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SYNTHESIS, MOLECULAR CHARACTERIZATION OF SILVER NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY AGAINST KLEBSIELLA PNEUMONIAE

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Abstract
Klebsiella pneumoniae bacteria have been known for causing a huge number of infections, such as pneumonia, wound or surgical site infections, meningitis, and liver abscesses. Traditionally, Klebsiella pneumoniae is considered to be the causative agent of many serious infections mainly in immunodeficient individuals, but the recent emergence and spread of hypervirulent strains have broadened the number of people susceptible to infections to include those who are healthy and immunosufficient. Moreover, Klebsiella pneumoniae strains have become progressively resistant to a lot of antibiotics treatment protocols and their infections are very challenging to treat. In our research work, we have done an assessment and evaluation of the anti-bacterial action of silver nanoparticles as an alternative for regular treatment protocols for the Klebsiella pneumoniae infections. The bacteria were isolated for soil and the procedures for the identification of bacteria have been achieved through using the both of the biochemical examination and polymerase chain reaction (PCR). The biochemical tests used were included tests like catalase, oxidase, urease, citrate utilization and methyl red test, in addition, polymerase chain reaction has confirmed the identification of the bacteria and the band position of Klebsiella pneumoniae was 130 bp after being visualized on 1.5% agarose gel. With respect to the synthesis and characterization processes of the silver nanoparticles, the nanoparticles were formed using a green synthesis approach through utilizing the Aspergillus niger to avoid pollution that related to the chemical synthesis approach, to obtain the physicochemical report of the silver nanoparticles, a number of characterization techniques have been done, like FTIR to acquire the infrared absorption spectrum of the the silver nanoparticles which acts as a fingerprint for the substance, TEM and SEM in the sake of displaying the morphology and giving information about the size and shape of the nanoparticles. With regard to the anti-bacterial activity of silver nanoparticles, agar well diffusion method has been done and the both of MIC and inhibition zone values have been obtained. The values were 156.25 µg/ml and 14 mm respectively.

Keywords: Silver nanoparticles, Aspergillus niger, Klebsiella pneumoniae, Antibacterial

1. Introduction

Klebsiella pneumoniae is believed to be one of the most clinically important microorganisms which have grabbed the attention of the scientific community. *Klebsiella pneumoniae* has been stated to be a member of Enterobacteriaceae family and it is considered as a one of the major opportunistic pathogens those causing a wide range of diseases, in addition to that, it is exhibiting a highly repeated acquirement of antibiotic resistance. It possesses the responsibility of causing about nearly 33% of all gram-negative bacterial infections, like cystitis, endocarditis, urinary tract infections,

pneumonia and septicemia [1]. It also causes wound or surgical site infections, liver abscesses and meningitis [2]. *Klebsiella pneumoniae* has been known for ramping up the mortality rates and causing a prolongation of hospitalization period which as a consequence to those actions can result in a very high cost per infection treatment [3]. Nowadays, the misuse of the bacterial infections treatment protocols through antibiotics has increased the issue of the antibiotic resistance which is provoked by bacteria in order to survive against treatment; the misuse issue has been announced to be one of the crucial reasons for the failure of infection treatment. Resistance is encouraged by alterations which have been made by bacteria to increase their refractoriness to the antibiotics through enzyme mediated hydrolysis or by reducing their therapeutic activity. Over the years, mutations in genes have appeared impulsively as a result of misusing or abusing antibiotics [4]. The high antibiotic doses and frequent administration of them have been participating notably in evoking the adverse side effects on humans. Bacterial tolerance has been expanded in the front of many types of antibiotics which are commonly used to treat bacterial infections [5]. In addition to that, no novel types of antibiotics have been discovered in the current years. Moreover, the production of new forms of antibiotics is a process that requires high cost and time to be achieved as it demands several clinical trials and approval [6]. Nanotechnology science is predicted to have a huge impact on fabrication of an alternative approach for common antibiotic protocols [7–10]. Nanomaterials have been recognized by their unique size as it falls within the range of 1–100 nanometres [11, 12]. The size of these materials allows them to have a unique set of physicochemical characteristics, which offer many advantages to them such as high surface area and special crystalline or amorphous structures [13]. Nanoparticles of metallic type such as silver nanoparticles have been utilized for sake of prophylaxis of burns and wounds induced infections [14]. Despite the fact that its mechanism of anti-bacterial action is not fully known, it has been assumed that silver ions invade the cell wall and membrane of bacteria through interaction with thiol groups and sulfur containing protein [15]. Once been in the cell, silver ions consider the DNA and respiratory enzymes as a main target for their anti-bacterial action which as a result is leading to destruction of the cell's replicating capabilities and finally cell death [16].

The overall purpose of conducting our research is to evaluate and investigate the anti-bacterial activity of biosynthesized silver nanoparticles against Klebsiella pneumoniae through the means of in silico studies which represented by molecular docking approach and in vitro studies those achieved through execution of agar well diffusion.

2. Materials and methods

2.1. Soil sampling

A total of 25 samples have been gathered at random different sites at the deepness of maximum 15 cm using an auger and then added together to get a combined sample. The composite sample then transferred in sanitary conditions using polythene bags to being analyzed in the laboratory. The samples were keeped at room temperature for further examinations [17].

2.2. Isolation of *Klebsiella pneumoniae* strains

As an initial step, serial dilution has been done and 9 ml of 0.9% sodium chloride solution was poured into each test tube. The test tubes were autoclaved at 121 °C (15 psi) for 15 min to achieve sterilization and then left to be cool [18]. The stock solution was prepared by dissolving 10 g of the dry soil in 90 ml of 0.9% sodium chloride solution and using the stock solution, 1/10, 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000 dilutions were formed. A volume of 100 µl from the concentrations of 1/1000, 1/100000, and 1/1000000 were inoculated on the modified mineral salt medium (MSM). The inoculum was incubated at 30 °C for 72 hours and the morphologically distinct colonies were identified and purified [19].

2.3. Identification of *Klebsiella pneumoniae* strains

2.3.1. Biochemical-based identification

A series of biochemical tests have been executed to assess the capability of *Klebsiella pneumoniae* to run certain biochemical reactions those provoke some changes in the media. The changes produced can involve production of gas, pH change and other end products excreted in the media which can be revealed by using certain indicators. The biochemical tests performed were methyl red test, indole, citrate, oxidase, urease and catalase. In coupling to those biochemical tests, gram staining technique was executed, and the results were noted accordingly [20].

2.3.2. Polymerase chain reaction (PCR)-based identification

The process of DNA isolation has been done through utilizing QIAamp DNA Mini kit (Qiagen, Germany, GmbH) in accord to the directions provided by the manufacturer. Unique primers have been employed in the sake of completion of the amplification process of *Klebsiella pneumoniae*; Primers used were supplied from Metabion (Germany) (table 1). The conditions offered for the PCR included: at 95°C and for 5 minutes, the initial step of denaturation has taken a place then a number of 35 denaturation cycles at 94°C for 30 sec. have been done. The step of annealing had been completed at 55°C for 30 sec. before the extension and final extension steps were done at 72°C for 30 sec. and 7 minutes respectively. PCR final products have been obtained detached from each other by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH).

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Prim. Den.	Amplification (35 cycles)			Final extension	Reference
					Sec. den.	Ann.	Ext.		
Klebsiella pneumoniae	*16S-23S ITS*	ATTTGAAGAGGTTG CAAACGAT	130	95˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	[21]
		TTCACTCTGAAGTTT TCTTGTGTTC							

2.4. Biosynthesis of silver nanoparticles

2.4.1. Culture of fungi

Aspergillus niger has been isolated from soil. After that a confirmation through morphological characteristics using a microscope was done. The sample was then inoculated on potato dextrose agar and left to grow at 25°C for 48 hours.

2.4.2. Nanoparticles biosynthesis

In regard of preparation of the biomass, *Aspergillus niger* was allowed to grow within aerobic conditions in potato dextrose broth. The flasks had been inoculated with the fungi before they were placed on orbital shaker at 25°C and the speed was set at speed of 120 rpm. The biomass then was filtered using Whatman filter paper No.1 after 72 hours. In addition to that, filtered biomass has been washed 4 times using distilled water to remove any remnants from the medium components. A nearly of 20 g of the biomass were mixed with 150 ml of distilled water for 24 hours at 25°C in Erlenmeyer flasks and then putted on the orbital shaker at speed of 120 rpm. The biomass has been passed through Whatman filter paper No.1 to separate the fungal filtrate. For the sake of synthesis of silver nanoparticles, A volume of 50 ml from silver nitrate which has 1 mM final concentration were putted with 50 ml of fungal filtrate in a 250 ml Erlenmeyer flasks. Another portion from the biomass has been separated from the fungal filtrate was combined with 50 ml silver nitrate solution with a concentration of 1mM then agitated using the orbital shaker at 25°C and 120 rpm in dark [22].

2.5. Characterization of silver nanoparticles

A series of techniques were performed to acquire the details of physicochemical characteristics of the biosynthesized silver nanoparticles such as FTIR analysis which provides the fingerprint of a substance through measurement of the wavelengths range that absorbed by the substance in the

infrared region, TEM and SEM techniques which have been used to display the morphology details and imaging of the nanoparticles.

2.6. Assessment of the anti-bacterial activity of silver nanoparticles

The Susceptibility examinations have been done according to directions provided by the National Committee for clinical laboratory Standards. Screening tests related to the inhibition zone assay were executed through the agar well diffusion method. Bacterial colonies were allowed to grow overnight in purpose to make the inoculum suspension. After that they were inoculated into Mueller-Hinton broth. Using a sterile swab immersion has been done in the suspension to inoculate Mueller-Hinton agar plates. Serial dilution has been done to silver nanoparticles to produce a series of concentration using dimethyl sulfoxide as a solvent (10, 5, 2.5…mg/ml) to determine MIC value. The measurement of the inhibition zone has been obtained around each well after 24h at 37^{o}C [23].

3. Results

3.1. Isolation of *Klebsiella pneumoniae* strains

Table 2: Number of Klebsiella pneumoniae isolated from soil samples

Total number of samples	Number of *Klebsiella pneumoniae* strains isolated
25	11

3.2. Identification of *Klebsiella pneumoniae* strains

3.2.1. Biochemical-based identification

Table 3: Biochemical examinations those have been executed to identify *Klebsiella pneumoniae*

Biochemical tests	Results
Catalase	+
Voges- Proskuer test (VP)	+
Citrate	+
Urease	+
Indole	-
Oxidase	-
Methyl red	-

3.2.2. Polymerase chain reaction (PCR)-based identification

A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The agarose gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

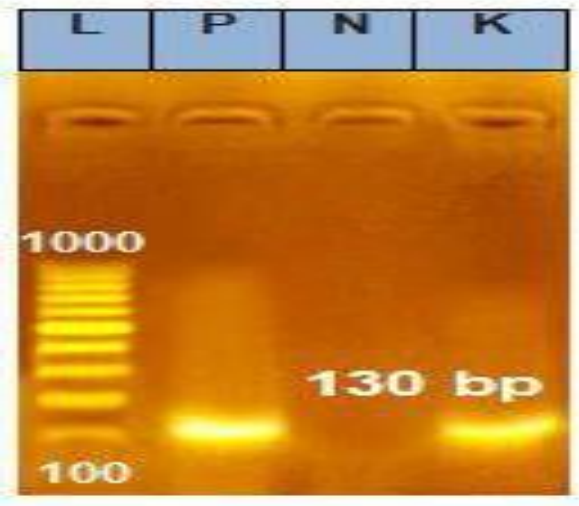

Figure (1) reveals the position of band on agarose gel for Klebsiella pneumoniae which is 130 bp

3.3. Characterization of silver nanoparticles

3.3.1. Fourier transformed infrared (FTIR)

The absorption spectrum which produced using FTIR analysis was displayed the peak range of 500-4000 cm-1. Sharp absorption peaks have been demonstrated at the wavelength of 548.59, 1638, 2074 and 3452 cm-1 which are representing several functional groups.

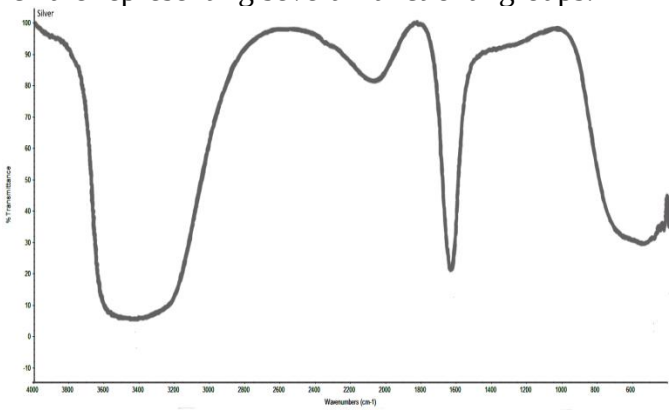

Figure (2) displays the infrared absorption spectrum of the silver nanoparticles

3.3.2. Transmission electron microscopy (TEM)

TEM was one of the most efficacious characterization techniques which used to display the size and shape of the nanoparticles. The results have demonstrated the spherical shape of nanoparticles with an average size range of 12–20 nm.

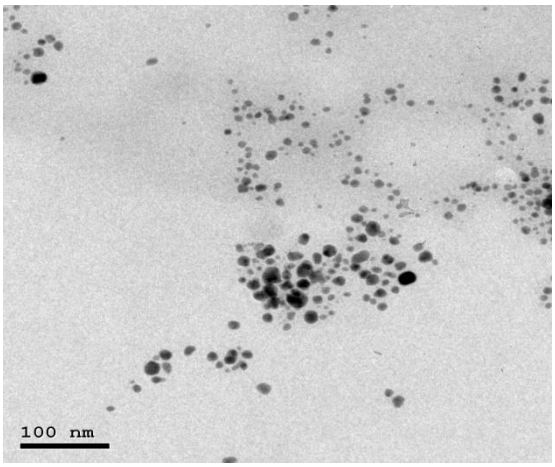

Figure (3) reveals the spherical shape and size range of the silver nanoparticles those have been obtained through using of TEM

3.3.3. Scanning electron microscopy (SEM)

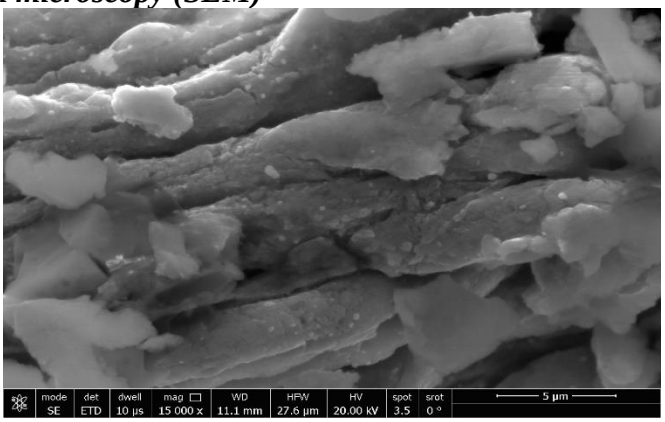

Figure (4) showed the results of utilizing SEM technique to characterize silver nanoparticles; the results confirmed the spherical shape of silver nanoparticles.

3.4. Assessment of the anti-bacterial activity of silver nanoparticles

In order to acquire information about the anti-bacterial activity of silver nanoparticles, both of MIC value and inhibition zone diameter have been obtained after execution of agar well diffusion method (table 4) (figure 5).

Table 4: Values of Inhibition zone and MIC of silver nanoparticles against *Klebsiella pneumoniae*

Tested microorganism	Inhibition zone diameter(mm)	MIC(µg/ml)
Klebsiella pneumoniae	14	156.25

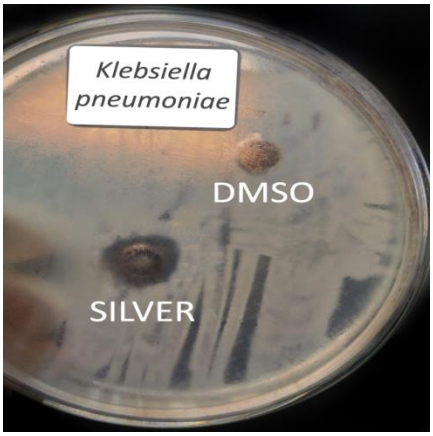

Figure (5) displays the inhibition zone diameter of silver nanoparticles against *Klebsiella pneumoniae*

4. Discussion

Klebsiella pneumoniae as it has been stated before in a lot of studies has newly gained popularity as a major infection causative agent due to the ramping up that happened to the number of clinically severe infections and the lack of effective treatment protocols. In addition to that, the concerns of scientific community have increased due to the existence of *Klebsiella pneumoniae* strains those developed mutations which lead to the formation of hypervirulent or resistant versions of the bacteria [24, 25]. Briefly, *Klebsiella pneumoniae* considered as a gram-negative bacterium that have a special feature which is having a capsule and its habitat can include soil, surface waters and on the medical devices [26, 27].According to what have been said in preceding studies on utilizing nanoparticles as anti-bacterial agents, nanotechnology has showed many beneficial features to be used in scientific applications and industrial processes. Nanoparticles have distinctive traits such as biological, physical and chemical traits those helping in grabbing the interest for being studied for many purposes. Nanoparticles use has resulted in great benefits for the microbiological applications including having an antimicrobial activity against bacteria, fungi, virus, and protozoa [28]. In our research we have examined the anti-bacterial effect of biosynthesized silver nanoparticles on *Klebsiella pneumoniae* strains which were isolated from soil, the isolation procedures have agreed with what have been mentioned before in other studies [29]. The bacteria have been identified by the means of both biochemical tests and polymerase chain reaction (PCR). The biochemical examinations include tests like oxidase, catalase, urease, citrate utilization test and methyl red test; additionally the polymerase chain reaction technique has confirmed the identification of *Klebsiella pneumoniae* and the products of it have showed the band position of 130 bp for the bacteria on agarose gel. All of the identification methodology has been confirmed with the previous papers those have been published in the same context [30, 31]. In concerning with the production and characterization of silver nanoparticles, the nanoparticles have been biosynthesized with the aid of *Aspergillus niger* utilizing a green synthesis approach which is considered environmentally friendly in comparable to chemical synthesis approach and to obtain the physicochemical report of silver nanoparticles, we have executed a number of techniques which involve FTIR to provide the fingerprint of the nanoparticles through formation of infrared absorption spectrum, TEM and SEM

for visualization and imaging of the silver nanoparticles, both of production and characterization processes are confirmed previously in the other studies [32]. The use of silver nanoparticles as an anti-bacterial agent has been evaluated using the agar well diffusion approach; the process involves using dimethyl sulfoxide (DMSO) as a control and the values of both inhibition zone and MIC have been obtained. The implementation of agar well diffusion approach as a method for assessment of the anti-bacterial action of silver nanoparticles is agreeing with a lot of former researches [33].

5. Conclusion

In our research work, we have evaluated and investigated the anti-bacterial activity of silver nanoparticles which are biosynthesized using *Aspergillus niger* on *Klebsiella pneumoniae* strains those are isolated from the soil. The identification process has taken place through two ways which are the biochemical examination and polymerase chain reaction (PCR). In regard to the synthesis and characterization steps of the silver nanoparticles, the nanoparticles have been biosynthesized using a green synthesis method through exploitation of *Aspergillus niger* to avoid toxicity and harmful environmental effects those are associated with the chemical synthesis methods and to achieve the characterization of silver nanoparticles, a number of techniques have been used, such as FTIR to provide the infrared absorption spectrum of the the silver nanoparticles, TEM and SEM for visualization of the size and shape of the silver nanoparticles. In respect to the anti-bacterial effect of silver nanoparticles, the assessment has been done using the agar well diffusion methodology and both of MIC and inhibition zone values have been obtained.

Data availability statement

The original contributions presented in the study are included in the article Material; further inquiries can be directed to the corresponding author.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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