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Antibiotic and antioxidant activities biogenic silver nanoparticles synthesized using *Escherichia coli* (VM1) bacterium

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Synthesis of silver nanoparticles (AgNPs) using microorganisms has gained considerable attention as an efficient alternative for conventional chemical synthesis. Earlier, we have demonstrated biosynthesis of silver nanoparticles using marine *Escherichia coli* VM1. Here, we investigated antibacterial, antifungal and antioxidant efficacy of the synthesized silver nanoparticles using standard techniques. The results have shown that the AgNPs synthesized using the marine *Escherichia coli* have potential antibacterial activity against both Gram positive (*Staphylococcus aureus*) and Gram negative (*E. coli*, *Klebsiella pneumoniae*, *S. paratyphi*, *Vibrio parahaemolyticus*, *V. cholerae* and *Proteus mirabilis*) pathogenic bacteria and moderate antifungal activity was observed against *Aspergillus niger*, *Penicillium* sp., *Trichothecium roseum* and *Trichophyton rubrum* . Further, *in vitro* antioxidant study was examined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. We observed significant effect on scavenging of free radicals suggesting that the AgNPs biosynthesized using *E. coli* could be an efficient and eco-friendly material for biomedical applications.

Keywords: Antibacterial, Antifungal, DPPH radical scavenging activity, Hydrogen peroxide radical scavenging activity

Nanobiotechnology is an interdisciplinary scientific approach in biochemical applications that focuses on the synthesis of nanoparticles using microbial sources to observe the improved antimicrobial, antioxidant and cytotoxic studies. Nanobiotechnology incorporates the efficient exploitation of the antimicrobial nature of silver in the form of nano-sized particles. Because of its unique mode of action, the nanobiotechnology has given a hope for the development of a new generation of antibiotics, to overcome multi drug resistance[1].

Metal nanoparticles, particularly, silver nanoparticles (AgNPs), have gained researchers' attention throughout the world in various fields, such as preparation of antimicrobials[2,3], therapeutics[4] and biomolecular detection[5]. Until now, there have been several investigations made on the application of AgNPs against different bacterial strains. Currently, topical dressings supplemented with silver are widely used to cure infections in open injuries and chronic wounds[6]. The importance of excessive free radicals in a body can be understood by observing the role they play in various degenerative diseases such as cancer and cardiovascular diseases[7].

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Chemosynthesis, a popular method for nanoparticle synthesis, involves toxic chemicals as reducing gents and leads to non ecofriendly derivatives. Awareness on biogenic nanoparticles has driven the need for alternative eco-friendly methods for nontoxic production of NPs[8]. In our previous study, we reported a simple and rapid technique for synthesis of AgNPs by a marine *E. coli* VM1. The biogenic AgNPs was examined against cell line and proved to be an effective anticancer agent[9]. In this work, we evaluated antioxidant activities of *E. coli* VM1 synthesized AgNPs by diverse processes. In addition, the antibacterial and antifungal activities of AgNPs against representative bacteria and fungi pathogens were also investigated.

Materials and Methods

Screening of bacterium, bio-reduction and characterization of AgNPs

For synthesis of AgNPs, the potential bacterium VM1 was isolated from marine sediments of Ennore coast, India. The strain was characterized as *E. coli* which was exploited for its capability to synthesize AgNPs. The resultant NPs were characterized by employing, UV–Vis spectroscopy, FTIR spectroscopy, X-ray diffraction and High Resolution Transmission Electron Microscopy (HRTEM) studies and results

were published by Maharani *et al.*[9]. The entire experimental work conducted to synthesize the *E. coli* based AgNPs and its applications described as a flow chart in Scheme 1.

Determination of antimicrobial assay

Pathogenic microorganisms of human origin were procured from the Raja Muthaihah Medical College, Annamalai University. These included bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi*, *S. paratyphi*, *Klebsiella oxytoca*, *E. coli*, *Vibrio parahaemolyticus*, *V. cholerae* and *Proteus mirabilis* used to investigate the antibacterial activity. The pathogenic fungal organisms, namely *Aspergillus niger*, *Penicillium* sp., *Trichothecium roseum*, *Trichoderma koningii*, *Rhizopus stolonifer*, *Hormodendrum hordei* and *Trichophyton rubrum* were used to examine the antifungal activity. To facilitate cultivable the pathogens in lab, nutrient broth and nutrient agar were employed for bacteria whereas for fungal pathogens, potato dextrose broth and potato dextrose agar were used. These were kept on corresponding agar slants at a temperature of 4°C.

The bacterial pathogens were inoculated into ~~a~~ sterile nutrient broth and incubated at 37°C for 18 h. The size of final inoculum was maintained at 10^5 CFU/mL by diluting fresh cultures with normal saline. For fungal pathogens, the culture was obtained by growing fungi on Potato dextrose agar (PDA) and incubating at 28±2°C. The inoculum was developed in potato dextrose broth by inoculating five different colonies (roughly around 1.0 mm in diameter) from 24 h old culture, and was perched in 5 mL of sterile 0.85% saline solution. The resultant suspension was vortex and the turbidity range was adjusted to yield 2×10^6 cells/mL (0.5 McFarland standards).

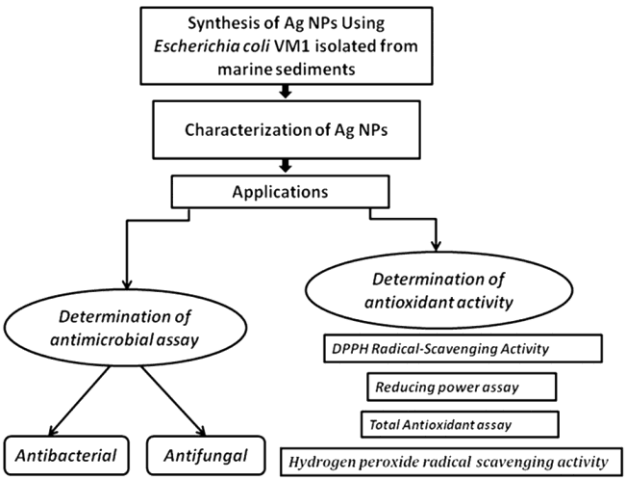

Fig. 1 — Flow chart of *E. coli* based Ag NPs synthesis and its applications

Antibacterial activity

Antibacterial properties of AgNPs were assessed by agar disc diffusion technique, by following the Guidelines of Clinical Laboratory Standard Institute guidelines (CLSI, 2018)[10]. The pathogenic bacterial suspensions (10^5 CFU/mL) were uniformly streaked in three different directions on the of Mueller Hinton agar plates using sterilized cotton swab. The sterilized filter paper discs (5 mm diameter) were dipped separately, in 50 µL of synthesized AgNPs (10, 20, 30, 40, 50 µg/mL) and dried at room temperature (28ºC). Later filter paper discs with different concentration of synthesized AgNPs were placed above the Mueller Hinton agar plates. Amikacin, 10 mcg/disc and Milli Q water was utilized as +ve and −ve. The plates were incubated at 37°C for 24 h and the zone of inhibition around each disc was observed. Experiments were performed in triplicate and average values of zone inhibition diameters were taken.

Antifungal activity

Pathogenic fungal growth inhibition by synthesized AgNPs was observed by the agar disc diffusion method, described by Bansod &Rai[11]. Sterile swabs were dipped in the fungal suspensions and uniformly applied on PDA medium. The sterile 5 mm diameter filter paper discs were dipped in the 50 µL of synthesized AgNPs having different concentrations 10, 20, 30, 40 and 50 µg/mL and dried at room temperature (28°C) and they were placed over the petridishes. Nystatin, was used as a +ve control for anti-fungal study and all plates were kept it in a incubator at 28±2°C for 72 h and the zone of inhibition around the individual disc was examined.

Determination of antioxidant activity

DPPH radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was assessed following the procedure of Ravikumar *et al.*[12] In brief, 1.0 mL of synthesized AgNPs at different concentrations 100, 200, 300, 400 and 500µg/mL was transferred to 1.0 mL of 0.16 mM DPPH methanolic solution. The same was blended for one minute and allowed to stand at 28°C for 30 min in dark. The absorbance was measured at a wavelength of 517 nm by using Spectra Max Plus384. The lower absorbance showed higher radical scavenging activity. The capability to scavenge the DPPH radical was measured with the following equation:

Scavenging activity (%) =

$$[(A_{control} - A_{sample}) / A_{control}] \times 100$$

where, $A_{control}$ denotes the absorbance of the control (DPPH solution without AgNPs), A_{sample} is the absorbance value of the test sample (DPPH solution and AgNPs). Butylated hydroxytoluene (BHT) was used as the standard.

Reducing power assay

The reducing ability of the AgNPs was examined by the procedure of Oyaizu[13] Briefly, 500 µL of biogenic AgNPs in increasing dilutions of 100, 200, 300, 400, 500 µg/mL was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide, separately. The resultant mixture was incubated at 50°C for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid **(**$C_2HCl_3O_2$) was added to it and then centrifuged at 650×g for 10 min. 2.5 mL of supernatant was transferred to another test tube and 2.5 mL of distilled water along with 0.1% of 0.5 mL ferric chloride were added to it. Photometric absorbance of the test samples was observed at 700 nm, with the help of a spectrophotometer. The rise in absorbance value in the reaction mixture indicated rise in reducing power. BHT was used as standard.

Total Antioxidant assay

For total antioxidant assay of AgNPs, 'Phospho-molybdenum Procedure' described by Ravikumar *et al.*[12] was used. To the increasing dilutions of the test solution (100, 200, 300, 400 and 500 µg/mL), reagents in solution form such as, 28 mM sodium phosphate, 0.6 M sulphuric acid, and 4 mM ammonium molybdate was added, separately. Later it was incubated at 95°C for 90 min. Then, it was cooled off at room temperature (28°C) and the photometric absorbance was observed at 695 nm in spectro-photometer. The mixture of 1.0 mL of reagent and 0.1 mL of distilled water was used as control. Ascorbic acid solution was used as standard. The total antioxidant potential was expressed as a number of gram equivalent of ascorbic acid.

Hydrogen peroxide radical scavenging activity

The hydrogen peroxide scavenging ability of the AgNPs was estimated using the method described by Avani Patel *et al.*[14]. About 300 µL sample of biogenic AgNPs in increasing dilutions of 100, 200, 300, 400, 500 µg/mL was mixed with hydrogen peroxide solution (0.1M) which was prepared in standard phosphate buffer (pH 7.4). The mixture was incubated along with control (phosphate buffer with hydrogen peroxide) and blank solution (phosphate buffer without hydrogen peroxide) at 36°C temperature for 10 min. The photometric absorbance value of hydrogen peroxide was observed at 230 nm in a spectro-photometer. BHT was used as standard. The percentage of hydrogen peroxide scavenging activity of Ag NPs was estimated using the subsequent formula:
The percentage of hydrogen peroxide scavenging activity (H_2O_2) = $[(A_o - A_1) / A_o] \times 100$, where A_o is the absorbance of the control and A_1 is the Absorbance of the test sample

Statistical analysis

Each experiment was performed in replicates and the results were expressed as mean±standard deviation was calculated using SPSS software version 16.0.

Results and Discussion

The silver nanoparticles (AgNPs) were synthesized by a *E. coli* bacterium isolated from marine sediments of Ennore coast. The synthesized AgNPs were crystalline in nature and the dimensions of the single nanoparticles have been in the range of 10-15 nm. The research work published by Maharani *et al*.[9] focused on the synthesis and characterization of AgNPs extracted from *E. coli*. The present study discusses the antimicrobial and antioxidant activity of AgNPs extracted from marine *E. coli*.

Silver ions and silver based compounds are renowned universal antimicrobial substances and are extreme bactericidal. Kędziora *et al.*[15] explained the possible resistance mechanism of antibacterial activity of silver ions. Briefly, the silver ions from AgNPs attach to the negatively charged bacterial cell wall. It results in denaturation of bacterial protein which leads to cell death indicatings that the antimicrobial efficacy of AgNPs vary according to the concentration used against pathogens under test. The observation was supported by Valodkar *et al*.[16] who had observed increase in zone of inhibition when concentration of AgNPs was increased. The biologically synthesized AgNPs showed excellent antibiotic activity against both Gram positive bacterium (*Staphylococcus aureus* and*)* and Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *S. paratyphi*, *Vibrio para-haemolyticus*, *V. cholerae* and *Proteus mirabilis*) pathogens (Table 1). Pazos-Ortiz *et al.*[17] observed the antibiotic activity of AgNPs against both Gram negative and Gram positive bacteria. Sarkar *et al.*[18] also observed that AgNPs possess superior bactericidal efficiency against *E. coli* and *S. aureus* as compared to penicillin. When AgNPs were used

against microorganisms, Gram negative bacteria showed highest zone of inhibition as compared with Gram positive bacteria. The difference in response to AgNPs, might be due to the thick cell wall composed of peptidoglycan layer of Gram positive bacteria. The peptidoglycan layer consists of linear polysaccharide chains, cross linked by short peptides which gives more rigidity to cell wall and makes it difficult for the penetration of AgNPs into the bacterial cell wall[19]. The present study also confirms that the antibiotic effect of AgNPs is dosage dependent and is more effective against Gram negative as compared to Gram positive bacteria.

The bactericidal mechanism of AgNPs is well recognized though the studies on the antifungal activities of AgNPs are inadequate. In present study on the antifungal activity of AgNPs of microbial origin, moderate activity was observed against *Aspergillus niger*, *Penicillium* sp., *Trichothecium roseum* and *Trichophyton rubrum*. No activity was observed in the remaining fungal pathogens (Table 2). The antifungal effect was also dosage dependent and the antifungal activity increased with the increased concentration of AgNPs in the test sample. Similarly, Kim *et al.*[20] have also observed significant antifungal activity of AgNPs against six clinical fungal isolates. They indicated the possible mechanism of AgNPs against fungal species. According to Kim *et al.* AgNPs exert disturbance in the arrangement of the cell membrane and it further affects the normal budding process due to the damage of the membrane integrity. Further, Roe *et al.*[22] have also studied the antifungal activity of plastic catheters covered with AgNPs, exhibiting excellent inhibition results for *Candida albicans*. Monteiro *et al.*[23] found excellent MIC results of AgNPs against *C. albicans* and *C. glabrata.* In the present study, the antibiotics such as Amikacin and Nystatin were used as a standard for observing antifungal activity. In the present study, the *E. coli* based AgNPs have emerged as a probable future biocidal against bacterial and fungal pathogens.

The antioxidant action of biosynthesized AgNPs was examined using different *in vitro* assays. The antioxidant activity of the AgNPs was compared with the standard i.e., BHT in the DPPH free radical scavenging assay. The antioxidants react with DPPH and modify it into 1,1-diphenyl-2-picryl hydrazine with decolonization. The DPPH radical-scavenging activity of AgNPs is shown in Fig. 2A. The results indicate that AgNPs produced by *E. coli* VM1, exhibited highest radical scavenging activity (75.83±0.37%), at 500 µg/mL of concentration. The DPPH activity of the NPs increases in a dosage dependent approach. The present study in which enhanced DPPH scavenging activity of AgNPs has been observed, some of the other researchers have reported similar results with metal nanoparticles[24,25].

Figure 2B confirms the reducing biocidal activities of AgNPs along with BHT as standard. The synthesized AgNPs of bacterial origin NPs exhibited significant reducing abilities ($P < 0.05$) in a dosage dependent manner. The maximum activity was found at 2.64±0.05 OD in 500 µg/mL and minimum activity was found at 0.81±0.03 OD in 100 µg/mL. The capacity of reducing ability from Fe^{3+} to Fe^{2+} conversion was examined in the presence of AgNPs.

Table 1 — Antibacterial activity of AgNPs against bacterial strains

Bacterial pathogens	Zone of inhibition (mm)				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
G-ve bacteria					
Escherichia coli	14.2±0.2	16.5±0.5	17.7±0.3	18.8±0.4	19.8±0.3
Klebsiella pneumonia	9.3±0.1	11.4±0.3	13.4±0.21	15.0±0.2	15.9±0.5
Salmonella typhi	-	-	-	-	-
Vibrio parahaemolyticus	12.3±0.03	14.2±0.2	14.9±0.05	15.6±0.3	16.5±0.3
Klebsiella oxytoca	-	-	-	-	-
Proteus mirabilis	7.0±0.03	8.5±0.02	9.2±0.23	9.5±0.3	10±0.5
Vibrio cholera	6.0±0.5	7.2±0.2	7.8±0.04	8.2±0.03	8.5±0.12
Salmonella paratyphi	-	-	-	-	-
G+ve bacteria					
Staphylococcus aureus	-	-	7.5±0.2	8.3±0.3	8.9±0.1
Streptococcus pneumoniae	-	-	-	-	-

[(-) = no zone of inhibition. Values are given as mean ± SD of three experiments]

Table 2 — Antifungal activity of AgNPs against fungal strains

Fungal pathogens	Zone of inhibition (mm)				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
Aspergillus niger	-	-	9.3±0.03	10.5±0.05	10.8±0.12
Penicillium sp.	-	-	8.5±0.3	9.0±0.5	9.7±0.5
Trichoderma koningii	-	-	-	-	-
Trichothecium roseum	-	-	8.3±0.12	9.4±0.2	10.0±0.3
Hormodendrum hordei	-	-	-	-	-
Rhizopus stolonifer	-	-	-	-	-
Trichophyton rubrum	-	7.8±0.05	9.5±0.2	10.3±0.3	11.2±0.4

[(-) = no zone of inhibition. Values are given as mean ± SD of three experiments]

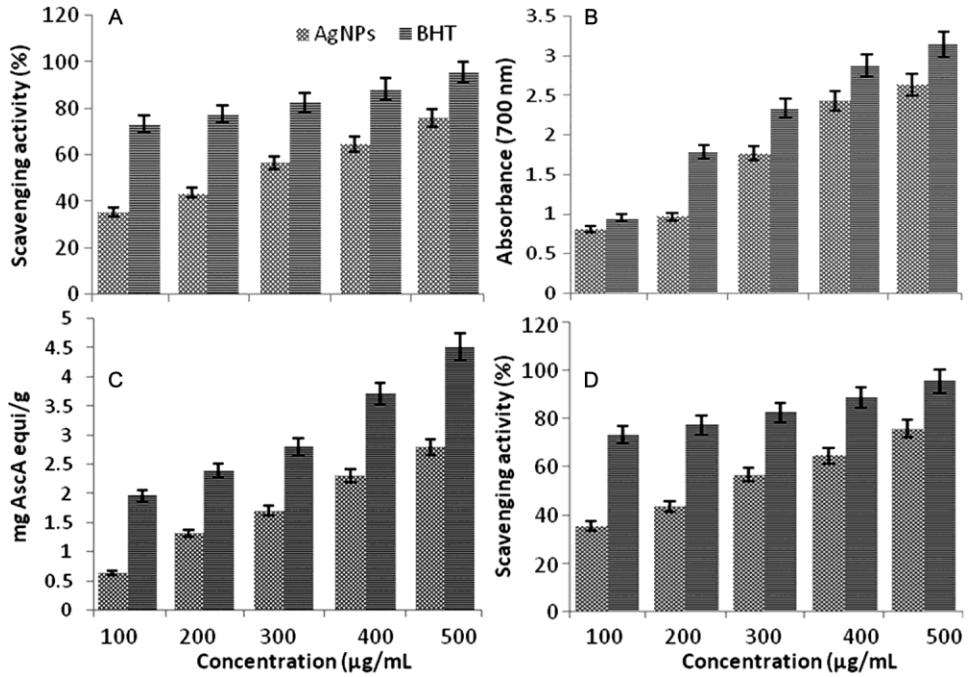

Fig. 2 — (A) Scavenging activity of AgNPs on DPPH radical; (B) Reducing power; (C) Total antioxidant activity; and (D) Hydrogen peroxide scavenging activity of AgNPs

The reducing capacity of AgNPs could serve as an important index for potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms, for instance, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging[26,27]. Similar to the DPPH scavenging activity, the reduction power of the AgNPs has been found to increase as the concentration increases. At all the concentrations of AgNPs, significantly higher bactericidal and fungicidal activities were observed. It indicates that AgNPs synthesized from *E. coli* AgNPs have greater reducing power. Husain *et al*[28] also support the present findings they made similar observations with AgNPs extracted from using *Nostoc muscorum* NCCU 442.

The total antioxidant activity of AgNPs is presented in Fig. 2C. The variations in antioxidant activity were observed. The antioxidant activity increases with increase in concentration ($P < 0.05$). The present experiment reveals that the antioxidant activity of the AgNPs increases with the increase in concentration of the ascorbic acid. Similar observations with enhanced total antioxidant activity have been observed by Husain *et al.*[28]. Shunmugam *et al.*[29] extracted gold nanoparticles by marine bacterium *Vibrio alginolyticus.*

The hydrogen peroxide radical scavenging assay of AgNPs is presented in Fig. 2D. An effective scavenging activity (72.83±0.3%) was observed at a concentration of 500 µg/mL of AgNPs. Once hydrogen peroxide enters into the cell, it is able to infiltrate rapidly into the cell membranes. H_2O_2 possibly reacts with Fe^{2+}, and Cu^{2+} ions to form hydroxyl radicals and this may be the cause of most of its toxic effects[30]. In the present study on the AgNPs of marine *E. coli* origin, it was observed that the hydrogen peroxide scavenging activity is dose dependent and it is statistically significant ($P < 0.05$). Similar types of observations were also made with AgNPs prepared from stem bark of *Shorea roxburghii*[31] and *Piper longum*[32]. The present findings as demonstrated by various *in vitro* antioxidant assays above clearly indicate that the AgNPs extracted from *E. coli* possess antioxidant activity.

Conclusion

In this study, effect of *E. coli* based synthesized silver nanoparticles (AgNPs) on bioactive applications was analyzed. The biogenic AgNPs have demonstrated significant antibiotic activity against bacteria, such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, S. paratyphi, Vibrio parahaemolyticus, V. cholerae* and *Proteus mirabilis*

and showed a moderate antifungal activity against *Aspergillus niger*, *Penicillium* sp., *Trichothecium roseum* and *Trichophyton rubrum.* Further, the *E. coli* based AgNPs exhibited highest free radical and hydrogen peroxide radical scavenging activity. The highlight of this study is that the *E. coli* based AgNPs showed better biocidal effect against the same *E. coli* species. However, it requires further investigation in detail to fully understand the mechanism behind this biocidal effect.

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Conflict of interest

Authors declare no competing interests.

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