Seripheidium quettense mediated green synthesis of biogenic silver nanoparticles and their theranostic applications

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ABSTRACT
Green synthesis of nanoparticles is an emerging field of nanotechnology, preferred over physical and chemical synthesis owing to their safety, cost-effectiveness nature, bio-compatibility, eco-friendly and scalable properties. The present study includes Seripheidium quettense aqueous extract mediated green synthesis, optimization of silver nanoparticles (Sq-AgNPs) and their pharmacological evaluations. Synthesis was done considering various optimization parameters including concentrations of AgNO₃, pH of the reaction mixture, extract to precursor ratio and temperature. Biosynthesis was carried out using our already optimized conditions. UV–visible spectrophotometer, FTIR, XRD and SEM analysis were used for characterization of Sq-AgNPs. The synthesized Sq-AgNPs (49.96–54.36 nm) were evaluated for their antibacterial, antifungal, anticancer and hemolytic potentials. The maximum antibacterial activity was found against Escherichia coli, Klebsiella pneumonia and Bacillus subtilis with their MICs of 11.1, 33.3 and 33.3 μg/mL, respectively. Aspergillus niger was found as the most susceptible fungal strain with the highest zone of inhibition (13.2 ± 0.72 mm). Sq-AgNPs inhibited proliferation of human liver cancer cell lines (HepG2) with median lethal concentration (IC₅₀) of 62.5 µg/mL. Results of the hemolytic assay showed that SqNPs are bio-compatible and have less effect on erythrocytes even at high concentration of 100 µg/mL.

1. Introduction
Nanotechnology is an emerging arena with extensive impending medicinal potentials and applications. Due to their distinctive surface chemistry and morphological aspects, it has been exploited in many fields of science such as electronics, medicine, nutrition, designing,
optics and microscopy (1, 2) Among the metallic nanoparticles, AgNPs have got substantial focus due to their better stability, exceptional catalytic potentials and better electrical conductivity (3). AgNPs have wide applications from drug and gene delivery for sensing of nucleic acids and pathogens (4). Moreover, AgNPs can be used as an alternative therapeutic remedy against microbes, tumors and other infectious diseases (5). Several physical and chemical methods are reported to synthesize these AgNPs (6). Using these approaches, the precursor silver nitrate salt is reduced via reducing agents such as borohydride, citric acid or other hydrocarbons (7). But these approaches are expensive and are associated with safety issues. Subsequently, focus has been directed to the formulation of more safe methods for the synthesis of metallic nanoparticles.

The green synthesis approach is rapid, scalable, eco-friendly and inexpensive approach (5). Moreover, stable and biocompatible nano particles (NPs) can be produced using the green approach (2). As particle shape and size are very crucial in many bio-medical applications, the biogenic synthesis provides well control over particle size and morphology as compared to chemical or physical methods of NP synthesis. This method utilizes various plants, Algae, Fungi or microbial metabolites for the capping, reduction, and stabilization of respective NPs (8). In comparison to microbes, plants provides many advantages (6). Despite their multiple advantages, the biogenic synthesis has few limitations which can be controlled via optimization of extract-metal salt solution concentrations, pH and temperature of reaction mixture (9).

Seriphidium quettense, family Asteraceae, is traditionally used as vermifuge to relieve fever, diabetes and joints pain. Seriphidium herba-alba are used for treatment of diarrhea and abdominal cramps (10). Methanol extract of S. quettense (aerial parts) shows antimicrobial potentials against S. aureus, K. pneumoniae and P. aeruginosa (11). To best of our knowledge, no studies are reported on the S. quettense aqueous extract mediated green synthesis of Sq-AgNPs, their optimization and their biological evaluation. In this study, we have explored the potential application of Sq-AgNPs against microbes and cancer cells with the purpose of finding their applications in field of theranostics.

2. Materials and methods

2.1. Plant collection and extract preparation

Fresh aerial parts of S. quettense were collected in July 2016 from kawas village of District Ziarat, Baluchistan, Pakistan. Plant was identified by Professor Dr. Zabta Khan Shinwari, at Department of Plant sciences, Quaid-i-Azam University, Islamabad. A sample was deposited at herbarium, Department of Biotechnology, Quaid-e-Azam University Islamabad, Pakistan with voucher no: 350. The plant was shade dried for one week and crushed to powder (12).

2.2. Extract preparation

Plant powder (10 g) was taken in 200 ml of distilled water, boiled for 10 min and double filtered by using Whatman filter paper No.1. The extract was stored at 4°C in the refrigerator and further used for phytochemical analysis and synthesis of Sq-AgNPs (13).

3. Quantitative phytochemical analysis

Total reducing power of extract was estimated using potassium ferricyanide colorimetric assay (14). Estimation of Total Phenolic Content (TPC), DPPH free radical scavenging potentials and Total Antioxidant Capacity (TAC) (Phosphomolybdenum method) were evaluated according to already reported procedures (15). Total Flavonoid contents (TFC) was estimated as we reported previously (16).

4. Optimization and biosynthesis of SqNPs

To optimize AgNO3 concentration, SqNPs synthesis was checked at increasing concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mM. Briefly, 100 µL of S. quettense extract was added to 1000 µL of each concentration of AgNO3 solution in separate Eppendorf tubes. For pH optimization, same procedure was followed but using plant extract at increasing pH of 4, 5, 5.7 8 and 9. Ratio based optimizations were done according to the previously reported protocol (17). Different ratios (Ext: AgNO3) of (1:1, 1.5, 1:10, 1:15, 1:20 and 1:25 v/v) were used. For temperature optimization, synthesis was done at increasing temperatures of 25°C, 30°C, 37°C, 50°C, 60°C and 70°C. In order to check the stability of synthesized Sq-AgNPs, UV absorbance was monitored for the duration of two months. The stability of Sq-AgNPs was checked at 15, 30 and 60 days post synthesis.

5. Characterization of Sq-AgNPs

UV-3000 double beam spectrophotometer was used to check the formation of Sq-AgNPs. The UV-spectra was obtained by scanning the samples in the range between 200 and 800 nm. The functional characterization of biomolecules present in the Sq-AgNPs was performed by FTIR spectrometry. The characteristic infrared (IR) absorption spectra of the different functional groups
were noted in the frequency range of 4000–5000 cm\(^{-1}\). Crystallographic structures of Sq-AgNPs were determined using X-ray diffraction (XRD) analysis. The morphological analysis of Sq-AgNPs was done via high-resolution SEM analysis (MIRA3 TESCAN).

6. Biological activities

6.1. Antibacterial assay

Antibacterial potentials of Sq-AgNPs were evaluated following standard well diffusion method previously reported (18, 19). The number of colony forming units (CFU) was determined by adjusting the turbidity of each bacterial inoculums to 0.5 McFarland standard solution (20). Fresh inoculum of each strain (100 µL) was swabbed onto previously sterilized tryptic soy agar plates and wells of 6 mm diameter were formed using sterile cork borer. A volume of 30 µL of Sq-AgNPs solution (100 µg/ml DMSO) was subsequently added to wells under laminar flow hood. Cefixime and DMSO were used Positive and negative control, respectively. The plates were incubated for 24 h at 37°C and the zone of inhibition was measured. Those bacterial strains having zone of inhibition of ≥10 mm were evaluated at lower concentration (1.1 µg/ml to 100 µg/ml) of Sq-AgNPs for determination of Minimum Inhibitory Concentration (MICs) using three-fold micro broth dilution method (21). Bacterial suspension of 50 µL of each strain was added to a sterile 96-well microplate containing increasing concentrations (1.1–100 µg/ml) of Sq-AgNPs. Bacterial suspension without Sq-AgNPs was used as control. The plates were incubated for 24 h at 37°C and were analyzed visually to evaluate the growth of bacteria. The concentration of Sq-AgNPs at which no viable bacterial growth was observed was MIC value.

6.2. Antifungal assay

Antifungal activity of Sq-AgNPs was evaluated for four pathogenic fungal stains following previously reported protocol (22). The fungal strains were grown on previously sterilized SDA (Sabouraud Dextrose agar) media. The spores of each fungal strain were suspended in Tween 20 (0.02%) solution and their turbidity was set according to standard (0.5) McFarland solution. SDA plates were prepared and swabbed with 100 µL of harvested spores. Sterile discs impregnated with 100 µg/disc of Sq-AgNPs were placed equidistantly on seeded SDA plates. Antifungal drug amphotericin B (250 µg/ml) and DMSO were used as positive and negative control, respectively. Plates were incubated at 28°C and growth inhibition zones were measured after 24–48 h with the help of vernier caliper. All experiments were performed in triplicates.

6.3. HepG2 cytotoxicity studies

6.3.1. Cell culture

Cytotoxic potential of Sq-AgNPs against HepG2 live cancer cells was evaluated using MTT assay (23). Cells were cultured in DMEM (pH = 7.2) containing 10% FBS (Fetal Bovine Serum) in a humidified atmosphere of CO\(_2\) (5%) at 37°C temperature. Cells were added to 96-wells plates (1 × 10\(^4\) in 100 µl of medium/well) and allowed to settle for 24 h. A volume of 100 µL of Sq-AgNPs suspension and extract solution of different target concentrations (7.8, 15.62, 31.25, 62.5 125, 250 and 500 µg/ml), were added to each well and left for 24 h. After incubation, 10 µL of sterilized 1 mg/mL MTT solution in distilled H\(_2\)O was added and the plate was incubated again for 4 h in humidified CO\(_2\) (5%) incubator. Thereafter, DMSO (100 µl) was added to each well and mixed with the cells systematically for the thorough suspension of formazan crystals. Cells without Sq-AgNPs and extract were used as a negative control. The absorbance was measured at 570 nm using a microplate ELISA spectrophotometer reader. The cell sustainability was articulated as a percentage of the sustainability of the control cells.

6.4. Hemolytic assay

Hemolytic assay was performed to check the effect of aqueous extract of S. quettense and synthesized Sq-AgNPs. One micro liter (1 ml) of fresh human blood was taken in 1.5 ml Eppendorf and centrifuged at 14,000 rpm to obtain erythrocytes (RBCs). After centrifugation, the supernatant was discarded and 200 µL from pellet (blood) was taken in a falcon tube. Subsequently, 9.8 ml of phosphate buffer saline (PBS) was added to it and centrifuged (10 min/2000 rpm) for washing. After centrifugation, the supernatant was castoff. This washing step was repeated three times. A volume of 100 µL of red blood cell suspension in PBS was placed in 96 well plate and different concentrations of Sq-AgNPs dissolved in D-water were added to each well. The plates were then incubated (1 h/35°C). Triton X-100 (0.5%) was used as a positive control, while PBS and water were used as a negative control. Percentage of hemolysis was estimated using the following formula;

\[
\text{Percent hemolysis} = \left(\frac{\text{OD of the test samples}}{\text{OD of the positive control}}\right) \times 100
\]
7. Data analysis
All the experiments were performed in triplicate. The data were presented as mean ± standard deviation (SD). The data was graphically presented using Origin Pro 8 and GraphPad Prism software’s.

8. Results and discussion

8.1. Phytochemical analysis and antioxidant capacity
The preliminary phytochemical screening of aqueous extract S. quettense aerial parts confirmed the presence of phenols and Flavonoids. In crude extracts, TFC of 56 ± 1.02 µg/mg extract and TPC of 40 ± 0.78 µg/mg extract were observed (Table 1). Flavonoids and phenolic compounds are known to have different biological properties, such as scavenging of free radicals and inhibition of lipid peroxidation. Certain flavonoids such as quercitin and luteolin are involved as bio-reductants during the synthesis of metal nanoparticles (24). The observed total antioxidant activity (TAA) and reducing power were 10.71 ± 0.65, 73.4 ± 0.11 µg/mg extract, respectively, whereas free radical scavenging activity was 30.6 ± 0.14%.

8.2. Optimization of Sq-AgNPs
Optimization is very cardinal step during the synthesis of metallic NPs (25). Optimization of various parameters such as concentration of the plant extract and silver salt solutions, pH of the reaction mixture, temperature at which the reaction is carried out and incubation period for the reaction can greatly affect the quantity, size, shape and biomedical applications of synthesized Sq-AgNPs (26, 27).

8.3. Characterization of SqNPs

8.4. AgNO3 concentration-based optimization
Synthesis was performed at increasing concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mM of AgNO3 solution.

Color change was observed which confirms the synthesis of Sq-AgNPs as shown in Figure 1(A). UV–visible spectrum of Sq-AgNPs is shown in Figure 2. Maximum absorbance was recorded at 428 nm for 4 mM of AgNO3.

8.5. pH optimization of Sq-AgNPs
Figure 3 shows the synthesis of Sq-AgNPs at different pH of the extract. The color of the reaction mixture became yellowish dark as the pH increased Figure 1(B). The extract reduced solution from colorless to light yellow due to the formation of Sq-AgNPs. The UV spectra show that maximum surface plasmonance (430 nm) was observed at alkaline pH (pH 8). It was observed that as the pH was increased the rate of Sq-AgNPs formation was also increased up to pH = 8 and then decreased. It might be owed to the ionization of phenolic composites in the extract of S. quettense (28). According to literature, acidic pH results in the formation of larger size particles while alkaline pH favors the formation of smaller size particles (29). High Sq-AgNPs formation was observed at basic pH than acidic pH. Our results are in agreement with previously published data (30), that basic pH results in the synthesis of stable NPs whereas at acid pH, NPs tend to aggregate.

8.6. Ext to AgNO3 ratio optimization
Like pH, the ratio of plant extract and AgNO3 also affect the size and morphology of synthesized NPs. Different ratios (Ext: AgNO3) of 1:1, 1:5, 1:10, 1:15, 1:20 and 1:25 v/v were used to determine the optimized ratio of extract and AgNO3. The color change was observed for each ratio Figure 1(C). Maximum absorbance was recorded at 448 nm for 1:5 v/v as shown in Figure 4. The absorbance shows that the quantity of Sq-AgNPs formed due to the reduction of silver ions.

8.7. Temperature-based optimization of Sq-AgNPs
Temperature plays a significant role in the production of Sq-AgNPs (28). NPs were synthesized at variable temperatures of 25°C, 30°C, 37°C, 50°C, 60°C and 70°C. Figure 1D shows the color change of the reaction mixture. The highest intensity peak (439) was observed at 60°C as shown in Figure 5. The absorbance increased with increasing temperature (26). It shows that the rate of NPs synthesis at room temperature can be boosted by increasing the temperature of the particular mixture. While on the other hand the particles tend to be poly-dispersed at high temperature.

Color change was observed which confirms the synthesis of Sq-AgNPs as shown in Figure 1(A). UV–visible spectrum of Sq-AgNPs is shown in Figure 2. Maximum absorbance was recorded at 428 nm for 4 mM of AgNO3.

Table 1. Phytochemical analysis of Seriphedium quettense extract.

| Phytochemicals            | µg/mg extract |
|---------------------------|---------------|
| Total flavonoids          | 56 ± 1.02     |
| Total phenolic content    | 40 ± 0.78     |
| Antioxidant capacity      | 10.71 ± 0.65  |
| Total reducing power      | 73.4 ± 0.11   |
| Free radical scavenging   | 30.6 ± 0.14   |

All values are expressed in mean ± standard error of three separate experiments. aQuercetin equivalent, bGallic acid equivalent, cAscorbic acid.
8.8. Stability of Sq-AgNPs

The stability of Sq-AgNPs was checked at 15, 30 and 60 days of synthesis as shown in Figure 6. It was observed that there are no vibrational changes at the surface plasmon resonance of the NPs. The formation of mono-disperse nanoparticles was shown by the appearance of sharp peak. In a study, AgNPs synthesized from Cassia roxburghii aqueous extract also showed stability for the duration of two months \((31)\).

8.9. Fourier transform infrared Spectroscopy (FTIR)

Bio-molecules involved in the capping and reducing of Sq-AgNPs were analyzed by FTIR Figure 7. FTIR study of Sq-AgNPs showed absorption peaks located at 3409, 3340, 2915, 2870, 2365, 1611, 1315, 1200, 1070 and 1020 which characterizes several functional groups like amine N–H stretching, alcoholic O–H stretching, C O of carboxylic anions, –C–H stretching, C≡N, nitriles, of – C≡C– designated to the amide 1 bonds of proteins, amine groups, (C–O), (eOCH) stretching and (C–N) receptivity.

8.10. X-rays diffraction

XRD spectra of Sq-AgNPs are given as Figure 8. XRD pattern of Sq-AgNPs shows the five main peaks at \(2\theta\) values of 32.29, 38.15, 44.55, 64.61, 77.67 corresponding to 101, 200, 200, 220 and 311 planes for silver indicated that these NPs were crystalline in nature. Scherrer's equation was used to calculate the mean particle size of silver nanoparticles (Sq-AgNPs); \(D = K\lambda/\beta \cos\theta\).
where $D$ represents the size of Sq-AgNPs, $\lambda$ represents the wavelength of the X-ray source (1.540) used in XRD, $\beta$ is the FWHM (full width at half maximum of the diffraction peak), $K$ is the Scherrer constant (values range from 0.9 to 1), and $\theta$ is the Bragg angle. It was found that the average grain/crystallite size of Sq-AgNPs from XRD data is 29.16 nm.

### 8.11. Scanning electron microscopy (SEM) analysis

The size and surface morphology of the synthesized Sq-AgNPs was characterized by SEM analysis. Figure 9 shows the SEM results of synthesized Sq-AgNPs. The micrograph shows that particles are of spherical shape and having various sizes ranging from 48.40 to 55.35 nm.
Most of the NPs were aggregated in the form of clusters, and only few of them were scattered, as observed under SEM. The average size was found to be of 53.3 nm.

9. Biological assays

9.1. Antibacterial potentials of Sq-AgNPs

Antibiotics resistance is becoming a global issue and search for effective antibacterial drugs is the need of current time (32, 33). The antibacterial activity of synthesized Sq-AgNPs was studied against Gram-positive and Gram-negative pathogenic bacterial strains. Sq-AgNPs were most active against *E. coli* and *B. subtilis* with MICs of 11.1 and 33.3 μg/mL, respectively. Sq-AgNPs were moderately active against *S. epidermidis*, *P. aeruginosa* and was inactive against *S. aureus* (Table 2). According to previous reports, AgNPs synthesized from *Chrysanthemum indicum* flowers showed good activity against *E. coli* and *K. pneumoniae* (34). The exact bactericidal mechanism of AgNPs is not very well understood yet. Some researchers believe that AgNPs might interfere with bacterial cell wall synthesis, inhibit the synthesis of vital proteins implicated in cell division, inhibit the synthesis of nucleic acids and interfere metabolic pathways (35). It is also reported that AgNPs induce plasmolysis and disturb the permeability of cell membrane which effect the respiration process (36, 37).

9.2. Antifungal potentials of Sq-AgNPs

Due to the emergence of resistant fungal infections, researchers are looking for more effective antifungal drugs from alternative sources. The antifungal potentials of green synthesized Sq-AgNPs were evaluated against four pathogenic fungal strains. High antifungal activity was observed against *A. niger* and *A. fumigatus* with inhibitory zones of 13.2 ± 0.72 and 12 ± 0.33 mm, respectively (Table 2). AgNPs synthesized via various plants extracts are reported to have considerable antibacterial and antifungal potentials (26, 38).

9.3. HepG2 cytotoxicity studies

In HepG2 liver cancer cell lines cytotoxicity assay, percent cell viability declined with an increase in the concentration of Sq-AgNPs (Figure 10). Median Inhibitory concentrations (IC₅₀) were 62.5, 251 μg/mL for Sq-AgNPs and extract, respectively. Sq-AgNPs were comparatively more active against HepG2 cells than crude extract. Previous studies also reported that AgNPs possess more cytotoxicity than plant extract (27). AgNPs synthesized using leaf extract of *Andrographis echioides* exhibited
The IC\textsubscript{50} value of 31.5 µg/mL against MCF-7 cells \cite{39}. The proposed mechanism of AgNPs-mediated cancerous cells toxicity is exhibited via induction of apoptosis \cite{40, 41}, generation of reactive oxygen species (ROS) leading too DNA damage and cell death \cite{42, 43}.

### 9.4. Hemolytic activity of Sq-AgNPs

The toxicity of synthesized Sq-AgNPs was evaluated against freshly isolated human RBC's. Figure 11 shows the percent hemolysis at increasing concentrations of Sq-AgNPs. Percent hemolysis of RBCs was observed to be dependent of concentration of Sq-AgNPs. The observed hemolysis at concentration of 12.5 and 100 µg/mL were 3.6 and 27.3%, respectively. The hemolytic potentials of NPs are highly dependent on its size and
Figure 9. SEM analysis of synthesized Sq-AgNPs.

Table 2. Antibacterial and antifungal activities of synthesis Sq-AgNPs from aqueous extract of Seriphedium quettense plant.

| Bacterial strains | Antibacterial activity of SqNPs | Fungal strains | Antifungal activity of Sq-AgNPs |
|-------------------|-------------------------------|----------------|--------------------------------|
|                   | (Sq-AgNPs) DIZ (mm) | MIC (μg/mL) | (CF) DIZ (mm) | (Sq-AgNPs) DIZ (mm) | (Amp) DIZ (mm) |
| B. subtilis       | 14.2 ± 1.05 | 33.3 | 17 ± 0.99 | A. fumigatus | 12 ± 0.33 | 25 ± 1.23 |
| S. aureus         | 10 ± 0.74  | 100  | 16 ± 0.91 | A. flavus  | 10 ± 0.41 | 22 ± 0.89 |
| S. epidermidis    | 14.2 ± 1.21| 11.1 | 27 ± 1.09 | A. niger   | 13.2 ± 0.72| 19 ± 1.09 |
| E. coli          | 11 ± 0.72  | 100  | 14 ± 0.21 | Mucor spp  | 11 ± 0.78 | 22 ± 0.67 |
| P. aeruginosa     | 13.2 ± 1.74| 33.3 | 19 ± 1.12 | –          | –          | –          |
| K. pneumonia      | 11 ± 0.72  | 100  | 14 ± 0.21 | –          | –          | –          |

Note: DIZ: Diameter of inhibitory zone, Sq-AgNPs: Seriphedium quettense mediated silver nanoparticles, CF: Cefxime, Amp: Amphotericin B, –: no activity. Values are expressed as (mean ± SD).
Figure 10. Cytotoxic activity of Sq-AgNPs.

Figure 11. Hemolytic activity of Sq-AgNPs.
size of 0.2 μm can enter RBCs causing its rupturing. Moreover, it is reported that NPs may bind to the thiol groups of the proteins or phospholipids of membrane cause their denaturation (44, 45).

10. Conclusions

Green synthesis of silver nanoparticles using plant extract is inexpensive, eco-friendly and cost effective approach. Silver nanoparticles were synthesized via an aqueous extract of *S. quettense* extract. Different optimization parameters like concentrating of AgNO₃ salt, extract pH, ratio of AgNO₃ and plant extract salt and temperature were optimized to synthesize AgNPs with desire size, shape and biological properties. The Sq-AgNPs (49.96–54.36 nm) were characterized by UV-visible spectrophotometer, FTIR, XRD and SEM. The Sq-AgNPs were found effective against *E. coli*, *K. pneumonia*, *B. subtilis* and *A. niger*. Sq-AgNPs showed considerable cytotoxic effects against HepG2 cells with IC₅₀ of 62.5 μg/mL. Further, Sq-AgNPs were bio-compatible with less cytotoxicity against RBCs. Further *in-vivo* studies on the Sq-AgNPs are recommended for more convincing results.

Acknowledgements

We are grateful for the Department of Biotechnology, Quaid-i-Azam University Islamabad for their Lab facilities to conduct this study. We are also thankful to Institute of space technology Islamabad and National center for physics for their help in characterization of nanoparticles.

Disclosure statement

No potential conflict of interest was reported by the authors.

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