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Antimicrobial effect of green synthesized silver nanoparticles (AgNPs) against multidrug resistant *Pseudomonas aeruginosa*

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ABSTRACT

Significant increase of mortality, morbidity and cost related to treatment caused by multidrug resistant (MDR) bacteria. Researchers are shifting towards nanoparticles in general and silver nano particles (AgNPs) in particular to solve the problem of emergence of MDR bacteria. Hence, AgNPs were produced by green synthesis method using green tea leaves *Camellia sinensis*, and were characterized by using UV spectroscopy,Fourier-Transform Infrared (FTIR) Spectroscopy,Particle Size Analyzer (PSA), Scanning Electron Microscope (SEM), and Energy Dispersive Spectroscopic (EDS). The antibacterial activity of the synthesized AgNPs were examined against Multidrug Resistant (MDR) bacteria of clinical importance(*Pseudomonas aeruginosa*) by means of Agar well diffusion, it was determined that AgNPs has a synergetic effect against *P aeruginosa*, which makes it a promising choice for use in drug products that may aid in prohibit spreading the MDR bacteria in various medical environments.

Keywords
Nanotechnology, Silver Nanoparticles (AgNPs), Green Synthesis, Multidrug Resistance (MDR), Pseudomonas aeruginosa.

Introduction

The broad prevalence of multidrug resistant (MDR) bacteria makes up a significant clinical issue [1]. One of the most commonly isolated gram negative and non-fermentative bacteria is *Pseudomonas aeruginosa* and it is one of the most frequent opportunistic pathogen. It could be easily noticed in patients with burn wound or lung infection and is a bacterium that predominant colonized in some medical devices. By taking advantage of its enzymes, toxins, structural components, and so on, *P. aeruginosa* incursion develops a violent neutrophil response and body tissue damage [2, 3]. Furthermore, formation of quorum sensing system and biofilm during the bacterial growth promotes adaptive resistance[4], which gives rise to MDR strains. The resistant microbes spread and infection are the reasons for the high mortality and morbidity and chronic disease status [5].

Decreasing antibiotics numbers in the development novel antibacterial approaches coupled with increasing antibiotic resistance [6]. Carbapenems were determined as the drugs' last resort for the treatment of infections caused by MDR *P. aeruginosa*. The appearance of resistance to carbapenems limits its use for treatment [7].

P. aeruginosa has a possibility to develop resistance, nearly to any antibiotics to which it is exposed, as a result of the presence of multiple resistance mechanisms and it becomes MDR strain. Infections caused by MDR *P. aeruginosa* are usually life threatening and serious; and nosocomial infections have frequently been reported to be caused by these strains. These MDR have been come out as a considerable problem in burn wards as burn injury disrupts many mechanisms of systemic host defense and the normal skin barrier as well which make burn patients the perfect hosts for opportunistic infections [8].

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MDR *P. aeruginosa* strains due to now has narrow range of susceptibility to antimicrobial agents. Therefore, researchers started to develop alternative therapies to aid patients to recover from the infections. Therefore, development or modification in antimicrobial compounds to improve bactericidal potential is a priority area of research in this modern era [9]. Nanotechnology provides a good platform to modify and develop the important properties of metal in the form of nanoparticles having promising applications in diagnostics, biomarkers, cell labelling, contrast agents for biological imaging, antimicrobial agents, drug delivery systems and nanodrugs for treatment of various diseases [10, 11]. Hence, researchers are shifting towards nanoparticles in general and AgNPsin particular to solve the problem of emergence of MDR bacteria [12].

AgNPsare one of the most widely used nanoparticles [13]. Recently, AgNPs have found excessive applications in different fields for instance antimicrobial agents, catalysis, biolabeling, filters, microelectronics, and sensors due to their distinct biological and physiochemical properties[14, 15]. These nanoparticles have inhibitory effects on the bacteria, virus and other prokaryotic micro-organisms growth with no toxic effects on humans [16]. Along with their specific properties, the cost of its production is slightly low [17]. Usually, chemical and physical methods have been used for the nanoparticles' synthesis [18, 19]. Fundamentally, the physical methods have low outputs and the chemical methods have hurtful effects on the environment as a result of the use of toxic solvents and the development of dangerous byproducts [20]. Currently, researchers concentrate on nanoparticles biosynthesis of using plants [21] fungus [22], and bacteria[23]. These biogenic processes are of high outputs and low cost, eco-friendly and safe in comparison with the chemical and physical synthetic procedures [24].

The technique of plant extraction is one of the feasible methods that has widely been utilized for producing the metallic nanoparticles including silver, gold, platinum and iron [25-28]. Also, biological synthesis of AgNPs by using plant extracts have been getting more consideration as a result of the presence of active phytochemicals. It has been seen that synthesis of AgNPs by plant extract is more stable than the ones synthesized using other organisms like fungi and bacteria [29].

Materials and Methods

Materials

All the chemicals were obtained from Al-Qasim Green University Laboratories in Babylon, Iraq. Silver nitrateand deionized water were purchased from Al-Ghadeer Company, Hillah City,Babylon, Iraq.Dried green tea leaves were purchased from the local market in Hillah City, Babylon, Iraq.

Tea extract's preparation

The dried green tea leaves were grounded by using a blender into a coarse powder.10 grams of the powder were dissolved in 100 mL of deionized water. The resulted solution was boiled at 100 ^{o}C for 10 min, then cooled down to room temperature, then filtered by using a Whatman No 1 filter paper first then by using syringe filter 0.45 micron. The resulted aqueous extract was refrigerated at 0–4 °C, and then used for further experiment.

Synthesis of AgNPs

One drop at a time, about 750 mL of (10 mM) silver nitrate was added into the 25 mL of the tea extract which prepared earlier, the solution was gently agitated during this process by using a

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magnetic stirrer at 30–50 °C for 120 min with the speed of 800 rpm. By using a centrifugal ultrafiltration, the AgNPs were concentrated and purified, then in order to remove any interactive biological molecules it were rinsed with deionized water. The AgNPs to be separated, the procedure was repeated three times, and then it was dried and ready to use in characterization. Formation of AgNPs was indicated by the appearance of signature brown colour of the solution, which is compatible with observations performed by [30].

Characterization of AgNPs

UV–Visible Spectroscopy

The UV-Visible spectrum analysis was used to check the Surface Plasmon Resonance (SPR) property of the synthesized AgNPs, and this property was used to differentiate the synthesized AgNPs from others. By using SPEKOL 1300 UV–visible spectrophotometer, the reduction of the pure AgNPs was recorded between 250 nm and 800 nm. The AgNPs dispersed in deionized water were observed for their SPR at 400-500 nm, respectively [31].

Fourier Transform Infrared (FTIR) spectroscopy

By using Fourier transform infrared (FTIR), the interaction between the synthesized AgNPs and green tea extract components was analyzed. In FTIR the chemical bonds vibration was measured due to the fact that chemical bonds can absorb the energy of infrared at specific wavelength or frequencies. The compound's basic structure was identified by spectral location of their infra red absorption. The documented FTIR range of the dried AgNPs by using Bruker Tensorwith the range of 400-4000 cm^{-1} following the procedure of [32].

Particle Size Analyzer

Light scattering was measured and an index of reflection was assumed by using laser diffraction particle size analyzers in order to calculate the distribution of particle size. The Mie theory of light was exploited, which relates the produced scattering pattern as light passes through a sample to the size of any particles present [33]. AgNPs sample was incubated in sonicator water bath at $35^{o}C$ for 30 min to prevent aggregation of particles before examination in size analyzer. By using laser beam scattering in beta sizer apparatus, emulsion of AgNPs sample were diluted by deionized water then putted in grove in apparatus and the size were measured during 5 min. The results were monitoring as screen of computer.

Scanning Electron Microscope Analysis (SEM)

The biological synthesis AgNPs were evaluated their size and shape by using SEM examination. Before loading the AgNPs onto a specimen holder, a drop of the AgNPs solution was placed on the carbon and kept until it gets dried. The micrographs were taken by analyzing the prepared grids at a voltage of 5-10 kV at different magnifications with low vacuum [34].

Energy Dispersive Spectroscopic (EDX or EDS)

The chemical microanalysis technique which is used in conjunction with SEM is called Energy Dispersive X-Ray Spectroscopy (EDX or EDS), This technique detects x-rays emitted from the AgNPs sample during bombardment by an electron beam to characterize the composition of the elemental of the analyzed volume. Phases or features as small as 1 μm or less can be analyzed. The dry AgNPs powder was used for this purpose using Hitachi S 3400 N operating at 30 kV of acceleration and at a magnification of 25k and the energy dispersive spectrum was recorded.

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Generally and due to SPR, metallic silver nanocrystals show typical optical absorption peak approximately at 3KeV [35].

Multidrug Resistant (MDR) clinical isolates of *P. aeruginosa*

Fifteen Multidrug resistant MDR *P. aeruginosa* were collected from burn wards of Al-Hillah Teaching Hospital, swabs were taken from the burn and then, in the laboratory, the identification and purification of each isolate was confirmed following standard microbiological methods [36, 37], and additionally confirmed by VITEK 2 to attain the very last diagnostic. By Kirby-Bauer disc method, the susceptibility was checked [38]. The strain that was resistant to three or more antibiotic class was considered as a MDR strain.

Antibacterial Assay of AgNPs

The antimicrobial activity of the AgNPs was checked by the use of well diffusion.

Agar Well Diffusion

The antibacterial activity of AgNPs was tested by Agar Well Diffusion. The inoculums used in this study were set by the addition of isolated colonies (three-five) that were grown up on a nutrient agar plate to five mL of usual sterile saline matched to the standard McFarland tube ($1.5X\ 10^8$ cells.ml^{-1}),the sterile swab was castoff to extract inoculum as of the suspension of the bacteria, theninoculumwas streaked on the Muller Hinton Agar plate then left-hand to dry, AgNPs(500,250,125,62.5,31.25. 15.6µg.ml^{-1}) were inserted into every well that made by cork borer at 8mm and permitted to position at room temperature for one hour to disperse to medium before incubation at 37 ^{o}C for twenty-four hours, zones of inhibition were assessed by a ruler or caliper and matched to the inhibition zones identified to determine the tolerance or sensitivity of the organism to AgNPs. In this study the usual inhibition diameters were castoff as prescribed by [39] and [40].

Results and Discussion

Visual observation

In agreement with previous researches, the colour change of the reaction mixture from colourless to brown colour confirmed the reduction of Ag+ to Ag0 as shown in Figure1.

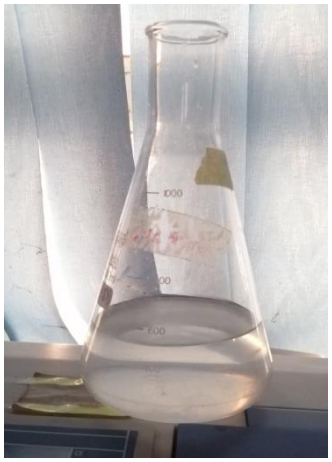
(**A**)  **(B)** 

Figure 1 Photograph showing change in colour before(A) and after (B) adding aqueous green tea extract to AgNO3

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UV-Spectroscopy

By using UV visible spectrophotometer and by obtaining a spectrum in the visible range of 250 nm to 800 nm, the presence of nanoparticles was confirmed. From this analysis, absorbance peak was found at around 450 nm as shown in Figure 2 which served as visual confirmation for the formation of nanoparticles. The UV–Vis absorption band in the current visible light region (420–450) nm is an evidence of the presence of SPR of AgNPs [41].

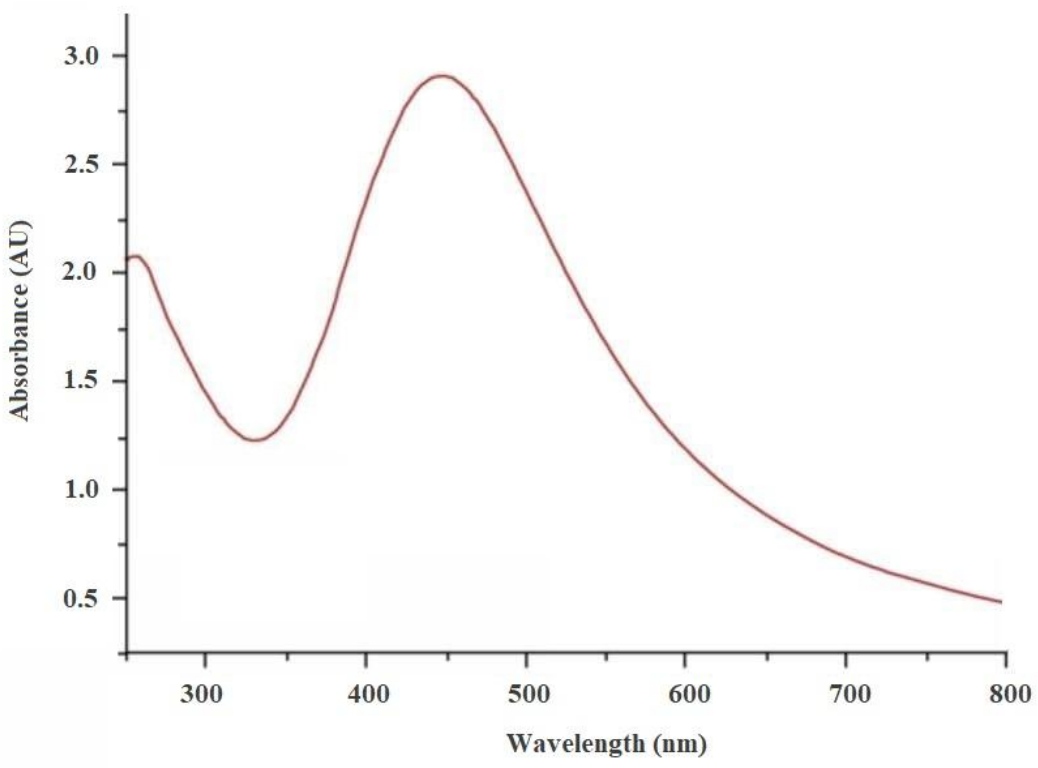

Figure 2 UV-VIS absorbance spectroscopy for AgNPs from green tea

Fourier Transform Infrared (FTIR) spectroscopy

The results illustrated FTIR spectrum of biological synthesized AgNPs in the wavelength range from 500 cm^{-1} to 4000 cm^{-1}. The band detected at 3400.52 cm^{-1} assigned to hydroxyl (OH) group and, the slight bands observed at 2920.50 $cm^{-1}$and 2851.15 cm^{-1} symbolize the methyl(CH) groups, the band detected at 2356.34 cm^{-1} referred to amine group (NH), the band detected at 1724.21 cm^{-1} represents the ester group (C=O) and the band detected at 1648.4 cm represents alkanyl (C=C) stretch. The bands at 1540.01 cm and 1435.19 cm related to carboxylate1098.17 cm^{-1} represents secondary alcohol (CO) stretch as shown in Figure 3. The FTIR analysis indicated the involvement of carboxyl, amino, amides groups and poly phenols in the synthesized AgNPs. It is well known that there are amino acid, polyphenols, and protein in tea. The tea extract's organic compounds could assign to the reduction of AgNO3 and the stabilization of AgNPs by the surface bound by the organic compounds[42]. Similar results were recorded for using plant extract as green synthesis method of AgNPs [43,44].

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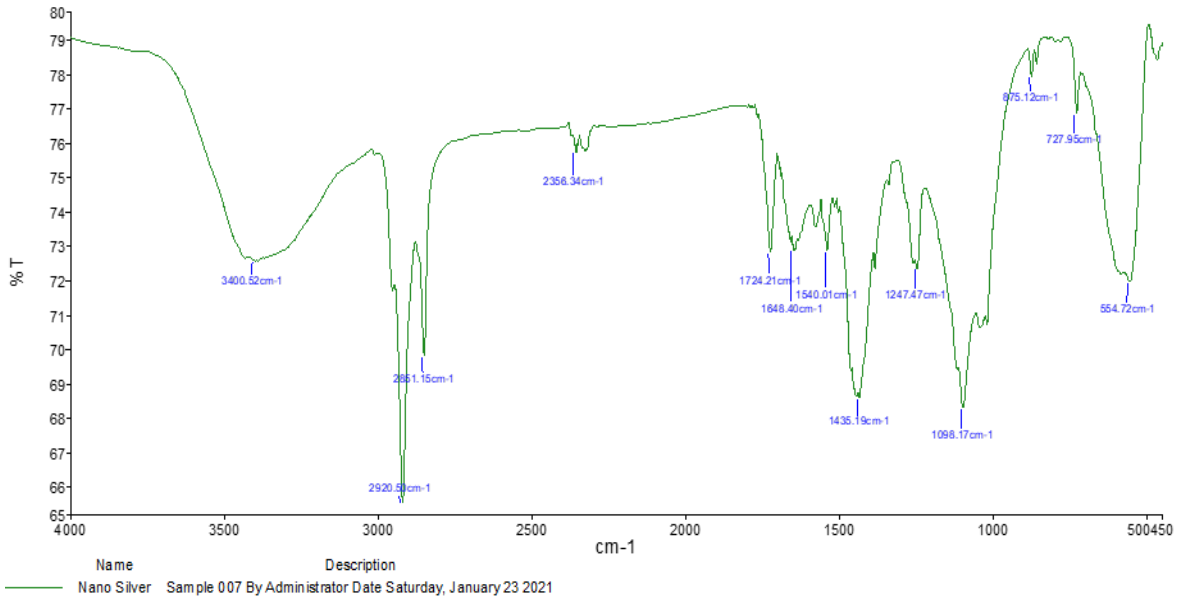

Figure 3 FTIR spectra pattern of dried powder AgNPs synthesized from green tea

Particle Size analyzer

By dynamic light scattering, the AgNPs size were determined. The size of the nanoparticles distribution analysis revealed the average of particles size was approximately 43.4 nm (Figure4).The AgNPs antibacterial activity influences of its size particles, it is well-known that the small particles are more effective than large one as antibacterial and antibiofilm [45]. Previous researches expressed that antibacterial activity is based on the size of AgNPs particles [46].

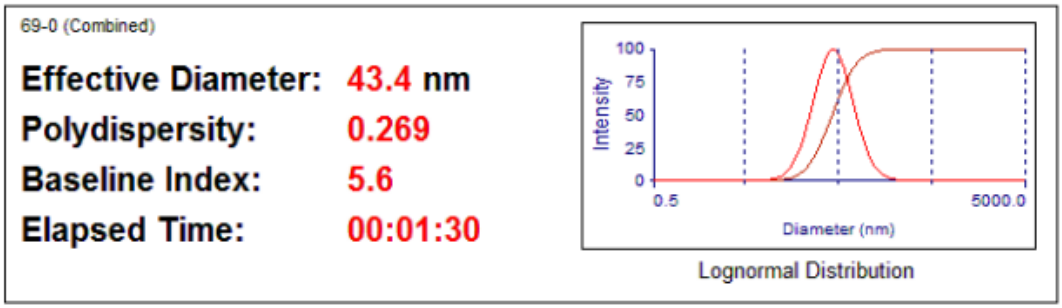

Figure (4) Size distribution analysis of AgNPs synthesized from green tea

Scanning Electron Microscope (SEM) analysis

Another technique used to determine the distribution, shape and size of synthesized AgNPs is SEM. Figure 5 shows the particles were spherical in shape and ranged 27–43nm, in terms of size, particles were different, which implies on this fact that the process introduced for AgNPs green synthesis is flexible in producing the nanoparticle with different size distribution profile and spectrum of particle size. Particularly, using sonication and stirring, both crack the particles' agglomeration and makes them [47]. The SEM image of AgNPswas resulted from the interactions of hydrogen bond and electrostatic interactions between the bio-organic capping molecules bound to the AgNPs [48].

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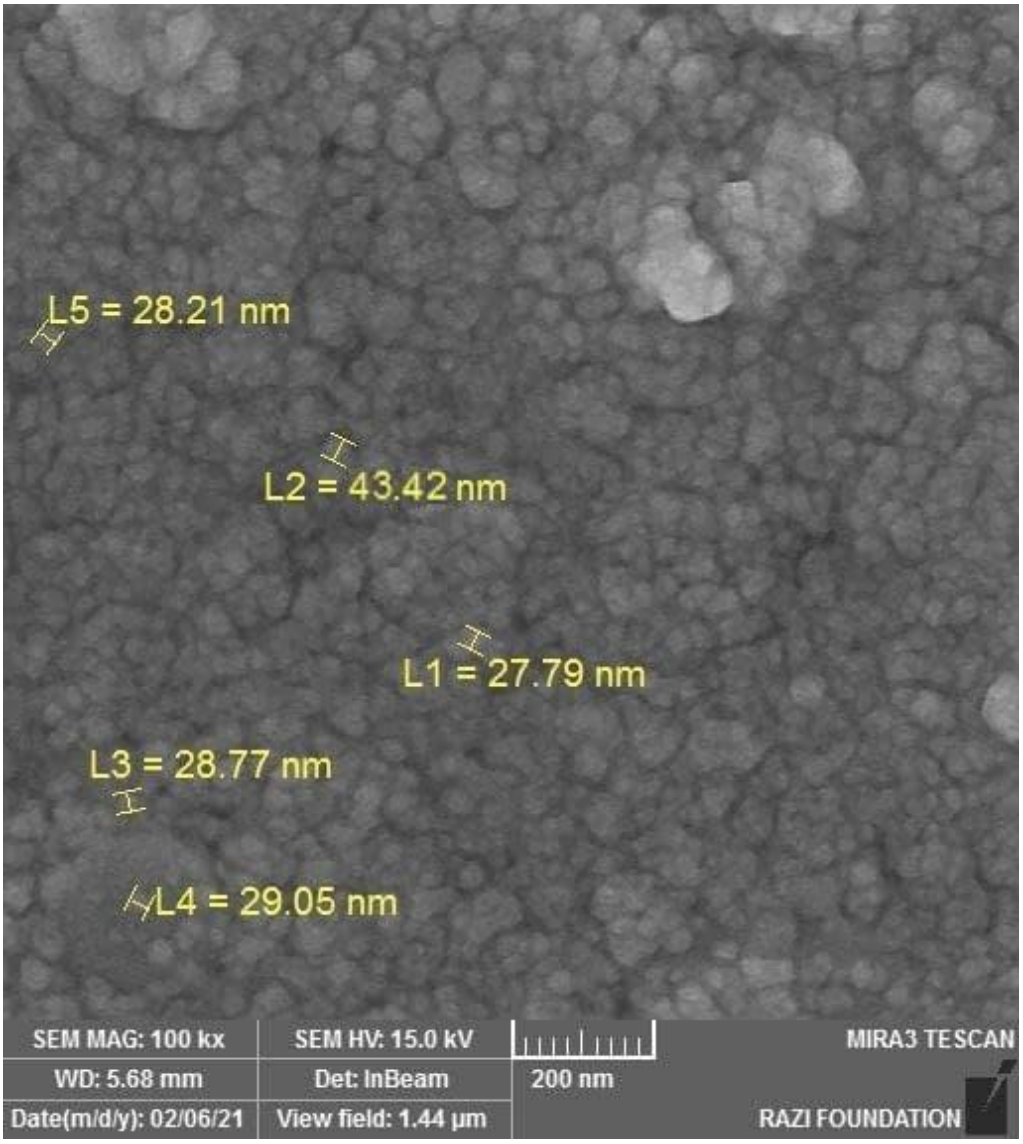

Figure 5 Image of SEM of AgNPssynthesized from green tea leaves.

Energy Dispersive Spectroscopic (EDX or EDS)

The EDX or EDS analysis of AgNPssynthesized from *Camellia sinensis* is shown in Figure 6,which proposedtha I the reaction mixture the silver ions was reduced to a silver element.

Approximately at 3keV, the most principal sharp signal was observed for silver, which is distingushing the crystalline absorption nature of biosynthesized AgNPs. Some of the weak peaks for Si, O, C , Cl, S and Al were also found. Due to the biomolecules that are bound to the surface of AgNPs, the present of the weak signal may possibly found. Approximately at 3KeV, generally the metallic silver nanocrystals show typical optical absorption peak due to SPR [35].

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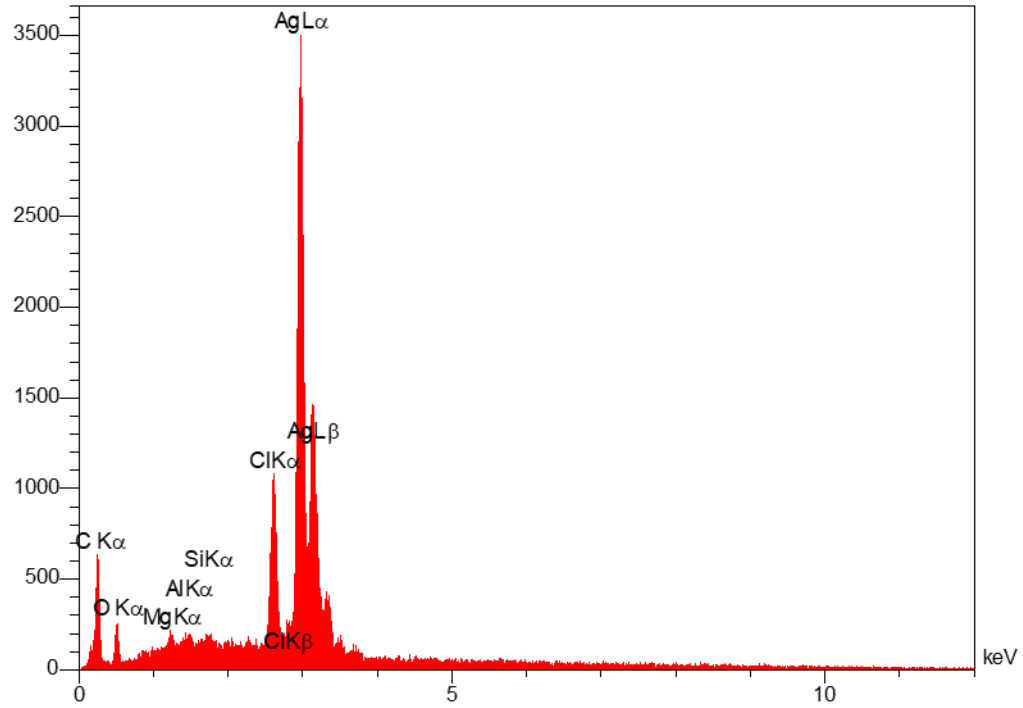

Figure 6 Image of EDS ofAgNPs synthesized from green tea

Antibacterial assay

Agar Well Diffusion

The inhibition zone in bacterial growth by the biogenic AgNPs in this study as shown in table1 and Figure 7 was depending on the medium concentration, the lowest concentration of 15.6 µg.ml^{-1} showed an inhibition zone of 6.13±0.80 mm which is the lowest inhibition zone, for the concentration of 31.6 µg.ml^{-1} the inhibition zone was 8.33±0.61 mm, for the concentration of 62.5 µg.ml^{-1} the inhibition zone was 10.40±0.82 mm, for the concentration of 125 µg.ml^{-1} the inhibition zone was 15.26±1.16 mm, for the concentration of 250µg.ml^{-1} the inhibition zone was 17.20±1.14 mm, and for the higher concentration of 500 µg.ml^{-1} the inhibition zone was 19.60±1.18 mm which is the largest, the effect of AgNPs was highly significant difference , $P<0.05$. Excellent antibacterial activity was revealed by the biologically synthesized AgNPs against bacterial pathogens [49]. The negatively charged bacterial cell wall may attach to the AgNPs discharge silver ions and rupture it, consequently leading to the denaturation of protein and death of the cell [50]. These massive changes in the bacteria membrane structure as a result of the silver cations' interaction cause the increased of the bacteria membrane permeability [51, 52].

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Table 1 Activity of AgNPs Against *P. aeruginosa* Isolates by Agar Well Diffusion Test. mean= mean, SD= Standard Deviation

AgNPs µg.ml^{-1}	Inhibition zone diameter in mm for *P. aeruginosa*
	mean± SE
15.6	6.12±0.80
31.2	8.33±0.61
62.5	10.40±0.82
125	15.26±1.16
250	17.20±1.14
500	19.60±1.18
P. value	0.001

There are three diverse theories described by [53] to explain the AgNPs effect on bacteria which is described. Firstly, AgNPs attribute to the cell membrane surface and distract its power functions, such as respiration and permeability [54]. The compulsory of the particles to the bacteria relies on the interaction of the available surface area. A large surface area will have a bactericidal influence, with a smaller particle size. Secondly, AgNPs are capable to enter the bacteria by may be interacting with phosphorus - and - sulfur containing composites like DNA and induce additional damage [55].Thirdly, the AgNPs discharge silver ions, which promote the bactericidal effect [56].

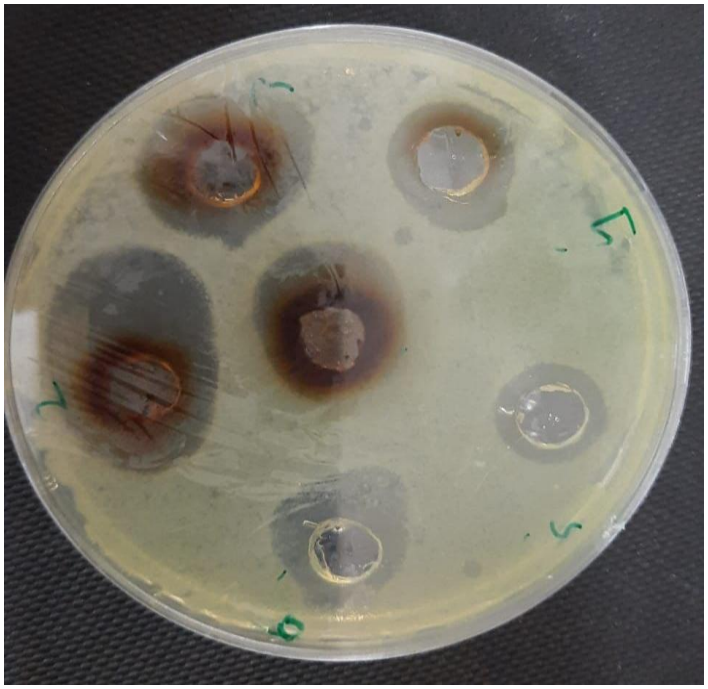

Figure 7 Zone of inhibition of AgNPsagainst*P. aeruginosa*

Conclusion

Green synthesis of AgNPs is an efficient way due to its cost-effective, eco-friendly, simple and productive protocol. The present study contained the green synthesis of AgNPs by using *Camellia sinensis* leaves extract, in which biomolecules acted as both stabilizing and capping agents. The reduction of silver nitrate to AgNPs was approved by UV–visible spectrophotometer, FTIR, PSA, SEM and EDS techniques. accordingly, nanoparticles can be synthesized by staying away from hazardous chemicals and utilizing the green protocol. The benefits of using a plant extract to synthesis AgNPs include cost effective, economic viability, quick synthesis method, toxicity free and ease in handling large scale synthesis. AgNPs demonstrated efficient antibacterial activity. Hence, they may be used as an antibacterial agent and could be used as an effective alternative to conventional antibiotics.

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