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Green synthesis, antimicrobial and cytotoxic effects of silver nanoparticles mediated by *Eucalyptus camaldulensis* leaf extract

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ABSTRACT

Objective: To investigate the environmental-friendly extracellular biosynthetic technique for the production of the silver nanoparticles (AgNPs) by using leaf extract of *Eucalyptus camaldulensis* (*E. camaldulensis*).

Methods: The NP were characterized by colour changes and the UV-visible spectroscopy. The cytotoxic effects of prepared AgNPs was detected against four types of pathogenic bacteria, including two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) by using agar well diffusion method.

Results: A peak absorption value between 400-450 nm for the extract and the colour change to dark brown were corresponding to the plasmon absorbance of AgNPs. On the other hand, aqueous extract of *E. camaldulensis* leaves could be effective against tested microorganisms which showed inhibition zones of 9.0-14.0 mm. Furthermore, biologically synthesized AgNPs had higher ability to suppress the growth of the tested microorganisms (12.0-19.0 mm).

Conclusions: Our findings indicated that extracellular synthesis of AgNPs mediated by *E. camaldulensis* leaf extract had an efficient bactericidal activity against the bacterial species tested. The exact mechanism of the extracellular biosynthesis of metal NP was not well understood. Further studies are needed to highlight the biosynthesis process of AgNPs and also to characterize the toxicity effect of these particles.

1. Introduction

Bionanotechnology has recently gained great interests as it combines with biotechnology and nanotechnology to develop a biological system for the synthesis of nanoscale materials by using a reliable and eco-friendly technique. NP are often referred to as particles with a maximum size of 1-100 nm and exhibit unique chemical, optical and mechanical properties which are quite different from bulk material[1]. Recently, the growing microbial resistance against metal ions, antibiotics, and the development of resistant strains has shifted the interests of many scientists to focus on metallic NP application which are the most potential compounds having a significatly high specific surface area and a high fraction of surface atoms, unique optical, electronic, catalytic, anti-bacterial and magnetic properties[2,3]. Among metals, silver exhibits higher toxicity to microorganism while it exhibits lower toxicity to mammalian cells. However, silver ions have the disadvantage of forming complexes and the effect of ions remains only for a short time. This disadvantage has been overcome by the use of silver nanoparticles (AgNPs) which are in an inert form and also exhibit antimicrobial function by inducing the production of reactive oxygen species such as hydrogen peroxide[1]. AgNPs, mainly in the range of 1-10 nm, exhibit strong toxicity to a wide range of microorganisms since their attachment on the surface of cell membrane significantly disturbs its proper function like respiration and permeability[4]. It has been found that AgNPs inhibited bacterial growth by a destructive effect on DNA, resulting in a loss of replication and degradation of DNA[5]. Antibacterial activity of AgNPs against *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P.*

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aeruginosa), *Escherichia coli* (*E. coli*), *Proteus* specie and *Klebsiella* specie has been documented[5,6]. On the other hand, it has been documented that the antifungal activity of AgNPs against sclerotium-forming phytopathogens especially *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sarracenia minor*, and two plant pathogenic fungi, *Bipolaris sorokiniana* and *Magnaporthe grisea*[7,8]. AgNPs can be synthesized by different methods such as chemical reduction of silver ions in aqueous solutions with or without solubilizing agents, radiation chemical reduction, photochemical, reverse micelles, electrochemical, microwave assisted methods, *etc.* However, most of these methods are tedious and pose a risk to the environment and health[9]. Above all, biological methods are currently gaining significance because they are eco-friendly, cost effective, and there is no use of any toxic chemicals in the synthesis process[10]. Microorganism cell or plant extract is considered as an exciting branch for biosynthesis of NP and used either as reducing agent or protective agent. Recently, the green synthesis of NP has evolved into an important branch of nanotechnology[11,12]. Biosynthesis of AgNPs is simple and large quantities of NP can be prepared in a short time. Previous studies already reported that AgNPs were prepared from the leaf extract of *Ziziphus spina-christi*[6], *Catharanthus roseus*[13], *Eucalyptus chapmaniana*[14], *Eucalyptus globulus*[15], *Eucalyptus angophoroides*[16], *Camellia sinensi*[17], seed powder extracts of *Cuminum cymimum*[18], *etc.* Furthermore, the ethanol extract of *Eucalyptus camaldulensis* (*E. camaldulensis*) was used as a natural reducing agent for the formation of magnetite-gold composite NP with size ranging from 6-20 nm[19]. In this study, *E. camaldulensis* was used as a agent for reducing and slicing the environmentally-friendly synthesis of AgNPs to form silver nitrate ($AgNO_3$) at room temperature. *E. camaldulensis* is an important ethno-medicinal plant belonging to Myrtaceae family. It is used as a therapy for sore throat and other bacterial infection of respiratory and urinary tracts[20]. Several reports have documented the antimicrobial activity of leaves extract and essential oil from *E. camaldulensis* and *Eucalyptus citriodora*[21-23]. Essential oils, particularly cineol, cuminal, phellandrene, aromadendral, valeraldehyde, geraniol, cymene, catechol, tannins, terpenes, isoprenoids, phenolics, cardiac glycosides, sterols, saponins and flavonoids are the phytochemical components detected in *E. camaldulensis* leaves[22,24]. In this study, since flavonoids are assumed to play an important role in the reduction process for biosynthesis of AgNPs, *E. camaldulensis* leaf extract was used to mediate reduction of the silver ions which present in the form of aqueous solution of $AgNO_3$[25].

2. Materials and methods

2.1. Materials

E. camaldulensis leaves were collected from Riyadh, Saudi Arabia. $AgNO_3$ was purchased from Merck Company (Darmstadt, Germany). Mueller-Hinton agar was purchased from Wateenalhyaa Company (Riyadh, Saudi Arabia) for the antibacterial assays.

2.2. Bacterial strains

Four bacterial species, *P. aeruginosa, E. coli, S. aureus* and *Bacillus subtilis* (*B. subtilis*) were obtained from the Department of Biology, Faculty of Science, Princess Nora Bint Abdulrahman University.

2.3. Synthesis of AgNPs

The aqueous extract of *E. camaldulensis* was prepared by mixing 10 g of dry leaf sample with 100 mL of highly purified water. The mixture was heated for 10 min at 80 °C to denature the enzymes in the extract. The solution was filtered through a Whatman filter paper No. 1 (pore size 125 mm). The supernatant (filtrate) was further filtered through a Whatman filter paper No. 1 (pore size 25 mm) to remove the remaining plant residues. For synthesis of the AgNPs, 12 mL aqueous leaf extract of *E. camaldulensis* were mixed with 88 mL of 1 mmol/L $AgNO_3$ solution in an Erlenmeyer flask and allowed to react at room temperature for 24 h. The AgNPs extract was stored at 4 °C until further analysis.

2.4. Characterization of AgNPs by UV-visible spectroscopy (UV-vis spectroscopy)

A sample volume of 0.1 mL was diluted with 2 mL deionized water in the cuvette. The reduction of pure Ag^+ ions was monitored by measuring the mixture using a UV-2450 double-beam spectrophotometer (Shimadzu, Tokyo, Japan) which was operated in the range of 200-800 nm.

2.5. Screening of antibacterial property in synthesized NP

The AgNPs that were synthesized using *E. camaldulensis* leaf extract was tested for antimicrobial activity against four types of pathogenic bacteria, including two Gram-negative bacteria (*P. aeruginosa* and *E. coli*) and two Gram-positive bacteria (*S. aureus* and *B. subtilis*) by using agar well diffusion method. Sterilized water was used as a control. Pure cultures of micro-organisms were sub-cultured on Mueller-Hinton agar. A sterile cotton swab was then used to spread the resulting suspension on the nutrient agar and allowed to dry for 10 min. Subsequently, four adequately spaced wells (holes) of 4 mm diameter each were made per plate at the culture agar surface using a sterile metal cup-borer. In each hole, 0.2 mL of each extract and control were put under aseptic conditions, and kept at room temperature for one hour to allow the biosynthesized extracts

to diffuse into agar medium and incubated accordingly. Distilled water was used as a reference negative control. The plates were then incubated at 37 °C for 24 h. At the end of the incubation period, the zones of inhibition were measured to the nearest millimeter[26]. The inhibition zone is the area surrounding the hole with no growth of inoculated microorganisms. Each test was performed in four replicates for confirmation of the results.

2.6. Statistical analysis

The data were analyzed using ANOVA. The significance of the differences between means was determined at $P<0.05$ using Duncan's multiple range test. Results were expressed as mean±SD for four replicates.

3. Results

In the present work, AgNPs as an antimicrobial agent has been biologically synthesized. Synthesis of AgNPs from $AgNO_3$ solution using leaves of *E. camaldulensis* was identified by change color of the solution (Figure1). It is observed that the color of the solution turned from colorless to brown, which indicated the formation of AgNPs.

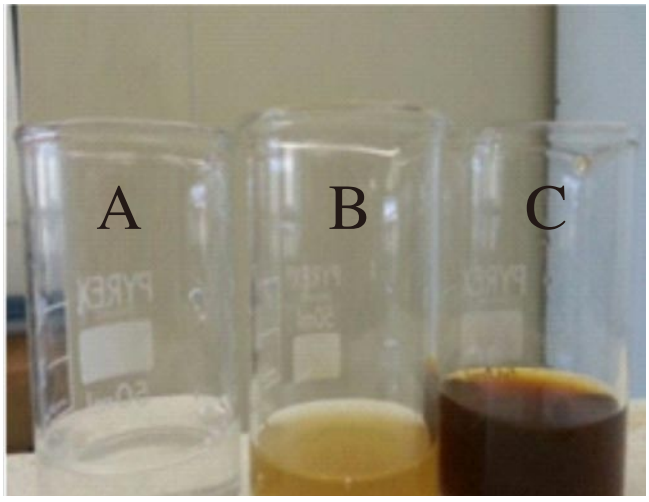

Figure 1. Synthesis of AgNPs by *E. camaldulensis* leaf extract.
A: Culture filtrate after exposure to $AgNO_3$ alone; B: *E. camaldulensis* leaf extract; C: *E. camaldulensis* leaf extract with $AgNO_3$.

Monitoring the process of the bioreduction of silver ions to AgNPs was applied in this study by UV-vis spectroscopy. Absorption spectra of AgNPs formed in the reaction media had shown silver surface plasmon resonance at 420 nm (Figure 2). The potential antibacterial activity of biologically synthesized AgNPs against Gram-negative bacteria *P. aeruginosa* and *E. coli* and Gram-positive bacteria *S. aureus* and *B. subtilis* using agar well diffusion method was detected and compared with the antibacterial activity of the aqueous extract of *E. camaldulensis* leaves. The results of antibacterial activity of AgNPs against tested microorganisms are shown in Table 1.

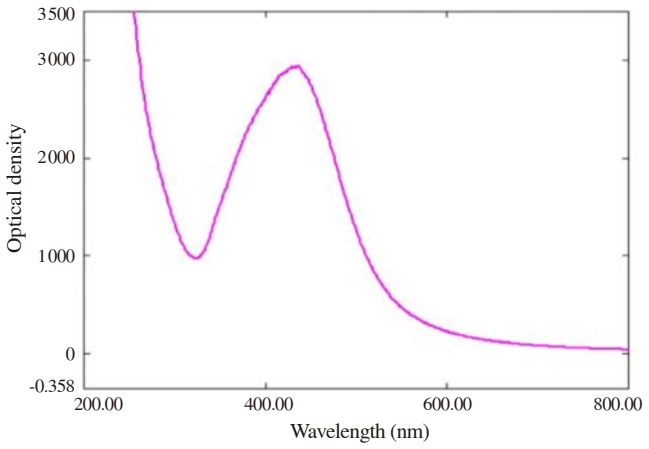

Figure 2. UV-vis spectroscopy of the formed AgNPs using *E. camaldulensis* leaf extract.

Table 1
Inhibition zone of biologically synthesized AgNPs against tested microorganisms.

Tested microorganisms	Inhibition zone (mm)	
	Aqueous extract	AgNPs
S. aureus	14.0±1.7^{a}	19.0±1.1^{a}
B. subtilis	12.0±0.9^{ab}	17.0±1.3^{a}
E. coli	9.0±0.9^{c}	12.0±0.9^{c}
P. aeruginosa	10.0±1.6^{b}	14.0±1.4^{b}

Values of four replicates are expressed as mean±SD. Different letters with in acolumn indicate significant differences. $P<0.05$.

The aqueous extract of *E. camaldulensis* has been found to be effective against all tested bacteria especially Gram-positive bacteria *S. aureus* and *B. subtilis* which showed 14.0 mm and 12.0 mm inhibitory zone, respectively. On the other hand, Gram-negative bacteria *P. aeruginosa* and *E. coli* showed 10.0 mm and 9.0 mm inhibitory zone, respectively. Furthermore, AgNPs showed higher ability to suppress the microbial growth than aqueous extract. The maximum antibacterial activity of AgNPs was recorded against *S. aureus* (19.0 mm), followed by *B. subtilis* (17.0 mm), then 14.0 mm for *P. aeruginosa* and 12.0 mm for *E. coli*. Generally, the aqueous extract of *E. camalduelnsis* leaves and biologically synthesized AgNPs showed good antibacterial capability against Gram-positive than Gram-negative bacteria.

4. Discussion

Since the last decade, resistance to antibiotics by the pathogenic bacteria has been perceptible. Therefore, the development of new antibacterial agents was a line of considerable interest for many researches worldwide. AgNPs as a promising applicant in the medical field was biosynthesized in this study. The biosynthesis of NP using biological agents like bacteria, fungi, actinomycetes, yeast, algae and plants was documented[27,28]. Synthesis of AgNPs from

$AgNO_3$ solution using leaves of *E. camaldulensis* was identified by color change. Many studies confirmed that the color change of the solution from transparent to brown is attributed to the surface plasmon resonance of AgNPs[9,29,30]. *E. camaldulensis* is rich in flavanoid and terpenoid[22]. They are the surface active molecules which play an important role in reducing and stabilizing process of AgNPs[25,31]. UV-vis spectroscopy might be used to detect the size and shape-controlled NP in aqueous suspensions[32]. Absorption spectra of AgNPs formed in the reaction media had shown peaks within the range of 400-450 nm, which provided a convenient signature for the formation of AgNPs[33]. NP prepared by *Eucalyptus chapmaniana* and *Eucalyptus hybrida* showed a surface plasmon vibrations peaks of 413 nm and *Eucalyptus globulus* was 412 nm[14,16,31]. Difference might be due to the variations in genotypes which were used for processing and preparation conditions. The specific action of metal NP extracellularly biosynthesis is still vague. However, it was assumed that nicotinamide adenine dinucleotide co-enzyme was efficient as an electron transporter to neutralize Ag^+[34].

Furthermore, the potential antibacterial activity of biologically synthesized AgNPs was detected and showed good antibacterial activity against tested bacteria. However, the antibacterial activity was species-dependent. The susceptibility of bacteria to plant extracts varied according to bacterial strains and species, which was well documented[35]. The water extract of *E. camaldulensis* has been found to be effective against all tested bacteria. Same range of inhibitory zones of *E. coli* and *S. aureus* were 7 and 14 mm, respectively, when aqueous extract of *E. camaldulensis* was applied[21]. This ability might explain the presence of tannins which are water soluble polymeric phenol[36]. Occurrence of tannins, saponins and cardiac glycosides in the leaf extract of *E. camaldulensis* were was well documented[22]. Furthermore, the antibacterial action of tannin was known for their capability to inhibit the cell protein synthesis by protein binding mechanism[37]. AgNPs showed higher ability to suppress the microbial growth than water extract, which was pronounced against Gram-positive bacteria than Gram-negative bacteria. Despite the extensive use of AgNPs, the mode of action of microorganisms is incompletely known. However, it has been reported that AgNPs are small in size and easily enter into the bacterial cell and affect the intracellular processes, such as DNA, RNA and protein synthesis[5,15]. Furthermore, it was also assumed that silver ions (particularly Ag^+) from AgNPs are able to interact with phosphorus moieties in DNA or react with sulfur-containing proteins which result in loss of cell viability that will led to cell death[38,39].

The current investigation demonstrated that the aqueous extract of *E. camaldulensis* leaves showed noticeable antibacterial potential and was also capable of producing AgNPs extra-cellularly. Furthermore, the biosynthesized particles had an excellent antibacterial activity against some Gram-positive and Gram-negative bacteria. Additional information are needed to characterize the mode of action of the plant to synthesize AgNPs and the mechanism involved in the antimicrobial activity of these particles. Improving our understandings to such important aspects of nanoparticle will definitely pave way towards maximizing the utilization of this multi-purpose nanotechnology.

Conflict of interest statement

We declare that we have no conflict of interest.

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