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Antibacterial activity of biostabilized silver nanoparticles

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Bioactive silver nanoparticles (Ag NPs) have been synthesized by reacting aqueous solution of silver nitrate ($AgNO_3$) with plant extracts which act as reducing and stabilizing agent at ambient temperature. The bio-reduction behaviour of extracts of different parts of plant such as *Plumbagozeylanica, Cassia tora, Kalanchoegastonis-bonnieri, Euphorbia milii, Tridaxprocumbens, Nyctanthesarbor-tristis, Psidiumguajava and Lantana camara* in the synthesis of silver nanoparticles have been studied. The size and size distribution of prepared NPs have been investigated employing UV-vis absorption spectrophotometer, Dynamic light scattering (DLS) and Transmission electron microscopy (TEM). Dispersion destabilization of NPs is detected by Turbiscan. Different parameters such as stirring effect, reaction time, temperature, silver nitrate concentration and amount of plant extract have been studied to find out the optimum condition for synthesis of Ag NPs. Furthermore, biologically synthesized Ag NPs are tested for their antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*.

Keywords: Bioactive silver nanoparticles, *Plumbagozeylanica, Cassia tora, Kalanchoegastonis-bonnieri, Euphorbia milii, Tridaxprocumbens, Nyctanthesarbor-tristis, Psidiumguajava, Lantana camara,* Antibacterial activity

Researchers are engrossed in NPs because properties of the nanoscale materials are very different from those at the larger scale. The properties of particles at the nanoscale are different because of two main reasons: 1) NPs have relatively large surface area which make materials more chemically reactive and affect their strength[1], and 2) Quantum effects can begin to dominate the behaviour of matter at the nano scale affecting the optical, electrical and magnetic behaviour of the materials. The development of reliable experimental protocols for the synthesis of highly monodispersed nanomaterials over a range of chemical compositions and sizes is one of the challenging issues in current nanotechnology[2-4].

A number of methods such as chemical, physical, electro- and photo-chemical reduction and heat evaporation are widely used for synthesis of nanoparticles but the use of toxic chemical species getting absorbed on the surface may have adverse effects in medical applications[5-7]. Currently biomimetic methods are emerging for generation of nanoparticles[4]. Biomimetic is a technique in which biological synthesis of nanoparticles involves natural phenomenon that take place in the biological systems. The importance of biological synthesis is being emphasized globally at present because physical and chemical methods are capital intensive, toxic, non-ecofriendly and have low productivity[4,8]. The biosynthetic method employing plant extracts have drawn attention as a simple and viable alternative to chemical procedures and physical methods. In this method, there is no need to use high pressure, energy, temperature and toxic chemicals[5-10]. Therefore, for environmental safety concerns, researchers in the field of nanoparticles synthesis and assembly have turned to the biological systems including bacteria, fungi, yeast and most importantly plants. Some well-known examples are extracellular synthesis of Au NPs by *Pseudomonas aeruginosa*, reduction of aqueous Ag^+ by *Klebsiella pneumonia, E. coli and Enterobacter cloacae* and Au NPs using endophytic fungus *Colletotrichum* [6].

The most widely synthesized nanoparticles are Ag, Au, Pt and Pd using bacteria, fungi and plant extract[11]. Among these, silver NPs plays a noteworthy part in the field of biology and medicine exhibiting attractive physiochemical properties including low toxicity and it also act as effective antimicrobial agent[10,11]. An eco-friendly process for rapid synthesis of silver nanoparticles was reported by Bar *et al.*[6] using aqueous seed extract of *Jatropha curcas*. They observed that most of the silver nanoparticles were spherical with diameter ranging from 15 to 50 nm. Silver nanoparticles of size 20-30 nm were also prepared by Krishnaraj *et al.*[11] using leaf extract of *Acalyphaindica.* Antibacterial activity of synthesized silver nanoparticles showed effective inhibitory activity against water borne pathogens viz.,

Escherichia coli and *Vibrio cholerae*. 10 μg/mL was recorded as the minimum inhibitory concentration (MIC) of silver nanoparticles against *E. coli* and *V.cholerae*. Savithramma *et al.*[12] synthesized silver nanoparticles from stem bark extracts of *Boswellia* and *Shorea*; and leaf extract of *Svensonia*. The Ag NPs synthesized from bark extracts of *Boswellia ovalifoliolata* and *Shorea tumbuggaia* showed toxic towards *Klebsiella* and *Aspergillus*; and *Pseudomonas* and *Fusarium* species respectively. Whereas the growth of *Pseudomonas* and *Rhizopus* species were inhibited maximum by the Ag NPs synthesized from leaf extract of *Svensonia hyderobadensis*, the results indicated that the silver nanoparticles might have an important advantage over conventional antibiotics.

In the present study, several plants are utilized for the production of Ag NPs having different constituents which may be responsible for reduction of Ag ions. Table 1 showed the components and role of different plants used in this paper. Several naphthoquinones and anthraquinones which are present in plant extract have excellent redox properties[11] and even proteins, polyphenols and carbohydrates are present in plants and might be responsible for synthesis of metal nanoparticles[13]. Proteins are reported to bind to the NPs either through free amine groups or cysteine residues present in the protein[14]. Many reports have shown that isolated quercetin (natural plant pigment), polysaccharide, terpenoids and water-soluble heterocyclic components such as flavones are mainly responsible for the reduction of silver ions[1,9,11-15] and also high phenolic content of the plants have strong antioxidant properties which help in the reduction of ions to NP[16]. The secondary metabolites present in plant systems may also be responsible for the reduction of silver ions and synthesis of nanoparticles.

In view of the above literature, attempts had been made to synthesize uniformly distributed stable silver nanoparticles using leaf, stem and flower extract of different plants which were highly effective for antibacterial activity. To the best of our knowledge, the use of these plants for biological synthesis of silver NPs has not been reported till date. Different parameters which affect particle size and size distribution were studied systematically. Turbiscan had been used to monitor stability of Ag NPs.

Experimental Section

Materials

$AgNO_3$ was purchased from Finar chemicals, India and used as received without further purification. Distilled water (Millipore, Elix, India) was used throughout the experiments for preparing the aqueous solutions and washing. Healthy leaves, flowers and the stem barks of *Cassia tora, Kalanchoegastonis-bonnieri, Psidiumguajava, Euphorbia milii, Plumbagozeylinica, Lantana camara, Tridaxprocumbens and Nyctanthesarbor-tritis* were collected from different areas of Surat (Gujarat, India).

Preparation of plant extract

Fresh leaves (20 g), stem (10 g) and flower (5 g) were washed to remove any dust particles that could interfere with binding of Ag ions to the biomass or formation of the NPs, then air dried and cut into fine pieces and added to 100 mL of boiled sterile distilled

Table 1 — Plants components and their role

Plant Name	Components	Role of plants	Reference
Plumbagozeylinica	Plumbagin	Antibiotic properties	[16,17]
Lantana camara	Secondary metabolites, lantadenes, terpenoids, steroids, alkaloids.	Antimicrobial, fungicidal, insecticidal, cancer, malaria, chicken pox.	[18-20]
Psidiumguajava	Polyphenols, carotenoids.	Antibacterial, cancer, inflammation, pain.	[21-24]
Euphorbia milii	Milin	Ornamental plant, folk medicine, Helps Prevent Schistosomiasis.	[25]
Nyctanthesarbor-tritis	Glycoside, B-sitosterol, flavonol, acids, mannitol, glucose.	Immunostimulant, antifungal, anti-inflammatory, antiviral, etc.	[26-28]
Cassia tora	Protein-rich, emodin, stearic, stigmasterol, quercitin, palmitic, etc.	Laxative, skin disease, antiperiodic, cough, etc.	[29]
Kalanchoegastonis-bonnieri	Bryophili A, bryophilin C, alkaloids, tannins, terpenoids, flavonoids, polyphenols.	Antibacterial, antiulcer, antitumor, insecticidal, inflammation.	[30]
Tridexprocumbens	Alkaloids, carotenoids, flavonoids, catechin, flavones, saponins, tannins.	Antiviral, antibiotic, wound healing activity, insecticidal and anti-inflammatory.	[30]

water at 100°C for 5 min for the release of intra-cellular material into solution. After boiling, the mixture was cooled and filtered to get the extract. The filtrate was used as reducing and stabilizing agent for 1mM of $AgNO_3$ solution.

Biosynthesis of silver nanoparticles using plant extract

$AgNO_3$ (1mM, 45 mL) was added to 5 mL of plant extracts separately to make up a final solution of 50 mL. A change in the colour from whitish yellow to brown indicated the formation of Ag NPs within 1 h. The reductions of Ag ions were monitored by measuring the UV-vis spectrophotometer. All the following procedures were performed at room temperature and under atmospheric pressure. Synthesis of nanoparticles were carried out by varying different parameters such as in presence and absence of stirring, reduction time, temperature of the mixture, extracts concentration and concentration of $AgNO_3$ and expressed in terms of particle size and size distribution.

Characterization of silver nanoparticles

The absorption spectra of Ag NPs were analysed at different time interval by UV-vis spectrophotometer (HACH, Germany). TEM images were obtained with a Philips tecnai-20, which at 200 kV provides 0.27 nm point resolution. The sizes of NPs were measured using dynamic light scattering (Malvern Zetasizer, Nano ZS 90, U.K.). NPs stability was analysed by turbiscan.

Antibacterial activity of silver nanoparticles

The antibacterial activities of Ag NPs were carried out by disc diffusion method of Kirby-bauer. Mueller-hinton agar plates were prepared, sterilized and solidified. After solidification bacterial cultures were swabbed on these plates. The sterile discs were dipped in Ag NPs solution and placed in the Mueller-hinton agar plate and kept for incubation at 37°C for 16-18 h. After incubation, MIC was recorded as the lowest concentration of the agent inhibiting the visible growth of microorganisms. The diameter of the zone of inhibition around the disk was measured to the nearest millimetre. The antibacterial activity of smallest size Ag NPs was studied against Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and Gram positive bacteria (*Staphylococcus aureus, Klebsiella pneumonia*).

Results and Discussion

UV-Vis absorption spectroscopy

After addition of plant extract in silver nitrate solution, the colour of entire mixture was gradually changed from water colour to yellowish brown and formation of Ag NPs was monitored by UV-vis absorption spectroscopy. The NPs obtained from stem extract of different plants showed absorbance peak between 400-470 nm and the NPs obtained from *Tridaxprocumbens* (*8*) stem extract showed strong absorbance peak compared to other plants. NPs formed from leaf extract of different plants showed peak between 420-470 nm and the NPs obtained from *Psidiumguajava (2)* leaf extract showed peak of higher absorbance compared to other plants. Green leaves are site of photosynthesis and more H^+ ions are available to reduce $AgNO_3$ into Ag NPs[31]. Therefore the NPs obtained from leaf extract had strong absorbance peak compared to NPs obtained from stem extract. Flower extract of *Euphorbia milii*, *Tridaxprocumbens* and *Lantana camara* used for synthesis of Ag NPs and showed absorbance peak between 430-450 nm. NPs obtained from *Tridaxprocumbens* (*3*) flower extract showed strong peak compared to others. Thus these plants were found to be promising in synthesis of Ag NPs. The difference in the rate of bio-reduction observed might be due to difference in the activities of components or enzymes present in plants used.

Dynamic light scattering

The size distribution and average particle size of bio-stabilized silver nanoparticles were obtained using dynamic light scattering (Table 2). AgNPs obtained

Table 2 — Particle size distribution and Z. Average of silver nanoparticles

Plant		Particle size distribution (nm)	Z. Avg. (nm)
Euphorbia milii	Flower	2.12-8.62	4.56
	Leaf	4.18-13.54	8.08
	Stem	13.58-43.82	26.15
Psidiumguajava	Leaf	2.01-6.5	3.88
	Stem	0.54-1.74	1.04
Nyctanthesarbor-tritis	Leaf	7.5-24.36	14.54
	Stem	4.52-21.54	13.58
Plumbagozeylinica	Leaf	3.61-10.1	6.39
	Stem	8.72-28.21	16.83
Cassia tora	Leaf	5.7-13.54	7.9
	Stem	1.29-3.62	2.29
Kalanchoegastonis-bonnieri	Leaf	5.6-18.17	10.84
	Stem	32.67-78.82	53.04
Lantana camara	Flower	3.6-13.54	7.64
	Leaf	3.12-10.1	4.2
	Stem	21.04-75.69	44.43
Tridaxprocumbens	Flower	2.01-4.85	3.26
	Stem	0.72-2.01	1.27

by stem extract of *Psidiumguajava* showed smallest particle size (0.54-1.74 nm) with average particle size of 1.04 nm. AgNPs obtained by stem extract of *Kalanchoegastonis-bonnieri* showed highest particle size (32.67-78.82 nm) with average particle size of 53.04 nm. AgNPs of sub-10 nm were used for antibacterial analysis.

Transmission electron microscopy

TEM images of AgNPs obtained by stem and leaf extract of *Psidiumguajava*, stem and flower extract of *Tridaxprocumbens,* stem extract of *cassia tora* and flower extract of *Euphorbia milii* were showed in Fig. 1a-f. From Fig. 1, it was observed that particles are spherical in shape with diameter regaining from 0.2-5 nm in size and particles are distinct and scattered in distribution.

Effect of different parameters on the formation of silver particles

Using stem extract of *Psidiumguajava* (*2*) mostly sub-10 nm Ag NPs were obtained. Hence effect of different parameters on size and size distribution were studied using same reaction mixture.

Stirring

Stirring influence the particle size and yield of the silver nanoparticles, thus it maximizes the efficiency of formation of Ag NPs in shorter period of time. It was found that without stirring, the particles were slightly agglomerated and particle size was larger. Ag NPs synthesized with and without stirring showed absorbance peaks at 430 and 450 nm which indicated blue shifting of the absorbance peak i.e. formation of smaller nanoparticles due to stirring in the system. Therefore, rest of the experiments were carried out under stirring condition.

Reaction time

Figure 2 showed that as the reaction time increased, more NPs were formed and the peak becomes sharper. Ag NPs formation was started at 10 min and intensity of the peak increased up to 1 h. With the progress of reaction, more and more Ag^+ were reduced which resulted increase in the size of silver hydrosol through diffusion controlled mechanism[32].

Temperature

AgNPs were prepared at different temperature, such as 15, 30 and 45°C, keeping all other parameters constant. At lower reaction temperature (15°C), slower would be the reaction rate and thus generated larger particles (average size was 4.41 nm). At room temperature (30°C), the rate of the reaction increased which resulted small particles (average size 1.36 nm, Fig. 3. Particle size again increased with increasing the temperature. Beyond 30°C, aids the growth of the crystal around the nucleus which leads to increase in the size of particles[10].

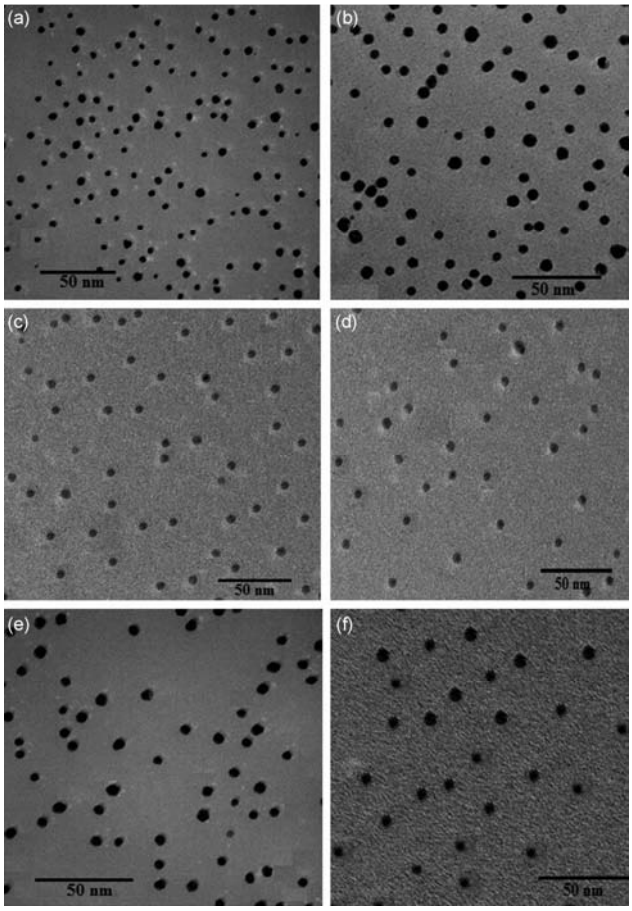

Fig. 1 — TEM image of Ag NPs synthesized from (a) *Psidiumguajava* stem; (b) *Psidiumguajava* leaf; (c) *Tridaxprocumbens* stem; (d) *Tridaxprocumbens* flower; (e) *Cassiatora* stem and (f) *Euphorbia milii* flower

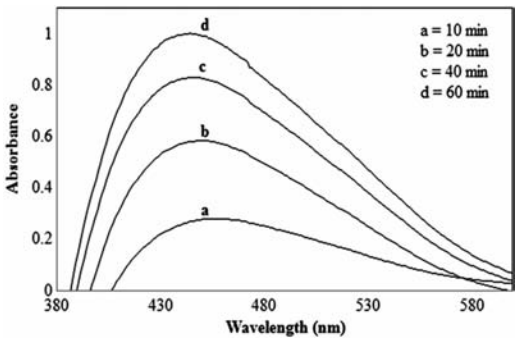

Fig. 2 — Effect of reaction time (a) 10 min; (b) 20 min; (c) 40 min and (d) 60 min on particle size and size distribution of Ag NPs

Silver nitrate concentration

Silver concentration was varied from 0.75-5 mM, keeping all the other parameters constant. Polydispersity index (PDI) is a parameter to define the particle size distribution of NPs and ranges from a value of 0.01-0.7 for monodispersed particles. Broader size distribution is observed for samples of PDI value > 0.7[33]. At 0.75 mM (lower concentration of silver) average particle size and PDI was found to be 3.28 nm and 0.232 (Table 3) respectively. It was observed that at 1 mM, the particle size and PDI decreased to 1.36 nm and 0.218 respectively and further increased with increasing silver concentration (3 and 5 mM).

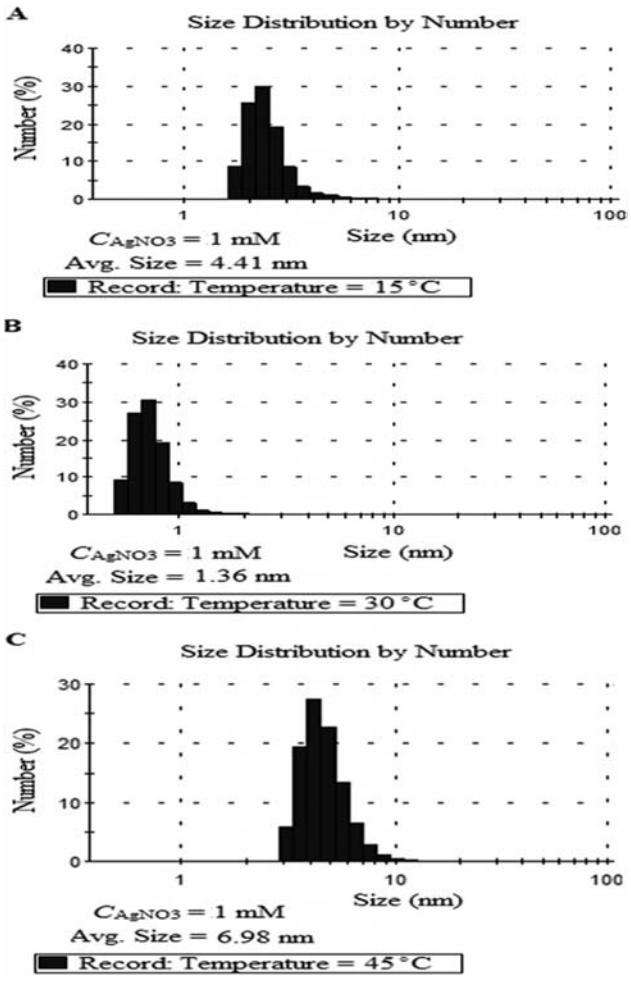

Fig. 3 — Effect of different temperature (A) 15°C; (B) 30°C and (C) 45°C on particle size and size distribution of Ag NPs

Extract volume

The volume of plant extract was varied from 2, 5, 7 and 10 mL, keeping all parameters constant (Table 3). It was observed that the 5 mL volume of plant extract was effective in the generation of Ag NPs, average size and PDI of NPs was 1.36 nm and 0.218 respectively. The absorption peak obtained with 5 mL extract was sharper compared to other volumes added in the reaction mixture. With further increase of amount of extract, particle size and PDI increased (Table 3).

Stability of silver nanoparticles

The stability of dispersion can be viewed in terms of the propensity for aggregation and in redispersibility of the final particles. Silver nanoparticles were scanned from bottom (0 mm) to the top of the vial (~70 mm) for a period of 20 min. Scanning was performed at different time intervals upto 24 h. It was observed that, Backscattering (BS) profiles of the AgNPs at different time intervals were superimposing. The stable position of absorption peak at 430 nm wavelength also indicated that NPs did not aggregate.

Antibacterial activity of silver nanoparticles

The mechanism of the bactericidal effect of Ag NPs against bacteria is not very well-known. Ag NPs may attach to the surface of the cell membrane and disturb its power function such as permeability and respiration. It is reasonable to state that the binding of the particles to the bacteria depends on the surface area available for interaction. The results of this study clearly demonstrate that the Ag NPs inhibits the growth and multiplication of the tested bacteria, including highly multiresistant bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* Rajesh *et al*.[34] observed that Ag NPs manifest antibacterial properties either by anchoring/penetrating bacterial cell wall and modulating cellular signaling by dephosphorylating putative key peptide substances on

Table 3 — Effect of $AgNO_3$ concentration and extract volume on particle size of Ag NPs

$AgNO_3$ (mM)	Avg. size (nm)	Polydispersity index	Extract volume (mL)	Avg. size (nm)	Polydispersity index
0.75	3.28	0.232	2	4.76	0.254
1	1.36	0.218	5	1.36	0.218
3	5.38	0.260	7	4.64	0.257
5	8.08	0.341	10	6.38	0.308

tyrosine residues and also reported that Ag NPs mediated antibacterial activity in much more efficient physiochemical manner than Ag^+ ions.

Ag NPs prepared by using different plant extract were used to study antibacterial activity. To compare antibacterial susceptibility of synthesized silver nanoparticles, modified Kirby-bauer diffusion method was used for the most devasting bacterial strains: *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Aspergillus niger*. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles.

The zone of inhibition seemed to be extremely good and showed a relatively large zone of inhibition in both gram positive and gram negative bacterial strains. Antibacterial activity of biogenic Ag NPs was evaluated by using standard Zone of Inhibition (ZOI) microbiology assay. Maximum ZOI was found to be 16 mm for *E.coli* and minimum of 11 mm for *P.aeruginosa* and *B.subtilis* (Table 4).

Ag NPs obtained by stem and leaf extract of *Psidiumguajava*, stem and flower extract of *Tridaxprocumbens,* stem extract of *cassia tora* and flower extract of *Euphorbia milii* with narrow size distribution (sub-10nm) were tested as antimicrobial agent (Table 5). Synthesized silver nanoparticles showed effective antibacterial activity against the test bacteria. No significant bacterial growth was observed at Ag NPs concentrations above 1.28 μg/mL. Ag NPs obtained from stem extract of all plant showed lower MIC (0.25 μg/mL). The Ag NPs obtained from stem extract of *Psidiumguajava* showed no activity against *B.subtilis.* Krishnaraj *et al.*[11] synthesized AgNPs of size 20-30 nm from *Acalypha* leaf extract and 10 μg/mL of Ag concentration was recorded on the MIC against *E.coil* and *V.cholerae*. Hence the synthesized Ag NPs showed better results than reported data.

Table 4 — Antibacterial activity of some plants extract by disc diffusion assay

	Zone of Inhibition (mm)					
Bacterial spp	*Psidium-guajava*		*Tridax-procumbens*		*Cassia tora*	*Euphorbia milii*
	Stem	Leaf	Stem	Flower	Stem	Flower
B.subtilis	-	11	12	13	13	12
E.coli	14	14	16	13	12	12
P.aeruginosa	12	11	13	11	12	11
S.aureus	12	15	15	15	14	15

Table 5 — MIC determination of antibacterial activity

	MIC (μg/mL)					
Bacterial spp	*Psidium-guajava*		*Tridax-procumbens*		*Cassia tora*	*Euphorbia milii*
	Stem	Leaf	Stem	Flower	Stem	Flower
B.subtilis	-	1.28	0.50	0.50	0.25	0.76
E.coli	0.25	0.25	0.25	0.50	0.76	0.76
P.aeruginosa	0.76	1.28	0.50	1.28	0.76	1.28
S.aureus	0.76	0.25	0.25	0.25	0.25	0.25

Conclusion

A rapid biological method has been used for synthesis of Ag NPs using the aqueous extract of different plants, which acted as both reducing and stabilizing agents. Different parameters are optimized including stirring effect, reaction time, temperature, concentration of $AgNO_3$ solution and volume of plant extract which had been identified as major factors affecting the size and size distribution of Ag NPs. The biosynthesized Ag NPs using *Tridaxprocumbens* leaf*, Tridaxprocumbens* flower*, Euphorbia milii* flower, *Cassia tora* stem*, Psidiumguajava* leaf *and Psidiumguajava* stem extract showed smaller particle size (sub -10 nm) and excellent antimicrobial activity. Ag NPs inhibited the growth and multiplication of the tested bacteria at very low concentration of Ag (lower than 1.28 μg/mL).

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