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Antiplasmodial activity of silver nanoparticles: A novel green synthesis approach

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ABSTRACT

Objective: To synthesize silver nanoparticles using silver nitrate by a green technique which involves different compositions of aqueous leaf extracts of *Azadirachta indica* (neem) and *Ocimum sanctum* (tulsi). **Methods:** Their shape and size were determined using transmission electron microscopy and UV-visible spectroscopy. Their antiplasmodial activity was studied using the malarial parasite strain (*Plasmodium falciparum*, 3D7). The parasite strain (3D7) was collected and revived *in vitro* using Trager and Jensen method in RPMI 1640 medium for 7-8 cycles. Half maximal effective concentration values were calculated by nonlinear regression analysis. **Results:** Transmission electron microscopy results confirmed the formation of silver nanoparticles with size ranging from 4.74-39.32 nm and their size differs by varying the concentrations from 20% to 100% of neem extract in neem and tulsi extracts. It was observed that samples B and C showed half maximum effective concentration of about 0.3 μM. **Conclusions:** It can be easily established that the aqueous leaf extracts of neem and tulsi in combination can be a good source for synthesis of silver nanoparticles with small size possessing appreciable antiplasmodial activity.

1. Introduction

Nanotechnology is a versatile branch of development and research which is growing at a tremendous rate from the past two decades. Nanoparticles are known for their numerous physical, biological, and pharmaceutical applications[1]. Silver nanoparticles (AgNPs), in particular, are being used as antimicrobial agents[2-5] as they exhibit interesting antibacterial[6-8] and antiplasmodial[9,10] activities. Several chemical methods for synthesis of nanoparticles have been reported[11-14]. However, the green synthesis methods are considered to be more versatile[15,16].

The use of green techniques for synthesis of nanoparticles is a rapid, low cost and eco-friendly process. The advantage of using plant extracts for synthesis of nanoparticles is that each plant extract, by virtue of its unique metabolites such as polyphenols, terpenoids and thiols, gives rise to a wide diversity of unique microenvironments. This influences the physico-chemical and biological properties of the AgNPs which are hence formed. Several

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plants of medicinal importance are commonly prevalent and have been used over centuries[17,18]. *Azadirachta indica* (neem) and *Ocimum sanctum* (tulsi) plants are commonly available and each part of these plants has been used as a household remedy against various human diseases from historic times[19]. These plants have also been found to show antimalarial and antiplasmodial[20-22] properties. AgNPs prepared from neem and tulsi leaf extracts individually show good antibacterial and antimicrobial properties. However, the combinatorial properties of the above two extracts in unison are still an unexplored niche.

In the present work, we have studied the antiplasmodial activity of the AgNPs synthesized from the combination of neem and tulsi plant leaf extracts when taken in different proportions. The AgNPs synthesized by novel combination of neem and tulsi plant leaf extracts have proved to be potential antiplasmodial agents. The knowledge of antiplasmodial activities of AgNPs based on the calculated half maximal effective concentration (EC_{50}) values would be helpful to understand their antimalarial properties.

2. Materials and methods

2.1. Materials

Fresh leaves of neem and tulsi were taken from the botanical garden of Sri Venkateswara College Campus. Silver nitrate was purchased from Merck India Ltd and 1 mM solution of the same was prepared. Other chemicals and solvents used were of analytical grade. All the glassware were washed with distilled water and dried in oven. Antiplasmodial activities were tested at National Institute of Malaria and Research, New Delhi.

2.2. Preparation of leaf extract

Collected leaves were first washed with distilled water and then air dried for 2 h. A total of 20 g of finely cut neem leaves were boiled in 150 mL distilled water for 30 min at 90 ℃ on an electric water bath and filtered through Whatman filter paper to obtain aqueous neem leaf extract. Also, 20 g of finely cut tulsi leaves were processed through the same procedure in order to obtain aqueous tulsi leaf extract. The tulsi extract obtained was centrifuged for 15 min at 5 070 *g*. The prepared extracts were stored at 4 ℃.

2.3. Synthesis of silver nanoparticles

For synthesis of AgNPs, 3 mL aqueous extract of neem leaves was mixed with 27 mL of 1 mM silver nitrate and a vivid color change was observed indicating the formation of AgNPs. Similarly, other samples were prepared by mixing plant extracts of neem and tulsi in different compositions as 100% tulsi (0% neem), 100% neem (0% tulsi), 80% neem (20% tulsi), 60% neem (40% tulsi), 40% neem (60% tulsi) and 20% neem (80% tulsi) namely A, B, C, D, E, F respectively. Keeping the ratio of extract(s) to 1 mM silver nitrate equal to 1:9, the effects of various physico-chemical parameters were examined by varying the reactant concentration, pH and reaction time. Reduction of ionic silver to AgNPs was monitored after diluting a small amount of the sample 20 times. Absorption spectra were recorded with UV/VIS/NIR spectrometer (Systronics-P C Based Double Beam Spectrophotometer - 2202). The excitation source was a 450 Watt CW xenon lamp. Completion of the reaction indicated by a change in color from colorless solution to a brown-yellow solution was observed, confirming the formation of AgNPs after 15 min in the presence of NaOH. Effect of pH was studied by varying the pH of all the samples by adding 2 drops of 1 M NaOH and adjusting its pH to 8. The pH of tulsi leaf extract was 7.0 and that of neem leaf extract was 6.5.

2.4. Characterization of the synthesized AgNPs

Synthesis of AgNPs solution with leaf extract can be easily confirmed by UV–Vis spectroscopy. The bioreduction of the Ag^+ ions in solutions prepared by leaf extract was monitored after 20 times dilution and measuring the UV–Vis spectra of the solution. UV–Vis spectra of these samples was monitored as a function of time of reaction on a Systronics-P C Based Double Beam Spectrophotometer-2202 in the 350–600 nm range operated at a resolution of 1 nm. Further, the reaction mixture was subjected to sonicator for 15 min before transmission electron microscopy (TEM). A small drop of sample was poured on 300 mesh copper grids for TEM.

Sample for TEM was prepared by drop coating purified AgNPs on carbon-carbon coated 300 mesh copper TEM grids at AIIMS, New Delhi. TEM measurements were performed on Techai G2 20 S-Twin with accelerating voltage at 200 kV.

2.5. Antiplasmodial activity

In-vitro parasite cultivation and drug sensitivity assay were performed as follows. The parasite strain (3D7) was collected from NIMR parasite bank, and revived *in vitro* using Trager and Jensen method in RPMI 1640 medium for 7-8 cycles. The culture of *Plasmodium falciparum* 3D7 parasites were maintained in human erythrocytes (AB+) at 2% hematocrit in RPMI medium 1640 supplemented with 50 mg/L gentamicin, 5% sodium bicarbonate 10% human serum. Parasites were synchronized with 5% *D*-sorbitol and maintained in ring stage. ELISA plates were used in triplicates with 20 μM of each sample of AgNPs dissolved in water serially diluted to seven fold concentrations. A total of 180 μL/well of parasites with 0.5% synchronized ring stage parasitemia and 2% fresh RBC hematocrit was added. Further plates were incubated for

72 h at 37 ℃ in CO_2 incubator (Flow Laboratories).

ELISA plates were initially pre-coated with 100 μL of 1.0 μL/mL primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) and incubated at 4 ℃ overnight. The plates were further dried and blocked with 200 μL of blocking solution (2% bovine serum albumin in PBS) followed by three times washing with the PBS-Tween 20 (0.05%). The plates were stored at -20 ℃ till the experiment was conducted.

For HRPII ELISA assay, 100 μL of hemolysed lysate of 72 h post infection (hpi) of parasite was transferred to pre-dosed primary IgM antibody coated plate and incubated at RT in humid chamber for 1 h. The content was discarded, plate was washed thrice with PBS-Tween 20 (0.05%) and tap dried. Followed by addition of 100 μL of 0.2 μg/mL concentration of IgG secondary antibody (MPFG-55P, Immunology Consultants Laboratories, Inc. Newberg, OR, USA) dissolved in 2% BSA and 1% Tween 20, IgG added plate was incubated for 1 h at RT in humid chamber, washed three times in PBS/Tween and dried. Further 100 μL 3,3',5,5'-Tetramethylbenzidine chromogen (Ameresco, US) was added and incubated for 10 min at RT in dark. The reaction was stopped by addition of 50 μL of 1M Sulphuric acid followed by absorbance recording of each plate using an ELISA plate reader (Spectrostar Nano, BMG LABTECH, Germany) at 450 nm[23,24]. EC_{50} value was calculated by nonlinear regression analysis. The software used for the study was based on a polynomial regression model and was freely available from http://malaria.farch.net.

3. Results

3.1. UV-Vis spectra analysis

The UV-Vis spectrum of silver nanoparticles (Figure 1) of samples A-F was recorded as a function of concentration in the range of 350-550 nm. λ_{max}(nm) range of the synthesized nanoparticles was found to be between 407-427 nm.

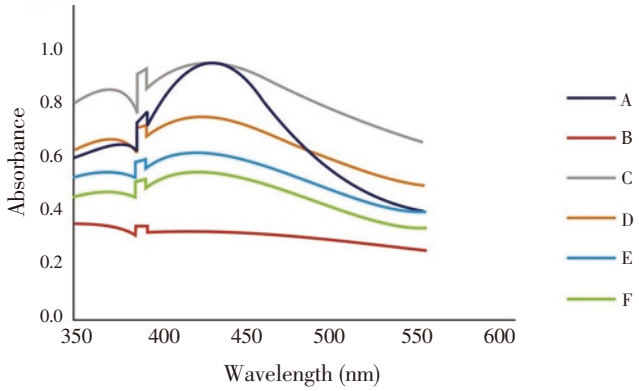

Figure 1. UV-Vis spectra of silver nanoparticles synthesized from aqueous leaf extracts of neem and tulsi in different combinations.
(A: 100% tulsi, 0% neem; B: 100% neem, 0% tulsi; C: 80% neem, 20% tulsi; D: 60% neem, 40% tulsi; E: 40% neem, 60% tulsi; F: 20% neem, 80% tulsi).

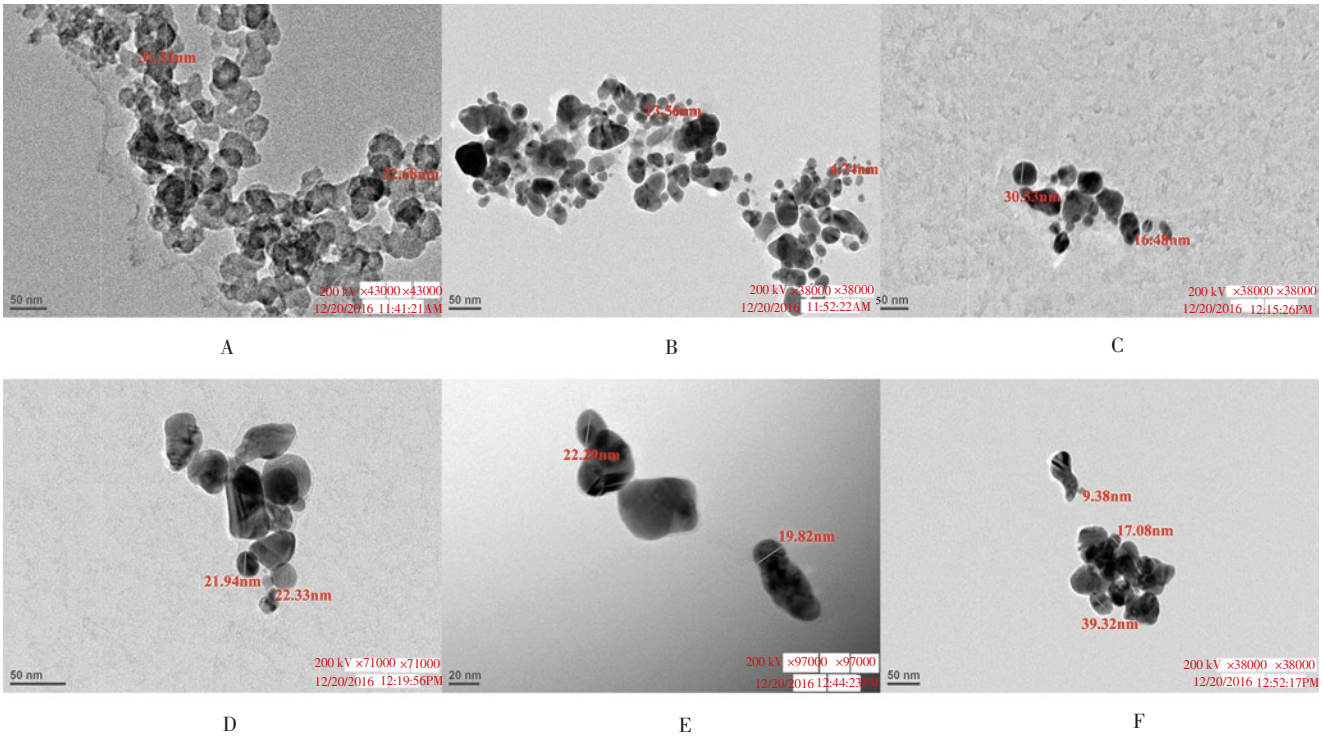

Figure 2. TEM images of AgNPs formed from different combinations of neem and tulsi extracts: Sample A (100% tulsi, 0% neem), B (100% neem, 0% tulsi), C (80% neem, 20% tulsi), D (60% neem, 40% tulsi), E (40% neem, 60% tulsi), and F (20% neem, 80% tulsi).

3.2. TEM analysis

TEM technique was employed to visualize the size and shape of AgNPs. Table 1 showed the corresponding sample combinations, particles size obtained, λ_{max}(nm), EC_{50} and R^2 values for the synthesized AgNPs. The optimum particle size was found to be < 40 nm. The TEM images of different samples were illustrated in Figure 2, confirming the formation of AgNPs between size range 4.74-39.32 nm. In addition, the TEM images showed that the nanoparticles were circular and spherical in shape (Figure 2).

Table 1

Particles size, λ_{max}(nm), EC_{50} and R^2 values of AgNPs synthesized from samples A-F.

Samples	% Composition of neem extract + tulsi extract	Particle size(nm)	λ_{max}(nm)	EC_{50}	R^2
A	100% tulsi + 0% neem	20.00-32.68	426.8	1.692	0.961 2
B	100% neem + 0% tulsi	4.74-13.33	411.6	0.313	1.000 0
C	80% neem + 20% tulsi	16.48-30.33	422.8	0.313	1.000 0
D	60% neem + 40% tulsi	21.94-22.33	420.4	0.742	1.000 0
E	40% neem + 60% tulsi	19.82-22.29	407.6	1.437	0.984 8
F	20% neem + 80% tulsi	9.38-39.32	420.8	0.513	0.987 6

3.3. Antiplasmodial studies

To determine the efficacy, the nanoparticles of neem and tulsi alone and in combination were incubated with synchronized ring stage *Plasmodium falciparum* 3D7 parasites. Each nanoparticle was screened in triplicates till 72 h post infection (hpi) using HRPII ELISA assay. The result indicated that 100% and 80% neem contained nanoparticles showed better value of EC_{50} about 0.3 μM among other combinations while other showed moderate activity. The EC_{50} values were mentioned in the Table 1 and belonged to 0.313-1.692 range.

4. Discussion

Reduction of silver ions into metallic silver when exposed to the plant extracts depicted a color change. The Surface Plasmon Resonance phenomena is accountable for the dark yellowish - brown color of AgNPs. For characterisation of the nanoparticles, UV-Vis spectroscopy substantiated to be a suitable technique for the analysis of nanoparticles. Reduction of Ag^+ ions in the aqueous medium to atomic silver in the presence of plant leaf extracts was correlated with the UV-Vis spectra.

The effect of AgNPs of neem and tulsi alone and in combination suggested that 100% and 80% neem exhibited EC_{50} value of about 0.3 μM. The observed anti-plasmodial activity could be due to presence of steroids, terpenes, coumarins, flavonoids, phenolic acids, lignans, xanthones and anthraquinones[25]. A simple green synthesis of AgNPs prepared from leaf extract showed effective antiplasmodial properties at concentrations 100% neem with 0% tulsi (Sample B) and 80% neem with 20% tulsi (Sample C). Pure neem and tulsi leaf extract have been reported[20,26] to show insignificant antiplasmodial activity with IC_{50} values ranging from 35-40 μg/mL whereas AgNPs synthesized by our group shows a manifold increase in antiplasmodial activity. This is probably because of the fact that AgNPs can penetrate more against *Plasmodium falciparum* owing to their small size and spherical shape. Further, a lot of clinical trials[1,27] are being carried out on antimalarial activity of AgNPs and our results would be helpful to understand the mechanism of their action.

In the present study we have found that plant extracts of neem and tulsi, when taken in different proportions in combination, give rise to novel AgNPs that are potential antiplasmodial agents. This knowledge of antiplasmodial activity of AgNPs based on the reported EC_{50} values would be helpful in understanding their antimalarial properties as well.

Conflict of interest statement

We declare that we do not have any conflict.

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