ARTICLE

Greens Synthesis of Antimicrobial Nanosilver using *in vitro* Cultured *Dioscorea bulbifera*

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ABSTRACT

Dioscorea bulbifera is a true yam species which is famous for its medicinal values. The plant is reported to possess anti-inflammatory, antidiabetic and antitumor properties. It has also been found that the *D. bulbifera* tuber extract is effective in synthesizing silver nanoparticles (AgNPs) because of its unique phytochemistry However, the plant is available in the rainy season only hence in this study *in vitro* system for maintenance of the *D. bulbifera* was developed using three media combinations namely basal Murashige and Skoog medium (MS), MS medium supplemented with 5 ppm kinetin (AN) and MS medium enriched with $CuSO_4 \cdot 5H_2O$ (CU). Aqueous extracts of these *in vitro* grown plantlets were found to have significant contents of phenolics, flavonoids and starch. These extracts were found to be effective in rapid synthesis of the AgNPs in 5 h with the optimum temperature of 50 °C and salt concentration equal to 5 mM. Fourier transformed infrared spectroscopy (FTIR) analysis revealed that the polyols in these extracts are responsible for bioreduction. AgNPs synthesized from extracts of *Dioscorea bulbifera* were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). AgNPs from plantlets growing on MS medium were found to have the smallest size and thus showed maximum antibacterial and antibiofilm potential towards *Pseudomonas aeruginosa* and *Vibrio harveyi.* The AgNPs synthesized from the extracts of plantlets growing on AN and CU medium were also found to be effective. The results also suggested the presence of variation in the mechanism of biofilm inhibition by AgNPs against these two bacteria as biofilm inhibition was found to be greater in *Vibrio harveyi.* To best of our knowledge no such study has been done before with the *in vitro* grown *Dioscorea bulbifera*.

KEYWORDS

Dioscorea bulbifera, *in vitro* Culture, Silver nanoparticles, *Pseudomonas aeruginosa*, *Vibrio harveyi*, Antibiofilm.

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INTRODUCTION

Dioscorea bulbifera is an arial yam species which is very well known for its medicinal properties. It is found effective in the treatment of a variety of chronic diseases such as cancer, type II diabetes and ischemic heart disease [1-3]. The plant is also used as a home remedy to treat diarrhea, conjunctivitis and dysentery [4]. In Ayurvedic medicine it is commonly used for rejuvenation and detoxification [5]. These medicinal properties of the plant are due to the presence of wide range of phytochemicals present in *Dioscorea bulbifera* [6]. This

rich phytochemistry of the plant has been exploited for the synthesis of silver, gold, palladium and plantinum nanoparticles [6-8]. Green synthesis of nanoparticles is the technique developed in the recent years for efficient and eco-friendly synthesis of nanoparticles using plant extracts. The technique involves plant derived phytochemicals such as phenolics, flavonoids and sugars as reducing agents and ascorbic acid as capping agent [9]. Thus, clearly phytochemistry plays an integral role in the process.

Like most of the other plants phytochemical profile of *D. bulbifera* has been found to vary with environmental conditions and geological location. Additionally various stress conditions like pathogen attack, drought, pollution also alter the phytochemistry [10]. Such variation may affect the nanoparticle synthesis potential. Plant tissue culture technique provides a way of reducing variations in the phytochemistry by using specific culture medium [11]. Moreover, the *in vitro* grown plantlets provide an additional advantage of availability of plantlets throughout the year. Thus, in this study, *D. bulbifera* plants were grown on different culture media and the effect of the phytochemical profile on nanoparticle synthesis was analyzed. The synthesized particles were characterized by UV-visible spectroscopy, Fourier transformed infrared spectroscopy (FTIR), transmission electron microscopy (TEM), dynamic light scattering (DLS).

Bacterial biofilms are communities of bacteria embedded in polysaccharide matrix. They enhance the virulence of the bacteria making it resistant to many conventional antibiotics [12]. *Pseudomonas aeruginosa* is one of such bacterias that forms biofilms in the lungs leading to cystic fibrosis [13]. Similarly, *Vibrio harveyi* is a marine pathogen that forms biofilms on shrimps [14]. AgNPs have long been known for antibacterial and antibiofilm potential. Thus, in this study, the synthesized AgNPs were assessed for their antibiofilm potential against biofilms of *P. aeruginosa* and *V. harveyi.* Recently, there are no reports on antibiofilm potential of nanoparticles synthesized from *in vitro* grown *D. bulbifera*.

EXPERIMENTAL

Growth media and culture conditions: *D. bulbifera* nodal segments collected from Parvati hill, Pune were surface sterilized and then inoculated on the following three media namely Murashige and Skoog (MS), kinetin (AN) and $CuSO_4 \cdot 5H_2O$ (CU). Each of these media had Murashige and Skoog basal medium supplemented with tryptone (0.5 g/L), L-cysteine HCl (10 mg/L), activated charcoal (0.2 %) and agar (0.8 %). Additionally, AN medium was supplemented with kinetin (5 ppm) + IAA (0.1 ppm) [15]. CU medium was fortified with kinetin (Kn) (5 ppm) + indole acetic acid (IAA) (0.1 ppm) + $CuSO_4$ (75 µM) [16]. The inoculated explants were incubated at 26-28 °C and exposed to 16/8 h light/dark cycle. Subculturing of the plantlets was done after every 22-25 days and after 3rd subculture these plantlets were used for synthesis of AgNPs.

Extraction of *in vitro* grown plantlets: Plantlets from all the three media (MS, AN, CU) were separately extracted. 5 g fresh weight of all three types of *in vitro* grown plantlets of *D. bulbifera* were boiled in 100 mL of water for 5 min. These three extracts namely DBE-MS, DBE-AN and DBE-CU were filtered through Whatman No. 1 filter paper and were stored at 4 °C [7].

Phytochemical analysis of the extracts: Phytochemical contents of all the extracts of the plantlets grown on MS, AN and CU medium were estimated by using standard biochemical tests.

Total phenolics content: 0.125 mL of the extract was mixed with 0.5 mL of distilled water and 0.125 mL of Folin-Ciocalteau reagent. This mixture was incubated for 5 min at room temperature and 1.25 mL of 7 % Na_2CO_3 solution was added to it. Volume of the solution was made up to 3 mL with distilled water. This solution was incubated for 90 min at room temperature. Absorbance was measured at 760 nm. Total phenolics content was determined by standard gallic acid curve [17].

Total flavonoids content: 0.5 mL of extract was mixed with 0.5 mL of 2 % $AlCl_3$ in methanol. This solution was incubated for 10 min at room temperature. Absorbance was recorded at 358 nm. Total flavonoid content was determined by standard quercetin curve [18].

Starch content: 0.5 mL of extract was diluted with 0.5 mL of distilled water. 4 mL of anthrone reagent was added to it and the solution was incubated in boiling water bath for 8 min. Absorbance was recorded at 630 nm. Starch content was determined by standard glucose curve [19].

Total reducing sugar content: 1 mL of the extract was mixed with 1 mL of 3,5-dinitrosalicylic acid reagent and it was incubated for 5 min at 95 °C. 10 mL of distilled water was added to this mixture and the absorbance was recorded at 540 nm. Total reducing sugar content was determined by standard maltose curve [20].

Diosgenin content: 5 mL 60 % perchloric acid was added to 1 mL of extract. This solution was incubated for 10 min at room temperature. Absorbance was recorded at 410 nm and diosgenin content was determined by standard diosgenin curve [21].

Synthesis and characterization of AgNPs: AgNPs were synthesized by adding 5 mL of extracts (DBE-MS, DBE-AN, DBE-CU) to 95 mL of $AgNO_3$ (1 mM) and incubated at 40 °C for 5 h on shaking incubator [7]. Bioreduction of Ag^+ ions was confirmed by analyzing the ultraviolet-visible spectra at regular intervals on a spectrophotometer (SpectraMax Molecular Devices Corporation, Sunnyvale, CA) operating at a resolution of 1 nm. Optimum temperature for the synthesis of AgNPs was determined by monitoring the synthesis of AgNPs at different temperatures ranging from 4 to 50 °C. Similarly, concentration optimization studies were carried out by screening the nanoparticle synthesis at different $AgNO_3$ concentrations from 0.3 to 5 mM. The AgNPs formed were characterized using transmission electron microscopy (TEM), dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR).

Antimicrobial activity: Antimicrobial activity of the AgNPs synthesized from the extracts of *D. bulbifera* grown *in vitro* on MS, AN and CU media was determined by agar well diffusion technique. Overnight grown cultures of *P. aeruginosa* (MTCC 2295) and *V. harveyi* (MTCC 7779) were used. 100 µL of each of the three nanoparticles (100 µg/mL) were added to the separate wells. Sterile distilled water was used as control.

These plates were incubated at 30 °C for 24 h and then observed for the appearance of zone of inhibition.

Antibiofilm activity: Antibiofilm activity of AgNPs was analyzed by crystal violet assay. In short, 20 µL of the bacterial cultures along with the 100 µL of each of the serially diluted nanoparticles were added to 200 µL of LB broth in each well. Control samples were without nanoparticles. After incubation for 24 h, the supernatant was removed and plates were washed with phosphate buffer saline. The biofilm growth was stained with crystal violet and quantification was done at 595 nm as per our earlier report [22].

RESULTS AND DISCUSSION

Phytochemical analysis: Phytochemical quantification of DBE-MS, DBE-AN and DBE-CU confirmed the presence of phenolics, flavonoids, diosgenin, reducing sugars and starch. High concentration of phenolics, flavonoids and starch were observed to be present in aqueous extract of *D. bulbifera* (Table-1).

TABLE-1
PHYTOCHEMICAL ANALYSIS OF AQUEOUS EXTRACTS OF *D. bulbifera*

Phytochemical component	Quantity (mg/mL)		
	DBE-MS	DBE-AN	DBE-CU
Phenolics	2.02	1.16	0.96
Flavonoids	1.08	0.97	0.83
Diosgenin	0.04	0.03	0.93
Reducing sugar	0.83	1.81	0.14
Starch content	0.35	0.28	0.18

Synthesis and characterization of AgNPs

UV-visible spectroscopy: The synthesis of AgNPs by reduction of Ag^+ ions was confirmed by development of yellow colour while on complete bioreduction after 5 h at 40 ºC. UV-visible spectra showed that the extracts of the plantlets grown in all three media showed a rapid building up of peak at 440 nm from 1 h till 5 h (Fig. 1). The synthesis was found to be rapid, steady and efficient. However, maximum peak intensity was observed in case of the synthesis mediated by DBE-MS extract.

Temperature and concentration optimization: Concentration optimization studies revealed that 5 mM of $AgNO_3$ solution was optimum for synthesis of AgNPs using the extracts of *D. bulbifera* grown *in vitro* on MS, AN and CU media (Fig. 2). Although 4 and 3 mM gave identical rate of synthesis with extracts grown in MS and AN, in case of CU, 4 mM was found to give higher rate of synthesis using all three extracts (DBE-MS, DBE-AN, DBE-CU). However, lower concentrations showed much lower rate of synthesis with all of the three extracts. Temperature optimization studies showed that 50 ºC was the most suitable temperature that gave highest rate of AgNPs synthesis (Fig. 3).

TEM analysis: TEM analysis exhibited spherical to irregular shaped AgNPs synthesized by extracts. AgNPs synthesized by DBE-MS were found to be spherical in shape with an average diameter ranging from 16 to 20 nm (Fig. 4). AgNPs synthesized by DBE-AN were found to vary in their shape and size considerably. The particles were in range from 9 to 45 nm. Nanorods, blund ended triangles along with nanospheres were also observed. Size of AgNPs synthesized by extracts of plantlets grown in copper medium were found to be comparatively larger in diameter ranging from 20 to 40 nm.

Particle size analysis: Particle size distribution demonstrated that AgNPs synthesized using DBE-MS ranged from 5 nm to 295 nm (Fig. 5). AgNPs synthesized using DBE-AN found to vary widely in the size ranging from 7 nm to 5 µm. Similarly AgNPs synthesized by DBE-CU were found in a

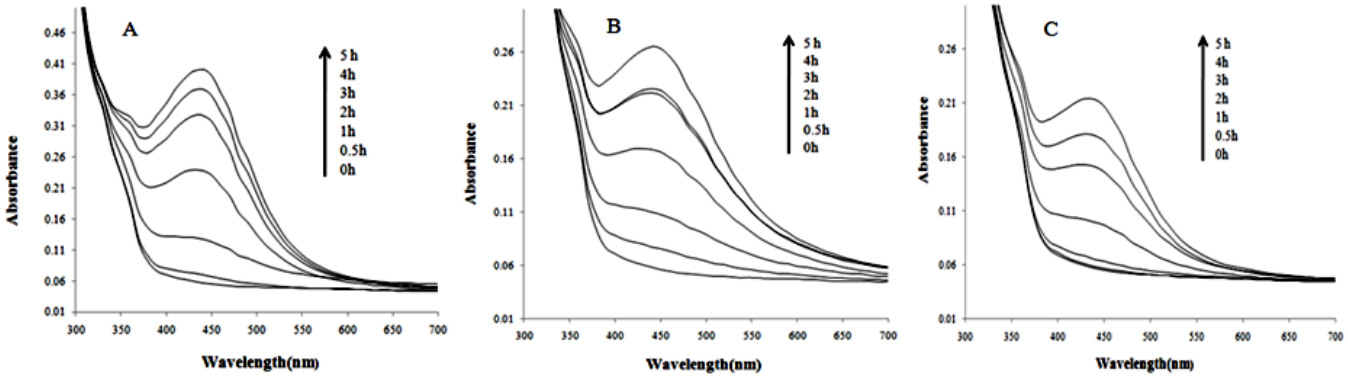

Fig. 1. UV-visible spectra recorded as a function of reaction time for synthesis of AgNPs by extracts of *D. bulbifera* plantlets (A) DBE-MS, (B) DBE-AN and (C) DBE-CU for 5 h at 40 °C

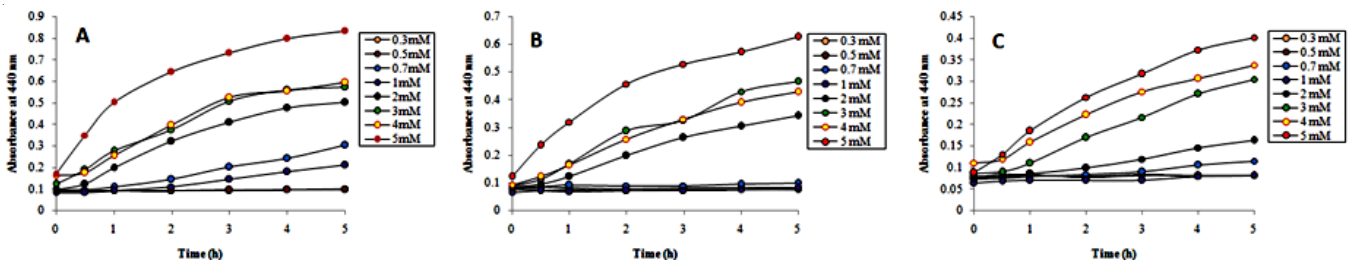

Fig. 2. Time course of AgNPs synthesis with varying concentrations of $AgNO_3$ by extracts from *D. bulbifera* plantlets (A) DBE-MS, (B) DBE-AN and (C) DBE-CU for 5 h at 40 °C

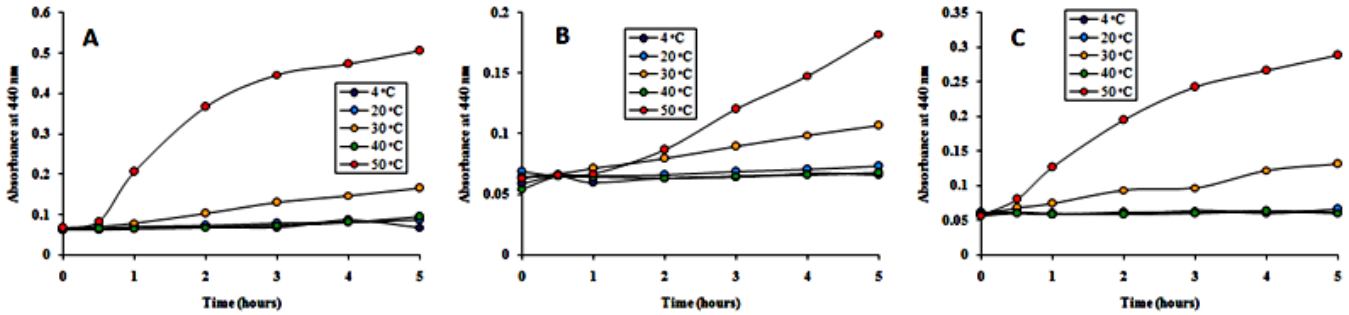

Fig. 3. Time course of AgNPs synthesis with varying reaction temperature at 1 mM concentration of $AgNO_3$ by extracts from *D. bulbifera* plantlets till 5 h. (A) DBE-MS, (B) DBE-AN and (C) DBE-CU

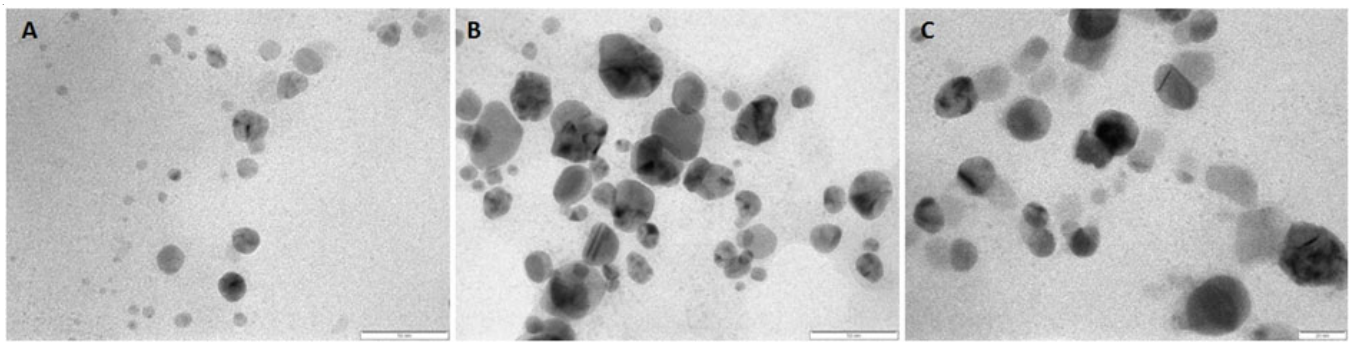

Fig. 4. TEM micrographs of AgNPs synthesized by extracts from *D. bulbifera* plantlets grown in (A) DBE-MS, (B) DBE-AN and (C) DBE-CU

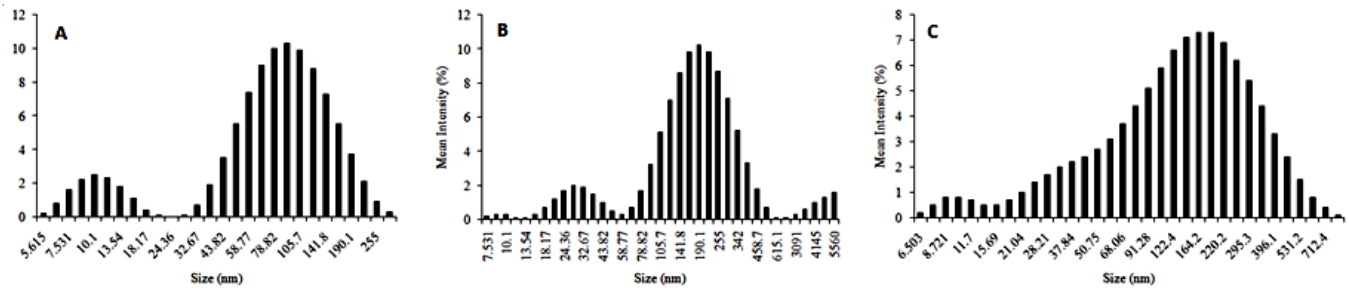

Fig. 5. Particle size distribution of AgNPs synthesized by extracts from *D. bulbifera* plantlets (A) DBE-MS, (B) DBE-AN and (C) DBE-CU

range between 6 nm to 825 nm. The larger dimensions might be formed by the slow agglomeration of the smaller particles in due course of maturation.

FTIR analysis: FTIR analysis was carried out for extracts as well as for nanoparticles to predict which phytochemical is involved in the synthesis of AgNPs (Fig. 6). In the formation of nanoparticles from DBE-MS, there is a disappearance of peaks ranging from 3501 to 3051 cm^{-1} which indicates the loss of –OH and increased intensity of the peak at 1724 cm^{-1} indicating the formation of carbonyl group in the nanoparticles. Thus, polyols present in the extract must be responsible for the bioreduction. Decrease in the intensities of peaks ranging from 3400 to 3200 cm^{-1} was observed in case of AgNPs synthesized using DBE-AN indicate the utilization of –OH group containing compound in the bioreduction. Appearance of new peak of 1197 cm^{-1} in the spectrum after DBE-AN mediated AgNPs synthesis indicates the involvement of ether linkage. This ether linkage containing compound must be acting as a capping agent for the AgNPs synthesis. Decrease in the intensities of peaks ranging from 3400-3200 cm^{-1} was observed after DBE-CU mediated AgNPs synthesis as compared to that in DBE-CU indicating the utilization of –OH group containing

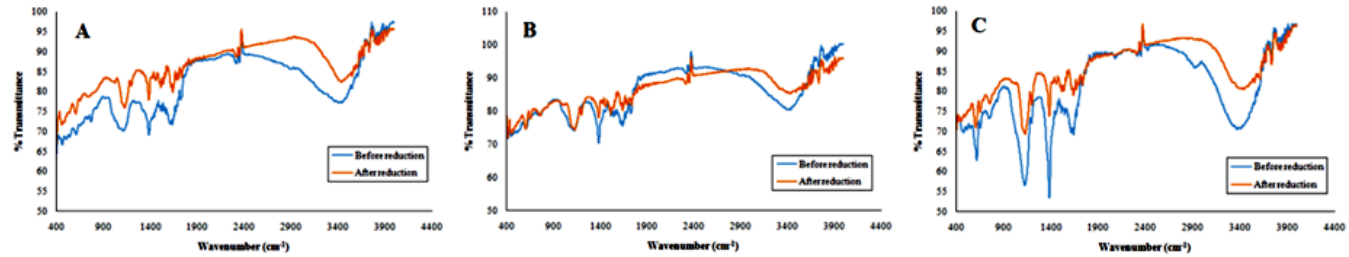

Fig. 6. FTIR of extracts before and after bioreduction of Ag^+ to AgNPs (A) DBE-MS, (B) DBE-AN and (C) DBE-CU

compound in the bioreduction. Appearance of the new peak of 3736 cm^{-1} after the nanoparticles synthesis corresponds to amide linkage. So, the nanoparticles must be surrounded by amino groups resulting in efficient stability owing to capping.

Antibacterial activity: Antimicrobial activity of AgNPs, using well diffusion method against both bacteria showed significant zones of inhibition (Fig. 7). All three AgNPs samples, synthesized from DBE-MS, DBE-AN and DBE-CU showed almost identical antimicrobial activity against *P. aeruginosa* and *V. harveyi*. Mean inhibition zone diameter for *P. aeruginosa* was found to be 9 mm and that for *V. harveyi* was 3 mm. This clearly indicates that the AgNPs exhibit greater cytotoxicity towards *P. aeruginosa* than that towards *V. harveyi*. The concentration of nanoparticles was found to be 30 µg/mL.

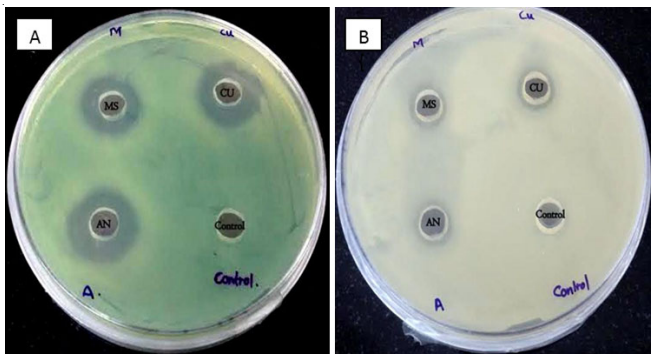

Fig. 7. Zones of inhibition by AgNPs synthesized from DBE-MS, DBE-AN and DBE-CU against (A) *P. aeruginosa* and (B) *V. harveyi*

Antibiofilm activity: In order to assess antibiofilm potential of AgNPs synthesized using DBE-MS, DBE-AN and DBE-CU, *P. aeruginosa* and *V. harveyi* were incubated for 48 h in the presence of AgNPs. The plate assay clearly showed that the AgNPs synthesized from the extracts of *in vitro* grown plantlets of *D. bulbifera* significantly inhibit the biofilm formation against both the bacteria. However, the percentage inhibition of biofilm by each of three types of AgNPs was found to be different. Highest biofilm inhibition was exhibited by AgNPs synthesized by DBE-MS against both the bacteria are shown in Table-2.

TABLE-2
BIOFILM INHIBITION (%) BY AgNPs

AgNPs (source)	Biofilm inhibition (%)	
	P. aeruginosa	*V. harveyi*
AgNPs (DBE-MS)	39.18	45.65
AgNPs (DBE-AN)	35.28	41.48
AgNPs (DBE-CU)	19.77	40.53

D. bulbifera is widely used in Ayurvedic and Chinese medicine. The plant grows in temperate region and the availability of the plant is restricted to rainy season only. Therefore, in this study *D. bulbifera* plantlets were grown *in vitro* using three media namely MS, AN and CU. This helped in maintaining the plants throughout the year. MS medium is classical Murashige and Skoog basal medium which is the combination of macronutrients, micronutrients, vitamins and carbon source required for the plant growth. AN medium was additionally supplemented with a cytokinin kinetin (5 ppm). The high cytokinin content supported the growth of the plantlets as described by Narula *et al*. [15]. The CU medium was enriched with $CuSO_4 \cdot 7H_2O$ as a source of copper ions as supplementation of divalent cations has been reported to promote growth in *D. bulbifera* [16]. The medicinal importance of *D. bulbifera* is due to the rich phytochemistry of the plant [6]. However, it has been demonstrated that the phytochemical profile of the plant varies with geographical locations and ecological conditions. Tissue culture techniques involving controlled growth conditions leads to production of plantlets with less variation in phytochemical profiles. Thus, in the present study, the plantlets grown *in vitro* on MS, AN and CU medium were extracted and their phytochemical profile was analyzed. Phenolics, polysaccharides and flavonoids were known to synthesize nanoparticles in eco-friendly method [23-26]. Thus the high levels of these phytochemicals in MS extract must be responsible for rapid synthesis of uniform AgNPs. DBE-MS showed highest phenolic and flavonoid content. Moreover, this diverse phytochemistry of *D. bulbifera* has also made the plant capable for green synthesis of variety of metallic nanoparticles. Therefore, the three extracts DBE-MS, DBE-AN, DBE-CU were analyzed for the potential to reduce $AgNO_3$ to AgNPs. The extracts of *in vitro* grown *D. bulbifera* plantlets resulted in the rapid synthesis of AgNPs in 5 h at the optimum temperature of 50 °C. The optimum $AgNO_3$ concentration for this synthesis was found to be 5 mM. This was in accordance with previous studies by Ghosh *et al*. [7] involving use of *D. bulbifera* tubers for AgNPs synthesis. However, in nature, the vegetative tubers serve as only mode of propagation of the plant. Further, the phytochemical contents in the tuber vary as per the age of the plant and season of collection. Additionally, TEM analysis revealed that DBE-MS, DBE-AN and DBE-CU produced more uniform AgNPs as compared to those by *D. bulbifera* tuber extracts. DBE-MS resulted in the production of the smallest AgNPs which can be attributed to high phenolic content. This result was further supported by the FTIR analysis which suggested the involvement of polyols in the bioreduction of $AgNO_3$ to Ag^0. Further, FTIR analysis also revealed that ether linkage groups and amide linkages provide capping in case of AgNPs synthesized by DBE-AN and DBE-CU, respectively. Thus, the *in vitro* grown plantlets possess phytochemicals which facilitate the formation of stable AgNPs.

The AgNPs are notable antimicrobial agents that are effective against broad spectrum of bacteria [27]. Thus, AgNPs synthesized by DBE-MS, DBE-AN and DBE-CU were analyzed for the antibacterial potential against *P. aeruginosa* and *V. harveyi*. All the AgNPs were found to exhibit efficient antibacterial effects against both *P. aeruginosa* and *V. harveyi.* Additionally, the AgNPs have been reported to be efficient against bacterial biofilms [28]. Biofilm is complex organization of bacteria in polysaccharide matrix. Thus, biofilms enhance the virulence of bacteria. The size of the nanoparticles has been previously correlated with the antibiofilm potential [29]. Smaller the nanoparticles greater is the uptake in the bacterial cells and thus more is the antibiofilm potential. In the present studies also AgNPs from DBE-MS showed highest antibiofilm activity followed by DBE-AN and DBE-CU. The biofilm inhibition by AgNPs from DBE-MS was more against *V. harveyi*

compared to *P. aeruginosa*. This must be due to differential mode of uptake of AgNPs in both the bacteria. Thus, this study, for the first time reported the synthesis of AgNPs from *in vitro* grown *D. bulbifera* plantlets and their antibiofilm potential.

Conclusion

The study reported the synthesis of AgNPs from extracts of *in vitro* grown *D. bulbifera* plantlets. The reaction parameters like $AgNO_3$ concentration and temperature were optimized. The characterization studies revealed that the synthesis of AgNPs having smaller size and fair stability could be achieved through this green route. Further the synthesized nanoparticles showed significant antibacterial and antibiofilm potential against *Psudomonas aeruginosa* and *Vibrio harveyi.*

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