Biosynthesis and evaluation of the characteristics of silver nanoparticles using *Cassia fistula* fruit aqueous extract and its antibacterial activity

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Received 6 July 2017
Accepted for publication 10 October 2017
Published 7 November 2017

Abstract

There are several ways to produce nanoparticles, but the biological method of nanoparticle production is considered most efficient by researchers due to its eco-friendly and energy saving properties. In this study, the biosynthesis of silver nanoparticles (AgNPs) via *Cassia fistula* fruit pulp extract was examined. Furthermore, its antibacterial effects were investigated both in vitro and in vivo. To achieve biosynthesis, 10 ml of *C. fistula* extract was added to 90 ml of aqueous solution of 1 mM silver nitrate. The solution was incubated in darkness overnight, at room temperature. After changing the color of solution, the production of AgNPs was examined by UV–Vis spectrophotometry, XRD and DLS methods. Finally, the antibacterial activity of AgNPs was investigated by using three methods: (1) agar well diffusion, (2) MIC determining and (3) effect on prevention of infection in wound on rat models. The results revealed that synthesized silver nanoparticles have strong antibacterial activity in vitro and in vivo conditions. Undeniably, further research is required to investigate the side effects of such particles.

Keywords: biosynthesis, silver nanoparticles, *Cassia fistula*, antibacterial

Classification numbers: 2.00, 2.03, 2.04, 2.05, 4.00, 4.02

1. Introduction

Nanoparticles are particles ranging from 1–100 nm in size that display various formations such as spherical, prismatic, amorphous etc, and have significant absorbency caused by their uncommon properties. Nanoparticles are of high importance owing to their very small size and large surface to volume ratio, and they display totally unprecedented specifications compared to gross particles of the mass substance [1, 2]. Silver nanoparticles (AgNPs) is known as having an inhibitory effect against several bacterial strains and microorganisms. As a result, AgNPs can be used in various industries including textile, pharmacy, medicine, food, and cosmetics [3].

There is increasing demand to develop more eco-friendly methods for the synthesis of nanoparticles that do not require the use of noxious materials. Many methods exist for the production of metal nanoparticles, including chemical, electrochemical, photochemical and heat evaporation; however, biosynthesis, using organic systems such as yeast, fungi,
bacteria and plant extracts are developing increasing favour [4-6]. Current techniques in nanoparticle biosynthesis employ the use of green agents such as Ocimum tenuiflorum leaves [7], Azadirachta indica leaves [8], Emblica officinalis fruit [9], Jatropha curcas seed [10], Pinus desiflora leaves [11], Ocimum sanctum root stem [12], Syzygium cumini leaf [13], Zingiber officinale rhizome [14]. Using plants for the synthesis of nanoparticles can be more expedient as since it reduces the risk of using chemical raw materials, likewise, it can be used for large-scale production of nanoparticles under non-uninfected conditions [15]. That said, nowadays many plants used for biosynthesized silver and silver conjugated nanoparticles, moreover, some new studies focused on biosynthesis nanoparticles via fungal endophytes. Endophytes are a kind of fungi that can have hereditary characters of its host plant [16].

Individual properties of nanoparticles have been identified as an important option for cancer treatment. Silver nanoparticles can selectively interfere in disruption of the mitochondrial respiratory chain by disruption of adenosine triphosphate synthesis through production of reactive oxygen species [17]. Moreover, the function of nanoparticles depends on their size and shape, so it is required to have a control on size and shape of nanoparticles, which can be done via changing in synthesis methods and reduction agents [18].

This publication outlines (i) the biosynthesis of silver nanoparticles using the aqueous fruit extracts of Cassia fistula (C. fistula), (ii) the techniques used in its synthesis of including UV–Vis spectrophotometry, TEM, XRD and DLS analysis, (iii) the evaluation its antibacterial effect, and (iv) an investigation of its mutagenic potential.

2. Materials and method

2.1. Plant material and preparation of the extract

Freshly collected C. fistula fruit pulps were dried in the dark and powdered. To extraction, 50 grams of the fruit pulp powder was boiled for 3 min in 100 ml sterile distilled water and filtered through Whatman No.1 filter paper (pore size 25). This first filtrate was subsequently filtered through 0.6 sized filters, and the final filtrate was used for the present study.

2.2. Synthesis of silver nanoparticles

1 mM aqueous solution of silver nitrate was prepared and used for the production of AgNPs. 10 ml of C. fistula extract was added in 90 ml of aqueous solution of 1 mM silver nitrate and incubated overnight at room temperature in darkness [19].

2.3. UV–Vis spectra analysis

The reduction of silver ions was checked by evaluating the UV–Vis spectrum of the reaction medium after overnight incubation. The color changes can be observed visually, so coloration was noticed at the synthesis phase. The concentration of produced AgNP was measured using a PerkinElmer UV spectrophotometer (model Lambda25), at of 1 nm resolution, between 200 and 500 nm [19].

2.4. X-ray diffraction analysis

In order to measure the biosynthesis of the AgNPs by C. fistula fruit extract, the AgNPs were used for x-ray diffraction (XRD) analysis. The diffracted intensities were recorded for AgNPs from 5° to 85° of 2θ angles. All the spectra were recorded in a PANalytical Automatic X-ray Diffractometer (model X’Pert PRO MPD) [20].

2.5. Transmission electron microscopy

The regular size and morphology of the AgNPs were determined via transmission electron microscopy (TEM). A drop of sample was placed on a carbon coated copper grid, and any excess solution was removed using blotting paper. The grid was dried under an infrared lamp. Micrographs were achieved on a Zeiss EM900 transmission electron microscope with 40–120 kV accelerating voltage, 0.4 nm resolution and a Gatan SC1000 camera [20].

2.6. Dynamic light scattering

Size reparation of biosynthesized AgNPs was measured via dynamic light scattering (DLS) (Zetasure Nano ZS ZEN3600, Malvern, UK). From this evaluation, the mean size of AgNPs inside the sample was acquired. Also, the relationship between the numbers of particles of a particular size contrasted with the size of the nanoparticles [21].

2.7. Antibacterial activity

2.7.1. Agar well diffusion. An antibacterial assay was used on gram-positive and gram-negative pathogenic bacteria which were obtained from the American Type Culture Collection (ATCC). The gram-positive bacteria used was Staphylococcus aureus (ATCC 6538), and gram-negative bacteriae were Acinetobacter baumannii (ATCC 19606) and Pseudomonas aeruginosa (ATCC 27853). Each bacteria was cultured in Muller-Hinton broth (Merck, Germany) medium and incubated at 37 °C, 200 rpm for 24 h. Each strain was subsequently streaked onto two Mueller-Hinton agar (Merck, Germany) media plates with a 10 mm cellulose membrane. AgNPs were placed on one of the two identical plates for each strain of bacteria. All plates were incubated at 37 °C for 24 h and inhibition zones were measured [20].

2.7.2. MIC determination. Biosynthesized AgNPs were analyzed for their antibacterial activity against A. baumannii, P. aeruginosa and S. aureus. Several aqueous solutions of AgNPs were made, comprising of the following concentrations: 500, 250, 125, 62, 31, 15, and 7 μl ml⁻¹. Stock cultures of A. baumannii, P. aeruginosa and S. aureus were grown independently in liquid nutrient broth medium. After 24 h, the bacterial culture suspensions (50 cells ml⁻¹) were added to seven flasks, each with 25 ml of nutrient broth and one of the aforementioned concentrations of AgNPs. The solutions were kept at 37 °C in an incubator for 24 h. A silver nitrate solution was used as a control. The minimum inhibitory concentration
(MIC) was determined as the lowermost concentration that inhibited an observable growth of the bacterium used [21].

2.8. Coating AgNPs treatment on cotton fabrics

The cotton samples were treated by primary concentration of AgNPs and 55 g 1−1 of 1,2,3,4-butanetetracarboxylic acid (BTCA) in the impregnating bath for 5 minutes. The concentration of SHP sodium hypophosphite (SHP) which was used in final stage of bathing was 60% of BTCA. The cotton samples were padded (90% wet pick up) and desiccated in 25 °C for 24 h. They were then desiccated in an oven at 180 °C for 3 minutes [22].

2.9. Antibacterial test on treatment fabrics

The antibacterial activities of silver coated cotton samples were tested against 
S. aureus (ATCC 66538), Acinetobacter baumannii (ATCC 19606) and P. aeruginosa (ATCC 27853) using the parallel streak method (AATCC 147–1998). The Mueller-Hinton agar solution (105437, Merck) was sterilized at 120 °C in an autoclave for 20 minutes then poured into disposable sterilized Petri dishes (100 mm diameter) for solidification. Each strain was cultured for 24 h in a sterilized modified tryptone soya broth (CM0989, Oxoid), and then 0.1 ml culture solution was diluted with 0.9 ml soya broth to a concentration of 6 × 107 CFU ml−1 (colony forming units in 1 ml), measured optical density (OD) by PerkinElmer UV spectrophotometer (model Lambda25) at 550 nm wavelength. One loopful of the diluted inoculum was streaked onto a plate in five parallel lines using a 4 mm inoculating loop. The cotton samples (2.5 × 5 cm2 in size) were gently pressed through the five inoculum streaks in the transverse fashion. Petri dishes were incubated at 37 °C for 24 h, and the zone of inhibition of growth along the streaks was measured [23].

2.10. In vivo animal trials and surgical protocols

The in vivo wound healing rate was evaluated by using 21 Sprague-Dawley 60 d aged female rats with a regular body weight of 250g. The rats were separated into 7 groups. Each rat was secured in a cage and fed individually. The rats were anesthetized via celiac injection of pentobarbital (30 mg kg−1 body weight). A circular (diameter 20 mm), full-thickness (depth 2.8–3.3 mm) incision was made through the skin of each rat on its flank using a pair of surgical scissors. Bacterial solutions of the pathogens Staphylococcus aureus (S. aureus), Acinetobacter baumannii (A. baumannii) and Pseudomonas aeruginosa (P. aeruginosa) were used to cause wound infection in the rats in 10−3 dilution of 0.5 McFarland turbidity standard (1.5 × 108 CFU ml−1). Rats in groups 1 and 2 were infected with P. aeruginosa, rats in groups 3 and 4 were infected via S. aureus, and rats in groups 5 and 6 were infected with A. baumannii. Rats in group7 were kept as negative controls (not infectious). Infection was confirmed by the bacterial culture of wound. Wound dressings were sterilized by ultraviolet light and applied onto the wounds of all rats as follows: the wound area of groups 1, 3 and 5 were covered with sterile gauze as positive controls and the wound area of groups 2, 4 and 6 were covered with AgNPs treated wound dressings as test groups. The control group was not treated with any wound dressing. Rats were pursued for 21 d and fresh dressings were applied if the old ones fell off. The percentage of post-surgical wound healing was calculated at day 7 (PWH7), at day 14 (PWH14) and day 21 (PWH21), as follows [23]

\[
\text{Wound healing (\%) = } \frac{A_0 - A_{\text{scar}}}{A_0} \times 100,
\]

where \(A_0\) is diameter of the wound in the initial time and \(A_{\text{scar}}\) is the diameter of the wound at day 7, day 14 and day 21, respectively.

3. Result and discussion

3.1. UV–Vis analysis

Cassia fistula fruits have been described to subtend compounds such as 2(3H)-furanone, rhein, thymol, oleic acid, inositol, palmitic acid, and 2-pyrrolidone [24]. The water-soluble components existing in aqueous extract are in charge of reducing metal ions and producing nanoparticles. Unlike results from previous publications, in this study the color of the solution at the onset of the reaction was dark brown,
and subsequently changed to slime green in about 24 h. These color changings are owed to the instigation of surface plasmon resonance (SPR) in metal nanoparticles, as metal nanoparticles such as silver have free electrons [25]. The biosynthesis of AgNPs was confirmed by UV–Vis spectrophotometry analysis. The examination displayed an absorbance peak of approximately 418 nm (figure 1), which was known for AgNPs.

3.2. XRD result
Bragg peaks resulting from the XRD study demonstrated the centered cubic structure of AgNPs. Intense peaks were detected at 38.20°, 44.29°, 64.48°, and 77.39°. The expanding of the Bragg peaks showed the production of AgNPs. The results were analyzed via X’Pert Highscore plus software and confirmed. The results are showed in figure 2.

3.3. TEM microscopy
TEM analysis demonstrated the production of AgNPs, as displayed in figure 3. The AgNPs were triangular, hexahedral and amorphous in form and their typical sizes were about 50–150 nm. Other studies showed different results of TEM method. As an illustration, Mittal et al reported the size of produced silver nanoparticles by Xanthium strumerium were about 20–50 nm with spherical shape. They suggested the cause of multi-size nanoparticles is attendance of various capping agents [26]. Another study that was done by Erjaee et al reported the size of biosynthesized nanoparticles about 24 nm. However, they did not mention to cause of differences between nanoparticles’ size [27]. In Ajayi et al investigation [28] they found that the size of nanoparticles was in the range 10–33 nm with spherical shape. Overall, with attention to previous studies, it can be understood that most of the biosynthesized nanoparticles are spherical, however triangular and hexahedral shapes were rare between nanoparticles. So, the mechanism of nanoparticles formation should be analyzed more accurately.

3.4. DLS analysis
The DLS templates (figure 4) showed that synthesized AgNPs have a Zeta average diameter of 31.16 nm with a polydispersity index (PDI) of 0.605. The observed size using DLS analysis is not the same as compared to the particle size detected from TEM micrographs because the DLS method measures the hydrodynamic radius. It seems, measurement nanoparticle by electron micrograph should be prudential done.

The results of Erjaee et al [27] approved our experiments. They expressed that the measured size by DLS test was substantially larger than TEM size. They concluded that these differences are due to the difference between TEM and DLS method in measurement. In TEM method, the based size distribution is measured only. However, in DLS method hydrodynamic diameter is measured which is included diameter of...
the particle and ions or molecules that are associated with the surface of nanoparticles [27].

### 3.5. The antibacterial effect of AgNPs

AgNPs demonstrated good antibacterial activity against *S. aureus*, *P. aeruginosa*, and *A. baumannii* as seen by the inhibition zone size (table 1). Various theories have been provided to explain the antibacterial effect of AgNPs, though the exact mechanism is not known. Hammouda et al. [29] and Dibrov et al. [30] reported that the positive charge on the silver ions is responsible for the antibacterial activity of AgNPs through the electrostatic interaction between the positive and negative charge of AgNPs and the cell membrane. In a different scenario the cell wall permeability of *Escherichia coli* was increased significantly by AgNPs due to the formation of pits within the cell wall [31]. The nanoparticles are thought to bind the bacterial cell wall, however, the exact mechanism of interaction between AgNPs and cell wall is still unknown. Govindappa et al. [32] indicated in their study that silver nanoparticles can inhibit the bacterial growth through demolishing bacterial cell wall.

The MIC of AgNPs against *P. aeruginosa*, *A. baumannii* and *S. aureus* were 62 µl ml⁻¹, 62 µl ml⁻¹ and 125 µl ml⁻¹, respectively. Antibacterial analysis showed an intense dose-dependent antibacterial activity versus all three of the test bacteria. Synthesized AgNPs were detected to exhibit more antibacterial activity toward gram-negative bacteria than toward gram-positive bacteria. Our MIC results were significantly more than Ebrahiminezhad et al. (37.5 µl ml⁻¹ for *S. aureus*) [33], however, was nearly equal to results in some previous studies (about 60 µl ml⁻¹) [31, 34, 35]. Rao and Tang suggested that silver nanoparticles can destroy bacteria via three primary paths: (i) binding to the surface of the cell membranes, (ii) entering into the bacterial cells, and (iii) affecting the cell respiration in bacteria [36]. Further research is necessary to elaborate on these findings in the future.

### 3.6. Antimicrobial activity of AgNPs treated fabric

The antibacterial activity of biosynthesized AgNPs treated fabric was investigated using parallel streak (AATCC-147) method via *S. aureus*, *P. aeruginosa* and *A. baumannii*. From figure 5 it is evident that, although there is no clear zone of inhibition in the first and second bacterial streaks, there is a clearly visible zone of inhibition in the remaining streaks. This zone of inhibition could be due to the decreased concentration of bacteria on the bottom lines. Nevertheless, bacterial growth was not observed in any of the lines residing under the treated fabric. These results are indeed consistent with the findings previously published by Scholz et al. [37], Tessier et al. [38] and Ali et al. [39].

### 3.7. In vivo antibacterial activity of AgNPs treated fabric

Most of the infected rats in groups 1, 3 and 5, whose wound was treated with sterile gauze died in the second week of the experiment, and none of these rat lived to see the third week. In contrast, the rats in groups 2, 4 and 6, whose wound was dressed with AgNPs treated fabrics, survived and showed marked wound healing (table 2). From this observation of the difference between groups, we can confer that AgNPs has bactericidal properties that result in the stunting of infection. Indeed, the rats who were treated with AgNPs treated fabrics demonstrated a significant reduction in the period of epithelialization.

**Figure 4.** DLS pattern of produced AgNPs with distribution by (a) number and (b) volume.

**Table 1.** Inhibition zone size using the paired samples t-test for AgNPs and *C. fistula* extract groups.

| Bacteria       | Dilutions | Inhibition zone size (mm) |
|----------------|-----------|---------------------------|
| *S. aureus*    | AgNPs     | 14.76 ± 0.88a             |
|                | *C. fistula* extract | 6.33 ± 0.66         |
|                | AgNO₃     | 5.33 ± 0.33               |
| *A. baumannii* | AgNPs     | 14.33 ± 0.88b             |
|                | *C. fistula* extract | 6.33 ± 0.33         |
|                | AgNO₃     | 6.00 ± 0.57               |
| *P. aeruginosa*| AgNPs     | 16.33 ± 0.88b             |
|                | *C. fistula* extract | 16.33 ± 0.88b        |
|                | AgNO₃     | 5.33 ± 0.33               |

a $p < 0.05$.

b $p < 0.01$. 

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Adv. Nat. Sci.: Nanosci. Nanotechnol. 8 (2017) 045019
There are several reports about the effects of nanosilver coated fabrics on burning wound healing in animal models. Liu et al [23] in 2009 studied in vivo wound healing on electrospun nanofibre membranes. Liu et al reported that polyvinyl alcohol linked AgNPs fabrics have very good antibacterial activity and result in significantly better wound healing than other textiles. Moreover, Leaper [40] in 2006 presented that AgNPs fabrics decrease infection incidence in open wounds and are effective against cross purulence. Given these promising findings, further studies would be of benefit, as advances in this research could have great implications in the field of medicine as it relates to morbidity and mortality secondary to wound infections.

4. Conclusion

It is concluded that the C. fistula fruit pulp extract is able to synthesize AgNPs at room temperature, and so biosynthesized AgNPs can improve the antibacterial effect of the extract. In the present study silver nanoparticles have shown a considerable antibacterial effect the in vitro and in vivo environments. Consequently, it can be concluded that AgNPs have a beneficial, measurable and significant effect in wound healing. Though the usage of nanosilver as an antimicrobial agent in textiles is growing and has shown promise, the possible side effects of metal nanoparticles remain unknown and could be a substantial issue. Therefore, further dedicated research is required to investigate the advantage and disadvantage of metal nanoparticles.

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