontoxic. The synthesis of nanoparticles using FPH will not only help to form cost effective and rapid synthesis of silver nanoparticles but also help to reduce the burden of recalcitrant feather waste.

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Conflict of interest

All authors declared no conflict of interest.

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