Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6 Issue 2, 2014

Research Article

QUORUM QUENCHING AND ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED FROM MEDICINAL PLANTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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Received: 23 Jan 2014, Revised and Accepted: 04 Mar 2014

ABSTRACT

Objective: The aim of this study was to evaluate quorum quenching and antibacterial activity of silver nanoparticles synthesized from medicinal plants against Methicillin-resistant Staphylococcus aureus (MRSA).

Methods: Development of efficient methods for green synthesis of silver nanoparticles using plant extracts has become a major focus area of nanotechnology. The importance of bactericidal nanomaterials is gaining due to the increased resistant strains of bacteria against most potent antibiotics. The discovery of bacterial communication system (Quorum-sensing system), which orchestrate important temporal events during the infection process, has afforded a novel opportunity to ameliorate bacterial infection by means other than growth inhibition. This has promoted research in the well-known bactericidal activity of Ag^+ ions and Ag-based compounds, including silver nanoparticles (AgNPs). In this study, extracellular biosynthesis of silver nanoparticles was carried out using aqueous extracts of nine different herbal plant leaves for the reduction of aqueous silver ions. The morphologies and structures of the nanoparticles were characterized by transmission electron microscopy, UV-visible spectroscopy and X-ray diffraction. Furthermore, the synthesized AgNPs were evaluavated for quorum quenching and antibacterial activity against MRSA.

Results: Among the nine selected medicinal plant leaf extracts *Aerva lanata* showed rapid reduction (2 min) of silver ions in the solution. Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD) analysis revealed the nanocrystalline phase of silver with the average particles size ranged from 37-47 nm and has spherical shape. Furthermore the synthesized AgNPs exhibited very effective antibacterial activity against Methicillin Resistance *Staphylococus aureus* (MRSA). Moreover, the stable silver nanoparticles found to inhibit violacein production, a quorum sensing regulated behavior in *Chromobacterium violaceum* CV026.

Conclusion: The presence of surface active molecules such as flavonoids, terpenoids in the plant leaves extracts stabilize the silver nanoparticles. Furthermore the green synthesized AgNPs established very effective antibacterial and quorum quenching activity against MRSA.

Keyword: AgNPs, Quorum quenching, Antibacterial activity, SEM, MRSA.

INTRODUCTION

Nanotechnology has gloriously developed as an important field in modern research with a focus on the synthesis of nanoparticles with the improved antimicrobial activities against life threatening diseases [1-3]. Nanoparticles are commonly synthesized using two steps: top-down and bottom-up [4]. The bulk materials are gradually broken down to nanosized materials in top-down approach. Atoms or molecules are assembled to molecular structures in nanometer range in bottom-up approach [5]. In bottom-up silver nanoparticles are synthesized by various chemical, physical and biological methods. Biological synthesis of nanoparticles is gaining importance because it is safe, cost-effective, biocompatible, non-toxic and eco-friendly whereas hazardous wastes from different physical and chemical processes leading to various environmental problems [6]. The natural sources like plants and microorganisms are used for synthesizing silver nanoparticles by biological approach [7]. Synthesis of nanoparticles using microorganisms consume more time for whereas using the plant extract mediated methods require less processing time [8] and also suitable for large scale production [9]. The silver nanoparticles are highly toxic to several pathogenic organisms and hence played a vital role in treatment of many diseases [10, 11].

Nowadays the multidrug resistance pathogens such as Methillin-Resistant *Staphylococcus aureus* (MRSA) have become a major public health concern [12]. Moreover, the expression of all virulent factors of *S. aureus* is controlled by the quorum sensing system AIP I, AIP II, AIP III, AIP IV. Furthermore, in most of the current research it was proven that multi-drug resistance bacterial has been developed due to the presence of quorum sensing molecules [13, 14]. The knowledge of the QS system and its major role in bacterial virulence and survival brings a new strategy to attack and inhibit bacterial pathogenicity [15].

Keeping this in view, the present study was focused to determine the antibacterial and quorum quenching activity of green synthesized AgNPs using medicinal plant leaf extracts against MRSA.

MATERIALS AND METHODS

Collection of plant samples

Nine medicinal plants such as *Aerva lanata, Enicostema axillare, Evolvulus alsinoides, Polygala chinensis, Gymnema sylvestre, Mukie scabrella, Ocimum sanctum, Phyla nodiflora and Spermacoce hispida* were collected randomly from Kumbakonam, Tamilnadu, India. Selection of plants was based on their common availability and ethnobotanical use categories relating to the infection. The collected plant leaves were thoroughly washed thrice with tap water and then with sterile double distilled water to remove dust particles, air-dried for a week under shade at room temperature, finely cut, milled into a fine powder and was stored in airtight containers for later analysis.

Preparation of aqueous plant extracts

10 gm of the leaf powder was mixed well with 100ml of double-distilled water and boiled for 15min to facilitate the formation of aqueous plant extracts. The extract thus obtained was filtered through Whatman No.1 filter paper and purified by centrifugation at 6000 rpm for 20 min and collected into autoclaved vials to ensure sterility of the samples. The filtrate was stored at 4° C for further use and used within a week. The filtrate was used as reducing and stabilizing agent for 1 mM of $AgNO_3$.

Phytochemical analysis of dried biomass

A fraction of leaf extracts of herbal plants was subjected to phytochemical screening as described by [16]. Tests for carbohydrates, amino acids, proteins, alkaloids, flavonoids, sterols,

terpenoids, saponins, tannins and phenolic compounds were carried out.

Green synthesis of silver nanoparticles

$AgNO_3$ was obtained from Sigma–Aldrich chemicals, India and were of analytical grade. Deionized water was used throughout the reactions. The green synthesis of silver nanoparticles was carried out in 250 ml Erlenmeyer flask containing 90 ml of 1mM $AgNO_3$ and 10 ml of leaf extract. The solution was kept at dark room at 37ºC, with continuous agitation at 100 rpm for 24 hrs and monitored for the formation of AgNPs. Periodic sampling after 30 minutes was carried out to monitor the formation of AgNPs.

After the period of incubation, the color of the solution changes from colorless to brown indicating the formation of AgNPs. Then the cell free supernatant solution containing silver nanoparticles was obtained by repeated centrifugation at 12,000 rpm for 20 min and stored in bottle at 4 ºC for further analysis.

Characterization of AgNPs

The formation of silver nanoparticles (AgNPs) was primarily confirmed by UV-visible spectrophotometric analysis. The surface structure and shape of the particles were analyzed by JEOL JSM-6701F Field Emission scanning electron microscopic (SEM) analysis. To analyze the crystalline nature of the synthesized particles, the lyophilized powder was subjected to X-Ray powder diffraction analysis by Bruker D8 Focus power X-ray diffractometer operated at 25˚C.

Antibacterial assays

In vitro antibacterial assay was carried out using Kirby-Bauer method. To screen out the antibacterial activity of AgNPs 15 ml of Muller Hinton agar (MHA) was prepared and poured into a pre-sterilized petri plates and left undisturbed to solidify. The pathogenic Methicillin Resistance *Staphylococcus aureus* at a concentration of 10^5 to 10^6 CFU/mL were spreaded on the surface of MHA plate. 10 µl of AgNPs (100 ppm) wad loaded on 6mm sterile disc and incubated for 24 hours at 37˚C. After the incubation time, the clear halo around the disc was resolved using high antibiotic zone scale.

Assay for the inhibition of violacein pigment in C*hromobacterium violaceum*

Well diffusion assay was performed with biosensor strain *Chromobacterium violaceum* CV026 to determine the violacein pigment inhibition. The biosensor strain, *Chromobacterium violaceum* CV026, is a mutant of the wild type strain and is unable to produce its own AHL signal, but responds to exogenous active signal molecules to produce a purple pigment; violacein. 0.1 ml of freshly grown cultures of *Chromobacterium violaceum* CV026 were aseptically transferred to Luria agar plates along with 30µg of AHLs as exogenous source of quorum sensing molecules. Wells of 6 mm diameter were made and impregnated with 10 µl of silver nanoparticles with different concentrations (50, 100, 150 and 200 ppm) and then the plates were incubated overnight at 30°C. Quorum quenching activity was detected as a clear halo zone around the well. The inhibition of violacein pigment by AgNPs indicates the quorum quenching activity of nanocolloids.

RESULTS AND DISCUSSION

Table 1 shows the phytochemical constituents of the aqueous extracts of nine medicinal plants. Our results revealed that the presence of the active phytochemical constituents like carbohydrates, amino acids, proteins, alkaloids, flavonoids, terpenoids, saponins, tannins and phenolic compounds. Moreover, in current research it was reported that the flavonoids and terpenoids present in these plant leaf extracts are the surface active molecules stabilizing the nanoparticles [17]. The time of addition of extract into 1mM $AgNO_3$ solution was considered as the starting point of the reaction. It is well known that silver nanoparticles exhibit a yellowish-brown colour in solution due to excitation of Surface Plasmon Resonance (SPR) vibrations which in turn is due to the presence of free electrons [18].

Table 1: Phytochemical analysis of aqueous leaf extracts of medicinal plants

Chemical Constituents	*Aerva lanata*	*Enicostema axillare*	*Evolvulus alsinoides*	*Gymnema sylvestre*	*Mukia scabrella*	*Ocimum sanctum*	*Phyla nodiflora*	*Polygala chinensis*	*Spermacoce hispida*
Alkaloids	+++	+++	+++	+++	+++	+++	+++	+++	+++
Tannins	+++	+++	+++	+++	+++	+++	+++	+++	+++
Phenols	+++	+++	+++	+++	+++	+++	+++	+++	+++
Flavanoids	+	+++	+++	+++	++	++	+++	+++	++
Saponins	++	+++	++	+++	+++	++	+++	+++	+
Terpenoids	+	+++	++	++	++	++	++	+++	+++
Cardiac glycosides	+	+++	++	++	++	++	+++	+++	+++
Carbohydrates	+	+	++	++	-	-	+++	+++	++
Proteins & Amino acids	-	++	++	+++	+++	++	+	++	-

(+), (++) and (+++) represents the presence of phytochemical constituents in the order of increasing intensity in colour and (–) indicates the absence.

Among the nine different selected medicinal plant extracts *A. lanata* shows rapid reduction of silver ion within 5 minutes, while *E. axillare, E. alsinoides, M. scabrilla, P. chinensis* and *S. hispida* shows reduction in 20 minutes and remaining three plants such as *P. nodiflora, O. sanctum* and *G. sylvestre* it takes 12, 16, 18 hrs respectively, indicating the color change from watery to yellowish brown and then gradually to deep reddish brown (Fig.1). This colour formation indicates that silver ions in reaction medium are converted to elemental silver having the size of nanometric range [19]. Deepening of colour further is due to increased concentration as well as growth of silver nanoparticles. After that there was no change in colour which is evidence for the completion of reduction reaction [20]. The possible explanation of difference in the reduction time could be due to the difference in their reduction potential for the metal ions [21]. Of all the plants studied, the intensity of colour development in the reaction mixture was significantly higher in *A. lanata* is due to excitation of Surface Plasmon Vibrations of silver nanoparticles.

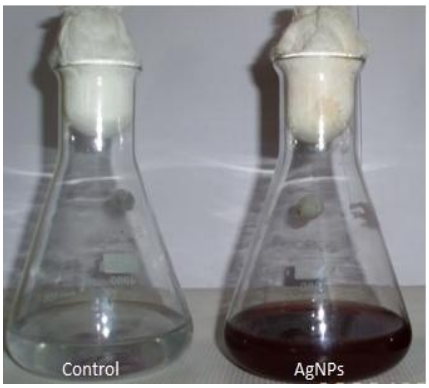

Fig. 1: Rapid biosynthesis of AgNPs using aqueous leaf extract of *Aerva lanata*

UV-Visible spectroscopy is an important preliminary technique to ascertain the formation and stability of metal nanoparticles in aqueous suspension. Thus formation of silver nanoparticles by reduction of aqueous Ag^+ during exposure to the aqueous extract of plants were followed and characterized by UV-Visible spectroscopy. The maximum absorption spectrum was found to be at 420 nm for the five different plant extracts such as in *P. chinensis*, *E. alsinoides*, *E. axillare*, *S. hispida,* and *A. lanata*. For all the remaining four plants it was found at 450 nm (Fig-2). It is generally observed that the frequency and width of surface plasmon absorption depend on the size and shape of nanocolloids in an aqueous suspension [22]. The broadening of peak and splitting of Surface Plasmon Resonance (SPR) is probably due to the dampening of the SPR and particle size distribution in colloidal solution [23]. Mostly all the AgNPs are known to exhibit a UV- Visible absorption maximum in the range of 400-500 nm and peak of absorption in this range clearly indicate the formation of silver nanocolloids [24].

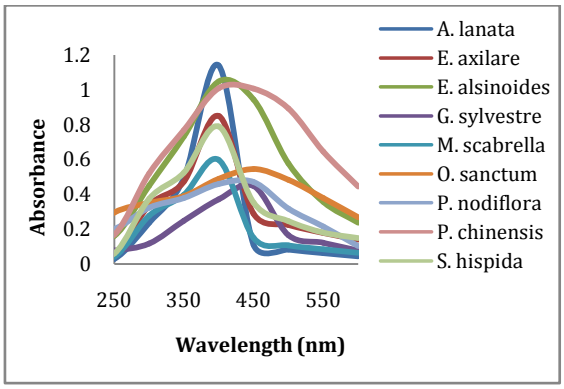

Fig. 2: UV-visible absorption spectra of AgNPs synthesized from aqueous leaf extract of medicinal plants

Scanning Electron Microscopy (SEM) has been employed to characterize the size, shape and morphology of synthesized silver nanoparticles. SEM provided further insight into the morphology and size details of the silver nanoparticles. The SEM image showed the presence of silver nanoparticles which are predominantly spherical shaped and polydispersed and ranges approximately from 37-47 nm in diameter (Fig-3). This is in agreement with the shape of the SPR band in the UV-Vis spectrum. Lower magnification image reveals the nanoparticles are uniformly embedded in a dense matrix which may be the organic stabilizing components of the extracts of *A. lanata*. This shows the even distribution of nanoparticles without any aggregation. Mostly spherical and near spherical shape is obtained. The results obtained from the SEM image gave the clear shape and size of the AgNPs produced from *A. lanata* which correlates with the work of [25].

The biosynthesized silver nanoparticles was further demonstrated and confirmed by the characteristic peaks observed in the XRD image. XRD analysis was carried out to study the crystalline nature of the silver nanoparticles. The presence of organic content associated with AgNPs was further confirmed by observing the sharp Braggs reflection in XRD spectrum. The diffracted intensities were recorded from 10˚ to 60˚ at 2 theta angles. Fig-4 revealing two peaks at degree (2θ) 27.226 and 42.346 corresponding to two diffraction facets of silver. The broadening of X- ray peaks observed is primarily due to the small particle size. The mean size of silver nanoparticles was calculated using the Debye- Scherre's equation. An average size of the AgNPs synthesized by *A. lanata* was 42 nm with size ranging from 37 – 47 nm. The typical XRD pattern revealed that the sample contains a mixed structure of silver nanoparticles. The observed peak broadening and noise were probably related to the effect of nanosized particles and the presence of various crystalline biological macromolecules present in the plant extract which may be responsible for the reductionof silver ions [26].

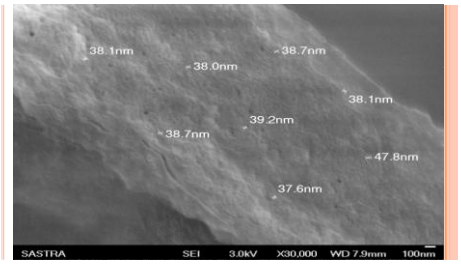

Fig. 3: SEM image of AgNPs synthesized from *Aerva lanata leaf extract*

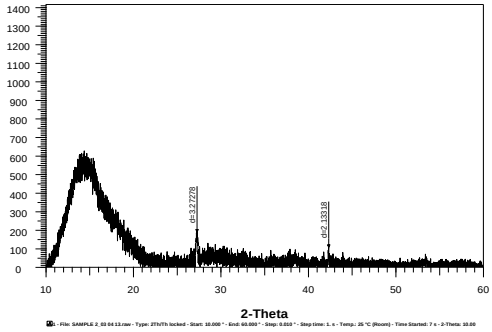

Fig. 4: XRD pattern of AgNPs synthesized using *Aerva lanata leaf extract Aerva lanata leaf extract*

The silver nanoparticles synthesized from the aqueous leaf extracts of *A. lanata, E. axilare, E. alsinoides, P. chinensis* and *S. hispida* exhibited excellent antibacterial activity against MRSA, whereas *M. scabrella, G. sylvestre, O. sanctum and P. nodiflora* show limited activity at a concentration of 100 ppm. It has been reported that antibacterial effect was size and dose dependent [27]. The antibacterial activities of colloidal silver particles are influenced by the dimensions of the particles. The tested silver nanoparticles not only inhibit bacterial growth but also kill bacteria. Ag^+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the dependent on the size and shape of nanoparticles [28]. Mostly, AgNPs were preferentially bound to the cytoplasmic membrane and disturb the cell membrane protein thereby it kill the bacteria [29]. The actual bacterial mechanism of AgNPs is not well known, some researchers agree that silver releases Ag^+ ions, and they interact with thiol groups of bacterial proteins affecting the replication of DNA [30].

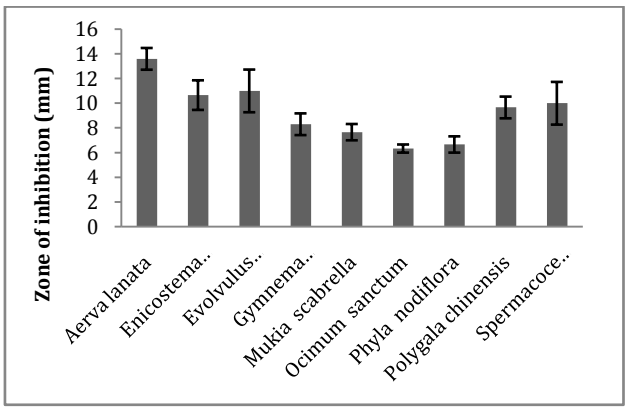

Fig. 5: *In vitro* antibacterial activity of AgNPs synthesized from medicinal plants.

Values are the mean of n=3 (mean ± Standard error)

Biosensor bioassay was performed with different concentration of AgNPs synthesized from nine different medicinal plants aqueous leaf extracts. A strong quorum quenching activity was observed in five plants such as *P. chinensis*, *E. alsinoides*, *E. axillare*, *S. hispida,* and *A. lanata* even at minimum concentration 50 ppm. However, a weak quorum quenching activity was observed in *M. scabrella, G. sylvestre, O. sanctum and P. nodiflora* at the concentration of 100, 150, 200 and 100 ppm respectively (Table-2).

Table 2: Assay for the inhibition of violacein pigment in C*hromobacterium violaceum*

S. No.	Name of the plant	Zone of inhibition (mm)			
		Concentration of AgNPs (ppm)			
		50	100	150	200
1	*Aerva lanata*	9	10	12	16
2	*Enicostema axillare*	7	8	10	11
3	*Evolvulus alsinoides*	8	10	12	13
4	*Gymnema sylvestre*	Nil	Nil	8	11
5	*Mukie scabrella*	Nil	7	10	12
6	*Ocimum sanctum*	Nil	Nil	Nil	5
7	*Phyla nodiflora*	Nil	7	9	10
8	*Spermacoce hispida*	8	10	11	13
9	*P. chinensis*	8	10	12	14

Our result revealed that the green synthesized AgNPs exhibited very effective toxicity against MRSA and also demonstrated varying level of AHL mediated violacein pigment inhibition in this reporter strain. The real mechanism of quorum quenching activity appeared to be a net effect of the ability of nanocolloids to interfere with the activity of quorum sensing molecules of *S. aureus*. Furthermore, the synthesized nanocolloids exhibited concentration-dependent inhibitory activity, which showed reduction in violacein production with the increase in concentration [31]. In most of the research, it appears that AgNPs have multiple modes of action in controlling microbial virulence and thereby it indirectly inhibits the QS-gene [32].

CONCLUSION

In our study we have demonstrated the rapid reduction of silver ion by the aqueous leave extract of *A. lanata*. The present study provides evidence that the leaves are good source for synthesizing stable AgNPs in lesser time. The presence of surface active molecules such as flavonoids, terpenoids in the plant leaves extracts stabilize the silver nanoparticles. Furthermore the green synthesized AgNPs established very effective antibacterial and quorum quenching activity against MRSA**.** Thus toxicity studies of AgNPs on Multidrug resistant bacteria open a door for a new range of antibacterial agents.

ACKNOWLEDGEMENT

The authors are very grateful to the management of SASTRA University and Department of Chemistry & Biosciences for providing facility to carry out our research works.

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