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Research Article

GREEN SYNTHESIS OF SILVER NANOPARTICLES: ITS EFFECT ON QUORUM SENSING INHIBITION OF URINARY TRACT INFECTION PATHOGENS

SHAHEEN SHEIKH, VIDYA TALE*

Department of Microbial Biotechnology, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University, Pune, Maharashtra, India. Email: vstale@gmail.com

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ABSTRACT

Objective: The present study was conducted to evaluate the antimicrobial and anti-quorum sensing (QS) ability of nanoparticles synthesized using fruit wastes.

Methods: QS is a system of response and stimuli correlated to population density. Bacteria by QS secrete certain signaling molecules called autoinducers. These bacteria also have a receptor that can specifically detect the signaling molecule known as an inducer. Since QS governs numerous processes in bacteria including virulence, QS inhibition promises to be an ideal target for the development of novel therapeutics. In the present study, silver nanoparticles (AgNPs) synthesized from the ginger, lemon peel, cinnamon, corn silk, pomegranate peel, and orange peel exhibited anti-QS properties by inhibiting violacein production in *Chromobacterium violaceum* assayed using agar well diffusion method.

Results: AgNPs were synthesized from various cost-effective fruit waste sources. These AgNPs exhibited significant antibacterial and anti-QS properties.

Conclusion: Hence, such sources can be explored for developing the effective therapy for urinary tract infections.

Keywords: Silver nanoparticles, Quorum sensing, Urinary Tract infection.

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INTRODUCTION

Quorum sensing (QS) is related to population density. Due to the QS activity, there is increase in population density which results in biofilm formation [1]. The reason for mechanical stability in biofilm is the extracellular polymeric substances (EPS) which mainly comprise polysaccharides, proteins, nucleic acids, and lipids helps in adhesion, forms a three dimensional network, and transiently immobilizes biofilm cells [2]. Biofilm is consortium of microorganisms enclosed in a robust exopolysaccharide matrix. Many Gram-negative pathogens have the ability to produce N-acyl homoserine lactones (AHLs) as signal molecules for QS [3]. This cell–cell communication system allows them to coordinate gene expression and regulate virulence. Detection of AHLs is important in the identification of QS capabilities in bacteria. *Chromobacterium violaceum* is a Gram-negative bacterium that produces the purple pigment violacein in response to the presence of the AHL N-hexanoyl homoserine lactone. By activating proteins belonging to the LuxR family of transcriptional regulators, these signal metabolites allow population density-dependent gene regulation within a species, as well as interspecies communication among different bacteria [4]. Gala and Desai have reported the potential ability of various plant extracts to disrupt bacterial QS which plays a key role in regulation of virulence in many Gram-negative and Gram-positive bacteria [5].

Nanoparticles can be used to reduce the QS activity which in turn will inhibit the biofilm formation and can be used as a therapeutic agent. Koh *et al.* reported the various types of plant-based natural products that exhibit anti-QS properties and their anti-QS mechanisms [6]. Plant parts such as pea (*Pisum sativum*) seedlings also produce exudates that contain compounds that can interfere with QS. Bhardwaj (2013) studied different model systems on various QS inhibition [7]. Al-Hussaini and Mahasneh reported antibacterial and antifungal activity of ethanol extract of different parts of medicinal plants against bacteria and fungi [8]. Mary and Banu reported anti-QS activity of methanolic leaf extract of *Vitex trifolia* against in *Pseudomonas aeruginosa* [9]. Markowska *et al.* described some anti-biofilm approaches using silver nanoparticles (AgNPs). Nanotechnological approaches to combat biofilm formation are based on the use of nanoparticles to functionalize the surface of biomaterials by coating, impregnation or by embedding nanomaterials [10]. One such technology with great potential is generation by the use of sonochemistry of nanoantibiotics that are more active and more effective than classical antibiotics against drug-resistant pathogens. The reason for the increased efficacy of these nanocompounds is likely to be due to improved permeability through the cell envelope. Nanotechnology along with biological sources results in generating of therapeutics which can be used to treat urinary tract infection (UTI).

METHODOLOGY

Isolation and characterization of UTI pathogens

Urine samples from UTI patients were collected from the hospital. The urine sample was inoculated using nutrient agar medium and incubated at 37°C for 24-48 hrs. Ten randomly selected colonies were characterized morphologically and biochemically. Gram Staining was performed. Biochemical tests such as IMViC test, Triple Sugar Iron agar, catalase test, oxidase test, nitrate reductase, and urease test were performed. Starch hydrolysis and hemolysis were performed using starch agar and blood agar medium, respectively.

The various selective and differential media were used for tentative identification of isolates including eosin methylene blue agar, Dettol agar, Mannitol salt agar, *Salmonella Shigella* agar, Brilliant Green agar, and MacConkey agar. The plates were incubated at 37°C for 48 hrs.

Screening biofilm forming ability

The biofilm formation ability of bacterial isolates was screened for using tube test described by Christensen *et al.* [11]. A loopful of test

organisms was inoculated in 10 mL of trypticase soy broth (TSB) with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hrs. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube [12]. The amount of biofilm formed was scored as weak/none (1), moderate (2), and high/strong (3).

Estimation of protein and carbohydrate

The amount of protein produced by bacteria was estimated by Folin-Lowry method. The overnight grown cultures of isolated bacteria (0.1 mL) were diluted with 0.9 mL distilled water to make up the volume to 1 mL. Reagent I (4.5 mL) was added and incubated for 10 minutes followed by reagent II (0.5 mL) and incubated for 30 minutes. The absorbance at 660 nm was measured [13].

Estimation of carbohydrate was carried out by phenol-sulfuric acid method. Overnight culture (10 mL) was grown in TSB broth with 5% sucrose. Overnight grown culture was centrifuged at 9000 rpm for 30 minutes at 4°C. The supernatant was collected in fresh tube. Two volumes of ethanol were added. It was kept overnight at 4°C. The pellet was dissolved in 5 mL distilled water. To 1 mL of EPS solution, 1 mL of 5% phenol followed by 5 mL of concentrated. H_2SO_4 was added. The mixture was incubated on ice for 20 minutes. Absorbance at 490 nm was recorded [14].

Synthesis of AgNPs

AgNPs were synthesized using peels of orange, lemon, and pomegranate and whole ginger. A volume of 20 g of each sample was cut into small pieces. The sample was dissolved in 200 mL of distilled water and boil for about 20 minutes. It was filtered with the help of muslin cloth. A volume of 40 mL of 1 mm $AgNO_3$ was added to the filtrate. The solution was kept at 60°C for 30 minutes followed by room temperature for 48 hrs. The solution was centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet was dissolved in distilled water and centrifuged at 8000 rpm for 15 minutes. The pellet was washed with 70% ethanol and centrifuged again. The pellet was again washed with distilled water and centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet was dried to obtain purified nanoparticles. It was then dissolved in appropriate amount of dimethyl sulfoxide (DMSO) [15].

Antibacterial assay

Agar well diffusion assay was performed to determine the antibacterial activity of AgNPs. Overnight grown culture of bacteria was spread on the sterile Mueller-Hinton agar plates and left to dry for 30 minutes. Wells were prepared and AgNPs (100 µl) dissolved in DMSO were loaded into the wells. DMSO acts as a negative control. The plates were incubated at 35°C ± 2°C for 24-48 hrs. After incubation, the zone of inhibition was measured and expressed as zone of sensitivity millimeter in diameter [16].

Extraction of natural C6-AHL from *C. violaceum* MTCC 2656

The method reported by Shaw *et al.* (1997) was used for the extraction of AHL. *C. violaceum* MTCC 2656 was grown in 200 mL LB broth on shaking incubator at 28°C for 18 hrs. The culture was then centrifuged at 8000 rpm for 1 hr, and the supernatant obtained was sterilized by membrane filtration. The filtrate obtained was extracted with acidified ethyl acetate (supernatant/acidified ethyl acetate, 7: 3, v/v) and finally concentrated and dried at 40°C and reconstituted in acetonitrile. The extract was stored at 4°C for further use in bioassay with *C. violaceum* MTCC 2656. The amount of extracted AHL needed for *C. violaceum* MTCC 2656-based assay was standardized by agar well diffusion plate assay [17].

Anti-QS activity of AgNPs using *C. violaceum* MTCC 2656

Anti-QS activity of nanoparticles using *C. violaceum* MTCC2656 was performed as reported by Khan *et al.* [18]. It was carried out by agar well diffusion method in the presence of appropriately standardized amount of natural C_6-AHL. LB agar plates were spread with 0.1 mL of appropriately diluted freshly grown culture of *C. violaceum* MTCC2656 (as reporter strain) and bacterial isolates (test organisms). AgNPs in three different combinations were loaded (100 µl) along with natural C6-AHL. Plates were incubated for 18-24 hrs at 28°C to check the inhibition of growth around the well.

RESULTS AND DISCUSSION

Morphological and biochemical characterization of the isolates

Ten randomly selected colonies were subjected to further characterization. Of ten isolates, 3 were Gram-positive and 7 were Gram-negative bacteria. The predominant organisms were *Pseudomonas* spp., *Escherichia coli*, *Staphylococcus* spp., *Klebsiella* spp., and *Salmonella* spp. The results of biochemical tests are shown in Table 1.

Screening for biofilm formation

Biofilm tube test was carried out to check for biofilm-forming capacity of the isolates, and the observations were recorded as high, moderate, and low biofilm forming capacity. Maximum biofilm formation was observed in *Pseudomonas* spp., *E. coli*, and *Staphylococcus* spp. However, *Salmonella* spp. showed moderate biofilm forming capacity. *Klebsiella* spp. is least in biofilm formation [19,20]. Protein and carbohydrate content of the organism is directly related to biofilm

Table 1: Biochemical characteristics of bacterial isolates

Biochemical test	Characteristics of isolated bacteria				
	***Pseudomonas* spp.**	***Staphylococcus* spp.**	***Klebsiella* spp.**	***Salmonella* spp.**	***E. coli***
Indole production	-	-	-	-	-
Methyl-red	-	+	-	+	+
Voges-Proskauer	-	+	+	-	+
Citrate utilization	+	+	+	-	-
Triple sugar iron agar test	Slant: Red Butt: Red Gas: Negative H_2S: Negative	Slant: Yellow Butt: Yellow Gas: Negative H_2S: Negative	Slant: Yellow Butt: Yellow Gas: Positive H_2S: Negative	Slant: Red Butt: Yellow Gas: Positive H_2S: Positive	Slant: Yellow Butt: Yellow Gas: Positive H_2S: Negative
Catalase	+	+	+	+	+
Oxidase	+	-	-	-	-
Nitrate reductase	+	+	+	+	+
Starch hydrolysis	+	+	+	-	-
Urease	-	+	+	-	
Blood agar	-	β	-	β	-

E. coli: Escherichia coli

formation since the biofilm is made up of exopolysaccharide matrix. This content is correlated to QS activity. Hence, protein and carbohydrate was estimated of the bacterial isolates.

Estimation of protein and carbohydrate

Protein estimation was done by Folin-Lowry method, and carbohydrates were estimated by phenol-sulfuric acid method. Amount of protein and carbohydrates produced by various isolates are shown in Tables 2 and 3. The experiment was performed in triplicates, and the mean observation of the each isolate is shown Table 2.

Green synthesis of AgNPs

Nanoparticles were synthesized from ginger, lemon peel and orange peel, pomegranate peel, corn silk, and cinnamon using 1 mm $AgNO_3$. Change in colour of solution from pale yellow to brown was observed after incubation of sample with AgNo3 at 60C for 30 mins followed by room temperature for 48hrs. . The AgNPs suspension was diluted ten times with deionized water and evaluated by ultraviolet-visible (UV-VIS) spectrometer within the range of 300-700 nm. The Gaussian peak was observed in the range of 400-500 nm for the different sources used during synthesis.

Table 2: Estimation of protein produced by the isolates

Bacterial isolates	Amount of protein (mg/mL)			Mean±SD
Pseudomonas spp.	0.899	0.905	0.894	0.899±0.005507571
Staphylococcus spp.	0.867	0.875	0.871	0.871±0.004
Klebsiella spp.	0.928	0.865	0.978	0.923±0.056624494
Salmonella spp.	0.788	0.79	0.821	0.799±0.018502252
E. coli	0.981	1.15	0.813	0.981±0.168500247

SD: Standard deviation, *E. coli: Escherichia coli*

Table 3: Estimation of carbohydrate produced by the isolates

Bacterial isolates	Total carbohydrate content (mg/mL)			Mean±SD
Pseudomonas spp.	0.147	0.15	0.146	0.147±0.002081666
Staphylococcus spp.	0.15	0.271	0.249	0.223±0.064454118
Klebsiella spp.	0.211	0.171	0.193	0.191±0.020033306
Salmonella spp.	0.159	0.183	0.178	0.173±0.01266228
E. coli	0.178	0.181	0.239	0.199±0.034385074

SD: Standard deviation, *E. coli: Escherichia coli*

Antibacterial assay of AgNPs

Antibacterial assay nanoparticles were carried out by agar well diffusion method. Zone of inhibition (mm) was recorded. The experiment was performed in triplicates, and the mean observation of the each isolate is shown in Table 4. The maximum antibacterial activity was shown by nanoparticles synthesized from ginger with maximum zone of inhibition of 18 mm against *Staphylococcus* spp. However, nanoparticles synthesized from all the sources significantly inhibit UTI pathogens. The results were similar to the reports given by Ghosh *et al.* showing QS inhibition by guava leaf extract [21].

Anti-QS assay

Anti-QS assay was done for the detection of QS capabilities of bacteria by agar well method using three different concentrations of the nanoparticles and natural C_6-AHL molecules (Table 5).

DISCUSSION

Pseudomonas spp., *Salmonella* spp., *E. coli, Staphylococcus* spp., and *Klebsiella* spp., were isolated from UTI infected urine samples. These bacteria are predominant pathogens responsible for UTI as per the reports [19,22]. In the present study, AgNPs were synthesized using cost-effective biological sources. These AgNPs exhibited significant antimicrobial activity against the isolates. The results are in accordance to the previous reports by Hassan *et al.* [15]. AgNPs also exhibited anti-QS activity by inhibiting the violacein production. Similar results are reported by Khan *et al.* [18] and Ghosh *et al.* [21]. This study focuses on using spices (cinnamon and ginger) and some value-added sources such as peels of lemon, orange, and pomegranate can be used as ideal source for generating nanotechnology-based therapeutics for treating UTI.

CONCLUSION

In the present study, effects of green synthesized AgNPs were studied on UTI isolates. Predominant bacteria isolated from urine sample of UTI-infected patients were *Pseudomonas* spp., *Salmonella* spp., *E. coli,*

Table 4: Antimicrobial activity of silver nanoparticles

S. No	Bacteria isolates	Zone of inhibition (mm)					
	Sources used for synthesis of silver nanoparticles	Pomegranate	Corn silk	Cinnamon	Orange	Lemon	Ginger
1	*Pseudomonas* spp.	-	-	14	-	-	-
2	*Staphylococcus* spp.	12.5	-	18	14.5	13	13
3	*Klebsiella* spp.	11.5	-	18	14.5	13	13
4	*Salmonella* spp.	13	-	13	14	14	16
5	*E. coli*	13	-	13	14	12	12
	Mean±SD	12.5±0.707106781	-	15.2±2.588435821	14.25±0.288675	13±0.816497	13.5±1.732051

SD: Standard deviation, *E. coli: Escherichia coli*

Table 5: Anti-quorum sensing activity of silver nanoparticles

Source of nanoparticle synthesis	Zone of inhibition (mm)																	
	Pseudomonas spp.			*Staphylococcus* spp.			*Klebsiella* spp.			*Salmonella* spp.			*E. coli*			*C. violaceum*		
	1*	2*	3*	1*	2*	3*	1*	2*	3*	1*	2*	3*	1*	2*	3*	1*	2*	3*
Pomegranate	11	10	8	13	12	10	15	13	10	12	9	7	12	10	9	-	-	-
Corn silk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cinnamon	15	14	11	12	7	6	-	-	-	16	14	11	13	12	9	13	15	-
Orange	16	13	12	13	10	8	14	10	7	13	10	8	10	8	7	11	10	10
Lemon	11	8	7	11	10	9	11	10	9	14	11	7	17	14	11	13	12	11
Ginger	12	8	6	13	12	11	13	12	10	14	13	12	12	10	9	11	10	-

SD: Standard deviation, *E. coli: Escherichia coli, C. violaceum: Chromobacterium violaceum*, 1*: 90 µl nanoparticles+10 µl C6-AHL, 2*: 70 µl nanoparticles+30 µl C6-AHL, 3*: 50 µl nanoparticles+50 µl C6-AHL

Staphylococcus spp., and *Klebsiella* spp. Biofilm formation ability of these isolates was checked. High biofilm formation was observed in *Pseudomonas* spp., *E. coli*, and *Staphylococcus* spp. Moderate to low biofilm forming activity was observed in *Salmonella* spp., *Staphylococcus aureus,* and *Klebsiella* spp. AgNPs were synthesized from various cost-effective fruit waste sources. UV-VIS characterization of these AgNPs indicated maximum absorption in the range 400-500 nm. These AgNPs exhibited significant antibacterial and anti-QS properties. Hence, such sources can be explored for developing the effective therapy for UTI.

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