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Synthesis of silver nanoparticles using leaves of *Catharanthus roseus* Linn. G. Don and their antiplasmodial activities

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ABSTRACT

Objective: To develop a novel approach for the green synthesis of silver nanoparticles using aqueous leaves extracts of *Catharanthus roseus* (*C. roseus*) Linn. G. Don which has been proven active against malaria parasite *Plasmodium falciparum* (*P. falciparum*). **Methods:** Characterizations were determined by using ultraviolet-visible (UV-Vis) spectrophotometry, scanning electron microscopy (SEM), energy dispersive X-ray and X-ray diffraction. **Results:** SEM showed the formation of silver nanoparticles with an average size of 35-55 nm. X-ray diffraction analysis showed that the particles were crystalline in nature with face centred cubic structure of the bulk silver with the broad peaks at 32.4, 46.4 and 28.0. **Conclusions:** It can be concluded that the leaves of *C. roseus* can be good source for synthesis of silver nanoparticle which shows antiplasmodial activity against *P. falciparum*. The important outcome of the study will be the development of value added products from medicinal plants *C. roseus* for biomedical and nanotechnology based industries.

1. Introduction

The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology[1,2]. The research on synthesized nanomaterials and their characterization is an emerging field of nanotechnology from the past two decades, due to their huge applications in the fields of physics, chemistry, biology and medicine[3]. Many techniques of synthesizing silver nanoparticles, such as chemical reduction of silver ions in aqueous solutions with or without stabilizing agents[4], thermal decomposition in organic solvents[5], chemical reduction and photoreduction in reverse micelles[6,7], and radiation chemical reduction[8-10] have been reported in the literature. Most of these methods are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks. Synthesis of silver nanoparticles has attracted considerable attention owing to their diverse properties like catalysis[11], magnetic and optical polarizability[11], electrical conductivity[12], antimicrobial activity[13] and surface enhanced Raman scattering (SERS)[14].

Biological methods of nanoparticle synthesis using microorganisms[15-17], enzymes[18], fungus[19], and plants or plant extracts[20-24] have been suggested as possible ecofriendly alternatives to chemical and physical methods. Sometimes the synthesis of nanoparticles using plants or parts of plants can prove advantageous over other biological processes by eliminating the elaborate processes of maintaining microbial cultures[20].

Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process[25,26]. The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds[27]. Further these biologically synthesized nanoparticles were found highly toxic against different multi-drug resistant human pathogens.

Green nanoparticle synthesis has been achieved using environmentally acceptable plant extract and ecofriendly reducing and capping agents. Plants and microbes are currently used for nanoparticle synthesis. The use of plants for synthesis of nanoparticles is rapid, low cost, eco-friendly, and a single-step method for biosynthesis

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process[28]. Among the various known synthesis methods, plant-mediated nanoparticles synthesis is preferred as it is cost-effective, environmentally friendly, and safe for human therapeutic use[29]. Many reports are available on the biogenesis of silver nanoparticles using several plant extracts, particularly neem leaf broth (*Azadirachta indica*), *Pelargonium graveolens*, geranium leaves, *Medicago sativa* (Alfalfa), *Aloe vera*, *Emblica officinalis* (Amla, Indian Gooseberry) and few microorganisms. Similarly different plant constituents such as geraniol possess reducing property and reduce Ag^+ to silver nanoparticles with a uniform size and shape in the range of 1 to 10 nm with an average size of 6 nm[30].

Catharanthus roseus (*C. roseus*) (L.) G. Don. (Apocynaceae) is one of the important medicinal plants, due to the presence of the indispensable anti-cancer drugs, *i.e.*, vincristine and vinblastine. Roots of this plant are the main source of the anti-hypertension alkaloid ajmalicine[31]. It is also a popular ornamental plant. There are commonly two varieties of this plant based on the flower colour *viz.*, pink flowered rosea and white flowered alba[32]. It is an erect bushy perennial herb and evergreen shrub containing latex. It is widely growing to 1 m tall at subtropical area. It contains more than 70 alkaloids mostly of the indole type. It has medicinal importance owing to the presence of alkaloids like ajamalicine, serpentine and reserpine, which are well known for their hypotensive and antispasmodic properties. *C. roseus* exhibited high *in vitro* antiplasmodial activity, which may be due to the presence of compounds such as alkaloids, terpenoids[33], flavonoids[34] and esquiterpenes[35] that were previously isolated from the plant. It also possesses known antibacterial, antifungal, antibiotic, antioxidant, wound healing and antiviral activities[36-38]. Hence the aim of the present study is to develop a novel approach for the green synthesis of silver nanoparticles using herbal plant *C. roseus* which has been proven active against the malaria parasite *Plasmodium falciparum* (*P. falciparum*).

2. Materials and methods

2.1. Materials

Fresh leaves of *C. roseus* Linn were identified and collected from Tamilnadu Agricultural University, Coimbatore, and Tamilnadu, India and the taxonomic identification was made by Botanical Survey of India, Coimbatore. The voucher specimen was numbered and kept in our research laboratory for further reference. Silver nitrate was obtained from the precision scientific co, Coimbatore, India.

2.2. Synthesis of silver nanoparticles

The fresh leaf of *C. roseus* broth solution was prepared by taking 10 g of thoroughly washed and finely cut leaves in a 300 mL Erlenmeyer flask along with 100 mL of sterilized double distilled water and then boiling the mixture for 5 min before finally decanting it. The extract was filtered through Whatman filter paper no 1 and stored at -15 ℃ and could be used within 1 week. The filtrate was treated with aqueous 1 mM $AgNO_3$ solution in an Erlenmeyer flask and incubated at room temperature. As a result, a brown-yellow solution was formed, indicating the formation of silver nanoparticles. It showed that aqueous silver ions could be reduced by aqueous extract of plant parts to generate extremely stable silver nanoparticles in water (Figure 1).

a b

Figure 1. Photographs showing color changes after adding $AgNO_3$ before reaction (a) and after reaction time of 6 h (b).

2.3. Characterization of the synthesized silver nanoparticles

Synthesis of silver nanoparticles solution with leaves extract may be easily observed by ultraviolet-visible (UV-Vis) spectroscopy. The bio-reduction of the Ag^+ ions in solutions was monitored by periodic sampling of aliquots (1 mL) of the aqueous component and measuring the UV-Vis spectra of the solution. UV-Vis spectra of these aliquots were monitored as a function of time of reaction on a Vasco 1301 spectrophotometer in 400-600 nm range operated at a resolution of 1 nm.

2.4. Scanning electron microscope (SEM) and energy dispersive X-ray (EDX)

2.4.1. Spectrometer (EDX) analysis

SEM is a type of electron microscope that images a sample by scanning it with a high-energy beam of electrons in a raster scan patterns. In this experiment after the synthesis of nanoparticles using the plants and then lyophilisation was done using VIRTIS BENCHTOP machine. SEM and EDX analysis was done using JEOL-MODEL 6390 machine.

Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

2.4.2. X-ray diffraction (XRD) analysis

The particle size and nature of the silver nanoparticle were determined using XRD. This was carried out using Shimadzu XRD-6000/6100 model with 30 kv, 30 mA with Cu k α radians at 2 θ angle. X-ray powder diffraction is a rapid analytical technique primarily used for phase identification

of a crystalline material and can provide information on unit cell dimensions. The analyzed material is finely ground, and average bulk composition is determined. The particle or grain size of the particles on the silver nanoparticles was determined using Debye Sherrer's equation.

$D = 0.94 \lambda / B \cos \theta$

2.5. Parasite sample collection

Malaria positive blood samples were collected from K.M.C.H Hospital, Coimbatore, Tamilnadu-641046, India. The samples were collected in EDTA tubes and stored at 4 ℃.

2.6. Staining and visualizing of parasites

The simple, direct microscopic observation of blood specimens to observe the malaria parasite is still the gold standard for malaria diagnosis. Microscopic diagnosis of malaria was performed by staining thick and thin blood films on a glass slide to visualize the malaria parasite. Parasites were stained using Leishman stain (0.15%). On light microscopic examination of the blood film the number, species, and morphological stage of the parasites can be reported.

In addition to providing a diagnosis of malaria the blood smear can also provide useful prognostic information; the parasite count, number of circulating pigment-containing phagocytes and the presence of late asexual stages of the parasite are all positively correlated with a fatal outcome.

2.7. Culture of parasites

Parasites are grown in human erythrocytes in a settled layer of cells in RP-C: RPMI -Complete. Incomplete medium (RP-I) was prepared by dissolving 16.2 g of powdered RPMI 1640 supplemented with L-glutamine and 25 mM HEPES buffer but without sodium bicarbonate. Complete medium (RP-C) was obtained by adding 4.2 mL of 5% sodium bicarbonate solution and 5 mL of 8% Albumax stock solution per 100 mL of RP-I. Parasites from cultures were added to the freshly washed erythrocytes to give a starting parasitemia between 0.1%-1.0%. The cultures were provided appropriate atmosphere using the candle-jar method with 1% O_2, 5% CO_2, and 94% N_2 with 24 h medium changes, requiring subculture by addition of fresh erythrocytes every 4-5 days[39].

2.8. In vitro antiplasmodial assay

The antiplasmodial activity of the extract and test compounds was performed in 96-well tissue culture plates as described by Rieckman[40] with modifications reported by Carvalho *et al*[41]. Two fold serial dilutions of test samples dissolved in sterile methanol were placed in microtiter plates and diluted with culture medium (RPMI 1640 plus 10% human serum). A suspension of parasitized erythrocytes (0.5%-1% parasitaemia, 2.5% haematocrit) containing mainly trophozoites was added to the wells to give a final volume of 100 mL. Chloroquine was used as positive control and uninfected and infected erythrocytes were included as negative controls. The plates were incubated at 37 ℃ and after 24 and 48 h the culture medium was replaced with fresh medium with or without test samples.

After incubation for 24 h, Giemsa-stained thick blood films were prepared for each well, and the percentage of inhibition of parasite growth was determined under microscope by comparison of the number of schizonts with three or more nuclei out of a total of 200 parasites with that of control wells.

The percent inhibition at each concentration was determined and the mean of the least three IC_{50} values of parasite viability was calculated using mathematical log-concentration-response probit analysis.

3. Results

3.1. UV-Vis spectra analysis

The UV-Vis spectrum of silver nanoparticles (Figure 2) was recorded from the reaction medium as a function of a reaction time (10, 15, 30, 60 min) using 10% *C. roseus* leaf broth with 1 mM $AgNO_3$. The samples showed similar behavior with maximum absorption peak ranging between 390-410 nm. The maximum absorption peaks for *C. roseus* and silver nanoparticles were 410 and 400 nm, respectively.

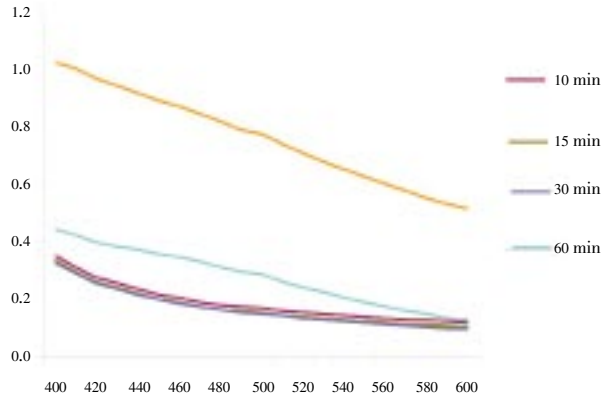

Figure 2. UV-Vis absorption spectra of aqueous silver nitrate with *C. roseus* leaf extract at different time intervals.

3.2. SEM and EDX studies

SEM technique was employed to visualize the size and shape of silver nanoparticles. In Figure 3, SEM images were obtained with 10% of *C. roseus* leaf broth. The SEM (JEOL-MODEL 6390) used SEM grids which were prepared by placing a small amount of sample powder on a copper coated grid and drying under lamp. The formation of silver nanoparticles as well as their morphological dimensions in the SEM study demonstrated that the average size was from 35-55 nm with inter-particle distance. The shapes of the silver nanoparticles proved to be spherical. EDX spectra recorded from the silver nanoparticles were

shown in Figure 4. From EDX spectra, it is clear that silver nanoparticles reduced by *C. roseus* have the weight percentage of silver as 20.16% and 16.41%.

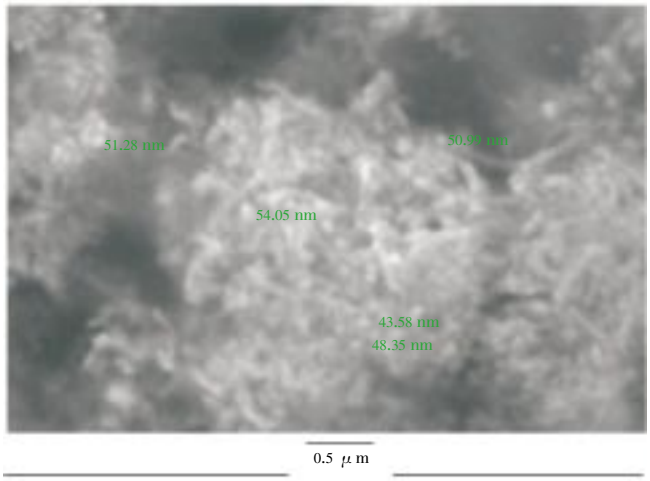

Figure 3. SEM image of silver nanoparticles formed by *C. roseus*.

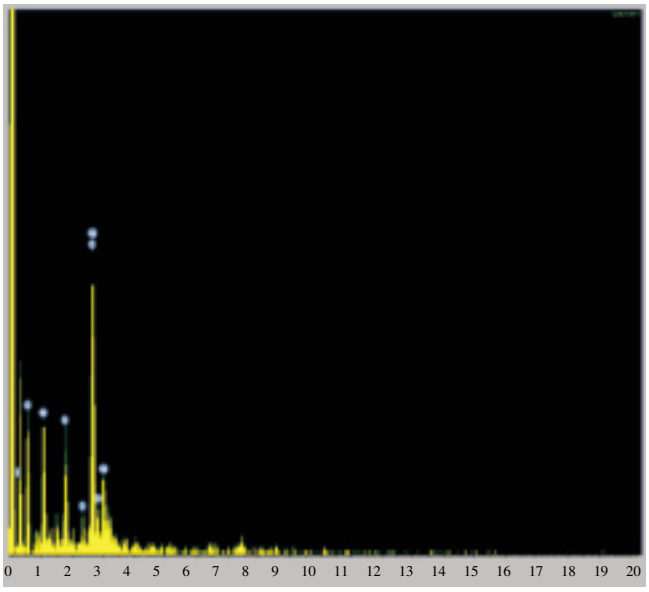

Figure 4. EDX spectra recorded from a film, after formation of silver nanoparticles with different X-ray emission peaks labeled.

3.3. XRD studies

Figure 5, 6 showed the XRD confirming the existence of silver colloids in the sample. The Braggs reflections were observed in the XRD pattern at 2 θ = 32.4, 46.4 and 28.0. These Braggs reflections clearly indicated the presence of (111), (200) and (311) sets of lattice planes and further on the basis that they can be indexed as face-centered-cubic (FCC) structure of silver. Hence XRD pattern thus clearly illustrated that the silver nanoparticles formed in this present synthesis are crystalline in nature.

In addition to the Bragg peaks representative of FCC silver nanoparticles, additional as yet unassigned peaks were also observed suggesting that the crystallization of bioorganic phase occurred on the surface of the nanoparticles.

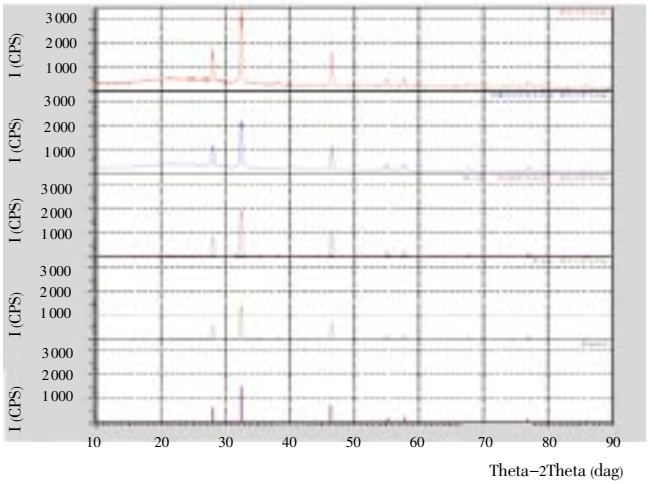

Figure 5. XRD pattern of silver nanoparticles formed after reaction of plant extracts *C. roseus*.

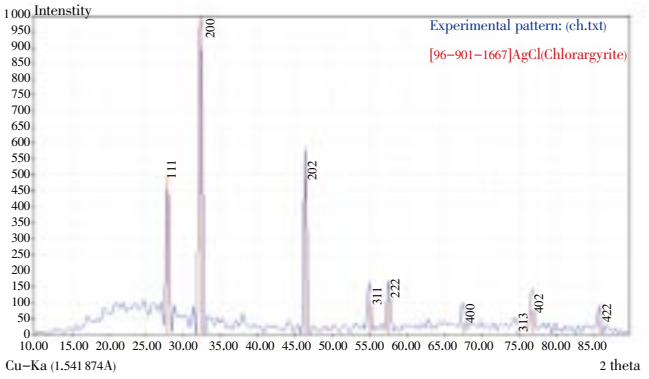

Figure 6. Phase matched XRD pattern of silver nanoparticles.

3.4. Antiplasmodial studies

Table 1 and Figure 7 showed that the activity of silver nanoparticles synthesized with *C. roseus* at different concentrations on the inhibition rate of the malarial parasites was observed after the treatment of plant extracts. Significant inhibition rates were observed after the treatment of plant extracts. Lowest parasitemia inhibition rate (20.0%) was observed in parasites at 25 μg/mL concentration of the silver nanoparticles from *C. roseus*. The parasitemia inhibitory concentration values for silver nanoparticles from *C. roseus* were 20.0%, 41.7%, 60.0%, and 75.0% for 25, 50, 75, and 100 μg/mL, respectively.

Table 1
In vitro antiplasmodial activity of silver nanoparticles synthesized using *C. roseus* against *P. falciparum*.

C. roseus + silver nanoparticles Concentration in μg/mL	Parasitemia inhibitory concentration (%)
25	20.0±0.7
50	41.7±0.3
75	60.0±0.6
100	75.0±1.1
Control	-

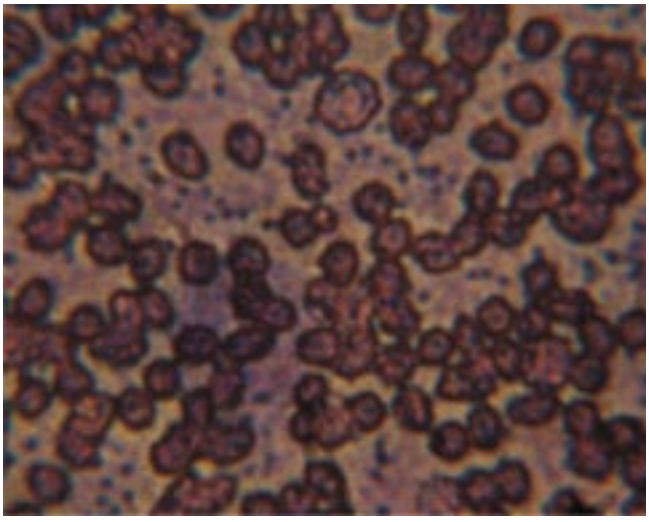

Figure 7. The antiplasmodial activity of silver nanoparticles against *P. falicifarum*.

4. Discussion

Reduction of silver ion into silver particles during exposure to the plant extracts could be followed by color change. Silver nanoparticles exhibit dark yellowish-brown color in aqueous solution due to the surface plasmon resonance phenomenon. The synthesis of nanoparticles is in lime light of modern nanotechnology. Biosynthesis of nanoparticles by plant extracts is currently under exploitation. The development of biologically inspired experimental processes for the synthesis of nanoparticle is evolved into an important branch of nanotechnology. The present study emphasizes the use of plants medicinal for the synthesis of silver nanoparticles with potent antiplasmodial effect.

The nanoparticles were primarily characterized by UV-Vis spectroscopy, which was proved to be a very useful technique for the analysis of nanoparticles. Reduction of Ag^+ ions in the aqueous solution of silver complex during the reaction with the ingredients present in the plant leaf extracts observed by the UV-Vis spectroscopy revealed that silver nanoparticles in the solution may be correlated with the UV-Vis spectra. As the leaf extracts were mixed with the aqueous solution of the silver ion complex, it was changed into dark yellowish-brown color due to excitation of surface plasmon vibrations, which indicated that the formation of silver nanoparticles[20]. UV-Vis spectrograph of the colloid of silver nanoparticles has been recorded as a function of time by using a quartz cuvette with silver nitrate as the reference.

In the UV-Vis spectrum, the broadening of peak indicated that the particles are poly dispersed. The reduction of silver ions and the formation of stable nanoparticles occurred rapidly within 2 h of reaction making it one of the fastest bioreducing methods to produce silver nanoparticles[42]. The surface plasmon band in the silver nanoparticles solution remains close to 380 nm throughout the reaction period indicating that the particles are dispersed in the aqueous solution, with no evidence for aggregation. It was observed that the nanoparticles solution was stable for more than six months with little signs of aggregation[43,44].

The silver nanoparticles formed were predominantly cubical with uniform shape. It is known that the shape of metal nanoparticles can considerably change their optical and electronic properties[45]. The SEM image showed relatively spherical shape nanoparticle formed with diameter in the range of 35-55 nm. Similar phenomenon was reported by Chandran *et al* and Udayasoorian *et al*[21,46]. Energy dispersive spectrometry (EDS) micro-analysis is performed by measuring the energy and intensity distribution of X-ray signals generated by a focused electron beam on a specimen. EDX spectra were recorded from the silver nanoparticles. From EDX spectra, it is clear that silver nanoparticles reduced by *C. roseus* have the weight percentage of silver as 20.16% and 16.41%.

XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles in plants tissues can be achieved by using XRD to examine the diffraction peaks of the plant. In our experiment the X-ray pattern of synthesized silver nanoparticles matches the FCC structure of the bulk silver with the broad peaks at 32.4, 46.4 and 28.0. These are corresponding to 111, 200, 311 planes, respectively[47]. In addition to the Bragg peak representative of FCC silver nanocrystals, additional and yet unassigned peaks were also observed suggesting that the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles[48]. The line broadening of the peaks is primarily due to small particle size.

The X-ray diffraction results clearly show that the silver nanoparticles formed by the reduction of Ag^+ ions by the *C. roseus* and *Cassia auriculata* leaf extract are crystalline in nature[28,46].

Parasitic diseases (like malaria, leishmaniasis, tryanosomiasis) are one of the major problems around the globe[49,50]. Antiparasitic chemotherapy is the only choice of treatment for these parasitic infections. The reason for this is that these infections do not elicit pronounced immune response hence effective vaccination may not be possible[51]. In spite of intensive efforts to control malaria, the disease continues to be one of the greatest health problems facing Africa. Although a number of advances have been made towards understanding the disease, relatively few antimalarial drugs have been developed in the last 30 years[52]. Meanwhile the traditional medicines have been used to treat malaria for thousand of years. The plant is commonly used in traditional medicine in Kenya to manage malaria[53,54]. Leaf extract of *Mentha piperita* (Lamiaceae) is very good bioreductant for the synthesis of silver and gold nanoparticles and synthesized nanoparticles are active against clinically isolated human pathogens, *Staphylococcus aureus* and *Escherichia coli*[55].

At least from ethnomedicinal use there is some evidence that the plant may be safe in humans. *Cassia occidentalis* leaves, *Cryptolepis sanguinolenta* (*C. sanguinolenta*) root bark, *Euphorbia hirta* (*E. hirta*) whole plant, *Garcinia kola* stem bark and seed, *Morinda lucida* leaves and *Phyllanthus niruri* whole plant produced more than 60% inhibition of parasite growth *in vitro* at a test concentration g/mL. Extracts from *E. hirta*, *C. sanguinolenta* and *Morinda morindoides* showed μ of 6 a significant chemosuppression of parasitaemia in mice infected with *Plasmodium berghei berghei* at orally given doses of 100-400 mg/kg per day[56]. The observed antiplasmodial activity may be related to the presence of terpenes, steroids, coumarins, flavonoids, phenolica acids, lignans, xanthones and anthraquonones[57]. Antibacterial activity of silver nanoparticles was exhibited by using stem derived callus extract of bitter apple *Citrullus*

colocynthis[58-61]. The antiparasitic activity was shown by the aqueous crude leaf extracts and synthesized silver nanoparticles of *Mimosa pudica*[62]. The antimalarial activities of the synthesized copper (II) nanohybrid solids were evaluated against *P. falciparum* isolate (MRC 2). These nanohybrid solids possessed distinct cell growth inhibitory activity. The IC_{50} values of $LCuCl_2$ and $LCu(CH_3COO)_2$ were found to be 0.025 and 0.032 lg/mL, respectively, almost the same potency as the standard drug chloroquine (IC_{50}, 0.020 lg/mL)[63].

The antimicrobial activity of synthesized silver nanoparticles was shown using *Euphorbia hirta* and *Nerium indicum* against six different bacteria such as *Escherichia coli*, *Streptococcus pyrogens*, *Staphylococcus auereus*, *Bacillus subtilis*, *Salmonella typhi* and *Citrobacter* sp[64]. Nanoparticles synthesized using *Trianthema decandra* are active against clinically isolated human pathogens *Escherichia coli* and *Pseudomonas aeruginosa*[65].

The bio-reduction of aqueous silver ions by the leaf extract of the *C. roseus* has been demonstrated. The reductions of the metal ions through leaf extract leading to the formation of synthesized nanoparticles are quite stable in solution. The control of shape and size of silver nanoparticles seems to be easy with the use of plant leaf extracts. In the present study we found that leaves can be good source for synthesis of silver nanoparticle. The synthetic methods based on naturally occurring biomaterials provide an alternative means for obtaining the nanoparticles. Use of plants in synthesis of nanoparticles is quite novel leading to truly 'green chemistry' route. This green chemistry approach towards the synthesis of nanoparticles has many advantages such as, process scaling up, economic viability and safe way to produce nanoparticles.

Conflict of interest statement

We declare that we have no conflict of interest.

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