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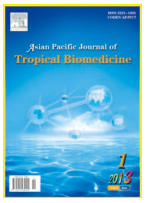

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Green synthesis, antimicrobial and cytotoxic effects of silver nanoparticles using *Eucalyptus chapmaniana* leaves extract

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PEER REVIEW

Peer reviewer

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Comments

The article is generally well written and present interesting results. Silver nano-particles were obtained with a biosynthetic procedure from *E. chapmaniana* leaves. The nano-particles are reported to inhibit the growth of various pathogenic bacteria and HL-60 promyelocytic leukemia cells.

(Details on Page 62)

ABSTRACT

Objective: To synthesize silver nanopaticles from leaves extract of *Eucalyptus chapmaniana* (*E. chapmaniana*) and test the antimicrobial of the nanoparticles against different pathogenic bacteria, yeast and its toxicity against human acute promyelocytic leukemia (HL-60) cell line. **Methods:** Ten milliliter of leaves extract was mixed with 90 mL of 0.01 mmol/mL or 0.02 mmol/mL aqueous $AgNO_3$ and exposed to sun light for 1 h. A change from yellowish to reddish brown color was observed. Characterization using UV-vis spectrophotometery and X-ray diffraction analysis were performed. Antimicrobial activity against six microorganisms was tested using well diffusion method and cytoxicity test using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole was obtained on the human leukemia cell line (HL-60). **Results:** UV-vis spectral analysis showed silver surface plasmon resonance band at 413 nm. X-ray diffraction showed that the particles were crystalline in nature with face centered cubic structure of the bulk silver with broad beaks at 38.50° and 44.76°. The synthesized silver nanoparticles efficiently inhibited various pathogenic organisms and reduced viability of the HL-60 cells in a dose-dependent manner. **Conclusions:** It has been demonstrated that the extract of *E. chapmaniana* leaves are capable of producing silver nanoparticles extracellularly and the Ag nanoparticles are quite stable in solution. Further studies are needed to fully characterize the toxicity and the mechanisms involved with the antimicrobial and anticancer activity of these particles.

KEYWORDS

Eucalyptus chapmaniana, Silver nanoparticles, Antimicrobial activity, Cytotoxic effect

1. Introduction

Nanoparticles are being viewed as fundamental building blocks of nanotechnology. The most important and distinct property of nanoparticles is 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. Metal nanoparticles have tremendous application in the area of catalysis, optoelectronics, diagnostic biological probes and display devices. Among the above four, silver nanoparticles play a significant role in the field of biological systems, living organisms and medicine[1-3].

Silver has long been recognized as having an inhibitory effect towards many bacterial strains and micro organisms commonly present in medical and industrial processes[4]. The most widely used and known applications of silver and silver nanoparticles include topical ointments and

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creams containing silver to prevent infection of burns and wounds[5]. Many attempts have been made to use silver nanoparticles as an anti-cancer agent and they have all turned up positive[6]. The role of silver nanoparticles as an anti-cancer agent should open new doors in the field of medicine.

Production of nanoparticles can be achieved through different methods, for example, reduction in solutions, chemical and photochemical reactions in reverse micelles, thermal decomposition of silver compounds[7], radiation assisted[8], electrochemical[9], sonochemica[10], microwave assisted method[11], and recently via biological routes. Biological methods of nanoparticles synthesis using microorganisms[12], enzyme[13], and plant or plant extract offer numerous benefits over chemical and physical methods. It is cost effective, environmental friendly, easily scaled up for large scale synthesis. In this method there is no need to use high pressure, energy, temperature and toxic chemical that may have adverse effect in the medical applications[14-16].

Although biosynthesis of silver nanoparticles by plants such as *Desmodium triflorum*[17], *Cinnamomum camphora*[18], *Moringa oleifera*[19], and *Eucalyptus hybrida*[20]have been reported, the potential of the plants as biological materials for the synthesis of nanoparticles is currently under exploitation.

In the present study, we report for the first time synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the methanolic extract of *Eucalyptus chapmaniana* (*E. chapmaniana*) leaves. Morphological characterizations are performed using X-ray diffractometer (XRD). The optical absorption spectrum of silver nano particles was recorded by using UV-visible spectrophotometer and furthers its efficacy to inhibit different pathogenic bacteria, yeast and toxicity against human acute promyelocytic leukemia (HL-60) cell line were evaluated.

2. Materials and methods

2.1. Materials

The chemical silver nitrate ($AgNO_3$), Mueller-Hinton agar (MHA) and Sabouraud Dextrose agar (SDA, Oxoid) were purchased from Merck, Germany. Penicillin and streptomycin were purchased from Bio Source International, Belgium. Tissue culture plastic wares were obtained from BD Bioscience (USA). All organic solvents used were of HPLC grade. RPMI 1640, fetal bovine serum (FBS) and MTT (3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of the extract

Leaves plant extract was prepared by Soxhlet extraction method. About 100 g of powder material was uniformly packed into a thimble and run in Soxhlet extractor. It was exhaustible extracted with methanol for the period of about 48 h or 22 cycles or till the solvent in the siphon tube of an extractor become color less. After that extracts were filtered with the help of filter paper and solvent was evaporated from extract in rotary evaporator to get the syrupy consistency. Then extract was kept in refrigerator at 4 °C for future experiments.

2.3. Synthesis of silver nanoparticles

Aqueous solution of silver nitrate ($AgNO_3$) at concentration of 0.02 mmol/mL or 0.01 mmol/mL was prepared and used for the synthesis of silver nanoparticles. Ten milliliter of *E. Chapmaniana* leaves extract was added into 90 mL of aqueous solution of 0.02 mmol/mL or 0.01 mmol/mL silver nitrate for reduction into Ag^+ ions and exposed to bright sunlight at 50 °C; the change of color takes place within few minutes from yellowish to reddish brown color.

2.4. Characterization of silver nanoparticles

UV-vis spectral analysis was done by using UV-vis spectrophotometer (PG- T80+ UV/Vis spectrophotometer, England) from 350-700 nm at a resolution of 1 nm. XRD measurements of the silver nanoparticles solution drop-coated on glass were done on a Shimadzu XRD-6000 model with 40 kV, 30 mA with Cu k α radiation at 2 θ angel. 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 crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula: $D= 0.94 \lambda / \beta \cos \theta$.

2.5. Evaluation of antibacterial activity

The silver nanoparticles synthesized using *E. chapmaniana* leaves extract was tested for antimicrobial activity by agar well diffusion method against different pathogenic microorganisms *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus volgaris* (*P. volgaris*) (Gram negative), *Staphylococcus aureus* (*S. aureus*) (Gram positive) and *Candida albicans* (*C. albicans*) (Yeast). The pure cultures of bacteria were subcultured on MHA and SDA for yeast. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Wells of 8 mm diameter were made on nutrient agar plates using gel puncture. Using a micropipette, 50 μL of nanoparticle solution was poured onto each well on all plates. After incubation at 37 °C for 24 h, the diameter of zone inhibition was measured in millimeter, and was recorded as mean±

SD of the duplicate experiments.

2.6. MTT assay

Cell viability was evaluated by the MTT colorimetric technique. Briefly, 100 μL of the yellow tetrazolium MTT solution (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) without phenol red (5 mg/mL in phosphate buffer solution) was added to each well. The plates were incubated for 3-4 h at 37 °C, for reduction of MTT by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. For solubilization of the MTT crystals, 100 μL of isopropanol or DMSO was added to the wells. The plates were placed on a shaker for 15 min to complete solubilization of crystals and then the optical density of each well was determined. The quantity of formazan product as measured by the amount of 545 nm absorbance is directly proportional to the number of living cells in culture. Each experiment was done in triplicate. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles as a vehicle was calculated as follow:

$$\text{Percentage of cell viability (\%)} = \left(\frac{\text{Sample absorbance}}{\text{Control absorbance}}\right) \times 100$$

2.7. Statistical analysis

The grouped data were statistically evaluated using ANOVA with SPSS/14 software. Values are presented as the mean±SD of the three replicates of each experiment.

3. Results

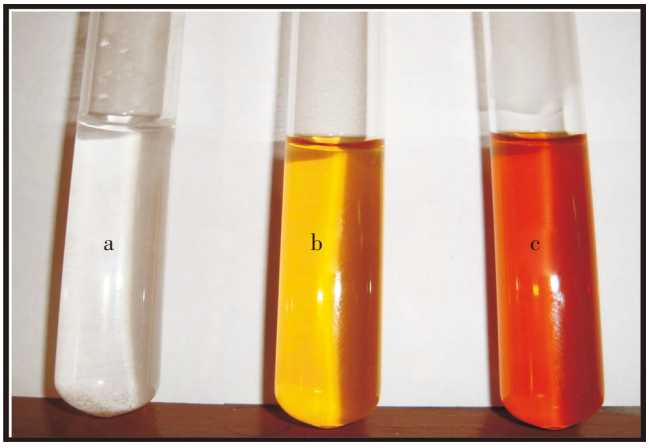

Figure 1. Photograph showing color changing.
(a) 0.02 mmol/mL $AgNO_3$ without *E. chapmaniana* leaf extract; (b) Aqueous leaf extract of *E. chapmaniana*; (c) Color changed from yellowish to reddish brown after adding 0.02 mmol/mL $AgNO_3$ and exposing to bright sunlight for 1 h.

Green synthesis of silver nanoparticles using 2 mmol/L $AgNO_3$ is shown in Figure 1. The fresh suspension of *E. Chapmaniana* was yellowish in colour. However, after addition of $AgNO_3$ and exposing to bright sunlight for 1 h, the suspension turned reddish brown. Formation of silver nanoparticles was confirmed using UV-vis spectral analysis and showed silver surface plasmon resonance band at 413 nm (Figure 2).

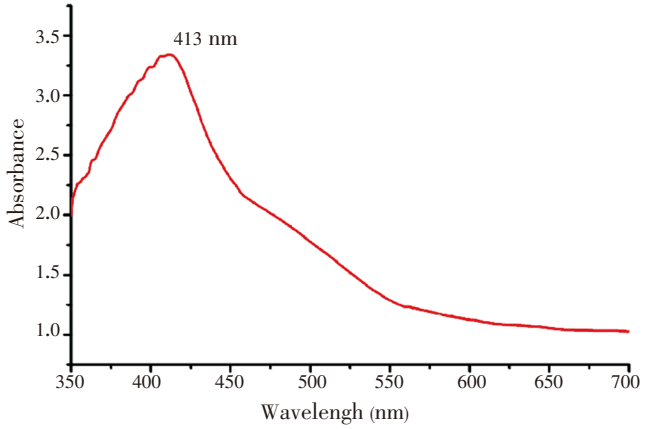

Figure 2. UV/Vis absorption spectra of reduction of silver ions to silver nanoparticles after 1 h of reaction.

The X-ray diffraction pattern of the biosynthesized silver nanostructure produced by the leaf extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Figure 3). The XRD pattern showed two intense peaks (38.50° and 44.76°) in the whole spectrum of 2 θ value ranging from 20 to 60 and indicated that the structure of silver nanoparticles is face-centered cubic (FCC). These are corresponding to (111) and (200) planes for silver, respectively. The lattice constant calculated from this pattern was a=4.086 A° and the data obtained was matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. The average grain size of the silver nanoparticles formed in the bioreduction process was determined using Scherrer's formula and was estimated as 60 nm.

Table 1
Size of the inhibition zone for silver nanoparticles synthesized by *E. chapmaniana* leaf extract against the tested microorganisms.

Pathogens	Zone of inhibition (mm)			
	Extract	$AgNO_3$	0.01 mmol/mL AgNPs*	0.02 mmol/mL AgNPs
S. aureus	18	14	23	27
S. pneumoniae	18	10	22	25
P. aeruginosa	18	12	20	23
K. pneumoniae	16	10	19	23
E. coli	16	12	20	23
P. volgaris	17	nil	20	23
C. albicans	19	13	24	25

* AgNPs: Silver nanoparticles,

The antimicrobial activity of silver nanoparticles against various pathogenic organisms including bacteria and yeast was investigated. Compared with the control, the diameters of inhibition zones increased for all the test pathogens (Table 1). It has been reported that antimicrobial effect was dose dependant. At 0.02 mmol/mL concentration of silver nanoparticles, the 27 mm clear inhibitory zone appeared around 100 μL against *S. aureus* after incubation for 24 h (Table 1 and Figure 4) followed by *C. albicans* and *S. pneumoniae* (25 mm), then 23 mm for Gram-negative bacteria *E. coli*, *K. pneumonia*, *P. aeruginosa* and *P. volgaris* suggesting that synthesized nanoparticles have

Table 2
Percent viability measured on HL-60 cells after treatment with silver nanoparticles for 6, 12 and 24 h, by MTT assay.

Treatment	6 h		12 h		24 h	
	Dead (%)	Viable (%)	Dead (%)	Viable (%)	Dead (%)	Viable (%)
HL-60+RPMI	2.00±0.14	98.00	3.00±0.24	98.00	2.00±0.16	98.00
HL-60+$AgNO_3$	18.00±0.80	82.00	20.00±1.24	80.00	23.00±1.70	77.00
HL-60+Extract	24.00±1.24	76.00	32.00±0.80	68.00	40.00±3.10	60.00
HL-60+0.01 mmol/mL AgNPs*	32.00±1.20	68.00	40.00±1.87	60.00	51.00±2.20	49.00
HL-60+0.02 mmol/mL AgNPs	50.00±1.23	50.00	74.00±2.20	26.00	85.00±1.80	15.00

*: Silver nanoparticles.

good antibacterial action against gram-positive organism than gram-negative organisms.

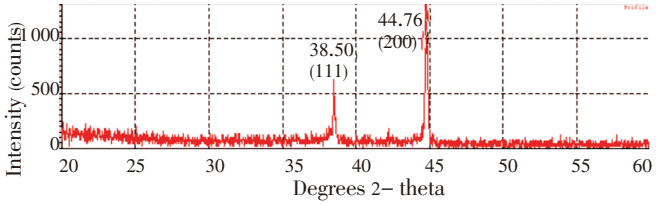

Figure 3. XRD patterns recorded from drop-coated films on glass substrate of silver nanoparticles synthesized by *E. chapmaniana* leaf extract with $AgNO_3$ aqueous solutions.

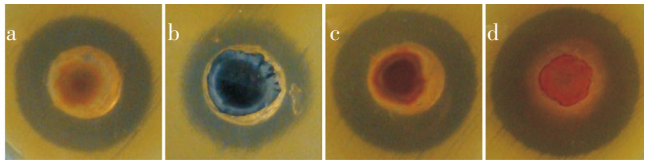

Figure 4. Antibacterial activity assay against *S. aureus*.
(a) *E. chapmaniana* leaf extract. (b) 0.02 mmol/mL $AgNO_3$ without *E. chapmaniana* leaf extract. (c) 0.01 mmol/mL $AgNO_3$/*E. chapmaniana* leaf extract. (d) 0.02 mmol/mL $AgNO_3$/*E. chapmaniana* leaf extract.

The *in vitro* cytotoxic effects of silver nanoparticles were screened against HL-60 cell line and viability of tumor cells was confirmed using MTT assay. The silver nanoparticles were able to reduce viability of the HL-60 cells in a dose-dependent manner as shown in Table 2. After six hours of treatment, the silver nanoparticles at concentration of 2 mmol/L decreased the viability of HL-60 cells to 50% of the initial level, and this was chosen as the IC_{50}. Longer exposures resulted in additional toxicity to the cells and reached to 85% dead cells after 24 h of incubation. However, the toxicity of AgNPs appeared much higher than that of $AgNO_3$ (23%) or *E. chapmaniana* leaf extract only (40%) at the same period of incubation (Table 2).

4. Discussion

The development of easy, reliable and eco-friendly methods helps to increase interest in the synthesis and application of nanoparticles that are beneficial for mankind[21, 22]. Reduction of silver ion into silver nanoparticles during exposure to the plant extracts could be followed by color change. Silver nanoparticles exhibited dark redish-brown color in aqueous solution due to the surface plasmon resonance phenomenon[23]. Dubey *et al.* synthesized silver nanoparticles by using the *E. hybrida* at 3 h of incubation and reported the flavanoid and terpenoid constituents of the leaf extract might are to be the surface active molecules stabilizing the nanoparticles[20]. Similarly, in the present study silver nanoparticles were synthesized using leaves extract of *E. chapmaniana*. Interestingly, silver nanoparticles were synthesized rapidly within 1 h of incubation period making it one the fastest bioreducing methods to produce silver nanoparticlesand there was no significant change afterwards.

The production of the silver nanoparticles synthesized from the leaf aqueous extract of *E. chapmaniana* was evaluated through spectrophotometry at a wavelength range of 350-700 nm; this revealed a characteristic peak for *E. chapmaniana* AgNPs at 413 nm for the extract and $AgNO_3$ mixture, which confirmed the formation of the silver nanoparticles. This is similar to the surface plasmon vibrations with characteristic peaks of the silver nanoparticles prepared by *E. hybrida*[20]. The result obtained in this investigation is very interesting in terms of the identification of potential forest plants for the synthesis of silver nanoparticles. The frequency and width of the surface plasmon absorption depends on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium[24,25]. It is generally recognized that UV-vis spectroscopy could be used to examine size- and shape-controlled nanoparticles in aqueous suspensions[19,26].

XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles in plants extracts can be achieved by using XRD to examine the diffraction peaks of the plant[27]. In present study the X-ray pattern of synthesized silver nanoparticles matches the FCC structure of the bulk silver and there was no obvious other phases as impurities were found in the XRD patterns. The X-ray diffraction results clearly show that the silver nanoparticles formed by the reduction of Ag^+ ions by the *E. chapmaniana* are crystalline in nature.

In this study, the application of silver nanoparticles as an antimicrobial agent was investigated and exhibited better antimicrobial activity against all human pathogens. However, the antimicrobial effect was dose-dependent, and was more pronounced against gram-positive bacteria than gram-negative bacteria. Additionally, the silver nanoparticles showed good inhibition activity towards *C. albicans*. The mechanism of inhibitory action of silver nanoparticles on microorganisms is not very well known[28, 29]. However, several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. It was also suggested that silver ions (particularly Ag^{+}) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or reacting with sulfur-containing proteins, leading to the inhibition of enzyme functions which results in loss of cell viability and eventually resulting in cell death [30-32].

In this study, we have employed a time and dose dependent approach to evaluate the toxicity of the nanoparticles on human acute promyelocytic leukemia (HL-60). The viability of HL-60 cells considerably decreased with increasing doses and time of incubation. The mortality data obtained in these results allow us to predict their potential not only because of the cytotoxic effect, but also in terms of the potential for tumor reduction. The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA[33,34]. Although the mechanism of action at a more detailed level needs more sophisticated experimental proofs, the nanoparticles appear to have a promising future.

In conclusion, it has been demonstrated that the extract of *E. chapmaniana* leaves are capable of producing silver nanoparticles extracellularly and the Ag nanoparticles are quite stable in solution. The biosynthesized silver nanoparticles showed excellent antimicrobial activity and possessed considerable cytotoxic effect against HL-60. The data represented in our study contribute to a novel and unexplored area of nano-materials as alternative medicine. Therefore, further studies are needed to fully characterize the toxicity and the mechanisms involved with the antimicrobial and anticancer activity of these particles.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by University of Technology, Baghdad, Iraq and Universiti Kebangsaan Malaysia (Grant No. DIP-2012-02).

Comments

Background

Nano-particle biosynthesis is an emerging technology and has potential applications in biomedical fields.

Research frontiers

The new method of synthesis of silver nano-particles from biological extracts of *E. chapmaniana* leaves is described. The nano-particles are studied for their anti-bacterial properties.

Related reports

Silver nano-particles from *E. chapmaniana* are reported to inhibit the growth of various pathogenic bacteria and HL-60 promyelocytic leukemia cells.

Innovations and breakthroughs

Silver nano-particles were synthesized from extracts of *E. chapmaniana* leaves and characterized by spectrophotometry and X-ray diffraction.

Applications

The method can be applied to the biosynthesis of other nano-particles, which can be tested for anti-bacterial or anti-cancer properties.

Peer review

The article is generally well written and present interesting results. Silver nano-particles were obtained with a biosynthetic procedure from *E. chapmaniana* leaves. The nano-particles are reported to inhibit the growth of various pathogenic bacteria and HL-60 promyelocytic leukemia cells.

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