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Biosynthesis of Silver Nanoparticles using *Pistacia lentiscus* Leaves Extract and Investigation of their Antimicrobial Effect

GHADIR A. EL-CHAGHABY* and ABEER F. AHMAD

Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), Giza (Egypt).
*Corresponding author: E-mail: ghadiraly@yahoo.com

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ABSTRACT

The biosynthesis of silver nanoparticles was successfully achieved using *Pistacia lentiscus* leaves extract and silver nitrate. The plant extract was mixed with silver nitrate, incubated at 50°C and synthesis of nanoparticles was followed using UV–Vis spectroscopy. The reaction was completed in three hours and the resultant nanoparticles were characterized using XRD and TEM techniques. The average size of the produced nanoparticles was found to 24-26 nm. The antifungal and antibacterial activities of the biosynthesized silver nanoparticles were investigated using disc diffusion method. The results showed that these nanoparticles displayed antimicrobial a good activity against fungal as well as bacterial cultures.

Key words: Plant extract; UV-Vis spectroscopy, XRD, TEM, Antimicrobial activity.

INTRODUCTION

In the recent years, nanotechnology has emerged as a new fast growing research area for several applications. With the increasing concern about the environment, using green technologies for the synthesis of nanomaterials is a key challenge.

Silver nanoparticles play a profound role in the field of biology and medicine due to their attractive physiochemical properties. Silver products have long been known to have strong inhibitory and bactericidal effects, as well as a broad spectrum of antimicrobial activities, which has been used for centuries to prevent and treat various diseases, most notably infections[1]. Due to the extraordinary antimicrobial properties of silver and the low toxicity of free silver ions to mammalian cells, the interest for food applications of these particles is increasing[2]. However, the EU safety regulation[3] which rules the presence of silver ions in food matrices and limits its amount to 0.05 mg Ag/kg must be satisfied.

Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles (Ag NPs) as stable, colloidal dispersions in water or organic solvents[4,5] .

Recently, the methods employing plants as mediators for the synthesis of nanoparticles have

drawn attention as cleaner and environmental friendly alternates for chemical procedures. Plants as autotrophs have this edge over others in terms of their morphological organization, molecular distribution, interaction of metabolites during metabolic fluxes (both primary as well as secondary). It is indeed their chemical constitutions (or metabolic status) which provides them strength to withstand environmentally diverse habitats. These chemical constitutions have enormous significance for the surviving humanity in various manners[6].

Plants such as *Nelumbo nucifera*[7], *Ocimum sanctum*[8], *Pongamia pinnata*[9], *Chenopodium album* leaf[10], *Mangosteen* leaf[11], *Mangifera indica* leaves[12] and others, have been reported to form silver metal nanoparticles.

P. lentiscus L. is an evergreen shrub of the *Anacardiaceae* family[13]. *P. lentiscus* (mastic tree) is well known in Mediterranean countries for its resin, mastic gum, used since antiquity for incense, as a chewing gum for pleasant breath, for spicing liqueurs and jam, and in the cosmetic industry[14]. The tree is widely distributed in Egypt.

P. lentiscus leaf extracts have been shown to exhibit moderate antimicrobial activities[15]. Yet to the best of our knowledge no study found in the literature have reported the use of *P. lentiscus* leaves in biosynthesis of silver nanoparticles. The aim of the present study was to investigate the biosynthesis of silver nanoparticles using *P. lentiscus* leaves and evaluation of the antibacterial effect of the prepared nanoparticles.

MATERIAL AND METHODS

Preparation of *Pistacia lentiscus* extract

Fresh leaves of *P. lentiscus* were collected, washed thoroughly in running tap water, and then the leaves were rinsed with Deionized water and cutted into small pieces. Plant extract was prepared following the procedure described by Jha *et al.,*[6]. Five g of the leaves were weighed into a 250 ml beaker containing 200 ml 50% Et-OH and were placed on boiling steam bath for 15–20 min till color of the solvent changes to light green. The extract obtained was filtered through Whatman No. 1 filter paper and the filtrate was collected in 250 ml Erlenmeyer flask and stored at 4 °C for further use.

Synthesis of silver nanoparticles (AgNPs)

Aqueous solution (0.025M) of silver nitrate, obtained from Aldrich, was prepared and used for the synthesis of silver nanoparticles (AgNPs). 30 ml of the previously prepared plant extract was added to 30 ml of 0.025M $AgNO_3$ solution. The reaction mixture was incubated at 50°C until visual color change was observed.

Characterization of silver nanoparticles

The bioreduction of silver ions was monitored by measuring the UV-Vis spectra of the reaction medium at different time intervals after diluting a small aliquot of 200 µL of the sample by 10 times. The UV- Visible spectral analysis was performed using a Labomed spectrophotometer UV-VIS Double Beam Model UVD-3500 operated at a resolution of 1nm.

The XRD spectra were taken with an X-ray diffractometer at room temperature, using Cu K_α radiation λ = 1.5406 Å over a wide range of Bragg angles ($10° \leq 2\theta \leq 80°$).

The size of the nanoparticles was calculated through the Scherer's equation[16].

$$D = K\lambda / \beta \cos \theta$$

Where *D* is the average crystal size, *K* is the Scherer coefficient (0.89), λ is the X-ray wave length (λ = 1.5406 Å), θ is Bragg's angle (2θ), β the full width at half maximum (FWHM) in degrees.

Transmission electron microscope (TEM) samples of the aqueous suspension of AgNPs were prepared by placing a drop of the suspension on carbon-coated copper grids and the films on the TEM grids were allowed to stand for 2 min, after which the extra solution was removed using a blotting paper and the grid was allowed to dry prior to measurement. TEM observations were performed on an Electron Microscope model JEM-100s Joel (Japan) operated at an accelerating voltage of 100 kV (Magnification: 70000 X). The size distribution of the resulting nanoparticles was estimated on the basis of TEM micrographs.

Antimicrobial assay

Antimicrobial activity of the biosynthesized AgNPs was determined using a modified Kirby-Bauer disc diffusion method[17]. Two fungal species (*Aspergillus flavus* and *Aspergillus niger)* and six bacterial species (*Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus faecalis*) were used in the study. Briefly, 100 µl of the bacteria grown in 10ml of fresh media until they reached a count of approximately 10^8 cells/ml for bacteria[18]. 100µl of microbial suspension was spread onto Mueller Hinton (MH) agar[19,20] in Petri plates corresponding to the broth in which they were maintained. An aliquot [10 µl] of each of the biosynthesized Ag nanoparticles was pipetted on a sterile paper disc (Whatman No. 1, 5.5 mm paper disc) on the agar surface. The plates were inverted and incubated for 24-48h at 37 °C in case of bacteria and for 48h at 25 °C in case of fungus. Microbial inhibition was determined by measuring the diameter of the clear zone of inhibition of growth around each disc and recorded as diameter of inhibition zone in millimeter. The zone diameters were measured with slipping calipers of the National committee for clinical Laboratory Standards[19].

RESULTS AND DISCUSSION

Visual observations and UV–visible spectroscopy

Few minutes after incubating the reaction mixture at 50°C, its color changed from pale green to light brown which turned to dark brown after 3 hours (Fig. 1). This change in color of the reaction mixture was taken as a primary evidence for the formation of silver nanoparticles. The characteristic brown color of silver solutions provided a convenient spectroscopic signature to indicate their formation[21].

Fig. 1: Visual observation for the change in color of the reaction mixture after incubation at 50°C for 3 h

Silver nanoparticles exhibits interesting optical properties directly related to Localized Surface Plasmon Resonance (LSPR) which is highly dependent on the morphology of the nanoparticles[22]. The UV–Vis spectra of AgNPs synthesized by *P. lentiscus* leaves are shown in Fig. 2. According to *Huang* and *Yang,*[23] a typical plasmon resonance band of AgNPs is confirmed by an absorption band in the range of 400-450 nm. As can be seen from the UV–Vis spectra the formation of AgNPs was detected by the distinct broad peak obtained at 450 nm. The broadness of this peak was taken as an indication on the polydispertion of the formed AgNPs [11]. The absorption of the obtained peak was found to increase by increasing the reaction time between *P. lentiscus* extract and silver nitrate solutions. As the duration of reaction increases, more silver nanoparticles are formed. Due to the instability of the silver nanoparticles formed, an optimum duration is required, as silver nanoparticles agglomeration after the optimum duration resulting in larger particle sizes 11. In our present study the optimum time required for the formation of AgNPs was found to be 3h.

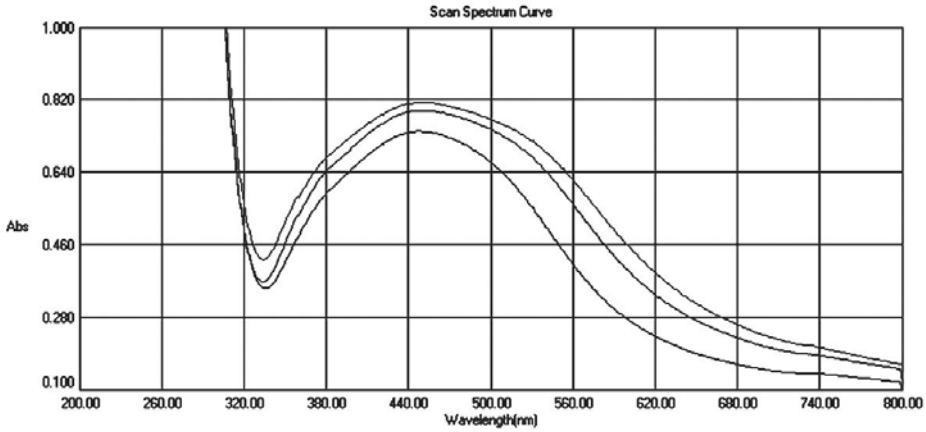

Fig. 2: UV-Vis absorption spectra of silver nanoparticles obtained from mixing equal volumes of *P. lentiscus* extract with 0.025M Silver nitrate and incubation at 50°C

X-ray diffraction (XRD) and Transmission Electron Microscopic (TEM) studies

The crystalline nature of AgNPs was confirmed from the analysis of the X-ray diffraction (XRD) pattern (Fig. 3). The XRD pattern was then analyzed and the FWHM was used with the Scherrer's formula to determine mean particle size of the biosynthesized Ag nanoparticles. The mean size of nanoparticles estimated by XRD was found to be 26 nm.

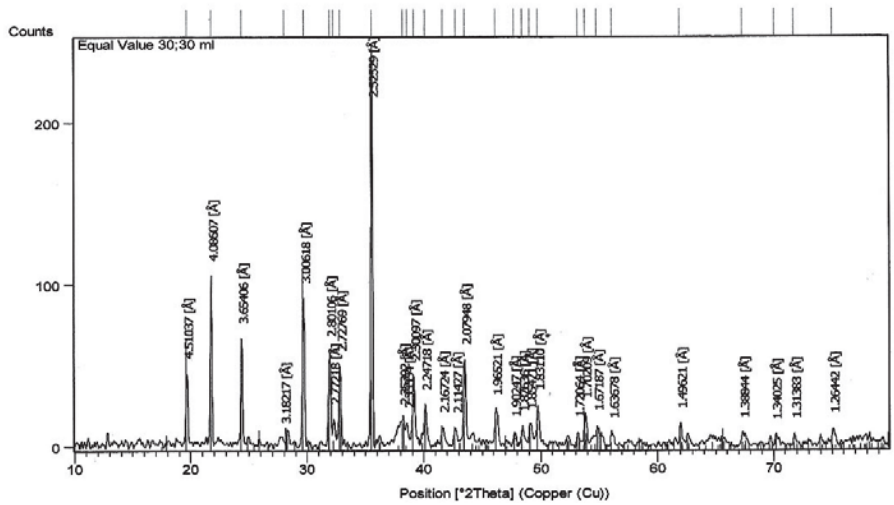

Fig. 3: XRD pattern of biosynthesized silver nanoparticles

Fig. 4 illustrates the TEM micrographs of the AgNPs being formed using *P. lentiscus* leaves extract. The micrograph was used to elucidate the shape and size of the resultant particles. Most of the formed nanoparticles were found to be spherical in shape and their mean size was determined to be 24 nm.

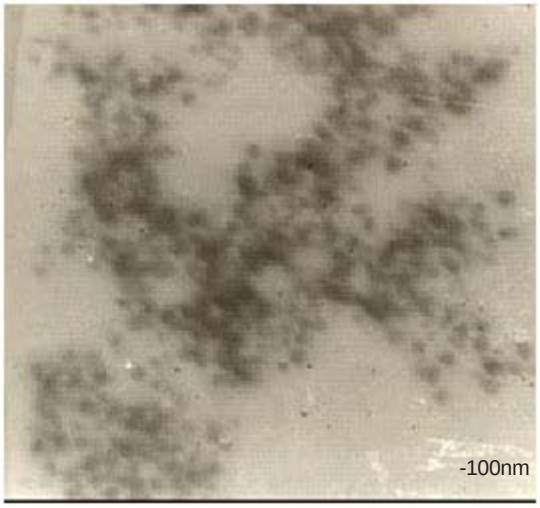

Fig. 4: TEM image of AgNPs biosynthesized using *P. lentiscus* leaves extract

The data obtained from XRD and TEM studies gave a further confirmation on the bioreduction of Ag^+ to Ag^0 upon the reaction between $AgNO_3$ and *P. lentiscus* leaves extract. The size of the formed nanoparticles as determined from XRD and TEM studies were found to be very close.

Antimicrobial efficacies of biosynthesized nanoparticles

The Antimicrobial effect of the biosynthesized AgNPs was examined using the disk diffusion assay which is mainly used to test the sensitivity of bacterial strains towards antibiotics[24] with a clear zone around the disk reflects the bacterial sensitivity towards antibiotics 25. The mean diameters of inhibition zones obtained in the present study and earlier studies are given in table (1). The results showed that AgNPs biosynthesized using *P. lentiscus* leaves extract showed good inhibition against the six studied bacterial strains and against the fungi *Aspergillus flavus* whereas they did not show any efficacy in the inhibition of *Aspergillus niger*. This observed antimicrobial activity could be explained by the fact that Ag NPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell[26] Smaller Ag NPs having the large surface area available for interaction would give more bactericidal effect than the larger AgNPs[26]. The interaction of silver ions with microbial cytoplasmic components and nucleic acids can inhibit the respiratory chain enzymes, and interferes with the membrane permeability, limiting the development of bacteria and yeasts[27]. It is also possible that AgNPs not only interact with the surface of membrane, but can also penetrate inside the bacteria[28]. The susceptibility of Gram positive and Gram negative bacteria to biosynthesized AgNPs was found to vary from one study to another. According to *Nagajyothi and Lee*[29], AgNPs were found to be significantly toxic against the fungal and gram positive microbes and exhibited mild toxicity against *E,coli*. Whereas *Antony et al.,*[30] reported that AgNPs had a considerably minimal microbicidal activity on Gram positive bacteria compared to Gram negative bacteria which they attributed to the high lipopolysaccharide and thick peptidoglycan layer of the microorganisms. The negatively charged

Table 1: Antimicrobial activity of silver nanoparticles biosynthesized from *Pistacia Lentiscus* leaves extract and other plant extracts found in litterature using disc-diffusion method

Microorganism (Gram reaction)	Inhibition zone diameter in mm
Bacillus subtilis (G^+)	14 (present study); 18[31]; 11[30]
Staphylococcus aureus (G^+)	13(present study) ; 14[31]; 14[30]; 12[33]
Streptococcus faecalis (G^+)	13 (present study)
Neisseria gonorrhoeae (G^-)	12 (present study)
Pseudomonas aeruginosa (G^-)	14 (present study); 12[30]; 7.3[32]; 9[33]; 4.7[34]
Escherichia coli (G^-)	13 (present study); 17[31]; 14[30]; 7[33]
Aspergillus flavus (fungi)	11 (present study)
Aspergillus niger (fungi)	0.0 (present study)

AgNPs can bind to Gram negative cell wall better. Our results revealed that AgNPs biosynthesized using *Pistacia Lentiscus* leaves extract exerted nearly similar antibacterial activity against both Gram positive and gram negative bacteria. In line with our results *Rastogi and Arunachalam*[25] noticed AgNPs synthesized using garlic extract produce equal sensitivities towards *P. aeruginosa* and *S. aureus.*

Silver ions were biologically reduced to metallic silver nanoparticles by mediation of *Pistacia lentiscus* leaves extract. The formation of the bioreduced silver nanoparticles was confirmed by visual observation of color change as well as by the distinct peak obtained at 450 nm in the UV-Vis spectra. The size of the biosynthesized nanoparticles was found to be 24-26 nm. The biosynthesized AgNPs using *P. lentiscus* leaves extract exerted good antibacterial activity against both *Gram positive* and *Gram negative* bacteria as well as a good antifungal efficacy towards *Aspergillus flavus.* The present work highlights the use of *P. lentiscus* leaves extract for the biosynthesis of AgNPs in an environmental friendly method. More studies could be further made for optimizing the biosynthesis reaction and also for the application of the biosynthesized AgNPs in food systems as preservatives.

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