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Antimicrobial activity of latex silver nanoparticles using *Calotropis procera*

Nadia Hussein Mohamed[1,2], Mady Ahmed Ismail[1], Wael Moustfa Abdel-Mageed[3,4], Ahmed Abdelfattah Mohamed Shoreit[1*]

[1]*Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt*

[2]*Biology Department, Faculty of Science and Art, Samtah, Jazan University, Saudi Arabia*

[3]*Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh 11451, P. O. Box 2457, Saudi Arabia*

[4]*Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt*

PEER REVIEW

Peer reviewer

Dr. Chiara Pelillo, PostDoc at Callerio Foundation Onlus, Trieste, Italy.
Tel: 00390405588635
E-mail: chiara.pelillo@libero.it

Comments

The research presented is very interesting and quite complete. The physico-chemical parameters of these latex silver NPs were well characterized. The antimicrobial activity was evaluated following the standard test used and against many different bacterial and fungi strains.
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ABSTRACT

Objective: To synthesize silver nanoparticles (AgNPs) by green methods using serum latex of *Calotropis procera* at 80 °C and evaluate them against bacteria, dermatophytes and phytopathogenic fungi comparing with the activity of untreated latex.

Methods: The synthesis of AgNPs was performed by mixing 3% latex serum extract with the same volume of silver nitrate (2 mmol/L) solution in round flask and heating in water bath at 80 °C. Characterization of silver particles were determined using UV-vis spectrophotometer, transmission electron microscopy (TEM), X-ray diffraction and Fourier transform infrared spectroscopy. The antimicrobial activity of the green synthesized AgNPs was determined against bacteria, dermatophytes and phytopathogenic fungi and compared to the crude untreated latex by agar-well diffusion methods.

Results: Biosynthesis of latex silver nanoparticles was successfully obtained by green method. The formation of AgNPs has been confirmed by UV-vis, TEM microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. TEM analysis showed that synthesized AgNPs are highly stable spherical shaped particles, well dispersed with a diameter ranged from 4 nm up to 25 nm and an average size of 12.33 nm. AgNPs showed strong antibacterial activity against Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia* sp.) and antifungal activity against *Trichophyton rubrum*, *Candida albicans* and *Aspergillus terreus*.

Conclusions: It can be concluded that serum latex of *Calotropis procera* was found to display strong potential for the synthesis of AgNPs as antimicrobial agents through rapid reduction of silver ions (Ag^{+} to Ag^{0}). The green synthesized AgNPs were found to show higher antimicrobial efficacy than crude latex.

KEYWORDS
Green synthesis, *Calotropis procera*, Latex, Silver nanoparticles, Antimicrobial activity

1. Introduction

Nanotechnology is an important tool in many fields, like health and medicine[1]. Nanotechnology is the technology of materials having particle size below hundred nanometers. The properties of materials below hundred nanometers usually differ from those in the bulk scales[1]. Silver nanoparticles (AgNPs) among all noble metals have been widely used in many pharmaceutical and biological applications because of its unique antimicrobial

*Corresponding author: Ahmed Abdelfattah Mohamed Shoreit, Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut, Egypt.
Tel: +201003406161
Fax: +2088 2342708
E-mail: ashoreit1968@ yahoo.com
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properties[1].

Biosynthetic green methods used for synthesis of nanoparticles usually involve using of medicinal plants and microorganisms such as fungi and algae to synthesis of nanoparticles for pharmaceutical and biological applications. It is eco-friendly, cost effective as compared to the other chemical and physical methods. It was also interesting to note that silver nanoparticles were able to exert inhibitory effect at a concentration that is below their cytotoxic limits. So they were regarded as safe to be used as antimicrobials[2-6]. The AgNPs prepared using green methods have high surface area, a smaller size and high dispersion and show a strong bactericidal and antibiotic activity. The AgNPs have several important applications in the field of antimicrobial agents, capable of purifying drinking water, degrading pesticides and killing human pathogenic bacteria[7-10]. Many recent reports were published on biosynthesis of AgNPs using plant latex[11-13], natural rubber latex[14,15], plant extract or by the whole plant showing promising biological activities[6,16], such as cytotoxic and antimicrobial activities.

Calotropis procera (family: Asclepiadaceae) (*C. procera*) is a cultivable wild xerophytic shrub found across Africa, Asia and South America[17]. It produces milky white latex that exhibits diverse curative properties[18-20]. Latex is found in special branching tubes called latex tubes[21,22], and has been the subject of interest due to its biological activities such as antibacterial[23], antifungal[24], antiviral[25], anticandidal[26] and anticarcinogenic activities[27,28]. More than 80% of the dry mass of the crude latex corresponds to rubber and the rest 20% covers soluble fractions rich in protein including antioxidant enzymes, cysteine protease with free thiol group and tryptophan[29,30].

In the present study, green nontoxic eco-friendly rapid method for synthesis of AgNPs using serum latex of *C. procera* plant at 80 °C was characterized and evaluated against bacteria, dermatophytes and phytopathogenic fungi and compared to the activity of untreated latex.

2. Materials and methods

2.1. Sampling of crude latex

Latex from *C. procera* grown in Assiut region, Egypt was drawn by sterile disposable syringe under sterile conditions into sterile Eppendorf tubes in July 2012.

2.2. Fractionation of plant latex

Fresh latex was centrifuged at 17 000 r/min for 20 min at 4 °C in SR4000 Prolabo centrifuge (made in France). It was separated into three layers: rubber, serum and lipids.

2.3. Synthesis of latex silver nanoparticles (LAg-NPs)

Separately, 25 mL of 3% latex serum extract diluted with deionized water was mixed with the same volume of silver nitrate (2 mmol/L) solution in round flask, heated in water bath at 60 °C with constant stirring for 15 min. The temperature was raised to 80 °C and the mixture was further incubated in water bath for 30 to 45 min until LAg-NPs was formed and the brownish yellow color of solution became stable.

2.4. Characterization of LAg-NPs

2.4.1. UV-vis spectral analysis

The reduction of pure Ag^+ ions by latex was monitored by periodic sampling of the reaction medium and measuring the UV-vis spectra of the solution at frequent time intervals using UV-vis spectrophotometer (Shimadzu model UV-1601) in range of 200 nm to 800 nm.

2.4.2. Transmission electron microscopy (TEM) analysis

The morphology of the AgNPs were investigated by TEM using JEOL-JEM-100 CXII instrument by drying a drop of the washed colloidal dispersion onto a copper grid covered with a conductive polymer.

2.4.3. Laser diffraction particle size analyzer

Particle size of AgNPs was analyzed on particle size analyzer system [Horiba LA-300 Light Scattering Particle Size Distribution Analyzer (Horiba Ltd, Kyoto, Japan)]. The average distribution of nanoparticles on the basis of intensity, volume and number weighting was studied comparatively.

2.4.4. X-ray diffraction (XRD) analysis

XRD was performed using X-ray diffractometer (Model PW 1710 control unit Philips Anode material Cu, 40 KV, 30 M.A, optics: Automatic divergence slit) with Cu Kα radiation λ=1.540 5 °A over a wide range of Bragg angles ($30° \leqslant 2\theta \leqslant 80°$). An elemental analysis of the sample was examined by energy dispersive analyses of X-rays with JED-2300 instrument.

2.4.5. Fourier transform infrared spectroscopy (FTIR) spectrophotometer

FTIR spectra of vacuum dried LAg-NPs were recorded as KBr pellet on Thermo Scientific Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, USA) Perkin Elmer RX1 model in the range of 4 000-400 cm^{-1}.

2.5. Antimicrobial activity of LAg-NPs

2.5.1. Test organisms

Fourteen species were used in this study as test organisms comprising five clinical isolates of bacteria [*Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Serratia* sp., *Bacillus subtilis* and *Staphylococcus albus*]; dermatophytic and opportunistic fungi [(*Trichophyton rubrum* AUMC 1804 (*T. rubrum*), *Microsporum canis* AUMC 2805, *Chrysosporium tropicum* AUMC 1807, *Candida albicans* AUMC 3880 (*C. albicans*) and *Aspergillus terreus* (*A. terreus*)] and phytopathogenic fungi (*Fusarium solani* AUMC 222, *Fusarium oxysporum* AUMC 3447, *Colletotrichum gloesporioides* AUMC 2779 and *Macrophomina phaseolina* AUMC 236). Bacterial and fungal strains were kindly obtained from Assiut University Mycological Centre (AUMC). All strains were sub-cultured on nutrient agar, nutrient agar (for bacteria) and Saboraud dextrose agar, Saboraud dextrose agar (for fungi) slants and stored at 4 °C until used.

2.5.2. Agar well diffusion method

The agar well diffusion method was used[12]. The agar plates nutrient agar for bacteria and Saboraud dextrose agar for fungi seeded with the test organisms were punched with a sterile cork borer (0.5 cm diameter) to make open wells. LAg-NPs were added into the open wells at different concentrations (5, 10, 20 μL for bacteria; 10, 25, 50 μL for fungi). The plates were incubated at 37 °C for 24 h for the bacteria, and at 25 °C for 6 d for fungi. The zones of inhibition were measured in mm and recorded. The lowest concentration of LAg-NPs that inhibited the growth of the test organisms was recorded as the minimum inhibitory concentration (MIC)[31].

2.5.3. Determining the growth curves of E. coli, P. aeruginosa and Serratia sp.

To study growth of bacteria in nutrient broth media, inoculations were given from fresh colonies on agar medium into 10 mL broth. These cultures were supplemented with crude latex (300 μL), and latex nanoparticles (LAg-NPs) (10 μL), control culture was treated in a similar fashion but without any treatments, Then the bacterial cultures were incubated at 37 °C temperature with rapid shaking at 150 r/min. The bacterial growth was determined by measuring the optical density at λ=600 nm at regular intervals using UV-vis spectrophotometer each hour. The growth curve was plotted between optical density and time.

3. Results

In the present work, in the synthesis of LAg-NPs with latex serum, it was found that 3% latex serum prepared from *C. procera* latex and 2 mmol/L aqueous silver nitrate solutions were optimum for obtaining monodisperse AgNPs. It has been observed that addition of latex serum to silver solution changes the white color of solution to yellowish brown after 30 min and turned dark brown after one hour and become stable at this color after 6 h (Figure 1).

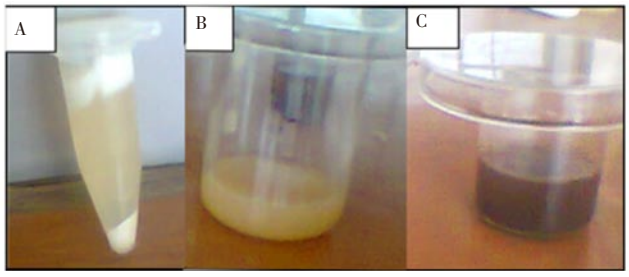

Figure 1. Silver nanoparticles synthesis using *C. procera* latex serum.
A: fractionated latex; B: LAg-NPs after 30 min; C: LAg-NPs after 6 h.

3.1. Characterization of LAg-NPs

3.1.1. UV-vis spectral analysis

UV-vis spectroscopic studies of the colored solution confirmed the synthesis of LAg-NPs as distinct surface plasmon resonance bands with a peak centered at around 290 nm were obtained (Figure 2). This color indicated reduction of silver ions, while no absorbance peak was observed in control (untreated latex serum).

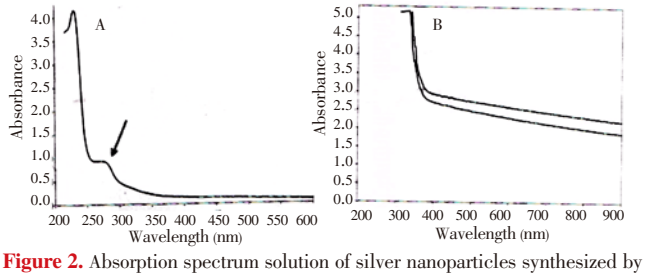

Figure 2. Absorption spectrum solution of silver nanoparticles synthesized by using *C. procera* latex serum and latex serum only after 1 h.
A: Using *C. procera* latex serum; B: Latex serum only (control).

3.1.2. TEM analysis

TEM image of LAg-NPs (Figure 3) showed that the particles were spherical in shape, well dispersed with a diameter range from 4 nm up to 25 nm and an average particle size of 12.33 nm.

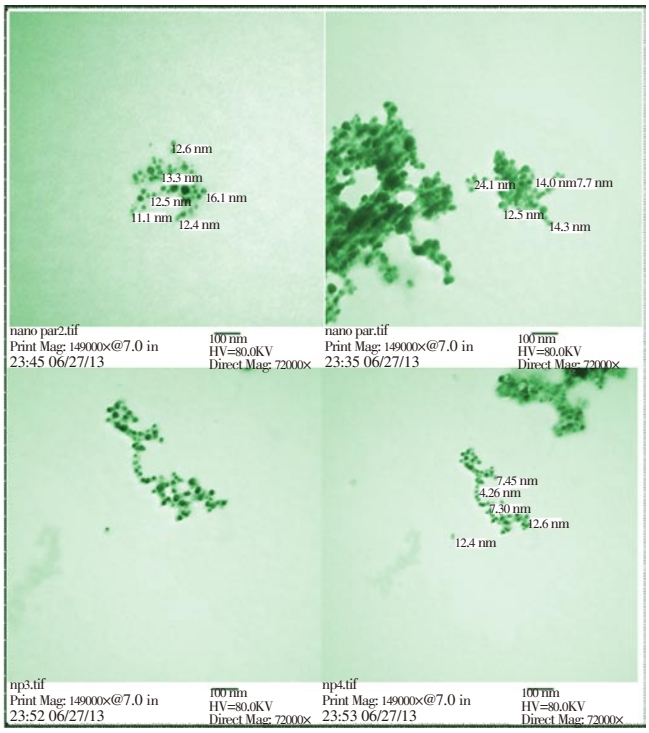

Figure 3. TEM of AgNPs synthesized using 3% *C. procera* latex serum after 2 h.

3.1.3. Laser diffraction particle size analyzer

Particle size analysis gives evidence of size and size distribution profile of AgNPs shown in Figure 4. It revealed that 80% of distributions of particles have small size with an average particle size 12.33 nm.

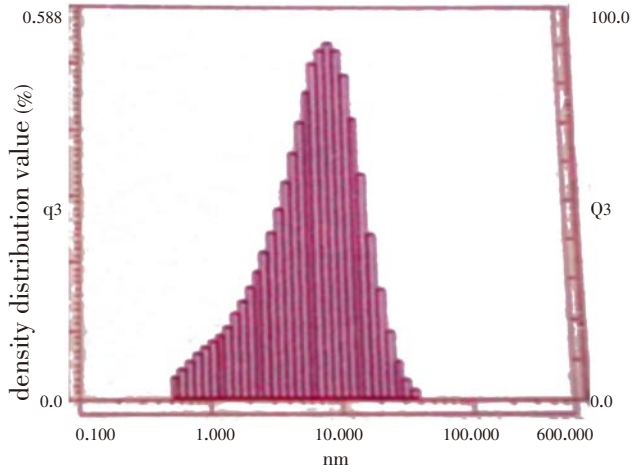

Figure 4. The particle size distribution histogram of LAg-NPs after 2 h.

3.1.4. XRD analysis

Further studies were carried out using XRD to confirm the crystalline nature of the particles. Figure 5 shows X-ray powder diffraction patterns of the synthesized AgNPs using latex serum extract of *C. procera* at 80 °C. The peak positions are consistent with metallic silver. This method is based on projecting a monochromatic X-ray beam onto the material at an angle theta. The angular positions and intensities of the resultant diffracted peaks as a result of varying the angle (θ) of the projected monochromatic beam (θ) result on a pattern characteristic of the samples. The peaks assigned in the AgNP sample were 111, 200, 202, 222 and 311. A number of Bragg reflections with 2θ values of 27.35°, 31.77°, 45.67°, 38.08°, 46.15°, 54.40°, 57.04°, 67.06°, 73.96° and 76.42°, are shown in Figure 5.

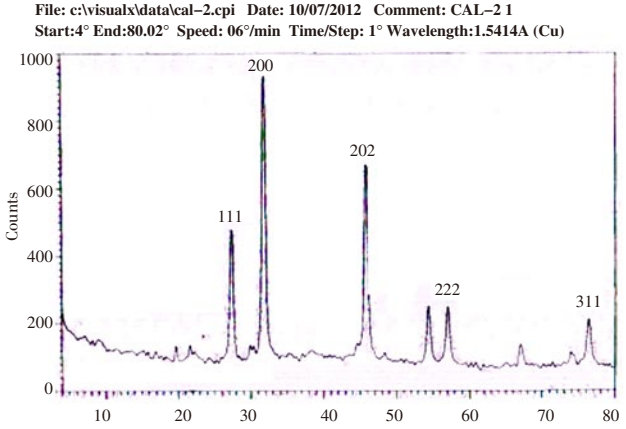

Figure 5. XRD pattern of synthesized LAg-NPs.

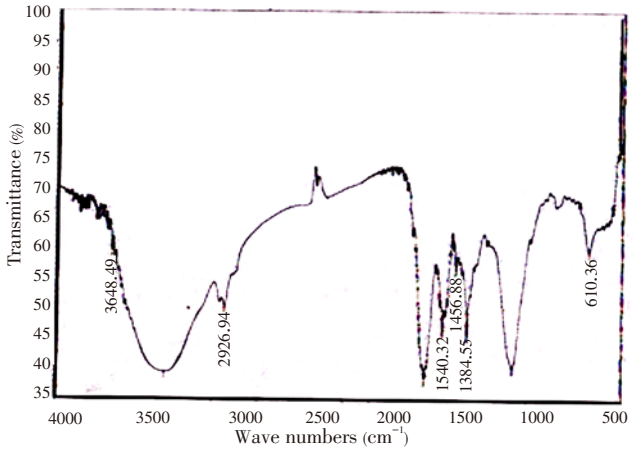

Figure 6. FTIR spectra of synthesized LAg-NPs.

3.1.5. FTIR spectrophotometer

FTIR is used to predict the role of bonding (stabilizing) and reducing capability of *C. procera* latex serum extract. The nature of the biomolecules involved in the reduction and formation of silver nanoparticle was studied by FTIR (Figure 6). The FTIR signals of LAg-NPs were observed at 3648, 3400, 2926,

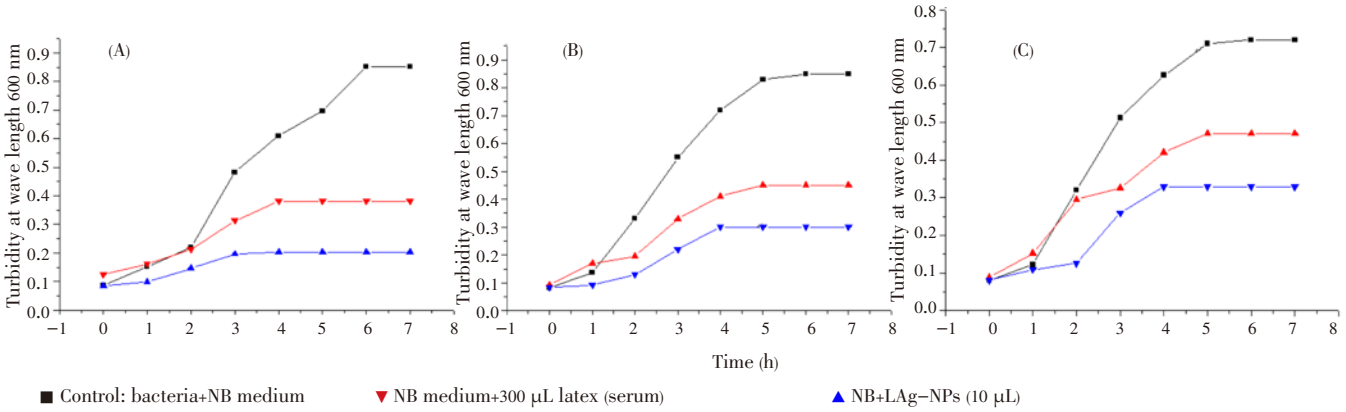

Figure 7. Growth curves of *E. coli*, *P. aeruginosa* and *Serratia* sp.
NB: Nutrient broth. A: *E. coli*; B: *P. aeruginosa*; C: *Serratia* sp.

1606, 1540, 1456, 1384, 1220 and 1091 cm^{-1}. The prominent bands at 1540 and 1606 cm^{-1} are attributed to N-H stretching possibly due to the presence of amide group, which is responsible for the reduction of $AgNO_3$ to Ag[32], The band at 1384 cm^{-1} is due to C-H bending vibration, the band at 2926 cm^{-1} may be due to the stretching of C-H group. Band at 1091 cm^{-1} is attributed to C-N stretching possibly due to the presence of amines group, band at 1220 cm^{-1} is attributed to C-O stretching possibly due to the presence of carboxylic acid group and also showed strong band for amines (N-H bond at 3400 cm^{-1}), alcohol (3648 cm^{-1}) and C-H bend alkynes at band 610 cm^{-1}.

3.2. Antimicrobial activity

The LAg-NPs exhibited good antibacterial activity against Gram-negative bacteria such as *E. coli*, *Serratia* sp. and *P. aeruginosa*. It also showed antifungal activity against dermatophytes and phytopathogenic fungi. Comparing with crude latex, LAg-NPs exhibited higher effect than the use of crude untreated latex (Table 1).

Table 1
Diameter of inhibition zone of crude latex and LAg-NPs on tested microorganisms and bacteria fungi (mm).

Tested microorganisms		Crude latex		LAg-NPs				
		300 μL	200 μL	50 μL	25 μL	20 μL	10 μL	5 μL
Bacteria	*E. coli*	15.80±0.70	0.00	NT	NT	16.80±0.70	13.50±0.10	11.00±0.20
	P. aeruginosa	11.00±0.80	0.00	NT	NT	11.50±0.70	10.50±0.17	0.00
	Serratia sp.	9.00±0.10	0.00	NT	NT	13.80±0.90	10.80±0.16	0.00
Fungi	*T. rubrum*	12.10±0.10	0.00	24.00±0.80	12.60±0.39	NT	0.00	0.00
	C. albicans	21.80±0.15	14.00±0.12	26.00±0.80	21.00±0.06	NT	14.00±0.80	0.00
	A. terreus	11.10±0.70	0.00	23.00±0.80	14.00±0.36	NT	0.00	0.00

Values are expressed as mean±SD. NT: Not tested.

3.2.1. Minimum inhibitory concentration of LAg-NPs for bacteria and fungi

The MIC of LAg-NPs for *E. coli* was 5 μL while for *Serratia* sp. and *P. aeruginosa* were 10 μL which were superior to that of crude latex (300 μL) (Table 1). For fungi, the MIC of LAg-NPs against *C. albicans* was 10 μL, but *T. rubrum* and *A. terreus* were 25 μL while in case crud latex MIC for *C. albicans*, *A. terreus* and *T. rubrum* were 300 μL (Table 1).

From these results it could conclude that the LAg-NPs were more effective at low concentrations than the crude latex alone. However, crude latex and latex nanoparticles exhibited no antibacterial and antifungal activity against some other bacterial and fungal strains tested, namely, *Bacillus subtilis*, *Staphylococcus albus*, *Chrysosporium tropicum*, *Microsporum canis*, *Fusarium solani*, *Fusarium oxysporum*, *Colletotrichum gloesporioides* and *Macrophomina phaseolina*.

3.2.2. Growth curves of bacterial cells treated with different treatment

In fresh liquid nutrient broth control medium, the growth curve was detected by increasing of turbidity at wave length 600 nm. It was clear that growth curve of each isolate tested decreased in the presence of either latex or LAg-NPs compared to control medium (Figure 7).

Bacterial growths of cells treated with crud latex were inhibited after 6 h, but bacterial cells treated with LAg-NPs were inhibited after 3 h as shown in Figure 7.

4. Discussion

The plant *C. procera* seems to be a potential source of hydrocarbons. The latex serum part contains various organic compounds such as alkaloids, cardiac glycosides, tannins, flavonoids, sterols and triterpenes[33]. These compounds may play role in the reduction of $AgNO_3$ to Ag.

Interaction between silver nitrate and latex serum lead to formation LAg-NPs. The produced brown color indicated the surface plasmon vibrations which is typical of AgNPs[12,13,34,35]. The control $AgNO_3$ solution (without latex serum extract) showed no color change.

UV-vis spectroscopic study of the colored colloidal solution confirmed the synthesis of LAg-NPs as distinct surface plasmon resonance band with a peak centered at around 290 nm was obtained. The UV-vis spectra for the latex serum of *C. procera* alone (control) showed no absorption peak. TEM image of LAg-NPs showed that the particles are spherical in shape, well dispersed with a diameter range from 4 nm up to 25 nm. Laser diffraction particle size revealed that the average particle size of particles is 12.33 nm[36,37].

XRD was used to confirm the crystalline nature of the particles. A number of Bragg reflections at 2θ values of 27.35°, 31.77°, 45.67°, 38.08°, 46.15°, are shown corresponding to 111, 200, 202, 311 and 222 plans respectively. Similar results were reported by Babu and Prabu and Sivakumar *et al*[38,39]. The XRD results show that the LAg-NPs formed by the reduction of Ag^{+} ions by latex of *C. procera* are crystalline in nature[40].

FTIR spectra showed various functional groups at different positions. The prominent bands at 1 540 cm^{-1} and 1 606 cm^{-1} are attributed to N-H stretching possibly due to the presence of amide group characteristic of proteins/enzymes that have been found to be responsible for the reduction of $AgNO_3$ to Ag[32]. This result showed similarity with those reported by Babu and Prabu, and Sivakumar *et al*[38,39].

AgNPs become an important application in the field of microbiology such as antibacterial and antifungal activities. *C. procera* is used in traditional medicine and biological activities as antibacterial[23], antifungal[24] and anticandidal activity[26]. Latex of this plant has successfully synthesized the LAg-NPs which exhibited good antibacterial activity against Gram-negative bacteria such as *E. coli*, *Serratia* sp. and *P. aeruginosa* as well as antifungal activity against dermatophytes and phytopathogenic fungi. This result showed similarity with Rai *et al*[41]. Comparing with crude latex, LAg-NPs exhibited higher antimicrobial activity than the use of crude untreated latex alone. The exact mechanism for strong antimicrobial activity of the AgNPs is still in debate. But several hypotheses mention that Ag-NPs may attach to the surface of the cell membrane leading to disturbance of permeability and respiration functions of the cell. The strong antimicrobial activity depends on the large surface area of the nanoparticles which give more surface area for interaction with the organisms than available with those for large particles. Moreover, it is possible that LAg-NPs not only interact with the surface of membrane, but also can penetrate inside the bacteria[42]. Other reported mechanisms are: uptake of free silver ions followed by disruption of adenosine triphosphate production and DNA replication, formation of reactive oxygen species and direct damage to cell membranes[42].

In the present study, biosynthesis of LAg-NPs was successfully obtained by green method of preparation which involves treatment of silver nitrate with latex serum extract of *C. procera* solutions. This method displayed that the plant latex of *C. procera* can be used as an effective stabilizing reducing agent for the synthesis of AgNPs. The methodology employed here is very simple, easy to perform, inexpensive, eco-friendly and is better alternative to chemical synthesis. The formed AgNPs are highly stable spherical shaped particles, showing strong antibacterial activity and antifungal activity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors thanks Assiut University for funding and technical support.

Comments

Background

The impact of nanotechnology in many fields of the research, like health and medicine, has been considerable. AgNPs, among all noble metals have been widely used in many pharmaceutical and biological applications because of its unique antimicrobial properties. Recently, a variety of biosynthetic green methods has been used to synthetize nanoparticles for pharmaceutical and biological applications. They are eco-friendly, cost effective, and avoid the use of any toxic agent.

Research frontiers

The current research proposes a green nontoxic eco-friendly rapid method to synthetize AgNPs using serum

latex of *C. procera* plant, a plant grown in Assiut region. The latex serum, rich in organic compounds (alkaloids, cardiac glycosides, tannins, flavonoids, sterols and triterpenes) interacts with silver nitrate and lead to the formation of LAg-NPs. These NPs are active against bacteria tested, dermatophytes and phytopathogenic fungi.

Related reports

Crude latex and latex nanoparticles exhibited no antibacterial and antifungal activity against some other bacterial and fungal strains tested while LAg-NPs showed an antimicrobic activity at low concentrations.

Innovations and breakthroughs

The innovation is due to the use of the latex serum collected from the *C. procera* plant that lead to the formation of AgNPs after having interacted with silver nitrate. This method is completely green and allows to obtain active AgNPs.

Applications

This research suggests this new biosynthetic green method can be used as an effective stabilizing reducing agent for the synthesis of AgNPs with highly stable spherical shaped particles and an average particle size of 12.33 nm. This methodology is suggested as an alternative to chemical synthesis to obtain AgNPs active against bacteria and fungi.

Peer review

The research presented is very interesting and quite complete. The physico-chemical parameters of these LAg-NPs were well characterized. The antimicrobial activity was evaluated following the standard test used and against many different bacterial and fungi strains.

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