BIOLOGICAL SYNTHESIS OF SILVER NANOPARTICLES USING RAPHANUS SATIVUS VAR. LONGIPINNATUS LEAF EXTRACT AND EVALUATION OF THEIR ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

Nanocrystalline silver particles have found tremendous applications in the field of diagnostics and therapeutics. In recent years, plant mediated biological synthesis of nanoparticles is gaining importance due to its eco-friendliness. In this study, the synthesis and characterization of silver nanoparticles was carried out by using Raphanus sativus var. longipinnatus leaf extract as reducing agent and evaluation of antibacterial and antioxidant activity. The synthesized silver nanoparticles were characterized with UV–Vis spectrophotometry (UV-Vis), Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy–Energy dispersive spectroscopy (SEM- EDX), Transmission electron microscopy (TEM) and X-Ray diffraction spectroscopy (XRD). The antibacterial activity of silver nanoparticles and in-vitro antioxidant activity have been evaluated. These synthesized silver nanoparticles were found to have significant antibacterial activity and antioxidant capacity, thus can be used as potential radical scavenger against deleterious damages caused by the free radicals.

KEYWORDS: Raphanus sativus var. longipinnatus Leaf Extract, Reducing Agent, Antibacterial Activity and In-Vitro Antioxidant Activity, Potential Radical Scavenger, Free Radicals

INTRODUCTION

Nanosized silver particles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnostics, antimicrobials, antioxidants and therapeutics, catalysis [1] and micro-electronics. Nanoparticles can be synthesized by various approaches like chemical and photochemical reactions in reverse micelles, microwave assisted, thermal decomposition, electrochemical, sonochemical process [2] and also by biological methods. Plant mediated biological synthesis of nanoparticles is gaining importance due to its simplicity, cost effective and ecofriendliness [3, 4].

Silver nanoparticles were proven to be most efficient as they possess good anti-microbial [5,6,7,8], anti-inflammatory [9,10,11], anti-plasmodial [12,13] anti-cancer [14] and anti-oxidant activities [15,16,17] etc. Bioreduction of silver ions to yield metal nanoparticles using plants such as Cajanus cajan [18], Cuminum cyminum [19], Pisonia grandis [20], Allium cepa [21], Parthenium hysterophorus [22], Ocimum basilicum [23], Murraya koenigii [24], Glycyrrhiza glabra [25], Coriandrum sativum [26] and Ocimum sanctum [27] have been reported. Nanoparticles attach to the cell surface of bacteria causes structural changes and damage disturbing the vital cell functions and finally leading to cell death [28]. Antioxidants are the substances which act as free radical scavengers by preventing and repairing damages caused by reactive oxygen species and can also enhance the immune defense and degenerative diseases [29].

Radish (Raphanus sativus var. longipinnatus) is a common vegetable crop in Asia. The plant produces a white and large, cylindrical, long, fleshy root which usually weighs up to 2-3kg. The leaves usually are medium, green and lobed
and have rough texture and has large amounts of vitamin B and C as well as pectin, phytin, manganese, iron and copper. Leaves are used to treat dysentery, asthma, cough, diarrhea and malnutrition [30]. It contains ferulic acid, gentisic acid, raphanin, erucic acid, sinapate, raphanin and sulforaphen. The seeds are carminative, diuretic and laxative. Roots have been used for treating syphilis, haemorrhoids, gonorrhoea, cancer [31] and urinary complaints.

In this investigation, green synthesis of silver nanoparticles were carried out using aqueous leaf extract of *Raphanus sativus var. longipinnatus* and characterized using UV-visible spectra, Fourier transform infrared spectra, Scanning and transmission electron microscopy, Energy X-ray diffraction spectra and XRD. The antibacterial activities have been investigated against Gram negative and Gram positive bacteria and evaluated in-vitro antioxidant properties. Results shown that RsAgNPs were effective bactericidal and antioxidants. To our knowledge green synthesis of silver nanoparticles by this plant leaf extract has not been reported so far.

**MATERIALS AND METHODS**

**Collection of Raphanus sativus var. longipinnatus**

*Raphanus sativus var. longipinnatus* leaves (Figure 1) were collected from the local market in Hyderabad, Andhra Pradesh, India. The leaves were rinsed with distilled water thrice followed by Milli Q water to remove the dust and other contaminants then dried at room temperature to remove the moisture for 2 hours.

![Raphanus sativus Plant](image)

**Figure 1: Raphanus sativus Plant**

**Preparation of Raphanus sativus var. longipinnatus Leaf Extract**

10gms of green fresh leaves were weighed and then sliced into small pieces. Then 100ml of Milli Q water was added and boiled for 15min at 60°C. After cooling the extract was filtered using whatman No.1 filter paper and stored at 4°C for further use.

**Preparation of 1mM AgNO₃ Solutions**

Accurate concentration of 1mM silver nitrate (Sigma, USA) was prepared by dissolving 0.0421gms AgNO₃ in 250ml of Milli Q water and stored in amber colored bottle.

**Synthesis of Raphanus sativus var. longipinnatus Leaf Silver Nanoparticles**

For the synthesis of silver nanoparticles from *Raphanus sativus var. longipinnatus* leaf extract, to 15ml of extract, 30ml of 1mM AgNO₃ solution was added and further heated up to 50°C for 30 minutes. The color change observed stands as a preliminary confirmation for the formation of silver nanoparticles. The solution was centrifuged at 20000rpm for 20min. The separated nanoparticles settled at the bottom were collected and washed thrice with Milli Q water, then dried in an oven at 60°C for two hours. The stabilized powder forms of the nanoparticles were stored for further characterization.
Characterization of *Raphanus sativus var. longipinnatus* Silver Nanoparticles (RsAgNPs)

An ELICO SL-159 UV-Vis spectrophotometer was used for the spectrometric analysis to confirm silver nanoparticles formation. The leaf extract was used as reference blank. The purified suspension was oven dried and the powder was subjected to FTIR spectroscopy analysis (Paragon 500, Perkin Elmer-RX1 spectrophotometer) in the diffuse reflectance mode at a resolution of 4cm⁻¹ in KBr pellet. Further the size and shape of synthesized AgNPs was characterized by Scanning electron microscope (SEM) in Zeiss 700 Scanning electron microscope and Transmission electron microscope (TEM) in Philips model CM 200 instrument operated at an accelerating voltage at 200 kV and the confirmation of the presence of elemental silver signal was characterized by energy-dispersive X-ray microanalysis spectroscopy (EDX; Sigma) and X-Ray diffractometer operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation.

Antibacterial Activity Using Disc Diffusion Method

The antimicrobial activity of synthesized silver nanoparticles was determined using disc diffusion method. Luria Bertani media was prepared and poured into sterilized petriplates and then plates were spreaded with of *Pseudomonas putida, Klebsilla pneumonia, Staphylococcus aureus and Bacillus subtilis* separately. Then sterile discs were kept and the samples were added to the disc and the plates were incubated at 37°C overnight. Then zone of inhibition was measured.

Antioxidant Activity of *Raphanus sativus var. longipinnatus* Silver Nanoparticles (RsAgNPs)

Determination of Total Antioxidant Activity

The Total antioxidant activity of the silver nanoparticles was assessed by the phosphomolybdenum reduction assay [32, 33]. The assay is based on the reduction of Mo (VI)–Mo (V) by the RsAgNPs and subsequent formation of a green phosphate/Mo(V) complex at acid pH. To various concentrations (20, 40, 60, 80, 100,120 & 140 μg/mL) of RsAgNPs diluted in methanol was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Then the tubes were incubated at 95°C for 90 min. Then the absorbance of the green phosphomolybdenum complex was measured at 695 nm using a UV-visible spectrophotometer against blank after cooling to room temperature. Methanol in the place of RsAgNPs is used as the blank. For reference, L-ascorbic acid was used as a control and prepared by dissolving 1mg of L-ascorbic acid in 1ml methanol. The following equation was used for calculating Total antioxidant activity expressed as gram equivalents

Estimation of Radical Scavenging Activity (RSA) Using DPPH Assay

The radical scavenging activity of silver nanoparticles was estimated using the method of DPPH assay[34]. A solution of DPPH (2,2-diphenyl-1-picrylhydrazly) 5mg in 100ml methanol was prepared and 3.0 ml of this solution was mixed with various concentrations (20, 40, 60, 80 & 100 μg/mL) of synthesized RsAgNPs. The reaction mixture was shaken vigorously and left in the dark at room temperature for 15 min. The absorbance was measured at 517 nm with ascorbic acid as standard. The following equation was used for calculating percentage inhibition:

\[
DPPH \% \text{ inhibition} = \frac{[(\text{Abs control} – \text{Abs sample})]/(\text{Abs control}) \times 100}
\]

Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + synthesized RsAgNPs solution/standard.

Estimation of Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity of the RsAgNPs was carried out by the method of Inbathamizh L [35]. Various concentrations of RsAgNPs was added with 1.0mL of EDTA solution (0.13 g of ferrous ammonium sulphate and
0.26 g of EDTA were dissolved in 100mL of water) and mixed with 1.0mL of DMSO (0.85%) in 0.1M phosphate buffer (pH 7.4) to initiate the reaction followed by the addition of 0.5mL of 0.22% ascorbic acid. The reaction mixture was kept in a water bath at 90ºC for 15 min and the reaction was terminated by adding 1.0 mL of ice-cold 1 7.5% trichloroacetic acid. Further 3.0mL of Nash reagent (75 g of ammonium acetate, 3.0mL of glacial acetic acid and 2.0mL of acetyl acetone in 1.0 L of water) was added to all the test tubes and incubated for 15 min for color development. Reaction mixture without ascorbic acid served as control. Absorbance was observed at 412 nm. The ability to scavenge hydroxyl radical was calculated by the following equation:

$$\text{Hydroxyl Radical scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})} \times 100$$

**Estimation of Hydrogen Peroxide Scavenging Activity**

Hydrogen peroxide scavenging activity of *Raphanus sativus var. longipinnatus* silver nanoparticles was estimated by replacement titration [36]. Aliquot of 1.0ml of 0.1m mole of H$_2$O$_2$ and 1.0ml of various concentrations (400µg/ml, 800µg/ml and 1200µg/ml) of *Raphanus sativus var. longipinnatus* silver nanoparticles were mixed, followed by 2 drops of 3% ammonium molybdate, 7.0ml of 1.8 M KI and 10ml of 2M of H$_2$SO$_4$. The mixed solution was titrated with 5.09 mM of Na$_2$S$_2$O$_3$ until yellow color disappeared. The percentage of scavenging of hydrogen peroxide was calculated as:

$$\text{% Inhibition} = \frac{(V_0 - V1)}{V0} \times 100$$

Where $V_0$ was volume of Na$_2$S$_2$O$_3$ solution used to titrate the control sample in the presence of hydrogen peroxide (without *Raphanus sativus var. longipinnatus* silver nanoparticles /Ascorbic acid), $V_1$ was the volume of Na$_2$S$_2$O$_3$ solution used in the presence of the *Raphanus sativus var. longipinnatus* silver nanoparticles/Ascorbic acid.

**RESULTS AND DISCUSSIONS**

The synthesis of silver nanoparticles is new technique in modern biotechnology and is evolving as an important branch of nanotechnology. This study deals with the synthesis and characterization of silver nanoparticles using leaf extract of *Raphanus sativus var. longipinnatus* (Figure 2A). Green synthesized silver nanoparticles were reddish brown in color. The color of the extract was changed from light yellowish to reddish brown after addition of AgNO$_3$ and on incubation for 5-10 min at 60ºC. The coloration was due to the excitation of the surface Plasmon vibration in the silver nanoparticles. Change in color after the reduction of silver ions to silver nanoparticles is shown in (Figure 2B). The reduction rate and formation of nanoparticles can be increased further by increase in incubation time.

![Figure 2: A. Plant Extract and Silver Nitrate Solution, B. Synthesis of Silver Nanoparticles](Image)

**UV-Vis Spectrophotometer**

The UV-Vis spectroscopy was the preliminary technique for the characterization of the silver nanoparticles. The
Biological Synthesis of Silver Nanoparticles Using *Raphanus sativus Var. longipinnatus* Leaf Extract and Evaluation of their Antioxidant and Antibacterial Activity

UV-Vis absorption was analyzed after centrifuging and redispensing the particles in deionized water, the maximum smooth and broad absorption peak was seen at 470nm. (Figure 3).

![UV-Vis Spectra of Silver Nanoparticles Obtained at Different Time Intervals](image)

**Figure 3:** UV-Vis Spectra of Silver Nanoparticles Obtained at Different Time Intervals

**FTIR Analysis of Silver Nanoparticles**

The FTIR spectrum indicates various functional groups present at different positions. FTIR spectroscopy study has confirmed that the carbonyl group of amino acid residues and peptides binds to RsAgNPs. The peaks in the region 3293 to 2917 were assigned to O-H stretching of alcohol and phenol compounds and aldehyde –C-H- stretching of alkanes. The peaks in the region 1584 corresponds to aromatic C=C with nitro group bending vibration, 1398 to 1087 corresponds to N-H group of primary and secondary amides and –C-N- stretching vibration of amines and –C-O- stretching of alcohols, ethers, carboxylic acids and anhydrides and peaks between 841 and 751 were assigned to alkyl halides (Figure 4). FTIR analysis reveals the dual function of biological molecules possibly responsible for the reduction and stabilization of silver nanoparticles in the aqueous medium.

![FTIR Spectrum of Silver Nanoparticles](image)

**Figure 4:** FTIR Spectrum of Silver Nanoparticles

**XRD Analysis**

XRD analysis of *Raphanus sativus var. longipinnatus* silver nanoparticles which showed diffraction peaks at 38.16°, 44.20°, 64.5° and 77.42°, indexing the planes 111, 200, 220 and 311 of the cubic face-centered silver (Figure 5). The lattice constant calculated from this pattern was a = 4.086Å and the data obtained was matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. Average grain size of the silver nanoparticles
formed in the bioreduction process was determined using Scherer’s formula, \( d = \frac{0.9 \times \lambda}{\beta \cos \theta} \) and was estimated as 22nm.

\[
\begin{align*}
\text{Figure 5: XRD Analysis of Silver Nanoparticles}
\end{align*}
\]

**SEM-EDX Analysis**

The morphology of the synthesized silver nanoparticles using *Raphanus sativus var. longipinnatus* leaf extract, the sample was spherical in shape and an average size of 22nm (Figure 6A). The EDS spectra shown that the sample (silver nanoparticle) contains 40.83% silver (Figure 6B and Table 1.)

\[
\begin{align*}
\text{Figure 6: A. SEM Analysis, B. EDS Spectra of Synthesized Silver Nanoparticles}
\end{align*}
\]

**Table 1: The Composition of Silver Nanoparticles Synthesized from *Raphanus sativus* Leaf Extract**

<table>
<thead>
<tr>
<th>Element</th>
<th>Weight%</th>
<th>Atomic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>-12.76</td>
<td>-70.83</td>
</tr>
<tr>
<td>O K</td>
<td>17.79</td>
<td>74.13</td>
</tr>
<tr>
<td>Na K</td>
<td>3.08</td>
<td>8.93</td>
</tr>
<tr>
<td>S K</td>
<td>4.52</td>
<td>9.41</td>
</tr>
<tr>
<td>Cl K</td>
<td>8.01</td>
<td>15.06</td>
</tr>
<tr>
<td>K K</td>
<td>8.52</td>
<td>14.52</td>
</tr>
<tr>
<td>Ca K</td>
<td>4.78</td>
<td>7.95</td>
</tr>
<tr>
<td>Ag L</td>
<td>66.06</td>
<td>40.83</td>
</tr>
</tbody>
</table>

**Total** | **100.00** |

**TEM Analysis**

The silver nanoparticles synthesized by the help of *Raphanus sativus var. longipinnatus* leaf extract when scanned using TEM from which we conclude that the average mean size of silver nanoparticles was in between 5-22nm and seems
Biological Synthesis of Silver Nanoparticles Using *Raphanus sativus* Var. *longipinnatus* Leaf Extract and Evaluation of their Antioxidant and Antibacterial Activity

to be spherical in morphology as shown in (Figure 7). Thus the transmission electron microscopy gave a detailed descriptive image of the silver nanoparticles synthesized with their structural details and their size.

![Figure 7: TEM Analysis and Particle Size Distribution](image)

**Antibacterial Activity by Disc Diffusion Technique**

Antibacterial activity of synthesized silver nanoparticles against Gram negative (*Pseudomonas putida* and *Klebsiella pneumonia*) and Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria was revealed and zone of inhibition was measured (Figure 8 and Table 2). The results indicated that silver nanoparticles synthesized from *Raphanus sativus* var. *longipinnatus* leaf extract showed effective antibacterial activity both in Gram negative and Gram positive bacteria which is compared with ampicillin.

![Figure 8: Antibacterial Activity of Silver Nanoparticles](image)

**Table 2: Zone of Inhibition (mm)**

<table>
<thead>
<tr>
<th>Name of the Organism</th>
<th>Zone of Inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampicillin (5µl)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>13</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>12</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>11</td>
</tr>
</tbody>
</table>

**Antioxidant Activity of *Raphanus sativus var. longipinnatus* Silver Nanoparticles (RsAgNPs)**

**Total Antioxidant Activity**

Total antioxidant capacity of *Raphanus sativus var. longipinnatus* silver nanoparticles is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method is quantitative, since the antioxidant activity is expressed...
as the number of equivalents of ascorbic acid. The antioxidant activity of the extract is in the increasing trend with the increasing concentration of the ascorbic acid and AgNPs.

At a concentration of 30µg/ml and 50µg/ml both RsAgNPs and Ascorbic acid showed similar antioxidant activity [Figure 9].

![Graph showing antioxidant activity](image)

**Figure 9: Total Antioxidant Activity by Phosphomolybdenum Assay**

**DPPH Radical Scavenging Assay**

The radical-scavenging activity of silver nanoparticles synthesized from leaf extract of *Raphanus sativus var. longipinnatus* was estimated by comparing the percentage inhibition of formation of DPPH radicals with that of Ascorbic acid. The silver nanoparticles showed moderate antioxidant activity when compared with Ascorbic acid. Radical scavenging activity of silver nanoparticles increased with increasing the concentration [Figure 10].

The IC 50 value was 99µg/ml for silver nanoparticles and 42µg/ml for Ascorbic acid. These results suggest that at concentration above 140µg/ml, the synthesized silver nanoparticles may serve as potent antioxidants.

![Graph showing radical scavenging activity](image)

**Figure 10: DPPH Radical Scavenging Activity**

**Estimation of Hydroxyl Radical Scavenging Activity**

The scavenging capacity of the silver nanoparticles from leaf extract of *Raphanus sativus var. longipinnatus* was shown in [Figure 11]. At a concentration of 100mg/ml, the silver nanoparticles showed 69.51% (IC50 -45µg/ml) hydroxyl radical scavenging activity with the standard Ascorbic acid activity being 85.58% (IC50-26µg/ml). The radical scavenging capacity of the sample might be attributed to phenolic compounds in the sample.
Biological Synthesis of Silver Nanoparticles Using *Raphanus sativus* Var. *longipinnatus* Leaf Extract and Evaluation of their Antioxidant and Antibacterial Activity

Estimation of Hydrogen Peroxide Scavenging Activity

Silver nanoparticles showed moderate inhibition against peroxyl radical which was less in comparison with Ascorbic acid. These results showed that silver nanoparticles synthesized from leaf extract of *Raphanus sativus* var. *longipinnatus* highly potent in neutralizing hydrogen peroxide radicals. Most of the hydrogen peroxide was scavenged by the RsAgNPs. IC 50 values for silver nanoparticles were 1120µg/mL, respectively whereas that of Ascorbic acid was 980 µg/ml [Figure 12]. H₂O₂ itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results showed that silver nanoparticles have less H₂O₂ scavenging activity than Ascorbic acid.

CONCLUSIONS

In this study, silver nanoparticles which were synthesized from *Raphanus sativus* var. *longipinnatus* leaf extract showed antibacterial activity and antioxidant activity. Thus it is proven from this study that the silver nanoparticles synthesized from *Raphanus sativus* var. *longipinnatus* leaf extract seem to be promising and effective antibacterial agent against bacterial strains and potent antioxidant. This biological chemistry approach towards the synthesis of silver nanoparticles is highly essential effort being addressed in nanomedicine because of its varied advantages. Plant extract being very eco friendly and cost effective can be used for the large scale synthesis of silver nanoparticles in nanotechnology processing industries.

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REFERENCES


