

**MARSILEA MINUTA(L.) PLANT EXTRACT MEDIATED SYNTHESIS OF GOLD NANOPARTICLE FOR CATALYTIC AND ANTIMICROBIAL APPLICATIONS**Niranjan Bala^{1,2}, Arpan Kool¹, Pradip Thakur^{1,3}, Sukhen Das^{1,4*}, Papiya Nandy⁵, Ruma Basu⁶¹Department of Physics, Jadavpur University, Kolkata- 700032, India.²Department of Botany, Sreegopal Banerjee College, Hooghly-712503, India.³Department of Physics, Netaji Nagar College for Women, Kolkata-700092, India.⁴Department of Physics, Indian Institute of Engineering Science and Technology, Howrah-711103, India.⁵Centre for Interdisciplinary Research and Education, 404B Jodhpur Park, Kolkata-700068, India.⁶Department of Physics, Jogamaya Devi College. Kolkata-700026, India***Corresponding author e-mail:** sdasphysics@gmail.com**ABSTRACT**

Gold nanoparticle (GNP) was synthesized using the plant extract of *Marsilea minuta* (L.) for catalytic and antibacterial applications. Formation of nanoparticle was confirmed by various physical and chemical characterization techniques. Morphology of synthesized NPs was analyzed using electron microscopy. GNP synthesized via this method was highly stable and their size was found to be ~ 25 nm. Green synthesized GNPs showed well catalytic activity in complete reduction of toxic para-nitrophenol (PNP) to para-aminophenol (PAP). GNP synthesized by this method showed better antibacterial potency against *Escherichia coli* and *Staphylococcus aureus*. It was observed that GNP synthesized via this method showed synergistic effect of chemically synthesized GNP and plant extract on antimicrobial activity.

Keywords: Gold nanoparticles; green synthesis; catalyst; para-nitrophenol; antimicrobial activity; synergistic effect.**INTRODUCTION**

During the course of improvement of nano-science, GNP drew the attention of research workers for their unique optical [1-2], chemical [3-4] and photochemical activities [5-6]. Along with these GNP were applied in biological fields like nano-medicine [7], drug delivery [8-9] and cancer therapy [10-11] for their noble nature and easily tunable size. Predictable synthetic approaches of gold nanoparticles are mainly involves toxic chemicals. Recently another approach has been become very attractive that using different organic compounds present in plants [12-13] like phenolic compounds, flavonoids, alkaloids, organic acids [14-16, 17-23] which can reduce Au³⁺ or Au¹⁺ into Au⁰ by donating electron and also stabilize the newly synthesized

GNPs simultaneously. Green synthesis of nanoparticles have drawn attention not only for avoiding the use of toxic chemicals during synthesis procedure, but for the reason that these nanoparticles are biocompatible, nontoxic, ecofriendly in nature and suitable for biomedical applications [14-16, 24]. Nitro-aromatic compounds like nitrophenols were abundantly distributed in environment [25]. PNP is one of the major members within the nitrophenols family produced as a by-product during industrial manufacture of pesticide, herbicide and synthetic dyes [26]. It was applied in agriculture, dyes and pigments production, manufacturing fungicide for leather [27-28]. PNP was also used for production of drugs like acetaminophen, a non- aspirin pain reliever, parathion and fluoridifen like pesticide [29]

PNP showed toxicity on aquatic organisms like algae, invertebrate, fish [30-32]. Widespread use of nitro-phenol PNP results in great hazards for all life forms of the ecosystem ranging from unicellular organism to human beings. Mainly, in water bodies these aromatic compounds reduce the photosynthetic potential of aquatic organisms by interfering with the entering sunlight and soluble gas balance, leading to the destruction of aquatic ecosystem [33].

In the present study inexpensive, highly stable GNP was synthesized by one step method using crude extract of a pteridophytic plant *Marsilea minuta*. Formation of the particles was confirmed by UV-visible spectroscopy (UV-vis) and X-ray diffraction (XRD). Morphology and size of NP were analyzed using dynamic light scattering (DLS), FESEM and HRTEM. Chemical constituents of synthesized NPs were analyzed by FTIR. Stability to salinity of these NP was compared with chemically synthesized GNP. Catalytic activity of biosynthesized GNP was studied in the reduction of environmentally toxic PNP and potency for antibacterial activities was examined against *Escherichia coli* and *Staphylococcus aureus* microorganism. Mechanism for antibacterial activity was confirmed by FESEM studies.

MATERIALS AND METHODS

Materials: *Marsilea minuta* (Indian native name: Sushni) a pteridophytic plant collected from Jadavpur University campus. Tetrachloroauric (III) acid (HAuCl_4) was purchased from Sigma Aldrich. PNP was purchased from Merck (Germany). All other chemicals used in this experiment were purchased from Merck (India).

Plant extract preparation: 1 gm of *Marsilea* leaves were washed 4-5 times thoroughly with distilled water and then surface sterilized with 90% ethanol. Subsequently, boiled with 20 ml double distilled water for 5 min and cooled to room temperature. Then the plant extract (pH7) was filtered with Whatman filter paper no.1 and stored at room temperature for further use.

Synthesis of gold nanoparticles: 2 ml aqueous solution of 0.01 M of Tetrachloroauric (III) acid (HAuCl_4) was mixed with 100 μl of the plant extract under continuous stirring. Initially, the colour of the solution was yellowish and after 30 min of continuous stirring red wine colour of GNP appeared indicating the formation of GNP (Fig. 1). The reaction mixture was allowed to 4 hours for complete reduction of gold ions. Optimal condition for the synthesis of green GNPs was checked against

different concentrations of plant extract to HAuCl_4 within a range of 25-200 μl . Citrate capped chemically synthesized GNP was obtained following the protocol of Polavarapu and Xu (2009) [34].

Characterizations: UV-Vis light spectra of synthesized nanoparticles were recorded in $\lambda 25$ spectrophotometer (Parkin Elmer, Germany). Particle size and particle distribution of aqueous solution of GNPs were analyzed by DLS with the help of Zetasizer-5000 (Malvern Instruments, UK). Morphology and particle size analyzed using FESEM (Inspect F50, FEI, Netherland) and HRTEM (JEM – 2100 HRTEM, JEOL, Japan). For FESEM study GNP solution was drop casted on glass cover slip and NP solution was sprayed on copper grid for HRTEM analysis. XRD pattern of synthesized green GNP was analyzed in the range of $2\theta \sim 35-80^\circ$ using powder diffractometer (D8, Bruker AXS,) by $\text{Cu K}\alpha$ radiation ($\lambda = 0.15425 \text{ nm}$). Biosynthesized GNPs solution was drop casted over cover glass and dried at 60°C for 2 hours for XRD analysis. FTIR spectroscopy recorded using a JASCO FTIR instrument-410 in the range of $4000-400 \text{ cm}^{-1}$ to investigate the chemical constituents of the plant extract and synthesized GNP after centrifugation and washing. The pellets were first prepared in Potassium Bromide (KBr) and samples were sprayed on it by drop casting.

Antimicrobial study: Investigate the effect of GNP on bacteria, antimicrobial studies were performed on *Escherichia coli* DH5 α (MTCC 1652) and *Staphylococcus aureus* (MTCC 96). Antimicrobial effect of the plant extracts and GNPs synthesized via green and chemical routes were studied following the standard protocol. 0.5 mg of lyophilized GNP was dissolved in 1ml of autoclaved water and different amount of the solution and plant extract (50-400 μl) were added to cultures of bacteria (10^7 CFU/ml) in 5 ml nutrient broth (0.5 % peptone, 0.1 % beef extract, 0.2 % yeast extract, 0.5 % NaCl, pH 7). So, the resultant GNPs concentration in nutrient broth was 5-40 $\mu\text{g/ml}$. The cultures were then incubated at 37°C for 24 h. Growth inhibition was followed by plating 50 μl of the treated culture on nutrient agar plates (nutrient broth with 1.5 % agar as the solidifying agent). Bacterial colonies were counted and compared with control after 24 h incubation at 37°C . The whole experiment was repeated thrice to ensure reproducible data. The same experiment was conducted with corresponding amounts of chemically synthesized GNP and crude plant extract of *M. minuta*.

The growth of bacteria was evaluated by counting colony forming unit (CFU) on agar plate. The

antibacterial efficiency was calculated using the equation 1.

$$M = \frac{B - C}{B} \times 100 \quad (1)$$

Where, M is the mortality rate (%), B is the mean number of bacteria on the control samples (CFU/sample) and C is the mean number of bacteria on the treated samples (CFU/sample).

Minimum bactericidal concentration (MBC) values were calculated using the standard plate count technique [35] with increasing concentrations of GNP.

Relative decrease of MBC values were calculated by the equation 2.

$$X = \frac{A - B}{B} \times 100$$

Where, X is relative decrease in MBC value, A is the mean value of chemically synthesized GNP/ crude plant extract and B is the mean MBC value of green synthesized GNP.

Mechanism behind GNP induced bacterial death was investigated by FESEM. For FESEM study bacterial samples were prepared following the standard protocol [36-37] protocol. Briefly, bacterial sample in mid exponential phase (10^7 CFU/ml) were treated with 20 μ g/ml of green GNP for 12 hour at 37°C. Control was prepared under similar condition but without GNP. Bacterial samples were then fixed with 2% glutaraldehyde. After washing with water, 1 μ l was placed on a silicon platelet (Plano, Wetzlar, Germany). Samples were passed through a gradient of ethanol dehydration steps followed by staining with 3% ethanolic solution (25 %) of uranyl acetate. Finally, the samples were washed with 0.1 M phosphate buffer solution (pH 7.2) and sputter coated with gold [36]. Microscopy was performed with Inspect F50 (FEI, Netherlands).

Catalytic Activity: Catalytic property of synthesized GNP was investigated by the reduction of para-nitrophenol (PNP) with sodium borohydrate. PNP is a common toxic byproduct of pesticide, herbicide and synthetic dye production [26]. PNP was easily reduced to para-aminophenol (PAP) by NaBH₄ in presence of gold in nano forms. [38]. Catalytic measurements were studied following the protocols of Pougou, Z. D. [39].

Stability to NaCl: Agglomeration is a big problem for colloidal nanoparticles. Stability of biosynthesized GNP was compared to chemically synthesized GNP. Stability of these particles were checked against very high salt concentration (5 M NaCl) following the protocol of S Pandey *et al.*[15]

RESULT AND DISCUSSION

Effect of crude plant extract on GNP synthesis:

GNP synthesis started after addition of aqueous plant extract to HAuCl₄. Initial colour of the reaction mixture was yellowish and gradually became reddish. Reduction of HAuCl₄ was complete after 4 h and wine red coloured green GNP was formed (Fig 1). Surface Plasmon resonance (SPR) band at 522 nm confirmed the formation of GNP by UV-VIS spectra (Fig. 2A). It was evident from the figure that complete synthesis of GNP took around 4 hours, the particles were quite stable and no agglomeration was detected even after 30 days. GNP formation started at above 1 % (v/v) plant extracts (Fig. 2B). With higher plant extract concentration SPR band intensity increased gradually indicating formation of more GNP and optimized at a concentration of 5% plant extract. Further addition of plant extract to the reaction mixture showed red shifting of SPR band. It was observed that 0.005M of HAuCl₄ is the minimum concentration for the green synthesis of GNP and GNP population optimizes at a concentration of 0.01M (Fig: 2C). Further increase in HAuCl₄ concentration did not show any significant difference, which means that the bioactive compounds present in plant extract was unable to synthesize more particles. To synthesize GNP (<40 nm) for biomedical application nearly 5 % (v/v) plant extract and 0.01M HAuCl₄ were required.

Functional groups involved in GNP synthesis:

FTIR spectrum of aqueous extract of *M. minuta* showed presence of characteristic bands for several functional groups (Fig. 4 C). IR peaks for phenolic – O-H Stretch were observed at around 3154 cm⁻¹ and 3122 cm⁻¹. Presence of phenolics were confirmed by C-C=C symmetric stretch, C-C=C asymmetric stretch, C=C-H asymmetric stretch and aromatic C-H bend at 1596, 1474, 3022 and 2848 cm⁻¹ respectively [14]. Amines were also present in the plant extract. Presence of aromatic amines (-C₆H₅NH₂) and aliphatic amines (R-NH₂) were confirmed by 1118 cm⁻¹ and 1390 cm⁻¹ bands. IR band at 1040 cm⁻¹ supports the presence of carbonyl (>C=O) groups of carboxylic acids. These findings are supported by some previous reports on phytochemical profiling of *M. minuta* [35]. Aqueous extract of plant contains phenolic compounds,

flavonoids, saponins, tannins and alkaloids. IR bands of these compounds justified their presence. FTIR spectra of bio-stabilized GNP was also carried out (Fig 4 D) to investigate whether any compound present in the extract with free carboxylic acid group (-COOH) or amino group (-NH₂) were attached to gold surface during GNP synthesis. Aqueous extract of *M. minuta* rich in compounds with free hydroxyl and amino group (-OH, -NH₂) can donate their electron to Au³⁺ ions to form Au⁰ and carboxylic and amino moiety can bind to Au⁰ to stabilize GNP [35].

Stability of nanoparticles: The stability of nanoparticles was the major factor for biomedical applications. Agglomeration was triggered by high

ionic concentration in body fluid. In our present work, we investigate the stability of green GNP and compared with chemical GNP. It was found that the stability of green GNP were much better than those of the chemical GNP (Fig. 5A & B). There was a shift of 6 nm in SPR band occurred in case of green GNP after addition of 5 ml of 5 M NaCl (Fig. 5 B), while SPR band shifted of 153 nm for chemical GNP after addition of only 800 µl of NaCl (Fig. 5 A). Green synthesized GNP synthesized using *M. minuta* extract shows minimum shifts in SPR band compared to that of the GNP synthesized by Pandey et al. [15]. From this observation it was clear that these green GNP were suitable for biomedical applications as it is stable in higher salt concentration.

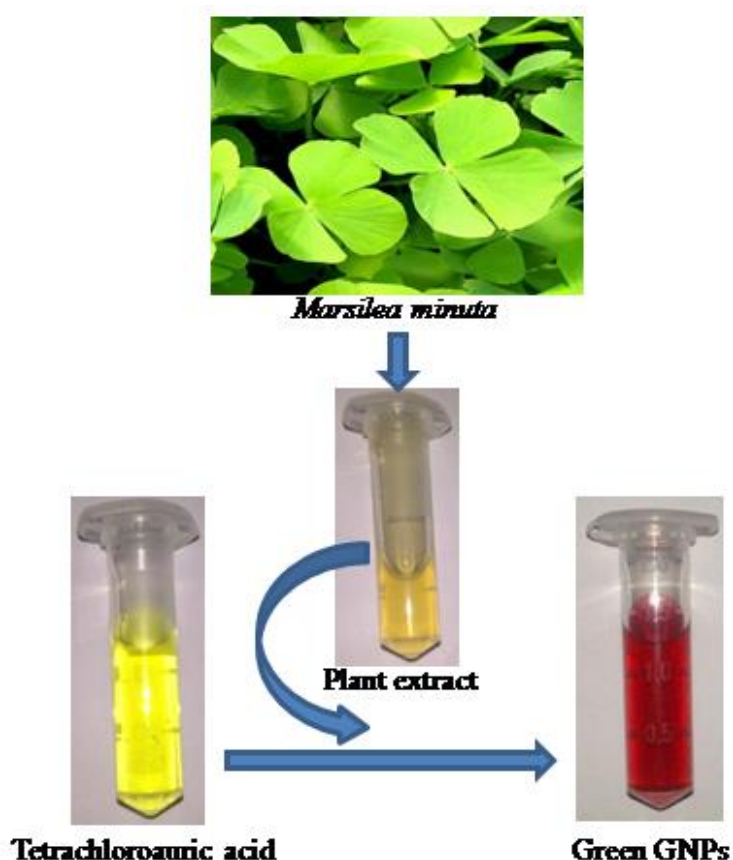


Fig 1: Overview of green GNP synthesis.

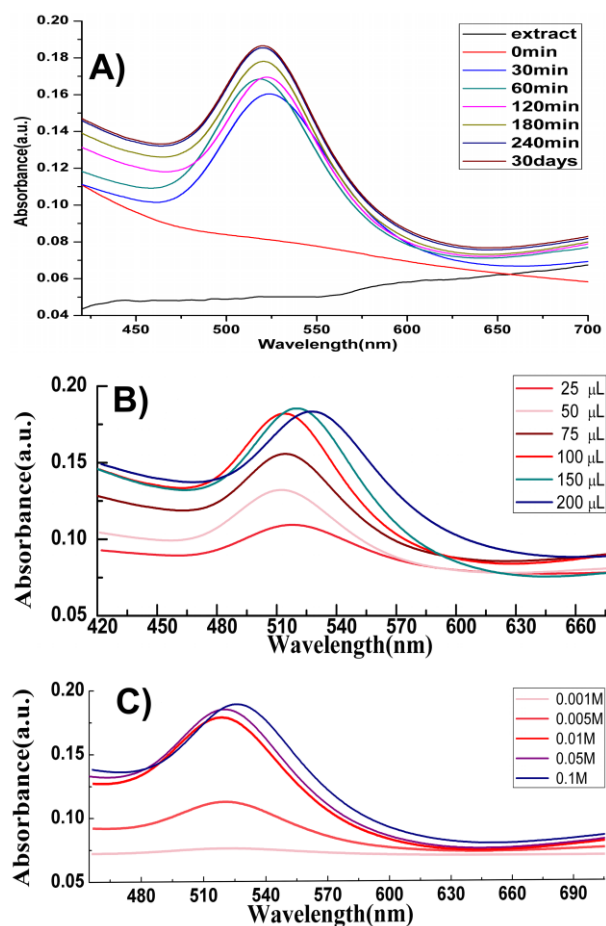


Fig. 2: Uv-vis spectra of green GNP; A) time dependent formation of green GNP, B) role of various concentration of plant extract and C) various concentration of HAuCl₄ in synthesis of green GNP.

DLS study confirmed the average particle size of 35 nm with the poly dispersion index of 0.389. The sizes of the particles were in the range from 10-60 nm (Fig 3 A). Average zeta potential of the particles was -14.35 ± 0.05 mV. Particle size and distribution obtained from DLS were further confirmed by HRTEM micrograph. The average size of the green synthesized particles was 25 nm with a size distribution from 15-35 nm (Fig 3 B). Nearly similar sized particles were observed in case of chemically synthesized GNP (Fig. 3 C). HRTEM micrographs of GNP shows particles were crystalline in nature and most of them were spherical in shape.

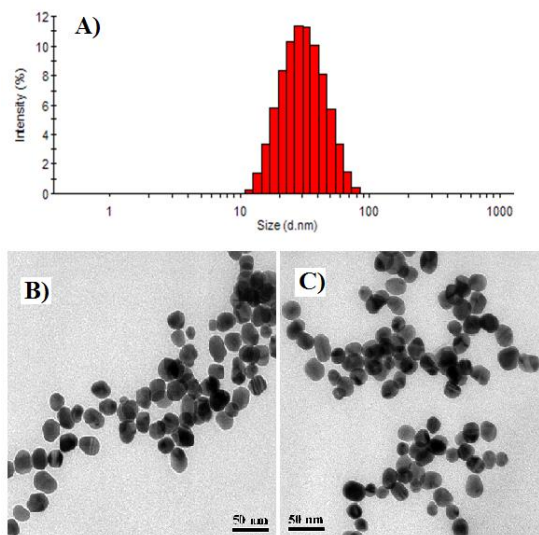


Fig. 3: A) Size distribution curve of green GNPs obtained from DLS study; HRTEM micrograph of B) green synthesized GNP at high magnification (scale bar 50 nm) and C) chemically synthesized GNP at high magnification (scale bar 50 nm).

FESEM micrographs confirmed well distribution of particles, spherical in shape (Fig 4A). Particle size was same as recorded in HRTEM. XRD patterns of the prepared samples are shown in Fig 4B. X-ray diffractogram show 2θ values at 37.46, 43.58, 64.22 and 77.08 corresponding to (111), (200), (220) and (311) planes which confirms the presence of GNP. All the peaks are duly assigned using JCPDS file No 04-0784. From the x-ray pattern it was observed that sample was well crystalline.

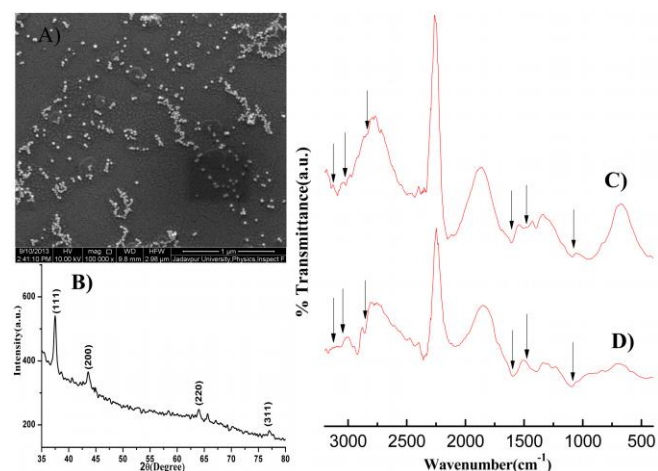


Fig. 4: A) FESEM micrographs, B) XRD pattern of synthesized green GNP; FTIR spectra of C) crude plant extract and D) GNP synthesized with plant extract

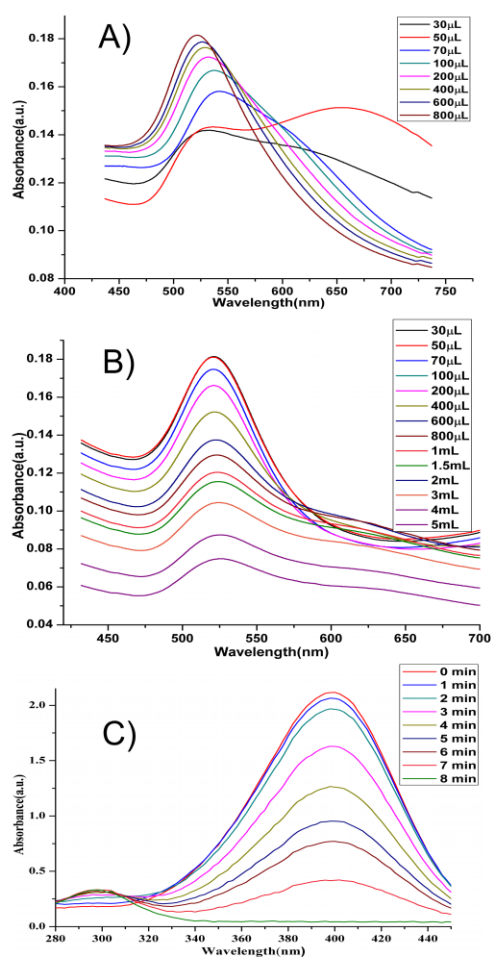


Fig. 5: B) stability of chemical GNPs to NaCl, C) stability of green GNP to NaCl and D) catalysis of PNP to PAP by green GNP.

Catalytic property: Reduction of PNP to PAP by NaBH₄ was enhanced by metallic catalyst. In presence of sufficient amount of GNP and excess NaBH₄, the reaction was pseudo-first-order reaction and depends only on the concentration of PNP in the reaction mixture. The role of GNP in this catalytic reaction was to bind PNP with two oxygen atoms of nitro group (-NO₂) [39]. PNP and PAP showed absorption peaks at 400 nm and 315 nm respectively. Results from UV-Vis spectra (Fig. 5C) clearly supported the view that no absorption peak at 315 nm in the beginning of reaction (t₀). There was a gradual decrease in absorption peaks at 400 nm and gradual increases in peak at 315 nm which is associated with PAP. Thus more and more PAP formation takes place until there is no PNP in the reaction mixture. In present experiments, 50 µl of GNP is enough to catalyze 200 µl of 100 mM PNP within 8 minutes.

Synergistic effect of *M. minuta* extract stabilized GNP in antimicrobial tests: Antimicrobial effect of aqueous extract of *M. minuta* was previously investigated [40-41]. GNP also exerts their bactericidal effects on *E. coli* and *S. aureus* [21, 42-43]. We investigated the antimicrobial effect of green GNP and compared its efficiency with the plant extract and also with chemically synthesized GNPs. From Table 1, it was clear that the MIC and MBC values for each strain of bacteria was lower in green GNP than each of chemical GNP and crude plant extracts. It was clear from Fig. 6 and Fig. 7 that green GNP shows much more bactericidal efficiency over chemical GNP and crude plant extract in bacterial sample *E. coli* and *S. aureus* respectively. Comparative analysis of mortality was shown in Fig 8 and it was evident that green GNP was more efficient against both the bacterial samples as compared to the crude plant extract and chemically synthesized GNP. MBC of *E. coli* and *S. aureus* for green GNPs were found to decrease by ~28.57% and ~33.33% relative to that of chemical GNPs while MBC decreased by ~44.44% and ~40% relative to that of the crude plant extracts. Result also suggested that, our green GNP has more effective antibacterial potency than GNP synthesized via other green routes [21, 43]. Annamalai et. al. (2013) showed that 200 µg/ml bacteria killed 88% *E. coli* cell [21], but green GNP synthesized by our protocol kills more than 90% bacterial cell at a concentration of 25 µg/ml.

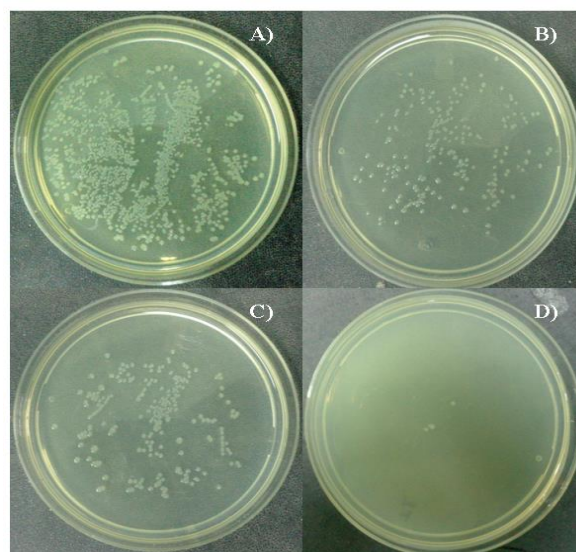


Fig. 6: Anti microbial effects on *E. coli*; A) control, B) with plant extract, C) with chemically synthesized GNP and D) green GNP.

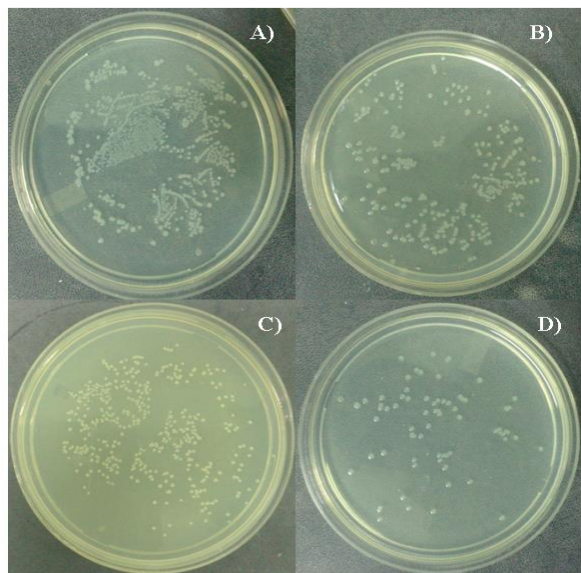


Fig. 7: Antimicrobial effects on *S. aureus*; A) control, B) with plant extract, C) with chemically synthesized GNP and D) green GNP

Table 1: MIC and MBC values of chemical GNP, crude plant extract and green GNP on *E. coli* and *S. aureus* respectively.

Bacterial strain	Chemically synthesized GNPs (µg/ml)	Crude plant extract (µl/ml)		Green GNPs (µl/ml)		
	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. coli</i> (MTCC 1652)	20	30	30	60	10	25
<i>S. aureus</i> (MTCC 96)	20	40	30	80	15	30

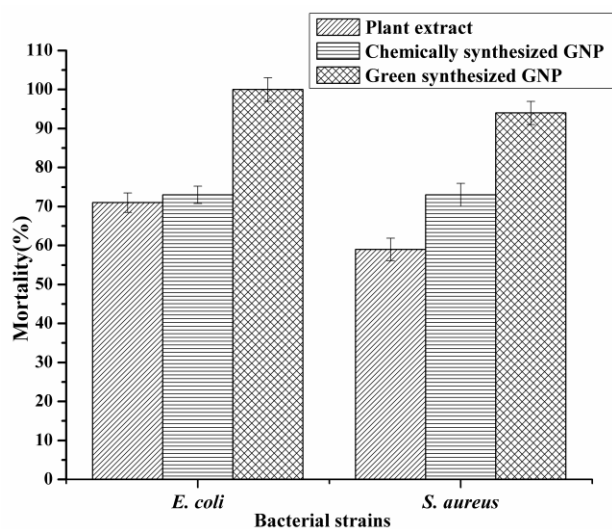


Fig. 8: Mortality (%) of bacterial samples treated with plant extract, chemically synthesized GNP and green synthesized GNP.

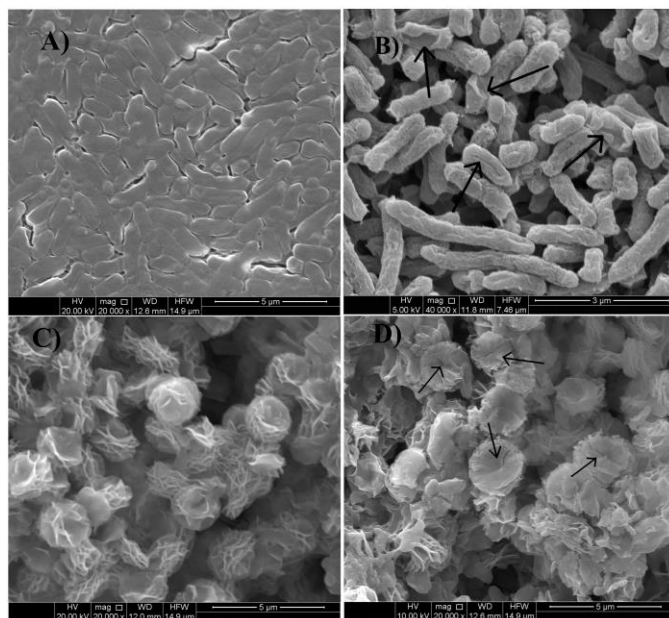


Fig. 9: FESEM micrographs of bacteria; *E. coli*. A) Without green GNP, B) treated with green GNP; *S. aureus*. C) Without green GNP, D) treated with green GNP.

FESEM micrographs of control and green GNP treated bacteria were analyzed (Fig 9). It was clear from the micrographs that, control *E. coli* showed rod like morphology, uninterrupted wall, confirming healthy nature of bacteria (Fig 9 A). GNP treated bacterial cell shows interrupted cell walls and cell wall breakage (showed with arrow) which reveals that, cells was dead (Fig 9 B). Lots of debris was also present in those micrographs due to cell rupture. Control set of *S. aureus* cell shows healthy morphology (Fig 9 C) but GNP treated cell showed unusual nature in cell shapes, breakage in cell walls were also observed (Fig 9 D). From the FESEM study of bacterial cells, it can be assumed that cell wall breakage and consequent ions and cell material leakage from the bacterial cell may be responsible for green synthesized GNP induced potential antibacterial activity.

Use of pteridophytic plants for curing bacterial infection in throat, boil and wound healing as folk medicine are well known [44]. The mechanism of antimicrobial effect of *M. minuta* extract was not known fully. The effect may be due to presence of phenolic compounds [45]. Flavonoids forms complex structures with cell wall materials and increases plasma membranes permeability, ultimately ion leakage induces cell death [46-47]. Hydroxyl group of polyphenols triggers hydroxylation of bacterial cell, which becomes toxic to bacterial cell [46].

Antimicrobial effects of GNP are well known. The possible mechanism of action was investigated by Yan Cui et al. [48]. GNPs change membrane potential and lower the production of ATPs leading to decrease in metabolism and ultimately cell deaths. Another mechanism was to inhibit ribosome from binding with t-RNAs as a result of which biological process collapsed. Mechanism of GNP induced antimicrobial activity was ROS independent which shows low cytotoxicity.

Green GNPs showed synergistic effects on antimicrobial efficiency. This is due to, green GNPs playing dual roles: plant metabolites like phenolics, flavonoids kill cell in a normal way and in addition GNPs exert their functions by down regulating ATP synthase activity and inhibiting ribosome-tRNA complex formation. Cell wall breakage and consequent ions and cellular material leakage from the bacterial cells were responsible for green synthesized GNP induced potential antibacterial activity.

Conclusion

Simple, eco-friendly and cost effective synthesis route of GNPs was developed without any involvement of hazardous chemicals. Green GNPs were merely 25 nm in size and were highly stable in high ionic concentration i.e. suitable for biomedical application. These particles showed good catalytic activity on transformation of toxic p- nitro phenol to nontoxic p- aminophenol and also have the potency

to kill bacteria more efficiently than chemically synthesized GNPs. GNPs synthesized using *M. minuta* shows better stability, antimicrobial potency compared to other green routes. It would act as a very effective catalyst and antimicrobial agent for biomedical applications.

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