OPTIMIZATION OF GREEN SYNTHESIZED SILVER NANOPARTICLES FROM CARALLUMA UMBELLATA

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INTRODUCTION
Nanoparticles have allured medicine and other fields due to its outstanding physical, chemical and biological properties [1]. Here, the nanodrugs act as carriers to improve the drug distribution to the targeted site by protecting the healthy cells [2]. The metal silver in the form of Rajata Bhasma had its attention from traditional medicine and is known to elevate the immune response [3]. Silver in the form of nanoparticles (AgNPs) are noble metals where their large surface area gives enhanced antimicrobial activity [4]. This exceptional property of silver has made its availability especially in biomedical industry and almost in all consumer products [5]. Plant-mediated green synthesis of AgNPs can be synthesized in a lucrative and eco-friendly manner [6] by replacing chemically derived reducing and capping agents with plants phytochemicals [7, 8], whereas the other methods tends releases noxious chemicals to the environment [9]. Also, plants efficacy can be increased by synthesizing AgNPs from its parts [10].

In this present study, the silver nanoparticles are synthesized from the tribal plant Caralluma umbellata (fig. 1) of family Asclepiadaceae. C. umbellata is the ground-dwelling succulent, xerophytic herb consisting of two parts-root and thornless stem [11]. Due to the presence of various phytochemicals and diverse therapeutic properties, C. umbellata is used to treat stomach ache and gastric ulcers by Kanni and Yanadi tribes of Tamil Nadu and Andhra Pradesh respectively [12, 13], the ethanolic extracts of C. umbellata serves as a remedy for liver toxicity [14], methanolic extract of this plant showed promising anti-diabetic activity [15]. Here, AgNPs were synthesized in an optimized route from novel stem extract of C. umbellata and the antimicrobial activity of the synthesized AgNPs (Cu-AgNPs) was compared with the pure stem extract of C. umbellata (Cu-SE).

MATERIALS AND METHODS
Materials
Silver nitrate (AgNO₃) from Fisher Scientific was used. Stems of Caralluma umbellata (AKAJ/MK101/09/2017) was collected in 12.1899 ° N, 79.9249 ° E, Auroville women's nursery (Marakkanam). Nutrient agar, Potato dextrose agar, antibiotic tetracycline and fluconazole, microbial strains E. coli, B. subtilis and A. niger.

Fig. 1: Tribal plant Caralluma umbellata

Stem extract preparation
The collected stems of C. umbellata was shade dried and grinded to fine powder. To that 5g of the powdered mixture, 100 ml of double-distilled water was added and was heated for 20 min. The pure stem extract was thus obtained by filtering the resulting concoction using whatman filter paper which turned yellow in colour.

Synthesis of silver nanoparticles (AgNPs)
To the 20 ml of the stem extract prepared, 20 ml of 0.55 mmol silver nitrate solution was added at a regular interval of 5 min (1:1 ratio) and stirred continuously for 20 min using magnetic stirrer. This resulted in the colour change of the stem extract of C. umbellata from yellow to dark brown which signifies the reduction of Ag⁺ ions in the solution to Ag⁻ ions [10].
Characterization studies of AgNPs synthesized from stem extract of C. umbellata

The synthesized AgNPs absorbance and stability was monitored using UV-Visible spectroscopy. Scanning electron microscopy (SEM) can be used to observe the size and shape of the AgNPs in high resolution. The reducing and capping agent present in the stem extract in the form of phytochemicals were identified by Fourier-Transform Infrared (FTIR) spectroscopy. The FTIR range was fixed between 500 cm\(^{-1}\) to 4500 cm\(^{-1}\). The X-Ray Diffraction (XRD) analysis were performed over 2θ range from 20° to 80°, where the crystal size and structure of the stem extract can be identified. The elemental silver presence along with its percentage of weight can be analysed using Energy Dispersive X-ray (EDX) analysis.

Antibacterial assay

The bacterial and fungal strains were obtained from The Institute of Microbial Technology, Chandigarh, India. The antibacterial activity was analysed for the Cu-SE and Cu-AgNPs against gram positive Bacillus subtilis (MTCC 441) and gram negative Escherichia coli (MTCC 739). Well diffusion assay was prepared by making 5 wells in the solidified nutrient agar swabbed with 24 h grown culture. With tetracycline as a positive control in the middle well and different concentrations of the test samples (250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml) were loaded to the four surrounding wells and incubated at 37 °C for 24 h. The inhibition zone diameter was measured after incubation to understand its inhibitory action [8].

Antifungal assay

Antifungal action of varying concentration (250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml) of Cu-SE and Cu-AgNPs were tested against Aspergillus niger (MTCC 5889) swabbed onto solidified potato dextrose agar (PDA) medium. Antibiotic fluconazole was used as a positive control. The zone of inhibition was measured after incubation at 37 °C for 2-3 d [16].

RESULTS

RSM optimization

The optimal conditions for the synthesis of AgNPs were carried out by Response surface methodology (RSM) integrated with Box Behnken Design (BBD) using Design Expert software (7.0.0 trial version). A total of 17 runs were experimentally conducted for the process parameters Silver nitrate (AgNO\(_3\)) concentration (mM), temperature (°C) and incubation time (min). The output responses observed through experiments was in close fit with the model predicted values (fig. 2). Also, p-value Prob>F (0.0001) in ANOVA represents that the model is significant. Using Design expert, the 3D plots (fig 3a, 3b, 3c) generated predicted the optimal conditions at the maximum absorbance (0.7398) to be 0.55 mmol AgNO\(_3\) concentration, 45 °C and 1442.50 min (24 h).
UV-Visible spectroscopy

The AgNPs synthesized from the Cu-SE can be observed visually by the colour change of the extract from yellow (fig. 4a) to dark brown (fig. 4b) on the addition of aqueous silver nitrate (AgNO₃) solution. The reduction of Ag⁺ ions to Ag⁰ ions by the phytochemicals present in the Cu-SE was initially analyzed by UV-Visible spectroscopy with the absorbance band near 425.5 nm (fig. 5).

Fig. 3 (A, B, C): 3D plots of the combined effect of two variables on C. umbellata plant-mediated synthesis of AgNPs

Fig. 4: Colour change of Cu-SE from (A) yellow to (B) dark brown (Cu-AgNPs)
Scanning electron microscopy (SEM)

The size and shape of the Cu-AgNPs can be detected by sputter coating the sample on the copper stub and the images of nanoparticles were studied using SEM [17]. From the SEM images, the spherical shape of the Cu-AgNPs with size ranging from 50 nm to 85 nm was observed (Fig. 6).

Energy dispersive X-ray spectroscopy (EDAX)

The EDAX analysis of Cu-AgNPs confirmed the presence of elemental silver without any contamination (Fig. 7). It was found that among all the elements present, silver is found to contain in higher percentage and its weight percentage was found to be 16.54%.
Fourier transform infrared (FTIR) spectroscopy

From the FTIR analysis, the natural reducing and capping agents of the Cu-SE was identified. From fig. 8a and fig. 8b, the functional groups present in the Cu-AgNPs and Cu-SE was analyzed for the spectra peaks at 3410.15 cm\(^{-1}\), 2939.52 cm\(^{-1}\), 1080.14 cm\(^{-1}\), 879.54 cm\(^{-1}\), 732.95 cm\(^{-1}\) and 2931.80 cm\(^{-1}\), 2376.30 cm\(^{-1}\), 1087.85 cm\(^{-1}\), 864.11 cm\(^{-1}\). The absorption peak at 3410.15 cm\(^{-1}\) suggests the O-H stretch, peak at 2939.52 cm\(^{-1}\), 2931.80 cm\(^{-1}\) and 732.95 cm\(^{-1}\) represents the saturated hydrocarbon C-H stretch, methylene C-H stretch and aromatic C-H bend respectively.

Peaks at 864.11 cm\(^{-1}\), 879.54 cm\(^{-1}\) represents C-C vibration and 1087.85 cm\(^{-1}\), 1080.14 cm\(^{-1}\) is due to primary amine C-N stretch. The hydroxyl group and the C-H stretch of alkane and alkene in Cu-SE are mainly involved in the reduction of Ag\(^+\) ions to AgNPs. Also, the presence of carbonyl group of amino acid residues and Aromatic amine residues acts as a capping agent to prevent agglomeration and stabilizes AgNPs [18].

X-ray diffraction (XRD)

The diffraction peaks at 2θ values 27.783°, 32.196°, 46.130°, 67.32°, 74.47° and 76.59° in the experimental diffractogram obtained through XRD have been identified to be due to the presence of noble metal silver in the sample and the corresponding hkl values are (111), (200), (200), (220), (311) and (311) respectively (fig. 9). All the peaks represent the Face-centered cubic (FCC) structure of the silver element. The average crystalline size ‘D’ of AgNPs was calculated by Debye-Scherrer formula and was found to be 26 nm and the average lattice constant of AgNPs is 4.5535 Å [19].

Antibacterial activity

A comparison of the antimicrobial activity of the Cu-SE and Cu-AgNPs was done to observe the inborn antimicrobial activity of the plant (Cu-SE) and its enhanced efficacy by synthesis of AgNPs (Cu-AgNPs). In table 1 and table 2, when gram positive strain Bacillus subtilis were tested against Cu-SE and Cu-AgNPs (fig. 10a, 10b), the diameter of the inhibition zone was higher for Cu-AgNPs ranging
from 12 mm to 16 mm than the Cu-SE which was found to be 11 mm to 13 mm. Also, when both the samples were tested against gram negative bacteria *Escherichia coli* (fig. 11a, 11b), enhanced antibacterial activity was observed with Cu-AgNPs showing zone inhibition from 11 mm to 14 mm when compared to Cu-SE with 10 mm to 14 mm diameter inhibition zone.

**Table 1: Zone of inhibition (mm) of gram positive *B. subtilis* by plant extract and AgNPs**

| Inhibition                  | Concentration (µg/ml) |
|-----------------------------|-----------------------|
|                             | PC        | 250     | 500     | 750     | 1000    |
| *C. umbellata* extract (mm) | 19±0.20   | 9±0.10  | 11±0.20 | 11±0.30 | 13±0.15 |
| AgNPs (mm)                  | 19±0.20   | 11±0.25 | 12±0.15 | 14±0.25 | 16±0.40 |

PC-Positive Control, AgNPs-Silver Nanoparticles, *C. umbellata*-Caralluma umbellate, (n=4, mean±SD)

**Table 2: Zone of inhibition (mm) of gram negative *E. coli* by plant extract and AgNPs**

| Inhibition                  | Concentration (µg/ml) |
|-----------------------------|-----------------------|
|                             | PC        | 250     | 500     | 750     | 1000    |
| *C. umbellata* extract (mm) | 18±0.10   | 10±0.30 | 10±0.40 | 11±0.25 | 12±0.35 |
| AgNPs (mm)                  | 15±0.30   | 11±0.15 | 12±0.10 | 12±0.40 | 14±0.10 |

PC-Positive Control, AgNPs-Silver Nanoparticles, *C. umbellata*-Caralluma umbellate (n=4, mean±SD)

**Antifungal activity**

Table 3 represents the antifungal activity of *Aspergillus niger* and the inhibition zone. The plant extract showed an inhibition diameter of 10 mm for varying concentrations (250 µg/ml-1000 µg/ml) of the sample. And the AgNPs showed a slight increase in the inhibition diameter from 10 mm to 11 mm with an increase in the concentration from 250 µg/ml to 1000 µg/ml (fig. 12a, 12b).
DISCUSSION

Under optimized condition 0.55 mmol AgNO₃ concentration, 45 °C and 24 h of reaction time, the synthesized particles were confirmed as silver in a spherical shape with a size 50 nm to 90 nm possessing FCC structure. The AgNPs synthesized showed enhanced inhibition against the tested microbes bacteria and fungus when compared to the C. umbellata stem extract. The electrostatic attraction between the positive charge of AgNPs and negative charge of the cell membrane enables Cu-AgNPs to attack the cell membrane thereby, inhibiting the growth of gram positive than gram negative bacteria by its efficacy to penetrate deep into the thick peptidoglycan layer of gram positive bacteria by building reactive oxygen species, causing lysis of cell [20, 21] and serving as an antimicrobial agent. In the case of B. subtilis, Cu-AgNPs damages the cellular membranes by losing the membrane integrity thereby, increasing the permeability causing protein dysfunction, increased reactive oxygen species (ROS) in cells [22], lowering the reductase activity and Phag-GFP expression levels leading to cell death [23]. Similarly, the inhibition of E. coli by Cu-AgNPs begins with the accumulation of envelope protein precursors causing dissipation of the proton motive force and membrane integrity [24] thereby, ceasing the exchange of phosphate ions and this causes an outflow of accumulated phosphate along with succinate, mannitol, proline and ghistamate [25]. Hence, the reduced susceptibility of gram positive bacteria to antibiotic [26] has been overcome by treatment with Cu-AgNPs. The inhibitory activity of Cu-AgNPs is higher when compared to Cu-SE where these Cu-AgNPs damages the membrane and forms complexes with bases of DNA followed by inhibition of the normal budding process and cell lysis [16].

CONCLUSION

The present work suggests that the chosen tribal plant and the synthesized AgNPs acts as wide spectrum inhibitors with good antibacterial and biocidal properties. Also, the Cu-AgNPs synthesized is of affordable and environmentally supporting product. The presence of varied phytochemicals in the plant produces AgNPs in an eco-friendly way which is suitable for targeted drug-delivery with minimal damage to the healthy tissues. Also, the phytochemicals present performs its role as capping agents, hence stabilized AgNPs is produced. The presence of antimicrobial property in C. umbellata as inborn makes the synthesized AgNPs even more effective and as a potent germicide. The antibacterial property of Cu-AgNPs can be used as wound healing products, in food storage packages or vessels, textile industry, consumer products, etc. Due to its biocidal properties, Cu-AgNPs can be used to destroy plant pathogens thereby preventing rotting of vegetables, fruits and use of artificial fungicides. The synthesized Cu-AgNPs is of low priced and can be further studied for its potent healing properties in the field of nanomedicine.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally.

CONFLICT OF INTERESTS

Declared none

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