The anti-Leishmaniasis activity of green synthesis silver oxide nanoparticles

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Abstract
The green biosynthesized nanoparticles are hydrophilic, biocompatible, and non-toxic and have an important application in different field of science. Leishmaniasis is a protozoan vector-borne disease and is one of the biggest health problems of the world. The anti-Leishmanial drugs have toxicity and the recent development of resistance. In the present study, Ag$_2$O NPs was synthesized using Ficus benghalensis prop root extract (FBPRE) and used against Leishmania (L) donovani. The UV–visible adsorption spectra showed that the absorbance peak is in the range of 430 nm, the vibrational modes of phytochemicals in the extract have been characterized by technique FTIR which allow the identification and information about material. TEM, SEM and XRD pattern have been used to confirm the morphology of silver oxide, which have a spherical nanoparticles shape, and crystalline size of 16 nm. Diluted concentration (25, 50, 100, 200, and 300 µg/ml) of Ag$_2$O NPs were used against L. donovani. The result indicated the effect of Ag$_2$O NPs on the parasite growth rate which clearly decreased compared with L. donovani treated with the same concentrations of the standard anti leishmanial drug (pentostam drug) and the control group. The viability percentage decreased to 66.3 ± 5.44 % compare with group that treated with the pentostam which was 270.0 ± 4.33 in 300 µg/mL concentration after 72 hours. The current results concluded that Ag NPs had an effect to inhibit L. donovani growth in vitro following the infection with parasite.

Keywords: Green biosynthesized, silver oxide nanoparticles, Leishmania donovani, antiparasitic effect.

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Introduction
Nano biotechnology has being developed using nano sized particles to treat many diseases including human tumors. Nano sized particles are very useful due to their nano-scale structure and size as well as their unique biological effects (1). In nanotechnology, green chemicals have been used for the synthesis of many metallic nanoparticles without addition of any external chemical material that is responsible for causing environmental contamination (2, 3). Green synthesis method of metallic nanoparticles produced by using extracts from different plants and their parts as reducing and capping agent(4).
Production of nanoparticles using the chemical methods is often expensive, toxic material, and time-consuming. The green method is eco-friendly, inexpensive, and less time consuming to produce nanoparticles(5). Silver oxide nanoparticles (Ag$_2$O NPs) have efficient and different applications in the nanotechnology such as catalytic, antibacterial properties and electrical conductivity (6).

*Ficus benghalensis* (FB) (Moraceae) is commonly known as the Banyan tree (7). This tree is grown in India, Bangladesh and Sri Lanka. Extracts of various plant parts of their anathematic are important as treatment analgesic, anti-inflammatory, antioxidant, anti-diabetic, and antimicrobial activities (8).

Leishmaniasis is a tropical disease that affects about 12 million people around the world, and it’s also becoming more common in developed countries (9). Among several drugs used in the treatment of leishmaniasis; the current therapy is pentavalent antimonial. This anti-parasite agent possesses many limitations: toxicity, high cost, and resistance or hospitalization requirement (10). Metal oxide nanoparticles have been used as anti-leishmania, antibacterial, antiviral and anti-carcinogenic effects. These results indicate that metal oxide nanoparticles are highly effective against both prokaryotic and eukaryotic infectious agents (11). The basis for the antimicrobial effect of silver, silver ions, and silver-containing compounds nanoparticles is their ability to produce reactive oxygen species (ROS) (12). *Leishmania* parasites are sensitive to ROS produced by macrophages, it can be forming a host cells, and it also produce high amounts of ROS in order to kill microbial agents (13). Nanoparticles anti-leishmanial activities of drugs are preferable as compared to any other parasitic disease mainly due to the fact that *Leishmania* parasite resides within the macrophages (14).

**Materials and methods**

**Chemicals and biological materials**

The AgNO$_3$ (99.9%) reactant was acquired from (Dae-Jung Chemicals, Seoul, South Korea), in stoichiometric amounts and was dissolved in aqueous solution as the metal precursor. *Ficus benghalensis* prop root. The extract was prepared by grinding the root into a fine powder after wash and dried. 5 g of this powder was added to 20 mL of deionized water in flask, mixed well, and maintained on a magnetic heating stirrer at 90°C for 1 hour. The extract yield was filtered for further use.

**Cell culture**

*L. donovani* strain (DUAA/ IQ/ 2005/ MRU15) was gained from the Biology Department, University of AL-Mustansiriya. They were maintained and sub-cultured every 7 days and they were cultured in RPMI-1640 medium with L-glutamine (Sigma, St Louis, MO). Medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron, using positive pressure rather than vacuum to minimize the loss of carbon dioxide, 90 ml of this filtering RPMI-1640 medium was taken and added 10 ml of Fetal bovine serum. 100 μg/ml of penicillin were added to the filtrated mixture, Liquid medium stored at 2-8°C. Cells (1 × 10$^6$ cells/mL) were added to a flask contain 5 mL of RPMI-1640 plus 10% FCS medium and the flask was incubated at 27°C. Cells were passage weekly (15).
Drug concentrations

Pentostam or Sodium stibogluconate (Sb) Pentostam present in liquid form as an injectable ampoule (100 mg/ml), it was manufactured by (Glaxo Operation UK Limited Barnard Castle, Member of the Glaxo Smith Kline group companies). The drug stored below 25°C and protects from light. It was gained from Al-Yarmok Teaching Hospital. A stock solution of Sb was used to prepare the following concentrations: 25, 50, 100, 200, and 300 µg/ml immediately before used.

Synthesis of silver oxide nanoparticles

For the synthesis of silver oxide nanoparticles (Ag₂O NPs), 10 mL of Ficus benghalensis prop root extract (FBPRE) was added to 90 mL of 0.458 g of silver nitrate solution in 250-ml (Erlenmeyer flasks) to form a reaction mixture, and the reaction was performed on magnetic heating stirrer at 80°C. Initial confirmation of silver nanoparticle production is by change in color from light yellow to dark brown in the reaction mixture during four hours (Fig. 1).

![Figure 1: Silver oxide nanoparticles formation](image)

Once silver nanoparticle production was complete, the obtained product was centrifuged for 30 min at 4000 rpm, followed by several washes with large amounts of deionized water and ethanol to ensure separation of free entities from the silver nanoparticles. The obtained material was dried at room temperature to make powder. The stock solution of Ag₂O NPs was serially diluted in phosphate buffered saline (PBS) (PH 7.4) to prepare the following concentrations: 25, 50, 100, 200, and 300 µg/ml immediately before used.

Determination of Ag₂O-NPs effects on the parasites proliferation

Determinations were carried out in culture of L. donavani. Every assay tube contained 3 mL of medium with 1 x 10⁶ cells/mL of L. donavani. Assays were divided into two groups: the first group was treated by Sb drug (positive control) in different diluted concentration, whereas the second group was treated by Ag₂ONPs. Control Leishmania culture was not treated to Sb drug or Ag₂O NPs and following that, each test tube was incubated at 27°C. Parasites were counted using Neubauer chamber improved bright-line hemocytometer (Marienfeld, Germany) at 24, 48, and 72 hours of incubation.
Statistical analysis

The Statistical Analysis System- SAS program was used to study the effect of difference factors in the experiment parameters. Least significant difference –LSD test was used to compare the significant values between means in this study.

Results and discussion

UV-Visible spectral

Silver oxide nanoparticle produce the through visual observation by changing color from light yellow to dark brown (Fig.1). The change in color is due to the changes in the excitation energy of the particles surface plasma resonance(16). The UV–visible spectrum (Fig.2) shows a surface Plasmon resonance peak (SPR) at 430 nm, which corresponds to Ag$_2$ONPs production(17). These results were consistent with the study of Velu Manikandan et al.(18). The band gap energy can be obtained in nanomaterials from maximum absorption. According to the theory of quantum confinement, it is equal Eg(2.4ev). The exact mechanism of the plant extract-mediated synthesis of silver nanoparticles is not well understood. It was hypothesized that the bioactive compounds presenting the plant extract, such as poly-phenols (flavonoids), have hydroxyl and ketonic groups that bind to the bulk metal silver to reduce it to nano-size (19).

![Figure 2: UV–visible adsorption spectra of Ag$_2$O NPs](image)

X-Ray Diffraction Studies (XRD)

The powder X-ray diffraction of the Ag$_2$O NPs is shown in Fig.3. It appear to be similar between XRD structure for all sample of Ag$_2$O NPs corresponds with what came in the card Joint Committee on Powder Diffraction Standards (JCPDS no 96-431-8189), it have a polycrystalline structure with cubic phase, and match that of the standard spectra of silver JCPDS standard card (no. 96-901-3049) (18), these results are in agreement with the results of references obtained by (1, 2, 17).
The pattern shows many Bragg reflection peaks; the detected \((h k l)\) peaks was at \(2\theta\) values of 27.8421° plane \((101)\), 32.2520° plane \((111)\), 46.0898° plane \((211)\) and 54.6560° plane \((220)\) as shown in Fig.3, which shows that the peaks of Ag NPs were cubic phase too, and correspond to \((111), (200), (220)\) and \((311)\) planes at \(2\theta\) \((38.1318, 44.0623, 64.5402, 77.3642)\) respectively, which is characteristic of mean particle diameter by applying Debye- Scherrer equation(20):

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

Where \(D\) is the mean crystallite size, \(\lambda\) is the wavelength of incident X-ray (1.5406 \(\text{Å}\)), \(\theta\) is the degree of the diffraction peak, and \(\beta\) is the full width at half maximum. The values of \(D\) lies within the nanoparticles range which the average crystalline size is 16.4 nm. The obtained sizes are smaller than the sizes reported by (19).

\[Figure 3: \text{X-ray diffraction patterns of synthesized Ag}_2\text{O NPs}\]

**Transmission Electron Microscopy (TEM)**

TEM was used for characterizing the morphology, size, and shape of synthesized Ag\(_2\)O NPs. Image showed a more spherical shape and mono dispersed Ag\(_2\)O NPS with an average particle size 15.73 nm Figs. 4 (a, b). The silver nanoparticle obtained from TEM is agreed with the size obtained from the XRD measurements. These results are in an agreement with the research of Taylor and Francis et al. (21).
Fourier Transform Infrared (FTIR)

FTIR measurements have been performed for identifying the possible bio-molecules in the extract of FBPRE and their possible involvement in the synthesis of Ag₂O NPs (Fig. 5). The spectrum was recorded in the range of 4000 - 400 cm⁻¹ showed several peaks as an indication. The bands appearing at 3441.07 cm⁻¹ assigned to the O-H stretching vibration of alcohol and phenols (18, 19). The peak at 2927.21 cm⁻¹ corresponds to C-H stretch of alkanes. The peak at 2359.27 cm⁻¹ corresponds to C-N stretching of aliphatic amines group (22, 23), the peak at 1632.05 cm⁻¹ corresponds to C=C aromatic, other peak 1034.06 cm⁻¹ corresponds C=O of carboxylic acid (24, 25), the peak 460.1 cm⁻¹ corresponds Ag-O (26, 27), amide groups on the surface of the FBPRE, which could be responsible for the reduction of Ag⁺. FBPRE mainly contains flavonoids, saponins, steroids, reducing sugars, alkaloids, antimony trioxide, glycosides, antimony trisulfide, 1,2-dihexanoyl-sn-glycero-3-phosphocholine, and 2-chloroethyl phosphonic acid, which was previously reported by Kumar et al. (28) and Omkar Paware et al. (29) might be involved in reducing the Ag⁺ to AgO.
Scanning Electron microscopy (SEM)

The SEM images of the $\text{Ag}_2\text{O}$ NPs are shown in Figs. 6 (a, b). It is seen that the root extracts being as reducing and capping agents formed spherical NPs and the size range (15-26) nm; this result agree with the result of Zainub et al. (30).

![FTIR spectra of $\text{Ag}_2\text{O}$ NPs](image)

**Figure 5: FTIR spectra of $\text{Ag}_2\text{O}$ NPs**

**Figure 6: SEM analysis shows that size and shape of $\text{Ag}_2\text{O}$ NPs**

a) At 200nm, b) 500nm
Antiparasitic activity

From the results shown in Fig.7 (a, b), and can be clearly seen the effect of different concentrations (25,50,100,200,and 300μg/ml) of Ag₂O NPs and Sp drug on promastigotes growth rate after 24, 48 and 72 h. The Ag₂O NPs showed a significant (P˂0.05) cytotoxicity in the growth rate of the promastigotes compared with the promastigotes treated with penostam and the untreated promastigotes as listed in Table1. The number of the parasites was (230, 200, 170,165, and 99.2×10⁴ cell/ml) respectively after 24 hrs. In comparison with the number of promastigotes treated with the same concentrations of pentostam which was (475, 445.8, 315.8, 283.3,265×10⁴ cell/ml) respectively and untreated parasites which was 610×10⁴ cell/ml, After 48and 72h the number of promastigotes treated byAg₂O decreased clearly reaching to (310, 266.7, 255.8,175, and 108×10⁴ cell/ml) and (225, 161.3, 156.3, 110 and 66.3×10⁴ cell /ml) respectively as shown in tables(2,3), compared with promatigotes treated with the same concentrations of pentostam which count (550,530,400, 333,and 276.7×10⁴ cell/ml) and (525,500, 390, 290 and 270×10⁴ cell/ml), respectively, and with untreated parasites which recorded 637×10⁴ cell/ml and 575×10⁴ cell/ml respectively. The IC50 of Ag₂O NPs was measured 2.435 μg/ml,2.354 μg/ml and 2.285μg/ml after 24h, 48h and 72hr respectively.

Figure 7: Cell viability of L. donavani treated with a) Ag₂O NPs after24, 48 and 72 hours, b)Sb drug after 24, 48 and 72 hours

### Table 1: Comparison between the effects of different concentration of Sb drug, Ag₂O NPs against L. donovani number after 24hr.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>No. of Leishmania (1×10⁴)</th>
<th>L.S.D value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sb</td>
<td>Ag₂O NPs</td>
</tr>
<tr>
<td>25</td>
<td>550 ± 15.87</td>
<td>310 ±7.63</td>
</tr>
<tr>
<td>50</td>
<td>530.0 ± 24.02</td>
<td>266.7±8.33</td>
</tr>
<tr>
<td>100</td>
<td>400.0 ± 66.14</td>
<td>255.8 ± 28.37</td>
</tr>
<tr>
<td>200</td>
<td>333.3 ± 41.67</td>
<td>175.0 ± 1.44</td>
</tr>
<tr>
<td>300</td>
<td>276.7 ± 19.70</td>
<td>108.3 ± 14.24</td>
</tr>
<tr>
<td>Control</td>
<td>637.5 ± 15.02</td>
<td>---</td>
</tr>
<tr>
<td>L.S.D</td>
<td>115.56 *</td>
<td>59.65 *</td>
</tr>
</tbody>
</table>

* (P<0.05).

### Table 2: Comparison between the effects of different concentration of Sb drug, Ag₂O NPs against L. donovani number after 48 hr.
Table 3: Comparison between the effects of different concentration of Sb drug, Ag$_2$O NPs against *L. donovani* number after 72 hr.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>No. of <em>Leishmania</em> (1×10$^4$)</th>
<th>L.S.D value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sb</td>
<td>Ag$_2$O NPs</td>
</tr>
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</tr>
<tr>
<td>Control</td>
<td>637.5 ± 15.02</td>
<td>---</td>
</tr>
<tr>
<td>L.S.D</td>
<td>115.56 *</td>
<td>59.65 *</td>
</tr>
</tbody>
</table>

* (P<0.05).

The results of this study shows that pentostam have lower efficacy on *L. donovani* promastigotes in all the used concentrations while Ag$_2$O NPs have shown higher efficacy through all days of treatment with concentrations (25, 50, 100, 200, and 300 µg/ml), this may be due to the fact that the pentavalent antimonials are required biological reduction to the trivalent form (SbIII) for anti-leishmanial viability (31). The site of infection and mechanism of reduction remain controversial.

Nanoparticles have unique physicochemical properties such as shape, small size, great surface area, and electrical charge. The nanoparticles are frequently used in medicine in drug delivery and cancer therapy (5). It was verified that the action of AgNPs-bio on *Leishmania* infected macrophages, observing a decrease in the amount of infected cells, intracellular parasites per macrophage and recovered promastigotes from both infected and treated cells. These results corroborate the studies by Baiocco *et al.* (32), and having reinforced that AgNPs-bio is able to act on...
the intracellular parasites or maximize the microbacterial machinery of macrophages (33). A study suggested that AgNPs-bio induced apoptosis-like events in treated promastigotes, a metal oxide nanoparticles are effective in inhibiting the enzyme of trypanothione metabolism that is vital in survival of Leishmania parasites (34). In addition, the antimicrobial efficacy of Ag₂O NPs is stimulated with impairing function of cell membrane by producing ROS (35). The Leishmania parasites are sensitive to ROS and it impairs their biological functions including metabolic activity, viability and survival within the host macrophages(36). Silver oxide nanoparticles that penetrate into bacteria are believed to interact strongly with molecules containing sulfur and phosphorus groups like DNA (37). On the other hand, the cell cycle halts at the G2/M phase owing to the DNA damage (38, 39). The cells get affected by oxidative stress, which is caused by inhibition of ATP synthesis and occurrence of ROS. As a consequence, apoptosis are included (40). Another reason for cell death after the exposure to Ag₂O NPs may be the release of silver ions from the nanoparticles. It is believed that after the penetration, of silver oxide nanoparticles make the bacterial enzymes inactive by releasing atomic Agº and ionic Ag clusters, and cause cell death by producing hydrogen peroxide and the other free radicals (41, 42).

**Conclusion**

This study reports the mechanism for the biosynthesis of Ag₂O NPs using Ficus benghalensis prop root extract (FBPRE). Biologically synthesized nanoparticles were 10-26 nm in size and spherical shape. The Ag-O NPS produced is an eco-friendly alternative with non-toxic as a safe mode alternative to physical and chemical methods. The molecules have been shown to be highly effective against leishmaniasis compared to pentostam drug with the same concentration when used in vitro assays.

**References**