GREEN SYNTHESIS OF SILVER NANOPARTICLES USING EUCALYPTUS CORYMBIA LEAVES EXTRACT AND ANTIMICROBIAL APPLICATIONS

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ABSTRACT

In this study biosynthesis of silver nanoparticles (AgNPs) using Eucalyptus corymbia and their antimicrobial activities have been reported. This work reveals that Eucalyptus corymbia leaf extract contains a variety of bio-molecules responsible for reduction of metal ions and stabilization of nanoparticles. These bio-molecules are believed to contain polyphenols and water soluble heterocyclic compounds. Optimized experimental conditions included using extraction temperature of 90˚C; plant extract pH 5.7 and silver nitrate to plant extract ratio of 4:1. These conditions favoured the formation of higher number of nanoparticles, which were stable within the study period. The synthesized nanoparticles were polydispersed with average mean size of 18-20 nm and were spherical in shape without significant agglomeration, as revealed from the TEM analysis. FT-IR spectra of the plant extract revealed that functional groups OH and –C=C– are responsible for reduction and stabilization of the nanoparticles. Anti-Microbial activity of the synthesized silver nanoparticles were studied against gram negative bacteria Escherichia coli (E.coli) and gram positive bacteria Staphylococcus aureus. In the medium treated with silver nanoparticles, E.coli and Staphylococcus aureus growth was inhibited, as these particles have an excellent biocidal effects and hence effective in inhibiting bacterial growth. These nontoxic nanomaterials, which can be prepared in a simple and cost-effective manner may be suitable for the formulation of new types of bactericidal materials.

Key words: Silver nanoparticles, Eucalyptus corymbia, Green synthesis, Escherichia coli, Staphylococcus aureus.

INTRODUCTION

Metal nanoparticles have received significant attention in recent years owing to their unique properties and practical applications. They exhibit properties that differ significantly from those of bulk materials as a result of small particle dimension, high surface area, quantum confinement and other effects [1]. Metal nanoparticles size and shape dependent properties are of interest due to wide applications as catalyst, optical sensors, in data storage and antibacterial properties [2]. Nanoparticles can be synthesised through different methods; chemical, physical and biological methods. Conventionally, chemical synthesis has been the method of choice because it offers faster synthetic route. However, chemical synthesis has raised environmental concerns because of the nature of chemicals used, such as reducing agents (sodium borohydride), organic solvents and non – biodegradable stabilizing agents (sodium citrate dehydrate). These chemicals are potentially hazardous to the environment and biological systems [3]. Majority of the conventional methods makes use of organic solvents because of the hydrophobicity of the capping agents. Capping and stabilizing agents are used to prevent aggregation which may hinder production of small sized silver nanoparticles [4] Due to the increasing interest in nanoparticles synthesis and applications; there is a need for eco-friendly approaches based on green chemistry principles [5].
Green method employs principles of green chemistry which involves exploitation of natural resources for metal nanoparticle synthesis, which is a competent and environmentally benign approach [6]. This involves three main steps, which must be evaluated based on green chemistry perspectives, including selection of solvent medium, environmentally benign reducing agent, and non-toxic stabilising agents [7]. These bio-inspired methods utilize plant extracts and micro-organisms for synthesis of nanoparticles intracellularly or extracellularly [8]. The use of plant in nanoparticles synthesis is more advantageous over environmentally benign biological processes because it eliminates elaborate process of maintaining cell cultures.

In addition, green synthesis using plants offers a better synthetic protocol because of the vast reserves of plants that are easily accessible, widely distributed, safe to handle with wide range of metabolites. Unlike conventional methods bio-inspired methods are economical and restrict the use of toxic chemicals and do not require high pressure, energy and temperatures.

The bioreduction of metal ions is done by combinations of biomolecules found in plant extracts (e.g. enzymes/proteins, amino acids, polysaccharides, and vitamins) in an environmentally benign, yet chemically complex process [9].

Depending on the origin there are three types of NPs: natural, incidental and engineered. Natural NPs have existed since the earth’s beginnings and still occur in the environment, for example volcanic dusts and mineral composites. Incidental NPs are typically represented by engine exhaust particles, coal combustion, or other fractions or airborne combustion by-products [10]. Engineered nanomaterials are defined as those nanomaterials that are designed with specific properties and intentionally produced via chemical or physical processes. They are further divided into four types [10], namely:

- Carbon-based materials, usually including fullerenes, single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). Fullerenes are made of pure carbon and represent a new carbon allotrope discovered in 1985 (Kroto et al., 1993).
- Metal-based materials such as quantum dots, nanogold, nanozinc, nanoaluminum, and nanoscale metal oxides like TiO₂, ZnO and Al₂O₃. Quantum dot is a closely packed semiconductor crystal comprised of hundreds or thousands of atoms, whose size is in the order of a few nanometers to a few hundred nanometers.
- Dendrimers, which are nanosized polymers built from branched units capable of being tailored to perform specific chemical functions. The surface of a dendrimer has numerous chain ends, which can be tailored to perform specific chemical functions.
- Composites, which combine nanoparticles with other nanoparticles or with larger, bulk-type materials.

Silver nanoparticles (AgNps) have been proven to have diverse importance and thus have been extensively studied. In the recent years, there has been an upsurge in studying AgNPs on account of their inherent antimicrobial efficacy. Many bacteria develop resistance to antibiotics hence the need to develop a substitute. So far no literature has reported any bacteria able to develop immunity against silver. Generally the nanoparticles are designed with surface modifications tailored to meet the needs of specific applications they are going to be used for [9].

The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known. However, it has been hypothesized that silver nanoparticles can act on microbes to cause the microbicidal effect through various ways. In one of the ways, silver nanoparticles are said to anchor on the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane. This leads to formation of ‘pits’ on the cell surface, and consequently accumulation of the nanoparticles on the cell surface [11]. It has also been proposed that silver nanoparticles can release silver ions (Feng et al., 2008) and these ions can interact with the thiol groups of many vital enzymes and inactivate them [12] i.e., Ag⁺ works through suppression of respiratory enzymes and electron transport components which interfere with DNA functions [13]. Silver ions are powerful antimicrobials but are easily sequestered by chloride, phosphate and other cellular components [14]. Silver
nanoparticles are less susceptible to being intercepted and therefore offer a more effective delivery mechanism [15]. Silver ions are released from the nanoparticles in presence of oxygen [14].

**EXPERIMENTAL SECTION**

**Materials and reagents**

100g Silver nitrate (AgNO₃) crystals and 2.5 Litres of HPLC grade Methanol, Ethanol and Diethyl Ether were purchased from Fischer Scientific Chemicals (United Kingdom). 50 g of oven dried AgNO₃ (Sigma Aldrich USA) was used as received for the study. Distilled de-ionized water and Nutrient broth (Sigma-Aldrich, USA) was obtained from the Biochemistry laboratory at the University of Nairobi. Folin-ciocalteus’s phenol reagent (2N), NaOH, FeCl₃, and Gallic Acid were purchased from Sigma-Aldrich (Germany).

**Extraction of polyphenols from Eucalyptus corymbia**

A leaf extract of Eucalyptus corymbia was prepared by weighing 5g of green leaves. The leaves were properly washed with distilled water, cut into fine pieces and transferred to 250ml Erlenmeyer flask containing 100ml of distilled water. The mixture was boiled for 5 minutes before filtering using a filter paper. The filtrate obtained was centrifuged at 15000 revolutions per minute for 10 minutes and stored at 4°C in a refrigerator for subsequent use within 7 days after extraction.

**Confirmatory test for phenolic compound in the leaf extract**

An aliquot of Folin-ciocalteus’s phenol reagent (2N) was added to 5mLs of the leaf extract and colour change recorded [16].

**Synthesis of silver nanoparticles**

1.7g of silver nitrate was dissolved in 10mL of de-ionised water. Aqueous solution of 1mM AgNO₃ was prepared by diluting 1 ml of 1M AgNO₃ in a litre of distilled water. Different volumes of the leaf extract were added slowly to varying amounts of aqueous silver nitrate solution with stirring [17]. This was repeated with 0.8mM, 0.6mM, 0.4mM and 0.2mM of silver nitrate solution. Analysis was done on the resulting solution.

**Procedure for calculating Percent yield of silver nanoparticles**

The efficiency of the synthetic procedure in this work was determined by calculating the percent yield of the synthesized silver nanoparticles. 10 ml aliquot of the mixture of plant extract and silver nitrate were centrifuged at 15000rpm and washed with distilled water, then dried in an oven at 60°C for 24 hours. The nanoparticles were weighed and the mass recorded in grams. The weight was divided by the mass of Ag ions in 10 ml of 1mM AgNO₃. The answer above was multiplied by 100 to get percentage yield;

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\text{Percent yield} = \frac{\text{Mass of Ag}^0}{\text{Mass of Ag}^+} \times 100
\]

**Uv-vis Spectroscopy procedure**

The solution for UV-Vis analysis was prepared by taking 1ml of silver nitrate –plant extract mixture and diluting it ten times. UV-VIS spectra analysis was performed using UV-VIS double beam spectrophotometer [UV-1700 pharmaspec UV-Vis spectrophotometer (shimadzu)] at university of Nairobi. Scanning of the spectra was done between 200-700nm at a resolution of 1 nm using quartz cuvette. Baseline correction was done using de-ionized water as the blank.

**FT-IR Spectroscopy Procedure**

Dry powder of the sample was crushed with KBr and the mixture pressed in a mechanical press to form a thin and transparent pellet. The collar and the pellet were put onto the sample holder. FTIR of plant extract was obtained by dropping a sample between two plates of sodium chloride (salt) and analyzed in a liquid cell. Finally, the dried nanoparticles were analyzed by FTIR-JAS-CO 4100 spectrophotometer in the range 4000–400 cm⁻¹.

**Transmission Electron Microscopy Procedure**

Samples for transmission electron microscopy (TEM) analysis were prepared by drop coating biologically synthesized silver nanoparticles solution on to carbon-coated copper TEM grids [18]. The films on the TEM grid were allowed to stand for 2 minutes. The excess solution was removed using a blotting paper and the grid allowed to dry under a lamp prior to measurement. TEM images were
acquired with Philips Technai-FE 12 TEM instrument, operated at an accelerating voltage of 120 kV, equipped with an Energy dispersive X-ray (EDAX) detector (Oxford LINK-ISIS 300) for elemental composition analysis and the EDAX spectra was measured at an accelerating voltage of 10 Kv.

**Determination of Antimicrobial Activity**

Nutrient broth (Sigma, St. Louis, USA) was prepared by adding distilled water to 3.25 gm of the powder to make 250 ml as recommended by the manufacturer. The medium was sterilized by autoclaving at 121°C for 15 minutes (All American, Hillsville, USA). *Escherichia coli* and *Staphylococcus aureus* cells were separately inoculated and cultured overnight at 37°C. Incubation was done in a thermo-shaker (Gallenkamp, London, England). A disk diffusion test was carried out according to the Kirby- Bauer disk diffusion susceptibility test protocol [19]. An inoculum of the bacteria culture was applied uniformly on the surface of Muller Hinton agar (MHA) plates.

Sterile paper discs of 6mm diameter were impregnated with 20μl nanoparticles of three different concentrations (0.6mM, 0.8mM and 1.0mM) of nanoparticles suspended in distilled water and placed on the plate with inoculum. A positive control was prepared by impregnating a sterile disc of 6mm diameter with an antibiotic (Kanamycin 10mg/ml)

The plates were incubated for 15 hours at 37°C in a research CO2 incubator ( LEEC limited, Nottingham, United Kingdom). The plates were observed at the end of the incubation period.

**Composition of Eucalyptus corymbia**

*Eucalyptus corymbia* leaf extract contains a variety of bio-molecules responsible for reduction of metal ions and stabilization of nanoparticles; among these bio-molecules are polyphenols and water soluble heterocyclic compounds [20], as shown in figure 1. These compounds have been used as reducing, capping and stabilizing agent in the synthesis of nanoparticles such as silver, gold among others [21].

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**Test for reducing capacity using Folin-ciocalteus’s phenol reagent (2N)**

When an aliquot of Folin-ciocalteus’s phenol reagent (2N) was added to 5mLs of the leaf extract the colour of phenol reagent changed from yellow to black. Folin-ciocalteus’s phenol reagent (2N) also called Gallic acid equipment method (GAE) does not only measure phenols, but also reacts with any reducing substance [22]. It therefore measures the total reducing capacity of a sample. Change of its color from yellow to black confirms the presence of

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Figure 1: structure of Gallic acid and catechin.
reducing compounds in *Eucalyptus corymbia* leaf extract.

**RESULTS & DISCUSSION**

The percentage yield of silver nanoparticles was 81.64%. The percent yield was calculated by dividing the mass of AgNPs by the mass of Ag+ ions in 10ml aqueous solution. The above calculated value demonstrated that 81.6 ± 0.3 % of the silver ions were converted to atomic state hence forming silver nanoparticles.

**Uv-vis analysis**

Formation of silver nanoparticles from the plant extract and AgNO₃ was noted by visual observation, a gradual colour change, which took less than ten minutes from colorless solution to yellow then deep red/brown on addition of the leaf extract of *Eucalyptus corymbia*, indicating formation of AgNPs which was further confirmed by Uv-Vis analysis (figure 2). The observed results are in accordance with what was reported earlier by Chandan Tamuly, et al. [23].

The biosynthesized silver nanoparticles were found to have absorbance peak at around 425nm as shown in figure 1. Typically AgNPs have surface plasmons resonance peaks with $\lambda_{\text{max}}$ values in the visible range of 400–500 nm [24].

![Figure 2: The absorbance spectra of silver nanoparticles synthesized with varying silver nitrate concentrations from 0.2 mM to 1mM at a wavelength range of 200nm to 700nm.](image)

The appearance of the deep red/brown color was due to collective oscillation of the conduction electrons in resonance with the wavelength of irradiated light [25].

**Transmission Electron Microscopy and energy dispersive spectroscopy results**

The grid for the TEM analysis of Ag-nanoparticles was prepared by placing a drop of the nanoparticles suspension on the carbon-coated copper grid and allowing the water to evaporate inside a vacuum dryer. Scanning under TEM (Philips CM-10) revealed that the average mean size of silver nanoparticles was 18-20 nm and the particles were spherical in shape without significant agglomeration (figure 3a).
Figure 3: (a) TEM image showing spherical silver nanoparticles and (b) An EDS spectrum showing two peaks of elemental silver in the silver region.

Energy Dispersive X-ray Spectroscopy was used to verify the presence of silver in the sample. Figure 3b showed two peaks at 3.0 keV and 3.15 keV, which are due to the elemental silver. The typical optical absorption band peaked nearly at 3 KeV confirms formation of metallic silver nanoparticles [26].

Fourier Transform Infra-Red (FT-IR) spectroscopy Analysis

The FT-IR spectra of *Eucalyptus corymbia* leaf extract and synthesized nanoparticles were done to identify the possible biomolecules responsible for the reduction of the Ag\(^+\) ions and capping of the bio-reduced Ag-NPs. Figure 4 shows the FT-IR spectrum of pure *Eucalyptus corymbia* leaf extract and bio-synthesized AgNPs.

Figure 4: FT-IR spectra of plant extract and silver nanoparticles.
The major absorbance bands present in the spectrum of *Eucalyptus corymbia* were at 3270.82, 1634.24, 428.15 and 422.09 cm\(^{-1}\). The extract containing AgNPs showed transmission peaks at 3260.7, 1634.62, 1376.62, and 1243.76 and at 425.25 cm\(^{-1}\). The broad and strong bands at 3260 and 3270 cm\(^{-1}\) were due to bonded hydroxyl (–OH) stretch from phenol group or alcohol group. The medium peak centered at 1634 corresponds to –C=C– stretch from alkenes. The peak at 1376 cm\(^{-1}\) and 1243 cm\(^{-1}\) is attributed to –C–H rocking and C–O from alkoxy group, respectively. The functional groups mainly OH and –C=C– are derived from heterocyclic compounds or alkanols e.g. alkaloid, flavones and tannins present in *Eucalyptus corymbia* leaf extract and are the capping ligands of the nanoparticles [27]. The peaks at 425 cm\(^{-1}\) suggests the presence of van der Waals forces of interaction between oxygen groups in alkanol structures in eucalyptus leaf extract on the surface of Ag-NPs [28].

Therefore, the FT-IR results imply that the (–C=C) and hydroxyl (–OH) groups of *Eucalyptus corymbia* leaf extracts are mainly involved in fabrication of AgNPs. On the other hand, additional research work is needed to pin down the specific phenolic compound responsible for the reduction of silver ions.

**Effect of Synthesized AgNPS on E.coli and Staphylococcus aureus**

Silver has been employed most extensively since ancient times to fight infections and control spoilage [29]. The antibacterial activity of green synthesized silver nanoparticles was tested on *E.coli* and multi-resistant strains, specifically methicillin-resistant *Staphylococcus aureus* (MRSA). Clear halos were observed for all nanoparticle concentrations used, i.e. 0.6mM, 0.8mM, 1.0mM and kanamycin 10 (mg/ml). This is a clear indication that the growth of the two microorganisms was inhibited by the synthesised AgNPs. However, more tests are required to establish the effective amount of nanoparticles and the expected kinetics.

![Figure 5: A Muller Hinton Agar (MHA) plate with *Escherichia coli* growth. Growth inhibition zones are indicated by the clear halos for the three AgNps concentration and a positive control (Kanamycin 10mg/ml).](image-url)
Figure 6: A Muller Hinton Agar plate with *Staphylococcus aureus* growth. Growth inhibition zones are indicated by the clear halos for the three AgNPs concentration and a positive control (Kanamycin 10mg/ml).

The results showed that in MHA medium treated with silver nanoparticles, *Escherichia coli* and *Staphylococcus aureus* growth was inhibited (figures 5 and 6). The diameters of zones of inhibition of nanoparticles, especially those of 0.8mM and 1.0mM concentration, compared relatively well with that of antibiotic kanamycin, an indication of their excellent biocidal effect.

This observation is in accordance with what was reported earlier that silver nanoparticles can release silver ions [30] and these ions can interact with the thiol groups of many vital enzymes and inactivate them [12], i.e., Ag⁺ works through suppression of respiratory enzymes and electron transport components which interfere with DNA functions [13]. In the present study silver nanoparticles were found to exhibit an excellent biocidal impact and effectiveness in inhibiting bacterial growth.

**CONCLUSIONS**

The use of *Eucalyptus corymbia* leaf extract offers a simple synthetic protocol devoid of chemicals either as reducing, stabilizing or capping agents which is in line with green chemistry principles. Ferric chloride and Folin-ciocalteu’s phenol reagent tests tested positive for presence of reducing compounds. FT-IR spectra of the plant extract revealed that functional groups OH and −C=C− could be the responsible candidates for reduction and stabilization of the nanoparticles. The particles were polydispersed with average mean size of 18-20 nm and were spherical in shape without significant agglomeration as revealed from the TEM analysis. EDX spectrum revealed the strong signal in the silver region, hence confirming the formation of silver nanoparticles. Moreover, the results showed that *E.coli* and *Staphylococcus aureus* growth was inhibited on MHA plates impregnated with known concentrations of nanoparticles. A similar observation was made when Kanamycin (10mg/ml) was used as a positive control. These non-toxic nanomaterials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidal materials.

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