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Research Paper

Green Synthesis of AgNPs Stabilized with biowaste and their antimicrobial activities

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Abstract

In the present study, rapid reduction and stabilization of Ag+ ions with different NaOH molar concentration (0.5 mM, 1.0 mM and 1.5 mM) has been carried out in the aqueous solution of silver nitrate by the bio waste peel extract of *P.granatum*. Generally, chemical methods used for the synthesis of AgNPs are quite toxic, flammable and have adverse effect in medical application but green synthesis is a better option due to eco-friendliness, non-toxicity and safe for human. Stable AgNPs were synthesized by treating 90 mL aqueous solution of 2 mM $AgNO_3$ with the 5 mL plant peels extract (0.4% w/v) at different NaOH concentration (5 mL). The synthesized AgNPs were characterized by UV-Vis spectroscopy, TEM and SEM. Further, antimicrobial activities of AgNPs were performed on Gram positive *i.e.* Staphylococcus aureus, *Bacillus subtilius* and Gram negative *i.e. E. coli*, *Pseudomonas aeruginosa* bacteria. The AgNPs synthesized at 1.5 mM NaOH concentration had shown maximum zone of inhibition (ZOI) *i.e.* 49 ± 0.64 in *E. coli*, whereas *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilius* had shown 40 ± 0.29 mm, 28 ± 0.13 and 42 ± 0.49 mm ZOI respectively. The MIC value of 30 μg/mL observed for *E. coli* Whereas, *Staphylococcus aureus*, *Bacillus subtilius* and *Pseudomonas aeruginosa* had shown 45 μg/mL, 38 μg/mL, 35 μg/mL respectively. The study revealed that AgNPs had shown significant antimicrobial activity as compared to Streptomycin.

Key words: Silver nanoparticles, biowaste, antibacterial activity, MIC, SEM, TEM.

Introduction

Recently, nanoparticles are used in multidisciplinary areas such as biomedicine, biocatalysis, electronics, chemistry and energy due to their extensive applicability. These particles have small size (1-100 nm) and elevated surface area which resulted in increase reactivity, spectacular alteration in optical, electronic and chemical properties which are significantly different from bulk materials (Catauro *et al.*, 2004; Stevanovic *et al.*, 2012; Vijayakumar *et al.*, 2013). Silver nanoparticles (AgNPs) have more concerned as compare to other metallic nanoparticles (MNPs) due to their unique properties like magnetic and optical polarizability, electrical conductivity and antimicrobial activities (Evanoff and Chumanov, 2005). As Inorganic agents (*i.e.* Ag) have already been used in various medical and industrial processes for an inhibitory effect towards many bacterial strains and microorganisms (Saxena *et al.*, 2010; Jain *et al.*, 2009; Latha and Kannabiran, 2006; Krishnamurthy *et al.*, 2012). AgNPs can be used to destroy microorganisms on textile fabrics (Vivek *et al.*, 2011; White *et al.*, 2012) or they can be employed for water treatment (Binupriya *et al.*, 2010). The capability of pathogenic bacteria to get resistance against antibacterial agents is a tremendous problem in medical practice which limits the efficacy of these drugs (Quelemes *et al.*, 2013). These drawbacks give researchers tremendous opportunities to develop new substances like AgNPs to combat them. Green synthesis of nanoparticles using plants or plant derived extracts is good option over chemical and physical methods because it is rapid, non toxic, eco-friendly, cost effective, don't require high pressure, temperature, toxic chemicals and compatible for pharmaceutical and biomedical applications (Vivek *et al.*, 2011). Plant-based nanoparticles synthesis has advantages

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over other biological methods because of their rapid reaction rate for the synthesis of nanoparticles (White *et al.*, 2012).

In the present study, rapid reduction and stabilization of Ag+ ions with different NaOH molar concentration in the aqueous solution of silver nitrate by the bio waste peel extract of *P.granatum* reported. Further, the anti-bacterial activity of these biologically synthesized nanoparticles performed against Gram positive (G+) and Gram negative (G-) bacteria.

Experimental

Punica granatum were collected from the National Institute of Ayurveda, Jaipur. Further, plant was identified and registered (Reg. No. RUBL21110) by Herbarium, Department of Botany, University of Rajasthan, Jaipur, India. *Punica granatum* peels were removed and dried under shade at room temperature for about 10 days. The dried peels were powdered by mechanical grinder and sieved to give particle size 50-150 mm. Powder (34 g) was filled in the thimble and extracted successively with 70% ethanol in soxhlet extractor at 40 °C for 48 h. The extracts were concentrated to dryness using rotary evaporator and used as reducing and capping agent. The stable AgNPs were synthesized by treating 90 mL aqueous solution of AgNO3 (2 mM) with 5 mL filtered (0.45 μm) peels extract (0.4% w/v) and 5 mL NaOH of different molar concentration (0.5 mM, 1.0 mM and 1.5 mM) at room temperature (25 °C) for 20 min (Vasireddy *et al.*, 2012). The obtained solutions were centrifuged at 15,000 rpm for 20 min (Vijayakumar *et al.*, 2013; Gan *et al.*, 2012) subjected to purification and dried for the analysis of the prepared AgNPs. UV-Vis spectral analysis was done between a range of 300-600 nm using a double-beam spectrophotometer (Hitachi, U-3010) with all the samples dispersed in distilled water and kept in a quartz cuvette with a path length of 10 mm (Vasireddy *et al.*, 2012). Scanning electron microscopy (Carl Zeiss EVO® 18 electron microscope) and Transmission electron microscopy (FEI Tecnai T20 TEM System) for the morphological analysis of the prepared AgNPs samples was performed (Vasireddy *et al.*, 2012).

Screening of nanoparticles using disc diffusion method

The antibacterial activities of the synthesized AgNPs were studied against four bacteria, viz. *Staphylococcus aureus* (G+), *Bacillus subtilis* (G+), *Escherichia coli* (G-), and *Pseudomonas aeruginosa* (G-) by discs diffusion method (Gould, 1952; Rios *et al.*, 1988; Kim *et al.*, 2007). Standard size Whatman No. 1 filter paper discs, 6.0 mm in diameter, sterilized by moist heat at 121 lb in an autoclave for 15 min were used to determine antimicrobial activity of AgNPs (Bhadauria and Kumar, 2012). Muller Hinton Agar (MHA) medium was poured into autoclaved petriplates and allowed to solidify. The homogeneous suspension (100 μL) of test inoculums 1-5 x 10^6 cfu/mL was used for inoculation over the respective agar medium plates. Sterilized filter paper discs were impregnated with 50 μL of AgNPs (100 μg/mL) and placed over the surface of agar plates containing bacterial culture. Negative controls were prepared in the same way but using 50 μL of pure solvent (autoclaved distilled water) on sterile discs. Similarly, The disc of control antibiotics *i.e.* Streptomycin sulphate (100 g/mL) for antibacterial activity were also aseptically placed over the seeded agar plates for comparison of antibacterial activity of AgNPs. The plates were incubated at 37 °C for 24 h after which the average diameter of the inhibition zone surrounding the disk was measured with a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for each type of nanoparticles (0.5 mM, 1.0 mM, 1.05 mM) and with each microbial strain were based on six replicates (Qi *et al.*, 2004). The activity index was calculated on the basis of the size of the inhibition zone by the following formula:

$$\text{Activity index} = \frac{\text{Inhibition zone of sample (mm)}}{\text{Inhibition zone of standart (mm)}}.$$

Determination of the minimum inhibitory concentration (MIC)

The lowest concentration of AgNPs that exhibits antibacterial activity was quantified by modified tube dilution method (Qi *et al.*, 2004). The MIC was determined based on batch cultures containing varying concentration of AgNPs in suspension (20-100 μg/mL) (Ruparelia *et al.*, 2008). Muller Hinton broth media were poured into a 16-by 125-mm glass tubes and autoclaved. A standard suspension of test bacterium (~0.5 McFarland standard), was prepared for bacterial inoculums (1-5 x 10^6 cfu/mL). The Ag-NPs solutions of different pH (9-11) and concentration were diluted with Mueller-Hinton broth and inoculated with the tested bacterial suspension. The tubes were then incubated to determine the MIC. The high rotary shaking speed was selected to minimize aggregation and settlement of the nanoparticles over the incubation period. Lower rpm setting during incubation may cause underestimation of the antimicrobial activity of the nanoparticles (Ruparelia *et al.*, 2008). All the experiments were carried out in triplicate. The average bacterial growth was measured as increase in absorbance at 600 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments included a positive control (tubes containing nanoparticles and Mueller-Hinton broth, devoid of inoculum) and a negative control (tubes containing inoculum and Mueller-Hinton broth, devoid of nanoparticles. The negative controls indicated the microbial growth profile in the absence of nanoparticles. Afterwards, the growth in all tubes at different concentrations of AgNPs was compared

with that of the nanoparticles-free control in order to determine inhibition after 24 and 48 hours of incubation.

Statistical analysis

Statistical analysis was carried out by SPSS version 16.0 software. The result express as arithmetic mean ± SD.

Results and Discussion

In the present study, the $AgNO_3$ solution immediately turned dark brown after the addition of P. garnatum peel extract as a reducing and stabilizing agent in all of the samples of different NaOH molar concentrations, which shows the formation of AgNPs (Figure 1). The oxidation reaction of phenol groups (Figure 2 a-d) in peel extract was responsible for the reduction of silver ions (Wang *et al.*, 2007; Soundarrajan *et al.*, 2012). It is observed that addition of 0.5 mM (I) NaOH showed broadening of the surface plasmon resonance (SPR) peak at 406 nm. Whereas, addition of 1.0 mM (II) NaOH shifted the absorption peak at 401 nm and 1.5 mM (III) NaOH resulted in a blue shift of λmax to 395 nm (Figure 3). Study revealed that increase in NaOH concentration may accelerate the nucleation process which increases the absorption intensity and the shifting of absorption peaks may be due to decrease in the particle size of Ag-NPs (Vasireddy *et al.*, 2012). Further, the phenolic groups of flavonoids and glycosides (Figure 2 a-d) of *P. granatumpeels* (Van Elswijk *et al.*, 2004; Jasuja *et al.*, 2012) act as a reducing agent may be ionized at higher molar NaOH concentration which leads rapid reduction reaction and synthesized spherical particles of AgNPs. The mechanistic reaction of the formation of AgNPs is expressed in Figure 4.

The electrons moves freely in conduction band and valence band which lie very close to each other in Metal NPs *i.e.* AgNPs. The collective oscillations of electrons

Figure 1 - (a) Colour change in $AgNO_3$ solution after addition of 5 mL (0.4%w/v) extract and 5 mL of 1.5 M NaOH (b) Natural biowaste peel extract of *P. granatum* (0.4% w/v) prepared by 70% ethanol in soxhlet extractor at 40 °C for 48 h (c) 2 mM $AgNO_3$ aqueous solution.

(Plasmon) generate surface plasmon resonance (SPR) absorption band (Taleb *et al.*, 1998; Noginov *et al.*, 2007; Link and El-Sayed, 2003; Kreibig and Vollmer, 1995) occurring due to the resonance with the incident light wave (Nath *et al.*, 2007). The electric field of an incident wave induces a polarization of these electrons with respect to much heavier ionic core of AgNPs (Das *et al.*, 2009). UV-Visible wave induces a polarization of the loosely bound surface electron due to low penetration depth (approximate 50 nm). As a result the net charge difference take place which acts as a restoring force. This creates a dipolar oscillation of all the electrons with the same phase (Inbakandan *et al.*, 2010). A strong absorption takes place when the frequency of the electromagnetic field becomes resonant with the coherent electron motion, which may be the origin of dark brown colour. Due to the localized SPR, Metal NPs Shows strong absorption peak while bulk metal particles shows propagating SPR. This absorption strongly depends on the particle size, dielectric medium and chemical surroundings (Noginov *et al.*, 2007; Link and El-Sayed, 2003; Umashankari *et al.*, 2012). The UV/Vis absorption spectra of the silver nano particles dispersed in water is shown in the Figure 1. When the size of particles is smaller than the average free path of the electrons (52 nm for silver metal (Abdullin *et al.*, 1998; Henglein, 1998), silver dielectric function modifies which leads to an increased Plasmon bandwidth with decreasing size of particle (Baset *et al.*, 2011).

SEM and TEM analysis

Figures 5 (A) and (B) showed the SEM and typical bright-field TEM micrographs of the synthesized AgNPs. The micrographs of AgNPs found polydisperse and mostly spherical in shape. In some places, Agglomeration of AgNPs may be due to possible sedimentation at a later time. The average size estimated was 15 nm for AgNPs. It is reported earlier that proteins can bind to nanoparticles either through free amine groups and therefore, stabilization of the AgNPs by protein is a possibility (Daniel and Astruc, 2004; Kawsar *et al.*, 2009; Shahverdi *et al.*, 2007; Chien *et al.*, 2007; Ahmad and Sharma, 2012).

Antibacterial activity

The study demonstrated the synergistic activity of AgNPs against gram-positive and gram-negative bacteria. The maximum inhibitory effects of AgNPs observed when prepared with higher NaOH (1.5 mM) molar concentration. The study revealed that AgNPs (50 µg/mL) had shown significant inhibitory effect against *E.coli* and *Bacilus subtilius* i.e. 49 ± 0.64 and 42 ± 0.49 mm when compared with Streptomycin (100 µg/mL) *i.e.* 43 ± 0.52 and 40 ± 0.31 mm respectively Figure 6 (a-d) and Table 1.

The MIC value of 30 µg Ag/mL was observed in *E. coli*. Whereas, *Staphylococcus aureus*, *Bacillus subtilius* and *Pseudomonas aeruginosa* had shown 45 µg/mL,

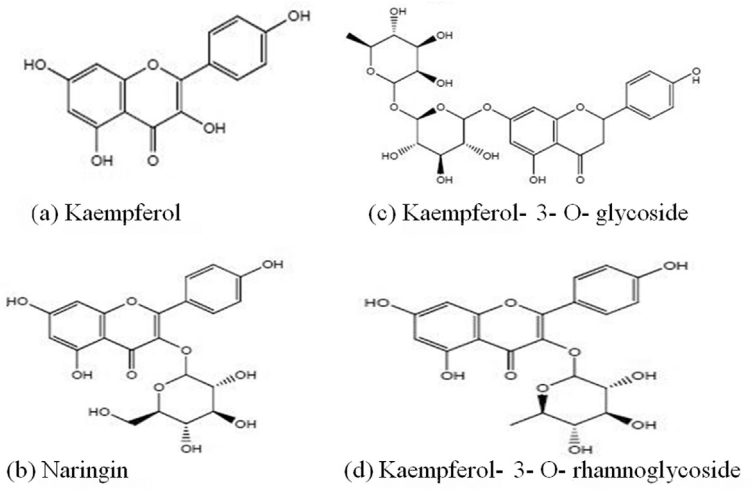

Figure 2 - (a-d) Flavonoids and their glycosides from *P. granatum* peels (Van Elswijk *et al.*, 2004; Jasuja *et al.*, 2012).

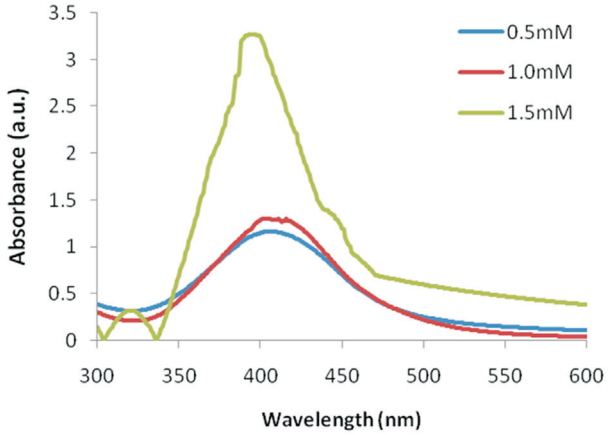

Figure 3 - UV-Visible spectra of AgNPs prepared at different NaOH (0.5 mM, 1.0 mM, and 1.5 mM) molar concentrations.

38 g/mL, 35 µg/mL MIC respectively (Table 2). AgNPs had shown more than 1 active index for *E. coli* and *Bacillus subtilius* when compared with standard drug (Figure 7).

The screening results indicated that AgNPs disc (50 µg/mL) were more active against gram-negative bacteria *i.e. Escherichia coli* with a mean zone of inhibition 49 ± 0.64 mm (Table 1). This may be due to the differences in the cell wall of gram-positive and gram negative bacteria. The cell wall of gram positive bacteria is wider than the gram-negative bacteria (Thiel *et al.*, 2007; Martinez-Castanon *et al.*, 2008; Kim *et al.*, 2007). Gram negative bacteria have a layer of lipopolysaccharide (LPS) which surrounded by a thin layer of peptidoglycan (7-8 nm) (Kreibig and Vollmer, 1995). The overall charge of bacterial cells at biological pH values is negative because of excess number of carboxylic groups, which upon dissociation make the cell surface negative (Raffi *et al.*, 2008). Weak positive charges present on silver nanoparticles (Schultz *et al.*, 2000) are attracted towards negative charges on the LPS. Moreover, Excess formation of free radicals may attack LPS which lead to a breakdown of membrane function. Increased permeability of the cell membrane or leakage of cell contents could be caused by Reactive Oxygen Species (ROS) (Mendis *et al.*, 2005). This also leads morphological changes of bacterial cells and growth inhibition (Amro *et al.*, 2000; Danilczuk *et al.*, 2006; Sondi and Salopek-Sondi, 2004). It is logical to state that binding of the nanoparticles to the bacteria depends on the surface area available for interaction. Nanoparticles have larger surface area available for interaction which enhances bactericidal effect than the large sized particles (Raffi *et al.*, 2008) eg. Inorganic substances or antibiotics; hence AgNPs exhibits more toxicity to the microorganism (Baker *et al.*, 2005).

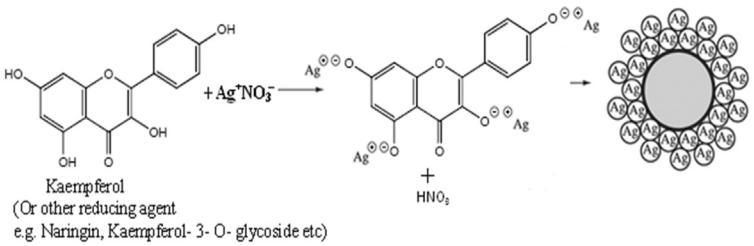

Figure 4 - Schematic diagram of reduction reaction of $AgNO_3$ by peel extracts to form AgNPs.

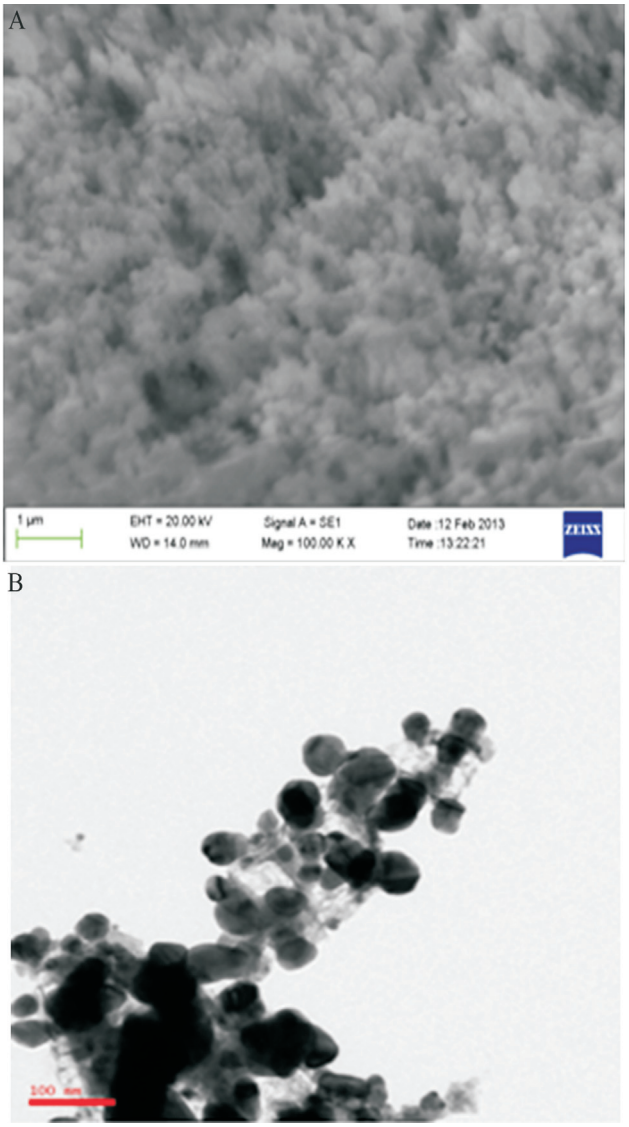

Figure 5 - (a) Scanning electron micrograph of AgNPs synthesized by green methods (b) Transmission Electron Microscopy (TEM) image of AgNPs (scale bar 100 nm).

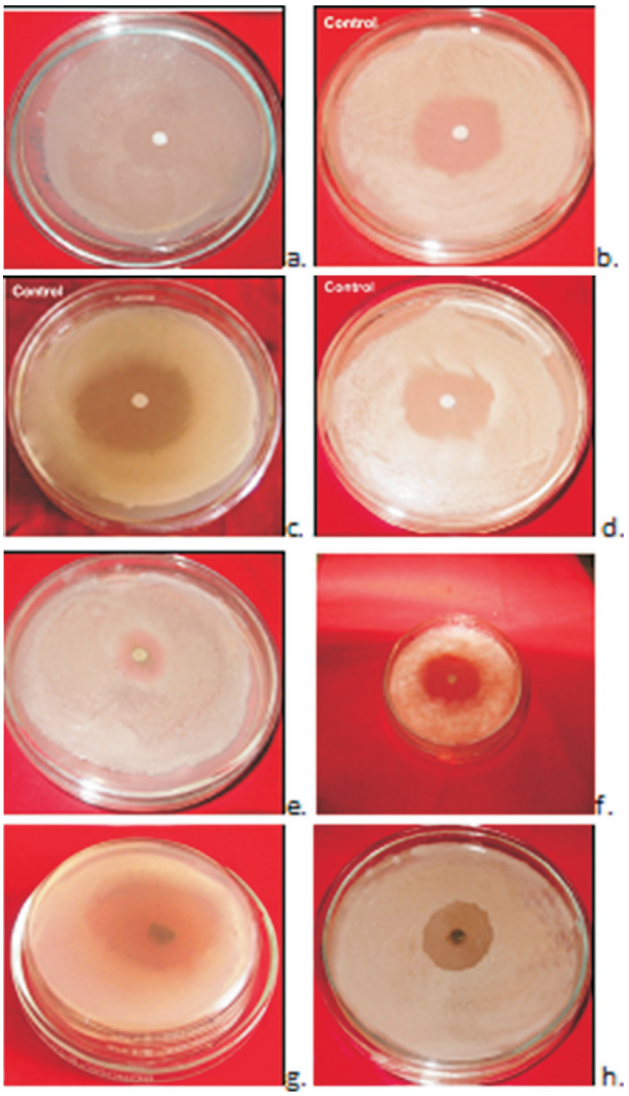

Figure 6 - (a-d) Antibacterial Activities of Streptomycin sulphate disc (1 mg/10 mL) on (a) *Staphylococcus aureus* (b) *Bacillus subtilius* (c) *E. coli* (d) *Pseudomonas aeruginosa*. (e-h) Antibacterial Activities of AgNPs (60 µg/10 mL) on (e) *Staphylococcus aureus* (f) *Bacillus subtilius* (g) *E. coli* (h) *Pseudomonas aeruginosa*.

Table 1 - Antibacterial activity of Streptomycin (100 µg/mL) and AgNPs (100 µg/mL) against bacterial species tested by disc diffusion assay.

Sr. No.	Name of organism	Agar-well diffusion (Zone of Inhibition in mm)			
		AgNPs (50 µg/mL)		Streptomycin	
		(0.5 mM NaOH)	(1.0 mM NaOH)	(1.5 mM NaOH)	(100 µg/mL)
1	*Staphylococcus aureus*	26 ± 0.33	26 ± 0.45	28 ± 0.13	28 ± 0.22
2	*Bacillus subtilius*	40 ± 0.64	41 ± 0.81	42 ± 0.49	40 ± 0.31
3	*E. coli*	45 ± 0.55	46 ± 0.21	49 ± 0.64	43 ± 0.52
4	*Pseudomonas aeruginosa*	39 ± 0.73	40 ± 0.12	40 ± 0.29	42 ± 0.11

Values are mean zone of inhibition (mm) ± S.D of three replicates.

Table 2 - Minimum inhibition concentrations (MIC) of AgNPs at different NaOH molar concentration.

Name of organism	Minimum inhibition concentration				
	AgNPs (μg/mL)			$AgNO_3$ (μg/mL)	Peel extract (mg/mL)
	(0.5 mM NaOH)	(1.0 mM NaOH)	(1.5 mM NaOH)		
Staphylococcus aureus	50	48	45	120	0.40
Bacillus subtilius	43	41	38	108	0.50
E. coli	38	35	30	102	0.85
Pseudomonas aeruginosa	44	40	35	105	0.45

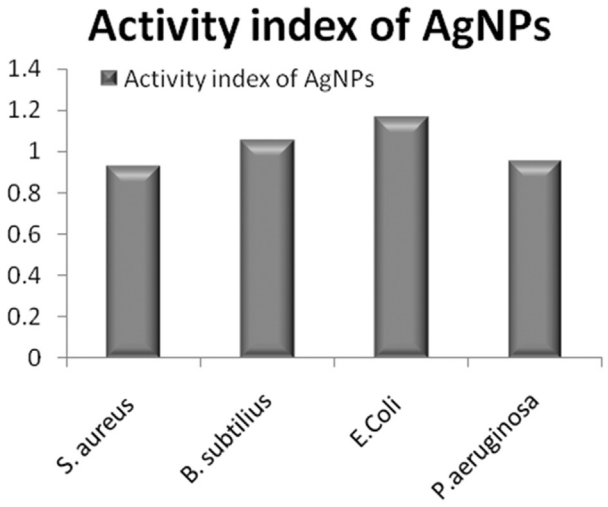

Figure 7 - Activity index of AgNPs compared with Streptomycin.

Conversely, the cell wall in gram-positive bacteria is composed of a thick layer (about 20-80 nm) of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides to form a three dimensional rigid structure (Wiley *et al.*, 2006). The rigidity and extended cross-linking not only provide the cell walls with fewer anchoring sites for the silver nanoparticles but also make them difficult to penetrate. Earlier studies also revealed that silver species release Ag+ ions which interact with the thiol groups of bacterial proteins, may retard or change the replication of DNA (Marini *et al.*, 2007; Martinez-Castanon *et al.*, 2008). Somehow, it may be the reason that the G+ *Bacillus subtilius* also inhibited by AgNPs significantly when compared with control antibiotics.

Conclusions

It is concluded that the extract of *P. granatum* are capable of producing stable AgNPs by reduction of aqueous Ag+ ions in to Ag0. This green chemistry approach toward the synthesis of AgNPs has various advantages i.e rapid reduction, economic viability etc. Applications of such eco-friendly nanoparticles in bactericidal, wound healing, medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic nanomaterials (Ankanna *et al.*, 2010) *e.g.* Au, Fe, Zn, Cu, Graphenes etc. The increase in zone of inhibition reported in this study was dependent on the concentration of nanoparticles due to higher NaOH molar concentration. Attachment of nanoparticles by cell wall of bacteria would be due to negative charges and specific functional groups on the bacterial surface. AgNPs after penetration into the bacterial cell may disturb the rigidity of cell wall or lipopolysaccharides membrane, inactivate their transport system, enzymes functioning, generate H_2O_2 which resulted in bacterial death. The silver nanoparticles synthesized via green route are highly toxic to G-ve and somehow for G+ve bacteria can be used in medical applications (Singh *et al.*, 2010).

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