TOXICITY ASSESSMENT OF BIOSYNTHESIZED SILVER NANOPARTICLES FROM SOLANUM VILLOSUM MILL. (SOLANACEAE): IN VITRO AND IN VIVO STUDY

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ABSTRACT

Objective: The aim of the study is an evaluation of toxicity assessment of biosynthesized silver nanoparticles (AgNPs) from Solanum villosum (Mill) using in vitro and in vivo study.

Methods: Biologically synthesized AgNPs are characterized by ultraviolet, scanning electron microscopy, energy dispersive X-ray spectroscopy, X-ray diffraction analysis, and its cytotoxicity effect against HepG2 cell lines was performed. Further, toxicity was confirmed by in vivo studies using Wistar albino rats. Various hematological, liver function marker enzymes and liver histopathology are investigated.

Results: The cytotoxic effect of S. villosum AgNPs (SV-AgNPs) was also concentration dependent and did not produce any toxicity to tested animals. The histopathological evidence is supported to biochemical observations.

Conclusion: So, biologically synthesized AgNPs are toxic only to cancer cells but not in animals were proved by the present study.

Keywords: Solanum villosum silver nanoparticles, HepG2 cell lines, Scanning electron microscopy, Energy dispersive X-ray spectroscopy, X-ray diffraction analysis.

INTRODUCTION

The field of nanotechnology is one of the upcoming areas of research in the modern field of material science. Nanoparticle shows completely new or improved properties, such as size, distribution, and morphology of the particles. Novel applications of nanoparticles and nanomaterials are emerging rapidly on various fields [1]. Nanocrystalline silver particles have been found tremendous applications in the fields of high sensitivity biomolecular detection, diagnostics, anticancer, therapeutics, catalysis, and microelectronics. However, there is still a need for economic commercially viable as well as environmentally clean synthesis route to synthesize the silver nanoparticles (AgNPs). Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes [2].

Green synthesis of metal nanoparticles is a growing research area because of their potential applications in nanomedicine. The synthesis of AgNPs using Solanum villosum leaves and its characterization is done using ultraviolet (UV), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD) analysis. In vitro cytotoxicity testing procedures reduce the use of laboratory animals. Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer death and accounts for 5.6% of all cancers [3].

The present study may open up several promising avenues of possible research. The S. villosum and related species are widely used as leafy herbs and vegetables, as a source of fruit and for various medicinal purposes. The S. villosum plant has been used in many ayurvedic medicines [4,5]. In spite of known uses in traditional medicines, no documental evidence is available on their biosynthesis of AgNPs and its activities. Hence, AgNPs of S. villosum are evaluated in a systematic manner to provide information for treating and preventing cancer and other diseases.

METHODS

Collection of plant material
The whole plant of S. villosum (Mill) was collected from the Thadagam hills, Coimbatore district, southern India. The plant samples were identified and authenticated by the Botanical Survey of India (Southern part Coimbatore, Tamil Nadu, India). The identification No. BSI/SRC/5/23/2014-15/Tech/255. Various stages of plant, S. villosum is shown in the Fig. 1.

Preparation of aqueous extract of S. villosum leaves
The dried S. villosum leaves powder 10 g was boiled in 100 ml of distilled water for 10 minutes. The extract was cooled to room temperature filtered through Whatman No. 1 filter paper (Pore size 25 μm). The filtrate was further filtered through 0.6 μm sized filters.

Synthesis of AgNPs
The AgNPs were synthesized using a constant volume of the plant extract under various experimental conditions. Aqueous solution of 1 mM AgNO₃ was prepared and used for the synthesis of AgNPs. 5 ml of S. villosum aqueous extract is mixed with 95 ml of AgNO₃ for the synthesis of AgNPs. The formation of AgNPs is confirmed by color change from greenish to reddish brown. The appearance of reddish brown color after 3 hrs it indicates the formation of AgNPs.

Separation of AgNPs
The synthesized AgNPs were separated by centrifugation (Spectrofuge 7M) at 13,000 rpm for 15 minutes. The process was repeated by dispersion of pellets in water, to obtain colored supernatant solutions. The sample was then stored at −4°C for further use.

Characterization of AgNPs
UV-vis spectra analysis
The reduction of pure Ag⁺ ions was monitored by measuring the UV-vis spectrum of the reaction medium at 15 10-12 hrs. UV-Vis spectral analysis was done using UV-vis spectrophotometer UV- 2450 (Shimadzu).
In vitro cytotoxic effect of \textit{S. villosum} AgNPs (SV-AgNPs) on HepG2 cell lines [6,7]

The human liver cancer cell line (HepG2) was obtained from the national center for cell science, Pune, and grown in eagle's minimum essential medium containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO$_2$, 95% air, and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediamine tetra acetic acid to make single cell suspensions, and viable cells were counted by trypan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of 1×10$^5$ cells/ml. 100 µl per well of cell suspension was seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO$_2$, 95% air, and 100% relative humidity. After 24 hrs, the cells were treated with serial concentrations of the test samples. They were initially dispersed in phosphate buffered saline and diluted to twice the desired final maximum test concentration with serum-free medium. Additional four, 2-fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following drug addition, the plates were incubated for an additional 48 hrs at 37°C, 5% CO$_2$, 95% air, and 100% relative humidity. The medium containing without samples were served as control and triplicate were maintained for all concentrations.

\texttt{\% Cell inhibition=100−Abs (sample)/Abs (control)×100.}

Nonlinear regression graph was plotted between % cell inhibition and log concentration, and inhibitory concentration at 50% (IC$_{50}$) was determined using GraphPad Prism software.
reaction medium changed rapidly from light greenish yellow to brown (Fig. 2a and b) due to surface plasmon resonance. This occurs due to the collective oscillations of the conduction electrons confined to metallic nanoparticles. The same mechanism was reported in ethanolic extract of leaves of *Pisonia grandis* [13]. It is well known that AgNPs exhibit a yellowish-brown color in aqueous solution due to excitation of surface plasmon vibrations in AgNPs. The approach employed in the production of these materials (AgNPs) is low cost and ecofriendly [14].

The major advantage of using plant extracts for the synthesis of AgNPs is that they are easily available, safe, and nontoxic, in most cases, have a broad variety of metabolites that can aid in the reduction of silver ions and are quicker than microbes in the synthesis. The main mechanism considered for the process is a plant-assisted reduction due to phytochemicals. The main phytochemicals involved are terpenoids, flavones, ketones, aldehydes, amides, and carboxylic acids [15].

The AgNPs were characterized by UV-Vis spectroscopy, one of the most widely used techniques and to confirm AgNPs formation by showing the plasmon resonance. The absorption spectrum of the AgNPs depicts in Fig. 3. The surface plasmon absorption band with a maximum of 200-800 nm, indicating the presence of AgNPs.

SEM has provided further insight into the morphological details of the synthesized AgNPs. SEM micrographs of the synthesized AgNPs using the aqueous extract of leaves of *S. villosum* fabricated on a glass substrate are shown in Fig. 4. The synthesized AgNPs were well dispersed without aggregation, possessing spherical shapes are confirmed by SEM.

In addition, elemental analysis of the synthesized AgNPs is further confirmed by EDX spectra with the absorption peak in the range of 3-4 keV (Fig. 5). This result is in accordance with the study of Jain et al. [16], who reported that the presence of optical absorption peak in the range of 3-4 keV is typical for metallic silver nanocrystallites. While weaker peaks like C, O, and Cl are likely due to X-ray emission from proteins/sugars may be present in the *S. villosum* leaf extract.

In this, XRD pattern (Fig. 6) showed the conformation of the existence of peaks belonging to AgNPs in the sample. The Braggs reflections were observed in XRD pattern at around 2θ=28°, 33°. Hence, the XRD pattern thus clearly illustrated that the AgNPs formed in this present synthesis. Peak obtained at around 33° indicated that the prepared samples were crystalline in nature. The crystalline size (D) was calculated using the Scherer’s Debye formula from the full-width half maximum (FWHM).

\[
\begin{align*}
D &= \frac{K \lambda}{\beta \cos \theta} \\
2 \theta &= \frac{1.47897}{\beta \cos \theta}
\end{align*}
\]

Where, KD constant=1.47897×10^{-10}, D is the average crystallite domain size perpendicular to the reflecting planes. λ is the X-ray wavelength, β is the full width at half

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**Fig. 2:** (a) *Solanum villosum* aqueous leaf extract; (b) Color changes after adding silver nitrate reaction time of 10-12 hrs

**Fig. 3:** Ultraviolet-visible spectra of *Solanum villosum* aqueous leaf extract reduction of silver nanoparticles

**Fig. 4:** Scanning electron microscopy micrographs for synthesized silver nanoparticles from aqueous leaf extract of *Solanum villosum*

**Fig. 5:** Energy dispersive X-ray spectrum analysis for biosynthesized silver nanoparticles from aqueous leaf extract of *Solanum villosum*
maximum and θ is the diffraction angle. The crystalline size was found to be around 17.63 nm. AgNPs were synthesized by various plant materials as capping agents such as papaya [17] and neem [18].

**In vitro cytotoxicity activity of SV-AgNPs**

In this present research work, we have employed a dose-dependent approach to evaluate the toxicity of the biosynthesized AgNPs against the human HCC (HepG2) cell line. Evaluation of cytotoxicity in liver HepG2 cells by SV-AgNPs was performed using MTT assay (viability assay). HepG2 cell line was previously used for various anticancer studies. SV-AgNPs (1.88-30 µg/ml) in a dose-dependent manner as seen in the MTT assay. We observed AgNPs treatment (24 hrs incubation) significantly decreased the percentage of cell viability in HepG2 cells.

The proliferation of HepG2 cell was significantly inhibited by SV-AgNPs. Fig. 7 and Table 1 shows the changes in the percentage of cell viability treated with SV-AgNPs (1.88, 3.75, 7.5, 15, and 30 µg/ml) in HepG2 cells. There was 100% cell death at 30 µg/ml concentration was observed. The IC₅₀ was fixed as 8.34 µg/ml. AgNPs mainly react with cancer cells, it is selectively involved in disruption of the mitochondrial respiratory chain and leading to the production of ROS and interruption of ATP synthesis, which, in turn, cause DNA damage. Mainly, the plant *S. villosum* contains the phytochemicals and antioxidants exhibited the synergistic effect against the cancer cells. The synthesized AgNPs cause cell damage with unique morphological and biochemical hallmarks.

AgNPs from plant extract can interfere with the respiratory chain at the cytochromes and can interact with the electron transport chain to activate the intrinsic signaling pathway to cell death through the activation of downstream processing.

The cytotoxicity of these nanoparticles depends on their shape, size, surface chemistry, etc., as spherical AgNPs and nanoparticles are almost nontoxic to human alveolar epithelial cells [8]. AgNPs seemed to be a defense mechanism of protection from the highly reactive behavior of silver ions (Ag⁺) to the cells. The incoming silver ions from the aqueous solution into the cells are converted to reduced state (Ag⁻). The synthesis of nanoparticles by this reaction in cells is attributed to the presence of an enzyme called nitrate reductase. The enzyme is found to be present in both aerobes and anaerobes [19].

**Toxicity assessment SV-AgNPs - in vivo study**

**Body weight and mortality changes**

During the treatment of SV-AgNPs, all the animals were observed daily for clinical signs and mortality patterns. After administration of different doses from 100 µg, 200 µg, and 300 µg/kg body weight, no clinical signs, mortality, and body weight changes of the rats. It is indicating the nontoxic effect of SV-AgNPs in minimum safety doses (Table 2).

**Hematological and biochemical assays**

In the repeated dose toxicity studies, administration of aqueous extract of SV-AgNPs in various experimental groups is carried out. All the groups did not produce any significant changes (p<0.05) in hematological parameters and biochemical parameters are showed in the Tables 3 and 4. There were no harmful changes found in the level of AST, ALT, ALP, total protein in serum and hematological parameters in blood of treated groups (II, III, and IV) when compared with control (Group I). SV-AgNPs did not affect liver enzymes and blood cells, and the lack of significant alterations is a good indicator of liver functions and hematopoietic mechanism. Hence, it is clearly indicated that SV-AgNPs are nontoxic to normal rats.

Lovric et al. reported that after absorption of nanosilver from the gastrointestinal tract, entered to blood systemic circulation, therefore, this particle can, potentially, interact with different metabolites such as: Plasma proteins, coagulation factors, platelets, red and white blood cells. The smaller diameters of the nanoparticles are the more its influence and interact to cells and its intracellular mechanisms will increase [20].

**Table 1: Effect of SV-AgNPs on HepG2 cell line (% inhibition)**

<table>
<thead>
<tr>
<th>Concentration of SV-AgNPs</th>
<th>% cell inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.88</td>
<td>-0.19</td>
</tr>
<tr>
<td>3.75</td>
<td>4.33</td>
</tr>
<tr>
<td>7.5</td>
<td>35.35</td>
</tr>
<tr>
<td>15</td>
<td>96.60</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

SV-AgNPs: *Solanum villosum* silver nanoparticles

**Table 2: Body weight of the rats before and after 28 days of treatment with SV-AgNPs**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (grams)</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>21.00±5.47</td>
<td>21.90±3.74</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>208.33±8.63**</td>
<td>217.33±5.62**</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>209.17±4.91**</td>
<td>218.00±4.19**</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>211.67±2.58**</td>
<td>220.33±3.23**</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD of six animals in each group. Statistical comparison: Group I versus Groups II, III, and IV. *Significant at 5% (p<0.05). NS: Not significant, SD: Standard deviation, SV-AgNPs: *Solanum villosum* silver nanoparticles.
Table 3: Effect of SV-AgNPs on the hematological parameters in blood of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hb (g%)</th>
<th>PCV (%)</th>
<th>WBC (10^6 µL)</th>
<th>RBC (10^12 µL)</th>
<th>Platelets (10^9 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>13.5±0.36</td>
<td>40.16±0.98</td>
<td>7.74±0.44</td>
<td>6.94±0.47</td>
<td>5.29±0.45</td>
</tr>
<tr>
<td>Group II</td>
<td>13.41±0.46</td>
<td>40.16±1.47</td>
<td>7.71±0.49</td>
<td>6.90±0.32</td>
<td>5.38±0.24</td>
</tr>
<tr>
<td>Group III</td>
<td>13.36±0.36</td>
<td>40.50±1.37</td>
<td>7.70±0.41</td>
<td>6.72±0.52</td>
<td>5.49±0.36</td>
</tr>
<tr>
<td>Group IV</td>
<td>13.41±0.33</td>
<td>40.00±1.26</td>
<td>7.90±0.53</td>
<td>6.58±0.58</td>
<td>5.66±0.41</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD of six animals in each group. Statistical comparison: Group I versus Groups II, III, and IV. *Significant at 5% (p<0.05). ns: Not significant. Units: WBC: White blood cell; PCV: Packed cell volume; RBC: Red blood cell; SV-AgNPs: Solanum villosum silver nanoparticles; Hb: Hemoglobin; SD: Standard deviation

Table 4: Effect of SV-AgNPs on liver function marker enzymes in serum of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST µ molar/L</th>
<th>ALT µ molar/L</th>
<th>ALP µ molar/L</th>
<th>Total protein g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>66.91±0.77</td>
<td>88±8.63</td>
<td>6.5±0.21</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>64±1.14</td>
<td>96±6.14</td>
<td>6.43±0.31</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>66.43±1.82</td>
<td>102±6.69</td>
<td>6.35±0.38</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>69.62±2.05</td>
<td>106±9.99</td>
<td>6.23±0.23</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD of six animals in each group. Statistical Comparison: Group I versus Groups II, III, and IV. *Significant at 5% (p<0.05). ns: Not significant. Units: AST, ALT - µ moles of pyruvate liberated/L; ALP - µ moles of phenol liberated/L; Total protein - g/dl; AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatases, SD: Standard deviation, SV-AgNPs: Solanum villosum silver nanoparticles

Histopathological investigations

All the control and experimental animals showed the normal architecture (Fig. 8) of the liver with central vein and cords of hepatocytes radiating from the central vein indicating the hepatoprotective activity. There was no cellular injury which indicates that the SV-AgNPs have no side effect at all the doses tested. The evaluation of histopathological changes in organs remains a cornerstone in safety assessment of medicines [21].

AgNPs synthesized from medicinal plant S. villosum and evaluated toxicity for HepG2 cell lines. The AgNPs significantly decreased the cell viability of HepG2 cells but did not cause any changes in the normal healthy rats. Nanoparticles of larger size have proven to be toxic to the cells. Larger size nanoparticles also do not bind with the specific receptors. The smaller size (5-20 nm) of the nanoparticle can easily interact with cancer cells cause damage. This particle size range has proven to be less toxic to the cells and also exhibit higher binding affinity toward the receptors. Similar results have been obtained when the particles were checked for interaction with HIV-1 [22]. Another reason, binding of AgNPs only with cancer cells but not the other body cells. One possible reason could be due to morphological and biochemical reaction differences between cancer cells and the other body cells.

The previous study reported that nanoparticle has difference in the curvature of the different-shaped nanoparticles. For example, the rod-shaped nanoparticles can have a larger contact area with the cell membrane receptors than the spherical nanoparticles when the longitudinal axis of the rods interacts with the receptors. The cancer cells are different in pore size when compared to the other normal cells and so a size controlled targeting of AgNPs can prove effective in the case of cancer treatment [23].

Current cancer treatments include surgical intervention, radiation and chemotherapeutic drugs, which often also kill healthy cells and cause toxicity to the patient [24]. The smaller size particles are attributed to the stability, catalytic activity, and enhanced adherence to the cells. Previous reports suggest that the increase in the concentration of AgNPs has resulted in the increase in cell death [25,26]. The effects of AgNPs on size-dependent toxicity with various concentrations already explained earlier [27].

CONCLUSION

The biological method of synthesis of AgNPs from S. villosum has proved to be an ecofriendly method than the chemical methods. The physical and chemical method involves use of hazardous chemicals and highly expensive approaches. The role of AgNPs is an anticancer agent in the field of medicine. The use of nanoparticles revealed that decrease the use of expensive drugs for cancer treatment. So, the use of AgNPs for cancer treatment is the novel and effective approach in the field of cancer biology. In conclusion, SV-AgNPs have anticancerous activity; it enhances the apoptotic property. Further studies on the mechanisms of AgNPs and the new drug discovery for synthesized AgNPs of the S. villosum for medical and industrial application. However, further in vivo studies are needed to fully characterise the antiproliferative potential of the biosynthesized AgNPs against RCC.

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