Green synthesis of silver nanoparticles using Coleus amboinicus lour, antioxidatnt activity and invitro cytotoxicity against Ehrlich’s Ascite carcinoma

INTRODUCTION
Nanoparticles are being viewed as fundamental building blocks of nanotechnology. The most important and distinct property of nanoparticles are that they exhibit larger surface area to volume ratio. The most effectively studied nanoparticles today are those made from noble metals, in particular Ag, Pt, Au and Pd [38, 6]. The development of green processes for the synthesis of nanoparticles involves the use of biological techniques. The green chemistry approach has been mainly useful in the treatment of malarial fever, hepato- opathy, renal and vesical calculi, cough, chronic asthma, hicouche, bronchitis, antihelminthic, colic and convulsions [37]. The green chemistry approach for the synthesis of nanoparticles deserves merit. Our present investigation describes the Coleus amboinicus Lour leaf extract mediated synthesis of silver nanoparticles, also analysed cytotoxicity and antioxidant activity.

MATERIALS AND METHODS

2.1. Boiling /Collection of the extracts
An herbs was included in this study (Coleus amboinicus Lour (Country borage)) and were collected freshly from the market (stored leaves are not used in this study). Primarily the leaves were washed and the cleaned leaves were dried with water absorbent paper. Then it was cut into small pieces (Note: Do not grind), dispensed in 100ml of sterile distilled water and boiled for one hour at 80ºC. Then the leaf extracts were collected in separate conical flasks by standard filtration method.

2.2. Preparation of Silver nanoparticles
1mM Silver nitrate solution was prepared and stored in brown bottles. 5ml of leaf extracts was taken in BOD bottle separately and to this 100ml of AgNO₃ solution was added [29, 23, 11]. The same protocol was followed for other four leaf extracts. The colour change of the leaf extracts from pale green to dark brown was checked periodically. Then the BOD bottles were incubated at room temperature for further incubation till 24 hours. The colour change to brown indicated that the silver nanoparticles were synthesized from the leaves and centrifuged at 10000rpm for 25 minutes where pellets used for antibacterial activity.

2.3. Biosynthesis of Silver Nanoparticles

2.4. UV–Vis Spectra Analysis
The biosynthesis of Ag nanoparticles was monitored periodically in a UV–visible spectrophotometer (Shimadzu, UV-2550, and Japan). For the analy...
sis. 0.1mL of the sample in a cuvette and was diluted to 2mL with deionized water. The UV–visible spectra of the resulting diluents were monitored as a function of reaction time and biomaterial dosage at a resolution of 1 nm.

2.5. XRD Analysis
A thin film of the silver nanoparticle was made by dipping a glass plate in a solution and carried out for X-ray diffraction studies. The crystalline silver nanoparticle was calculated from the width of the XRD peaks, using the Debye-Scherrer formula

\[ D = \frac{0.9\lambda}{\beta \cos \theta} \]

Where D is the average crystallite domain size perpendicular to the reflecting planes, \( \lambda \) is the X-ray wave length, \( \beta \) is the full width at half maximum and \( \theta \) is the diffraction angle [8].

2.6. Observation of Silver Particle Size
XRD patterns were analyzed to determine peak intensity, position and width. Fullwidth at half-maximum (FWHM) data was used with the Scherrer’s formula to determine mean particle size. Scherrer’s equation is given by

\[ d = \frac{0.9\lambda}{\beta \cos \theta} \]

where \( d \) is the mean diameter of the nanoparticles, \( \lambda \) is wavelength of X-ray radiation source, \( \beta \) is the angular FWHM of the XRD peak at the diffraction angle \( \theta \) [8].

2.7. SEM Observation of Silver Nanoparticles
The plant extract biomass after reaction spontaneously precipitated at the bottom of the tubes. After the precipitation, the suspension above the precipitate was sampled for SEM observation. SEM samples of the aqueous suspension of silver nanoparticles were fabricated by dropping the suspension onto clean electric Stubs and allowing water to completely evaporate. SEM observations were carried out on a ZEISS EVO 40 EP Electron microscope.

2.8. EDX Observation of Silver Nanoparticles
Energy dispersive X-ray spectrometers take advantage of the photon nature of light. In the X-ray range the energy of a single photon is just sufficient to produce a measurable voltage pulse X-ray, the output of an ultra low noise preamplifier connected to the low noise are a statistical measure of the corresponding quantum energy. By digitally recording and counting a great number of such pulses with in a so called Multi Channel Analyzer, a complete image of the X-ray spectrum is building up almost simultaneously. This digital quantum counting technique makes the energy dispersive spectrometry exceedingly reliable. A semiconductor material is used to detect the x-rays together with processing electronics to analyses the spectrum. EDX observations were carried out by a Bruker LN2 free X-Flash 4010 SDD Detector and analytical Software was QUANTAX 200.

2.9. Estimating Antioxidant Activity

2.10. DPPH Photometric Assay
This method was given [5] and later modified [28]. It is one of the most extensively used antioxidant assay for plant samples. Recently the assay has been used to determine antioxidant activity in Tanacetum [34], Moldavian balm [9] and Phyllanthus amarus [21]. This method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour. This delocalization is also responsible for the deep violet colour, characterized by an absorption band in ethanol solution at about 520 nm. Representing the DPPH radical by \( Z^* \) and the donor molecule by AH, the primary reaction is: \( Z^*+AH \rightarrow ZH+A^* \)

2.11. FRAP Ferric-Ion Reducing Antioxidant Power
Ferric reducing ability of plasma (FRAP) assay is a technique to determine the total antioxidant power interpreted as the reducing capability. The FRAP assay was first given [28]. This assay was very recently used [25],. The FRAP reagent is prepared by adding 200ml of acetate buffer; 20 ml TPTZ, 20ml FeCl3 and 24 ml distilled water. Keep the reagent in desiccators a discard if the solution tube blue and prepare fresh solution after rinsing the bottle thoroughly with demineralised water. Mix and keep in water bath at 37°C. Run a set of blank and washed down cuvettes with distilled water. Add 1 ml FRAP reagent vigorously to each cuvette and mix the contents thoroughly and set the spectrophotometer at 593 nm. The temperature is kept at 37°C for 4 min. After 4 min, zero the blank and press run record.

\[ \text{Fe (TPTZ) 3} + ArOH \rightarrow \text{Fe (TPTZ) 2} + ArOH}^+ \]

2.12. In vitro cytotoxicity test
EAC tumour cells from intra peritoneal cavity of tumour induced mice was aspirated out and transferred it into the tube containing PBS. These cells were washed three times with PBS. It was resuspended into known volume of PBS. Checked the viability of the cells using trypan blue dye exclusion method. The cell suspension (1×10⁶ cells) was added into tubes containing various concentrations of the test compound and the volume was made up to 700µl using PBS. Control tubes were also maintained, which is containing only cell suspension in PBS. The assay mixture was incubated at 37°C for 3 hours. Then the percent of dead cells were evaluated by trypan blue dye exclusion method.

\[ \text{Percentage of dead cells} = \frac{\text{Number of dead cells}}{\text{Total number of cells}} \times 100 \]

3. RESULTS AND DISCUSSION

Characterization of silver nanoparticles Colour change
The aqueous extract of Coleus amboinicus lour was mixed with silver nitrate solution and incubated in dark in rotary shaker. Samples showed changed in colour from almost colourless to brown, this is a clear indication of the formation of silver nanoparticles in the reaction mixture. The intensity of the colour was increased during the period of incubation. The appearance of brown colour was due to the excitation of surface Plasmon vibrations [1]. Control showed no change in colour of the mixture when incubated in the same conditions. Results are reported in Figure 1.

![Figure 1. Periodical colour change from green to brown of Coleus amboinicus lour with 10 mM Agno₃](image)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Herbs</th>
<th>Dose (mM Agno₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0.94</td>
</tr>
<tr>
<td>10 mins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30 mins</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1 hr</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2 hr</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>4 hr</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>8 hr</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>16 hr</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>24 hr</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>28 hr</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

- No colour change; + Dark Green; ++ Reddish green; +++ Red; ++++ Reddish brown; ++++++ Brown threads
Synthesis of colloidal silver nanoparticles was initially performed by UV-Visible spectroscopic analysis. In UV–Visible spectrum, a strong peak was observed between 560 nm, indicating the presence of silver nanoparticles [12, 30]. UV – visible spectra is reported in Figure 2.

The silver nanoparticles were observed to be extremely stable even after 28 hours. Rapid synthesis of stable silver nanoparticles using (25gm leaf extracts) coleus amboinicus lour (1mM aqueous AgNO₃) have been reported, where bioreduction was found to be completed within 28 hours. The difference in the rate of bioreduction observed may be assign to the differences in the activities of the enzyme present in the extracts [12, 30]. UV – visible spectra is reported in Figure 2.

Visible spectroscopic analysis. In UV – Visible spectrum, a strong peak was observed between 560 nm, indicate the presence of silver nanoparticles [12, 30]. UV – visible spectra is reported in Figure 2.

FRAP Ferric reducing ability of plasma (FRAP, also Ferric ion reducing antioxidant power) is an antioxidant capacity assays which uses Trolox as a standard [36]. The FRAP assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured aqueous solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. Results of DPPH reduction by extract is shown in Table 1. The antioxidant activities of the individual compounds, present in the extract may depend on structural factors, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features [24]. It is found that IC₅₀ value of Coleus amboinicus lour is 96mg/ml which indicates the remarkable antioxidant activity of the ethanolic extract. This indicates a highly potential as free radical scavengers (Table 1).

FRAP reduction by extract is 350µmoles/mg. This result is evident for the majority of the ethanolic extract, hereby the greatest increase was observed.

Table-1 DPPH Scavenging of Coleus amboinicus lour silver nanoparticles.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>IC₅₀ mg/ml</th>
<th>Coleus amboinicus lour (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5mg</td>
<td>10.59±0.10</td>
<td></td>
</tr>
<tr>
<td>25mg</td>
<td>15.58±0.12</td>
<td></td>
</tr>
<tr>
<td>50mg</td>
<td>26.87±0.17</td>
<td></td>
</tr>
<tr>
<td>100mg</td>
<td>50.56±0.20</td>
<td></td>
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</tbody>
</table>

Values are mean ± SD of three samples in each column.

FRAP Ferric reducing ability of plasma (FRAP, also Ferric ion reducing antioxidant power) is an antioxidant capacity assays which uses Trolox as a standard [36]. The FRAP assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols. It is reduced in the presence of an antioxidant molecule. Use of FRAP provides an easy and rapid way to evaluate antioxidant activity. Results of FRAP reduction by extract is 350µmoles/mg. This result is evident for the majority of the ethanolic extract, hereby the greatest increase was observed.

Invitro Cytotoxicity of silver nanoparticles

The cytotoxicity of the silver nanoparticles was evaluated in vitro against EAC cell line at different concentrations (10, 20, 30, 40, 50 µg/ml). Our cytotoxicity analysis of the sample shows a direct dose-response relationship; cytotoxicity increased at higher concentrations (Figure 5). The samples
demonstrated a considerable cytotoxicity against the EAC cell line. The concentration necessary to produce 50% of the cytotoxicity was 30µg/ml for the silver nanoparticles. As shown in Figure 1, in the lowest tested concentration (10 µg/ml), silver nanoparticles were able to inhibit the cell line’s growth by less than 30%. In contrast the presence of 50 µg/ml of silver nanoparticles significantly inhibited the cell line’s growth (> 70%).

Figure-5 In vitro Cytotoxicity of Coleus amboinicus lour silver nanoparticles silver nanoparticles

4. CONCLUSION
The rapid biological synthesis of silver nanoparticles using Coleus amboinicus lour (country of borage) provides a simple and efficient route for the synthesis of nanoparticles with tunable Optical properties directed by particle size. The green chemistry approach towards the synthesis of silver nanoparticles has many advantages such as ease with which the process can be scaled up, economic, viability etc. It was concluded that the aqueous silver ions exposed to the herb extract was reduced and the nanoparticles were synthesized. The presence of nanoparticles were confirmed by the brown colour formation. A decrease in pH indicates the synthesis of nanoparticles. Coleus amboinicus lour (country of borage) should be decrease in pH which indicates the synthesis of nanoparticle. The aqueous extract of Coleus amboinicus lour (country of borage) was tested for anti oxidants activity and their cytotoxicity were noted to have a significant amount of DPPH and FRAP reducing capacity.

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