Biosynthesis, characterisation, free radical scavenging activity and anti-bacterial effect of plant-mediated zinc oxide nanoparticles using *Pithecellobium dulce* and *Lagenaria siceraria* Leaf Extract

M. Jeevan Prakash, S. Kalyanasundharam*

Department of Chemistry, Poompuhar College (Autonomous), Melaiyur - 609 107, Nagapattinam District, Tamil Nadu, India

*E-mail address: skalyanasundharam@gmail.com

ABSTRACT

The study involved synthesis of Zinc Oxide nanoparticles using biological and chemical reducing agents. The aim was to compare the yield, nature and antimicrobial activity of nanoparticles. Nanoparticles synthesized by the two methods were characterized by FTIR Spectroscopy, Scanning Electron Microscopy (SEM), Energy Dispersive Xray (EDAX), Transmission Electron Microscope (TEM) and X-ray diffraction (XRD). In biological method, *Pithecellobium dulce* and *Lagenaria siceraria* leaf extract; and in chemical method, sodium hydroxide was used as reducing agents. Antibacterial study was carried out on gram-positive and gram negative bacterial strains by disc diffusion method. The biologically synthesized ZnO Nanoparticles showed better antimicrobial activities with respect to the activities of synthetic drugs than chemical method. Antioxidant potential of synthesized nanoparticles was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.

**Keywords:** ZnO; gram-positive and gram negative bacterial; *Pithecellobium dulce*; *Lagenaria siceraria*
1. INTRODUCTION

In the fields of nanoscience and nanotechnology, the largest activity has been focused on the synthesis of new nanoparticles with different sizes and new shapes, which have strong effects on their widely varying properties. Nanoparticles are attracting increasing attention due to their unusual and fascinating properties, which are strongly influenced by their size, morphology and structure [Gopalakrishnan, K. et al, 2012]. Often chemical synthesis methods like sol-gel process, micelle, chemical precipitation, hydrothermal method, pyrolysis, chemical vapour deposition etc. lead to the presence of some toxic chemical species adsorbed on the surface that may have adverse effects in medical applications. Several methods have been used for the green synthesis of NPs using various biological materials as reducing agents such as microorganisms, marine organisms, micro-fluids, and plant extracts [Susan Azizi et al, 2013].

Figure 1: Plant Images of (A) Pithecellobium dulce leaf (B) Lagenaria siceraria leaf
Among the most important bioreductants are plant extracts, which are relatively easy to handle, readily available, low cost, and have been well explored for the green synthesis of other nanomaterials. Thus, their phytochemicals include hydroxyl, carboxyl, and amino functional groups, which can serve both as effective metal-reducing agents and as capping agents to provide a robust coating on the metal nanoparticles in a single step [Mahdavi, M et al, 2013]. In this study, we report the novel synthesis of ZnO nanoparticles by biological method using *Pithecellobium dulce* and *Lagenaria siceraria* leaf extract solution. We have also synthesized ZnO by chemical method using sodium hydroxide as reducing agent for a comparative study of yield, nature of synthesized particles and antimicrobial activities of particles obtained by both methods. The phytochemicals responsible for the synthesis of nanoparticles are terpenoids, flavonoids, phenols, carbohydrates, saponins, alkaloids and proteins (Sharma PV et al, 1996). The term phenolic compound embraces a wide range of plant substances that bear in common an aromatic ring with one or more hydroxyl substituents. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [G. Bhumi et al, 2014].

*Pithecellobium dulce* (P. dulce) Benth (Fabaceae) is a small to medium sized, evergreen, spiny tree, up to 18 m height, native of tropical America and cultivated throughout the plains of India and in the Andamans. It is known as ‘Kodukkapuli’ in Tamil. The bark of the plant is reported to be used as astringent in dysentery, febrifuge and it is also used in dermatitis and eye inflammation. In traditional medicine practice, the leaves of *P. dulce* are used for the treatment to fearache, leprosy, pepticulcer, toothache, venereal diseases and also act as emollient, anodyne, larvicidic (Sugumaran, M et al, 2008) and abortifacient, and antidiabetic properties in folk medicine. Various parts of plant are used for different purposes like, leaf as astringent, seed oil as spermicidal, anti-inflammatory, anti-oedema, fruit and seed as edible, bark for tannin (Mohammed et al., 2004).

*Lagenaria siceraria* leaf, Bottle gourd (*Lagenaria siceraria* (Mol.) standley, family Cucurbitaceae) commonly called as Dudhi or Ghiya is widely cultivated in the tropical and subtropical regions of the world, *Lagenaria siceraria*, which has diuretic and anti-swelling effects, is used as food (H.X. Wang et al, 2000). A decoction of *Lagenaria siceraria* is employed in the treatment of anasarca, ascites and beriberi (B. Anandh et al, 2014).

2. MATERIAL AND METHODS

2.1. Materials

Zinc Nitrate and sodium hydroxide and starch were analytical grade purchased from Merck and used without further purification. All aqueous solutions were prepared using de-ionized water. All glass wares were cleaned with chromic acid followed by thorough washing with de-ionized water and then acetone for prior use.

2.2. Preparation of aqueous leaf extract

*Lagenaria siceraria* and *Pithecellobium dulce* leaf were collected from rural areas of Chidambaram, Tamil Nadu, India. Leafs were washed thoroughly with tap water and dried in the shade. Dried leafs were cut into small pieces and ground coarsely using pulverizer. Soxhlet extraction was then performed to obtain the crude aqueous extract which is stored in
the refrigerator for further use. 30 ml of these extracts was used as reducing agents to synthesize nanoparticles. 3 g of zinc nitrate was added to both extracts separately and kept at 60 °C under vigorous stirring until dried. The resulting powder was ground and calcined at 570 °C in muffle furnace (Maensiri S et al. 2008).

2. 3. Synthesis of zinc oxide nanocrystals – by chemical method

Zinc Oxide nanoparticles were synthesized by this method by adding 0.6 g of zinc nitrate in 100ml of 0.1% starch solution and kept under constant stirring. To this solution, 60 ml of 0.2 M NaOH was added drop wise and the reaction was allowed to proceed for 2h after addition of NaOH. The solution was then allowed to settle overnight. Supernatant was discarded and the remaining solution was centrifuged and washed several times before drying at 80 °C overnight (Behera J L 2009).

2. 4. Analytical methods

High resolution Scanning Electron Microscopy (HRSEM) and Elementary Dispersive X-ray (EDX) analysis experiments were carried out on a FEI Quanta FEG 200 instrument with EDX analyzer facility at 25 °C. TEM images of metal oxide nanoparticles were obtained using a transmission electron microscope (PHILIPS CM200 model) at an Operating voltages: 20-200 kv Resolution: 2.4 Å. XRD spectra was recorded on the X’PERT PRO model X-ray diffractometer from Pan Analytical instruments operated at a voltage of 40 kV and a current of 30 mA with Cu Ka radiation. Fourier transform infrared spectroscopy. FT-IR spectroscopic studies were carried out to identify the possible functional groups in the leaf extract responsible for capping leading to efficient stabilization of the ZnO. The FT-IR spectra of powdered ZnO and the dried leaf extract were mixed with KBr pellets and are recorded in the 4,000-400 cm⁻¹ range on a Shimadzu FTIR-8400s.

2. 5. Antimicrobial activity

In the present study, in vitro antimicrobial activities were carried out by the using of disk-diffusion method (H.W. Seely et al, 1975; A. L. Barry, 1976). This method followed the following procedure: First of all, Petri plates were prepared with 20 mL of sterile Muller Hinton Agar for bacteria and 20 mL of Potato dextrose agar for fungi. Then, the 24 h prepared test cultures of inoculums were swabbed on the top of the solidified media and allowed to dry for 10 min. chemically and biosynthesized ZnO nanoparticles impregnated disks at the concentrations of 50 µg/mL for bacteria and fungi were placed aseptically on sensitivity plates with appropriate controls. The loaded disks were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvent. Ciprofloxacin (5 µg/disk) was used as positive control for bacteria and Amphotericin-B (10 µg/ disks) was used as positive control for fungi. All the plates were then incubated at 37 °C for bacteria and 28-35 °C for 24 h for fungi respectively. The sensitivity was recorded by measuring the clear zone of growth inhibition on agar surface around the disks in millimeter.

2. 6. Free radical scavenging activity

Biosynthesized Au-NPs and S. nigrum were tested for the scavenging effect on DPPH radical according to the method of Blois [Blois, 1958]. Different concentrations (50, 100 and
150 µL) of S. nigrum and biosynthesized Au-NPs were added, in equal volume, to 0.1 mM metabolic DPPH solution. The reaction mixture was incubated for 30 min at room temperature under shaking condition and the absorbance was recorded at 517 nm. The synthetic antioxidant butyl hydroxyl toluene (BHT) was used as positive control. All determinations were performed in triplicate. The DPPH radical scavenging activity (RSA) was expressed in percentage of inhibition using the following formula.

\[
RSA = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where: \(A_{\text{sample}}\) is the absorbance of the blank control and \(A_{\text{control}}\) isthe absorbance of the test sample.

3. RESULTS AND DISCUSSION

3.1. Powder X-Ray diffraction (ZRD)

The X-Ray diffraction pattern of ZnO nanoparticles prepared by *Lagenaria siceraria* and *Pithecellobium dulce* leaf extract and chemical method is shown in Figures 2(A), 2(B), 2(C) respectively. It is evident from the graphs that, the 5 peaks observed in all the graphs are similar and their peak values coincide with that of reference values. The peak position with 2θ values of *Pithecellobium dulce* mediated synthesized ZnO were 31.73°, 36.23°, 47.48°, 56.58°, 62.83° and 67.88° and the peak position with 2θ values of *Lagenaria siceraria* mediated synthesized ZnO were 31.72°, 36.22°, 47.52°, 56.57°, 62.87° and 67.92° in the case of chemical synthesized ZnO were 31.82°, 36.27°, 47.51°, 56.62°, 62.87° and 67.91°. The peaks obtained from biological and chemical method synthesized ZnO were indexed as (100), (101), (102), (110), (103) and (112) planes, which are in good agreement with those of powder ZnO obtained from the International Center of Diffraction Data card (JCPDS-36-1451) confirming the formation of a crystalline monoclinic structure. All diffraction peaks can be readily indexed to wurtzite hexagonal ZnO structure. No peaks due to impurity were observed, which suggest that high purity zinc oxide was obtained. The average crystallite size of the synthesized zinc oxide nanosheets was calculated to be 20 nm using Debye-Scherrer equation.

3.2. FT-IR

FT-IR analysis was performed. FT-IR spectrum of *Pithecellobium dulce* and *Lagenaria siceraria* leaf extract and chemically synthesized ZnO NPs are shown in Figs. 3a, 3b and 3c. The presence of functional groups in samples a, b and c are nearly same, with the change in transmittance percentage from one another. The FT-IR spectrum of ZnO NPs synthesized from both method showed strong absorption bands at 2848, 2854 and 2862 cm\(^{-1}\) can be ascribed to the stretching mode of C-H bonds of *Pithecellobium dulce* and *Lagenaria siceraria* leaf extract and chemically synthesized ZnO NPs. The strong band at 1618, 1622 and 1643 cm\(^{-1}\) is attributed to the \(\text{C}=\text{C}\) stretch in aromatic ring and \(\text{C}=\text{O}\) stretch in polyphenols. The band at 3437, 3402 and 3433 cm\(^{-1}\) denoted the presence of -OH stretching vibration.
Fig. 2. XRD patterns of (A) *Pithecellobium dulce* leaf extract synthesized ZnO (B) *Lagenaria siceraria* leaf extract synthesized ZnO (C) Chemically synthesized ZnO.
FTIR spectrum also shows the characteristic vibration band at 559, 540 and 526 cm$^{-1}$, which was correspond to E$_2$ mode of hexagonal ZnO wurtzite structure [Shoeb M et al, 2013].

3. 3. SEM and EDX
The SEM of ZnO nanoparticles synthesized from green and chemical method has been represented in Figure 4a, 4b and 4c. The SEM analysis was used to determine the structure of the reaction products that were formed. SEM image has showed individual zinc particles as well as a number of aggregates. This agglomeration is due to polarity and electrostatic attraction of ZnO nanoparticles [Zhang J. et al, 2002]. The SEM image showed relatively spherical shape nanoparticle formed with diameter range nm. The EDAX results also confirm the formation of ZnO nanoparticles synthesized from green and chemical method nanoparticles Figure 6a, 6b and. 6c

Transmission electron microscopy was used to examine the morphological characteristics of the ZnO nanoparticles obtained using Pithecellobium dulce and Lagenaria siceraria leaf extract and chemical route respectively (Figure 5a, 5b, 5c). ZnO nanoparticles obtained from Pithecellobium dulce and Lagenaria siceraria leaf extract and chemical route (Figure 5a, 5b, 5c) shows the presence of spherical shape with average diameter of ~ 4.3 nm, 34.7nm and 120nm.

3. 4. Antibacterial properties

Antibacterial activity results revealed that Pithecellobium dulce and Lagenaria siceraria leaf extract mediated synthesized ZnO nanoparticles acted as excellent antibacterial agents against both Gram-positive and Gram-negative bacteria when compared chemically synthesized ZnO nanoparticles.
Figure 4. SEM Images of (a) *Pitheclobium dulce* leaf Extract synthesized ZnO; (b) *Lagenaria siceraria* leaf Extract synthesized ZnO; (c) Chemically synthesized ZnO

Figure 7 shows the zone of inhibition produced by green and chemically synthesized ZnO nanoparticles against both Gram-positive and Gram-negative bacterial strains. *Pitheclobium dulce* ZnO nanoparticles exhibited maximum (15 mm) bacterial growth inhibition against *B. subtilis* and *Lagenaria siceraria* ZnO nanoparticles exhibited maximum (14 mm) bacterial growth inhibition against *B. subtilis*, in the form of zone-of-inhibition studies, where diffusion of nanoparticles on nutrient agar plates inhibits growth. In contrast, chemically synthesized ZnO nanoparticles showed zones of inhibition of 11 mm against *B. subtilis*.

In the case of *E. coli* maximum growth, inhibition zones were found to be the following: 17, 12, and 06 mm for *Pitheclobium dulce*, *Lagenaria siceraria* leaf extract, and chemical synthesized ZnO respectively (Figure 7 & Table 1).

Similar patterns were observed in the case of *P. aeruginosa*, Streptococcus pyogenes and *S. aureus*, where the maximum zone of inhibition was exhibited by *Pitheclobium dulce* followed by *Lagenaria siceraria* and Chemical ZnO. Nanoparticles tend to adsorb on the bacterial cell and undergo dehydrogenation due to respiration process which occurs at the cell membrane of bacteria.
Figure 5. (a) TEM Images of *Pithecellobium dulce* leaf Extract synthesized ZnO; (b) *Lagenaria siceraria* leaf Extract synthesized ZnO; (c) Chemically synthesized ZnO.
Figure 6. EDAXImages of (a) Pithecellobium dulce leaf Extract synthesized ZnO; (b) Lagenaria siceraria leaf Extract synthesized ZnO; (c) Chemically synthesized ZnO.
Figure 7. The antibacterial effect of (3) *Pithecellobium dulce* leaf Extract synthesized ZnO; (2) *Lagenaria siceraria* leaf Extract synthesized ZnO; (1) Chemically synthesized ZnO.

Graph 1. Antioxidant activity of *Pithecellobium dulce* leaf Extract synthesized ZnO, *Lagenaria siceraria* leaf Extract synthesized ZnO and Chemically synthesized ZnO.
Table 1. Antibacterial activity of *Pithecellobium dulce* leaf extract synthesized ZnO, *Lagenaria siceraria* leaf extract synthesized ZnO and Chemically synthesized ZnO.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Bacteria</th>
<th>ciprofloxacin</th>
<th>Zone of inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chemical Method ZnO</td>
</tr>
<tr>
<td>1</td>
<td><em>Bacillus subtilis</em></td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia coli</em></td>
<td>27</td>
<td>06</td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td><em>Staphylococcus aureus</em></td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td><em>Streptococcus pyogenes</em></td>
<td>26</td>
<td>09</td>
</tr>
</tbody>
</table>

Table 2. Antioxidant activity of *Pithecellobium dulce* leaf extract synthesized ZnO, *Lagenaria siceraria* leaf extract synthesized ZnO and Chemically synthesized ZnO

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentrations (µL)</th>
<th>Chemically synthesized ZnO nanoparticles</th>
<th><em>Lagenaria siceraria</em> mediated synthesized ZnO nanoparticles</th>
<th><em>Pithecellobium dulce</em> mediated ZnO nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 µL</td>
<td>21.20 ±0.28</td>
<td>38.35 ±0.12</td>
<td>43.23 ±0.63</td>
</tr>
<tr>
<td>2</td>
<td>100 µL</td>
<td>26.16 ±0.20</td>
<td>49.26 ±0.68</td>
<td>51.56 ±0.44</td>
</tr>
<tr>
<td>3</td>
<td>150 µL</td>
<td>33.46 ±0.45</td>
<td>56.51 ±0.72</td>
<td>59.15 ±0.81</td>
</tr>
</tbody>
</table>

3.5. DPPH radical scavenging activity

Plants contain specific metabolites that are acknowledged to perform a range of purposeful activities. It is well known and also reported in literature that plant mediated nanoparticles synthesis involves sequential reduction followed by capping with these constituents of plants [K Halil M. M. H. et al, 2010]. The reducing activity of ZnO nanoparticles synthesized from chemical and leaf extract of *Pithecellobium dulce* and *Lagenaria siceraria* was quantified spectrophotometrically by changing the DPPH color from
purple to yellow. Percent of inhibition of DPPH radical scavenging activity was presented on Table 2 and Graph 1. Biologically synthesized nanoparticles were found to be potent free radical scavenger when compared to chemical method. The DPPH radical scavenging activities were 21.20 ±0.28, 26.16 ±0.20 and 33.46 ±0.45% in 50, 100 and 150 µL for chemically synthesized ZnO nanoparticles and 38.35 ±0.12, 49.26 ±0.68 and 56.51 ±0.72% in 50, 100 and 150 µL for Lagenaria siceraria mediated synthesized ZnO nanoparticles whereas value obtained 43.23 ±0.63, 51.56 ±0.44 and 59.15 ±0.81% in 50, 100 and 150 µL for Pithecellobium dulce mediated ZnO nanoparticles, respectively. The average percentage inhibition of Pithecellobium dulce mediated synthesized ZnO nanoparticles was showed maximum of 59% as compared to that of chemically synthesized ZnO nanoparticles and Lagenaria siceraria synthesized ZnO nanoparticles (33% and 49%) at different concentrations used in this study and the activity increased with increasing concentrations of ZnO nanoparticles. This antioxidant activity may be due to the capping constituents present in plant extract and present on metal surface.

4. CONCLUSION

Synthesis of ZnO nanoparticle is basically a reduction process. This reduction is carried out by chemical or biological reducing agent. In the case of plant extract terpenoids, flavonoids and other phenolic compounds are responsible in the synthesis of nanoparticles, whereas in the case of chemical method, reducing agents involved. Pithecellobium dulce and Lagenaria siceraria mediated synthesis of ZnO nanoparticles approach is a fast, green and economical method which produces highly stable ZnO NPs. Plant is known to contain flavonoids, phenolic, tri-terpenoids and coumarins compounds that seem to play important role in synthesis, stabilization of ZnO NPs. The plant mediated synthesized nanoparticles have better antibacterial and antioxidant activity than chemically synthesized nanoparticles due to capped phenolic compounds.

References


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