Antibacterial activity of biosynthesized silver nanoparticles against *Pseudomonas aeruginosa in vitro*

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Summary

This study was conducted for the synthesis of silver nanoparticles by using olive leaves aqueous extract and evaluating its antibacterial activity against *Pseudomonas aeruginosa in vitro*. The synthesis and characterization of silver nanoparticles was confirmed by Ultra Violet Visible – spectrophotometer and Scanning Electron Microscopy. Well diffusion method was used to show the antibacterial action of silver nanoparticles against *Pseudomonas aeruginosa in vitro* in comparison with standard antibacterial silver sulfadiazine by using different concentrations of each agent ranged from 12.5-200 µg/ml. The results of this study showed it possible to produce silver nanoparticles in eco-friendly and easy process and UV-Visible absorption spectra of the silver nanoparticles revealed maximum absorbance at 420 and 430 nm. The Scanning Electron Microscopy analysis demonstrated that the mean of the silver particles diameter was 26 nm. The antibacterial findings of the synthesized silver nanoparticles against *Pseudomonas aeruginosa in vitro* showed that the silver nanoparticles were more effective than silver sulfadiazine against *Pseudomonas aeruginosa*. It could be concluded that olive leaves extract can be used effectively in the production of silver nanoparticles and these synthesized nanoparticles had considerable antibacterial activity against *Pseudomonas aeruginosa in vitro*.

Keywords: Silver nanoparticles, *Pseudomonas aeruginosa*, Antibacterial activity.

Introduction

Nanotechnology was predictable to open certain novel viewpoints to treat and prevent diseases by atomic measure skills of materials. Capability to discover the construction and role of bio-systems at the nano size encourages investigation lead to progress in medicine field (1). Nanoparticles (NPs) were gatherings of atoms in the scale of 1-100 nm. (2). Silver nanoparticles (AgNPs) were the most rapidly growing classes of nanoproducts (3). Silver has been used for many years in different fields of medicine as broad-spectrum antimicrobial agents (4), in cancer therapy (5) and in wound healing (6). The activity of AgNPs. was higher than silver ions because of the very small size and high surface area (7). Due to the environmental effects and energy consumption by using chemical and physical methods for AgNPs. production, the choice of biological method was most desirable, because this method was found to be non-toxic and eco-friendly (8). Biogenic synthesis of AgNPs. by using plants and micro-organisms was beneficial because its decreases ecological effect in comparison with the other methods of synthesis, and it can be used to yield high amounts of NPs with no contamination and have a distinct morphology and size (9 and 10). The potent antibacterial properties of AgNPs. have been studied widely and provide hopeful findings for upcoming antibacterial agents (11). With the increasing number of infections due to multidrug resistant bacteria, there has been a need for effective antimicrobial alternatives. Thus, silver was a great alternative because it was an antiseptic that targets a wide range of G+ and G- bacteria (12 and 13). The present study was conducted to synthesis the AgNPs biologically by olive leaves extract and evaluate its antibacterial activity against *P. aeruginosa in vitro*.

Materials and Methods

Freshly leaves of olive collected from local olive trees in Baghdad. Later these plant leaves were washed under tap water, and then dried in room temperature at shade. The dried leaves were crushed to a fine powder by an electrical grinder. The plant classification was done in the Ministry of Agriculture/ State Board for Seeds Testing and Certification S.B.S.T.C in

Abu Ghraib /Baghdad at certificate No. 1077 in 26/ 3/ 2014. The extract was made by putting 50 gm. of prepared powder in 500 ml of sterile distilled water then the mixture heated for 10 min. till the color of the mixture become faint yellow. At that point the obtained extract cooled to room temperature and filtered (14).

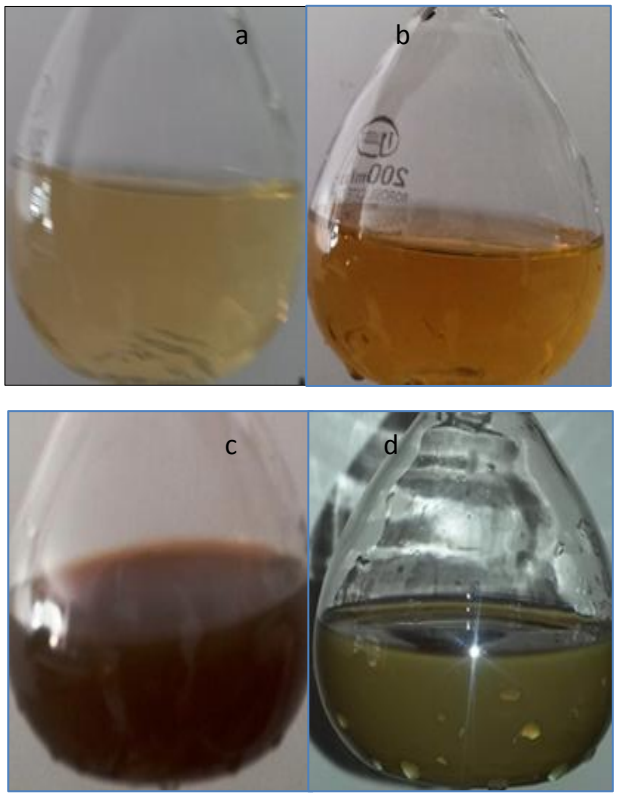
The synthesis of AgNPs was made by mixing 100 ml. of 10^3M AgNO3 solution with 5ml. of olive leaves extract with stirring to give a faint yellow solution at room temperature. The blend heated in a water bath at (40℃ and 60℃). The changing in color of the blend was monitored at different temperatures and times as explained here: 40℃ and 5 min of reaction time, 60℃ and 10 min. of reaction time and 60℃ and 15 min. of reaction time. The AgNPs obtained by centrifugation of the blend at 15,000 rpm for 10 min. then re-disposed in sterile distilled water to eliminate of any awkward materials (14).

UV-vis. Spectrophotometer was used to study the optical features of biologically synthesized AgNPs. This apparatus used to confirm creation and constancy of AgNPs in sterile distilled water in wavelength ranged from 200 to 800 nm. The reduction of Ag+ to Ag° was checked by spectrophotometer after mixing the extract with $AgNO_3$ solution, and the measuring was at regular intervals from 0 till 15 min. (15). The shape and size of synthesized AgNPs. were examined at advanced nano search center by using Scanning Electron Microscopy (SEM) as following: Dropping very small amount of the sample on specialized grid (carbon coated copper) (16).

The agar well diffusion method was adopted according to (17), for assessing the anti- *pseudomonas* activity of the prepared AgNPs. and standard antibacterial silver sulfadiazine (SSD). After mixing the bacteria with the agar, 6 wells with 6 mm in diameter were made in each agar, after that wells were filled with 100 microliter of each concentration of AgNPs and SSD which ranges from 12.5-200 µg/ml i.e. 12.5, 25, 50, 100 and 200 µg/ml for each antibacterial while the sixth well filled with distilled water. The plates were then incubated in the upright position at 37°C for 24 hours. Three replicates were carried out for each concentration of antibacterial and the activity was determined by measuring the diameter of inhibition zone around each well by millimeter against the tested organism.

Results and Discussion

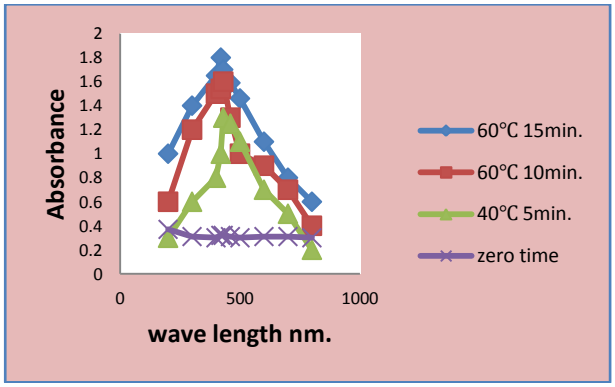
The current study provided evidence that the olive leaves were great source for synthesizing stable AgNPs in lesser time. When olive leaves extract was mixed with the $AgNO_3$ solution at room temperature gave pale yellow color, after 5 min. of reaction time at 40℃ the color of the solution altered from faint yellow to profound or deep yellow color at 40℃ and 5 min. of reaction time. This change in color indicating formation of AgNPs. due to reduction of Ag+. When the temperature of water bath increased to 60℃ for 10 min. the color of blend altered to deep brown color, and finally the color of the mixture became grey-black at 60℃ after 15 min. of reaction time (Fig. 1).



Figure, 1: Color change during synthesis of silver nanoparticles a- Pale yellow color when olive leaves extract was mixed with the silver nitrate solution. b- Deep yellow color at 40℃ after 5 min. of reaction time. c- Deep brown color at 60℃ after 10 min. of reaction time. d- Grey-black color at 60℃ after 15 min. of reaction time.

The mechanism by which the plant extract could be synthesized AgNPs may be explained by the higher total content of phenols and flavonoids (18 and 19). These phenols and flavonoids have high reducing capacity which leads to formation of the AgNPs (20 and 21). These NPs. exhibited yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in AgNPs, and this result has been previously obtained by several investigators (22-24). Increasing the temperature of water bath to 60℃ and 10 min. of reaction time, the color of mixture changed to deep brown color and at 15 min. of reaction time at 60℃ the deep brown color changed to grey-black, this further color change was due to increased concentration of NPs. (25) with assisting of temperature .

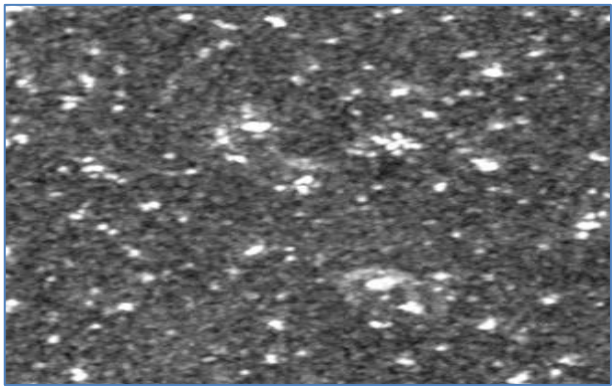
The resulted color of mixing olive leaves extract with AgNO3 solution was pale or faint yellow color, examination the mixture at this time which considered zero time by Uv-vis spectra revealed no sign and no peak for the synthesis of AgNPs. At 40℃ after 5 min. of the reaction time, the color altered from faint yellow to deep yellow because of surface plasmon vibrations of the AgNPs. became excited which produced a peak centered at 420 nm. which corresponds to the absorbance of AgNPs. Also at 60℃ after 10 min. of the reaction time and 60℃ after 15 min. of the reaction time the peak of the absorbance at 430 nm. and 420 nm. Respectively, (Fig. 2). This result agreed with several studies showed that the AgNPs surface plasmon vibrations peak at around 420 nm. (23, 26 and 27).



Figure, 2: UV-Vis absorption spectra of AgNPs synthesized by olive leaves extract at different times and temperatures.

NPs. have optical properties that were sensitive to size, shape, concentration and agglomeration state and strongly interact with specific wavelengths of light. This strong interaction with light occurred because the conduction electrons on the metal surface undergo a collective oscillation when they were excited by light at specific wavelengths and this oscillation is known as a surface plasmon resonance (SPR), and it causes the absorption and scattering intensities of AgNPs to be much higher than identically sized non-plasmonic NPs., so at zero time, Uv-vis. spectra showed no peak because the AgNPs not formed yet i.e. there was no any concentration of AgNPs, whereas at 5, 10 and 15 min. after reaction, there was increase in absorbance due to increase NPs. concentration and the Uv-vis. spectra showed peak at 420 nm. and 430 nm, which corresponds to the absorbance of AgNPs (28). These peaks appeared single and narrow which mean the biologically synthesized AgNPs. possibly were stable and didn't aggregated, which may be due to AgNPs. had negative surface charge and this high negative charge of particles increases its stability (more than 1 year) due to repulsion between the particles that avoid its aggregation (29).

SEM gave further understanding into the size and shape details of the synthesized AgNPs. The outcomes demonstrated that the mean diameter of produced AgNPs. were around 26 nm. Also the image showed relatively spherical shaped NPs. (Fig. 3).



Figure, 3: SEM image showed synthesis of AgNPs with mean diameter 26 nm with relatively spherical.

Antibacterial activity of green synthesized AgNPs and SSD against *P. aeruginosa* at different concentrations showed dose-

dependent antibacterial activity against the tested bacterium. It was seen that, when the concentration of antibacterial agent increased, bacterial growth decreases (Table, 1 and Fig. 4). The comparison between two antibacterial agents (AgNPs and SSD) revealed that the AgNPs were more effective than SSD against *P. aeruginosa*, and there was significant difference in antibacterial activity of all concentrations of AgNPs as compared to concentrations of SSD at (P<0.05). Also the results showed there was significant difference between concentrations (12.5, 25, 50, 100 and 200 µg/ml) of SSD against *P. aeruginosa* at (P<0.05) in preventing bacterial growth (inhibition zone) except between 12.5 µg/ml and 25 µg/ml concentrations, which produced restraint zone 11.40±0.18 and 11.50±0.11 mm. respectively, which statistically not different.

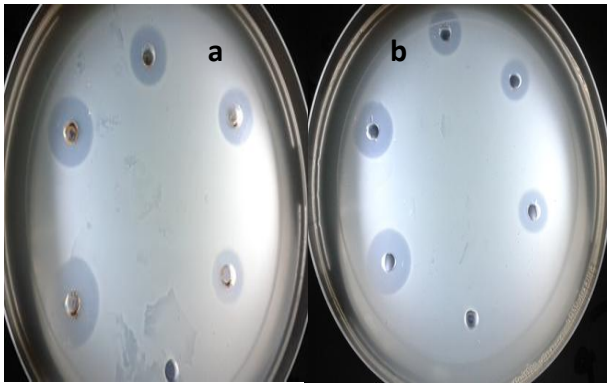
Also there was significant difference between the antibacterial effect of different concentrations (12.5, 25, 50, 100 and 200 µg/ml) of AgNPs against *P. aeruginosa* at (P<0.05) and the concentration 200µg/ml produced highest zone of inhibition against *P. aeruginosa* which was (21.40±0.33 mm) as compared with inhibition zone of the other concentrations of AgNPs (12.5, 25, 50 and 100 µg/ml) which were (13.00±0.45, 14.50±0.16, 15.60±0.25 and 19.00±0.38 mm) respectively. The anti- *pseudomonas* effect of AgNPs may be due to the ultrafine size and large surface area, the utilization of AgNPs to increase the surface-to-volume ratio has been reported to be successful in enhancing the bactericidal efficiency of the silver ions. The surface-to-volume ratio improvement is accompanied by an increase in contact area with the bacteria. In order for the silver to exhibit its antibacterial activity, it needs to be in its ionic form. Thus, the increase in the contact area of the NPs. is able to generate more silver ions able to interact with the bacteria, so as to damage them via its multiple pathways (4). Also the positively charged AgNPs bind to the negatively charged bacterial cell wall, causing disruptions in membrane permeability (30), or due to inhibition ATP synthesis by binding to the enzyme associated with ATP generation in the cell.

In conclusion, the olive leaves were good source for synthesis of stable AgNPs. in short time, with simple, low cost and eco-friendly method. Also we reported that the temperature and time played important role in accelerating the synthesis of AgNPs. The Antibacterial activity of the synthesized AgNPs against *P. aeruginosa in vitro* was dependent on the concentration of the AgNPs. There was proportional relation between the concentration and growth inhibition of the tested bacterium.

Table, 1: Mean of inhibition zone diameter of silver nanoparticles and silver sulfadiazine at different concentrations against *P. aeruginosa.*

Con. / Antibacterial	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml
AgNPs	21.40 ±0.33 Aa	19.00 ±0.38 Ba	15.60 ±0.25 Ca	14.50 ±0.16 Da	13.00 ±0.45 Ea
SSD	19.00 ±0.45 Ab	17.70 ±0.23 Bb	14.00 ±0.10 Cb	11.50 ±0.11 Db	11.40 ±0.18 Db
Distilled water	0.00 ±0.00 c	0.00 ±0.00 c	0.00 ±0.00 c	0.00 ±0.00 c	0.00 ±0.00 c

The different capital letters refer to a significant differences between concentrations at (P<0.05). The different small letters refer to a significant differences between treatments at (P<0.05). Values represent Mean ± SE. N = 3



Figure, 4: Diameter of inhibition zone a- Diameter of inhibition zone of AgNPs at concentrations 12.5, 25, 50, 100 and 200 µg/ml against *P. aeruginosa*. b- Diameter of inhibition zone of SSD at concentrations 12.5, 25, 50, 100 and 200 µg/ml against *P. aeruginosa*.

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التأثير المضاد الجرثومي لدقائق الفضة النانوية المخلقة حيوياً ضد جرثومة الزائفة الزنجارية في المختبر

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الخلاصة

أجريت هذه الدراسة لتخليق دقائق الفضة النانوية باستعمال أوراق الزيتون وتحديد الفعالية المضادة لجراثيم الزائفة الزنجارية (*Pseudomonas. aeruginosa*) في المختبر. وُصّفَت دقائق الفضة النانوية المخلقة حيوياً بواسطة جهاز المطياف الضوئي والمجهر الإلكتروني الماسح. وحُددت فعاليتها المضادة لجراثيم الزائفة الزنجارية بواسطة طريقة الانتشار بالأطباق مقارنة مع المضاد الحيوي سلفاديازين الفضة باستعمال تراكيز مختلفة لكل منهما تراوحت بين 12.5-200 مايكرغرام/مل. وأظهرت نتائج هذه الدراسة أنه بالإمكان تخليق دقائق الفضة النانوية بطريقة سهلة وصديقة للبيئة، كما أنّ أقصى امتصاص لأطياف هذه الدقائق كان عند 420 و430 نانومتر بجهاز المطياف الضوئي. كما أظهر تحليل المجهر الالكتروني الماسح ان متوسط قطر هذه الدقائق كان 26 نانومتر. وأوضحت نتائج فعالية الفضة النانوية المضادة لجراثيم الزائفة الزنجارية في المختبر ان هذه الدقائق كانت أكثر فعالية من سلفاديازين الفضة ضد هذه الجرثومة. يمكن أن نخلص إلى أن مستخلص أوراق الزيتون يمكن استعماله استعمالاً فعالاً في إنتاج دقائق الفضة النانوية، وكان لهذه الجسيمات النانوية المخلقة نشاطا فعالا مضاد لجرثومة الزائفة الزنجارية في المختبر.

الكلمات المفتاحية: دقائق الفضة النانوية، الزائفة الزنجارية، الفعالية المضادة للجراثيم.