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Original article

Antimicrobial and antibiofilm effect of silver nanoparticles on clinical isolates of multidrug resistant *Klebsiella pneumoniae*

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ABSTRACT

Background: Multidrug-resistant (MDR) *Klebsiella pneumoniae* (*K. pneumoniae*) causes life threatening infections. Pharmaceutical nanotechnology is anticipated to produce new therapeutic products for biomedical uses. One of these compounds, silver nanoparticles (AgNPs), is with promising antibacterial and anti-biofilm characteristics. **Aim of the work:** To evaluate the anti-microbial and anti-biofilm activities of AgNPs against MDR *K. pneumoniae* clinical isolates in Ain-Shams university hospitals. **Methodology:** The study was conducted on fifty MDR *K. pneumoniae*. The isolates were retrieved from Ain Shams university Microbiology laboratory. The biofilm-forming activity was tested by a microtiter plate based on crystal violet staining, the AgNPs were synthesized biologically and characterized by different methods. The antibacterial and antibiofilm activities of biosynthesized AgNPs were investigated against selected strains using standard methods. **Results:** The results revealed that all isolates were resistant to tested antibiotics and biofilm-forming ability was detected in 28/50 (56%) isolates. The antibacterial activities of AgNPs showed that all isolates were susceptible to AgNPs with Minimal Inhibitory Concentration (MIC) ranging from 15.625 µg /ml to 125 µg /ml. Also, AgNPs significantly reduced the biofilm formation as 26/28 of isolates became non-biofilm producers (0) and 2/28 became weak. Silver nanoparticles showed minimal cytotoxic concentration (conc.) up to 100 percent and 99.42 percent viability on normal human lung fibroblast cells (MRC-5) cell lines treated with AgNPs at conc. of 2 µg/ml and 3.9 µg/ml respectively. **Conclusion:** Multi drug resistant *K. pneumoniae* is a rising problem and the rate of biofilm formation in these isolates is high. Silver nanoparticles exhibit good antibacterial and antibiofilm activity against them.

Introduction

One of the most common causes of community-acquired infections is *Klebsiella pneumoniae (K. pneumoniae*). It is also a cause of hospital-acquired infections [1]. It has several virulence factors, including fimbriae, lipopolysaccharides, capsules, and biofilm formation,

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and represents a major threat due to high rate of drug resistance among them [2,3].

One of *K. pneumoniae's* virulence factors that contributes to resistance is biofilm formation which acts as a barrier to the antibiotic action and can also develop resistance due to cell contact and DNA acquisition from surrounding bacterial communities [4].

Although new antimicrobial generations have been developed, none have yet demonstrated to be completely effective against multidrug-resistant (MDR) bacteria or biofilm forming bacteria, such as *K. pneumoniae*, making treatment selection challenging to physicians [5]. Finding an alternative therapy is therefore crucial [6].

Pharmaceutical nanotechnology is anticipated to produce new therapeutic products for biomedical uses. Nanotechnology has already developed new possibilities for providing effective treatment alternatives for resistant bacteria [7].

A promising agent is silver nanoparticles (AgNPs), which have the efficient physicochemical characteristics needed to fight microorganisms. Different studies have examined its antibacterial effectiveness against various microbes during the past 20 years [8].

The mechanism of action of AgNPs is challenging for bacterial resistance. They have a bactericidal effect due to their capability to attach and penetrate the cell wall of bacteria, as well as alteration of cell membrane structures. Its high surface-to-volume ratio and nanoscale size both contribute to their effectiveness [9].

Few studies have also shown that AgNPs are effective against biofilm formation . The high rate of surface capacity of AgNPs prevent the formation of biofilm components that is required for protection of bacteria from antibiotics [10,11].

Silver nanoparticles are a good choice for unusual antibacterial applications. These particles can be extracted and created using a wide range of techniques, as solvent-based extraction, microwave-assisted extraction, and maceration extraction. The ideal extraction technique should be easy to use, affordable, rapid, and feasible in laboratory [12].

So, the present study aimed to evaluate the anti-microbial and anti-biofilm activities of biologically synthesized AgNPs against MDR *K. pneumoniae c*linical isolates in Ain Shams University Hospitals.

Material and Methods

Klebsiella pneumoniae isolation and identification

The present study was done starting from January to August 2022 on fifty *K. pneumoniae* strains retrieved from the main Microbiology Laboratory of Ain-Shams university hospitals. The identification of isolates was done by using conventional methods according to **Collee et al.** [13] including, microscopic examination and colonial identification by examining colonies on culture media and biochemical reactions (triple sugar iron test, urease test, indole test and citrate utilization test,).

Silver nanoparticle biosynthesis [14]

To synthesize AgNPs, 250 ml of distilled water and 25 g of dried *E. camaldulensis* (red gum) (Camphor) leaves extract was mixed. The mixture was then boiled at 80 °C for three hours while being constantly stirred, and then filtered through Whatman grade (1) filter paper (Sigma-Aldrich). Also, to reduce the silver ions, 90 ml of an 11.77 mM aqueous silver nitrate AgNO3 solution (Sigma-Aldrich) and 10 ml of plant extract were added in a 250 ml Erlenmeyer flask. At 40 °C, a magnetic stirrer was used to stir the reaction mixture-containing flask continuously for 10 minutes.

Silver nanoparticles characterization [14]

Characterization of AgNPs was done by color change observation, X-Ray Diffraction Spectroscopy (XRD), Ultraviolet Visible spectrometry (UV-Vis), and Transmission Electron Microscopy (TEM). Change of color was observed. Also, the surface plasmon resonance (SPR) of AgNPs was demonstrated using (UV-Vis) spectroscopy using a Shimadzu Japan UV-3101PC UV-VIS-NIR Spectrophotometer in order to demonstrate and validate the bioreduction of silver ions to AgNPs. The size and appearance of AgNPs were assessed using (TEM- JEOL 1010 Japan). An X-ray diffractometer (Panalytical X'Pert Pro, Netherlands) was used to evaluate the phase and crystalline structure of the biosynthesized AgNPs, and those measurements were compared to the JCPDS standard of AgNPs.

Moreover, the Fourier Transform Infrared Spectroscopy (FTIR) spectrum of biosynthesized AgNPs was recorded on a model Nicolet™ iS50/iS50R FTIR Spectrometer (Thermo Scientific company) (USA) (4000-400 cm-1) with a spectral resolution of 1 cm^{-1} to find out the functional groups present in the phytoconstituents responsible for the reduction and capping of AgNPs.

Antimicrobial susceptibility

The 50 isolates were tested for antimicrobial susceptibility by disc diffusion method. The following antibiotics were used according to Clinical and Laboratory Standards Institute guidelines (CLSI 2022) [15] (ampicillin 10µg, amoxicillin / clavulanic acid 20/10µg, , ampicillin/sulbactam 10/10 µg, piperacillin/ tazobactam 100/10µg, aztreonam 30µg, cefotaxime 30µg, cefepime 30µg, cefoxitin 30µg, ceftazidime 30µg, ciprofloxacin 5µg, levofloxavin 5µg, tobramycin 10µg, gentamycin 10µg, amikacin 30µg, meropenem 10µg, imipenem 10µg and trimethoprim sulphamethoxazole 23.75 1.25µg (Oxoid, England)). A MDR organism was defined as being resistant to three or more antimicrobial classes in addition to ampicillin.

Antibacterial effect of biosynthesized AgNps against *K. pneumoniae* isolates.

According to the CLSI standard [15], the Minimal Inhibitory Concentration (MIC) of AgNPs was determined using broth microdilution method. Preparation of two-fold serial dilutions of AgNPs in nutrient broth was done to achieve 1 ml of each concentration in each tube. Then, each 0.2 ml of the bacterial suspension was added. Negative controls for broth and AgNPs were used. Tubes were then incubated for 24 hours at 37 °C. The MIC of AgNPs against isolates was determined using the resazurin-based turbidimetric assay. The MIC was defined as the concentration at which there was no colour change after 4 hrs incubation of the overnight culture with 0.3 % resazurin [16,17]**.**

Biofilm formation assay [2]

After overnight cultures of the tested organisms a loopful was added to 5 mL of tryptic soy broth (TSB) (Lab M Ltd, UK) with 1% glucose and incubated at 37 °C for 24 hours. By using sterile 96-well, flat-bottom polystyrene tissue culture plate, each well was filled with 200 µL of the bacterial solution (Sigma-Aldrich Co. LLC, USA). A broth was provided as a negative control in order to test the sterility. The plates underwent a 24-hour incubation period at 37 °C. After incubation, the contents of each well were removed with a gentle tap, and the wells were then cleansed three times with 300 L of sterile saline. The remaining adhering bacteria were fixed by using hot air for 60 minutes at 60 °C. Then 150 L of crystal violet stain was added to each well. After 15 minutes, decantation was used to remove the excess dye from the plate by adding 95% ethanol equal to 150 mL volume. After 30 minutes, the optical densities (OD) of stained adherent bacterial films were measured at 620 nm using a microtiter plate reader.

The test was performed three times, and the average of the outcomes was obtained. The cut-off value (ODc) was obtained after the OD values for all tested strains and negative controls were computed. The following categories of strains were used to interpret the findings: non-biofilm producer (0): $OD \leq ODc$, weak biofilm producer (1+): $ODc < OD \leq 2 \times ODc$, moderate biofilm producer (2+): $2 \times ODc < OD \leq 4 \times ODc$, strong biofilm producer (3+): $4 \times ODc < OD$ [18].

The antibiofilm effect of AgNPs was performed using the previously described method with the addition of AgNPs. The inhibition of biofilm formation was calculated using the equation below :

Inhibition rate = 1- (OD treatment/OD control)*100 [19].

Antiproliferative activity

Several concentrations (10, 20, 50, 75, 100, and 150 µg/ml) of biosynthesized AgNPs were applied to lung fibroblast cells (MRC-5). The distilled water was used to prepare the dilutions. Cells were incubated for 24-hour in CO2 incubator, after which the morphology of the cells was examined under the microscope. Then, 20 µL of the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT reagent (5 mg/ml) was added, and the mixture was then incubated for 4 hours at 37 °C. Following incubation, the formazan was solubilized by the addition of 100 µL of distilled water. Cell viability was determined using the following formula after the absorbance at 570 nm was recorded using a 96-well plate read

[mean Abs (treated sample) - blank Abs/mean Abs(control) - blank Abs] ×10

Statistical analysis

Analysis of the results was done using SPSS version 22. Quantitative data were expressed in the form of mean and standard deviation or median and range. Paired t-test was used to assess the significance of the inhibitory activity of AgNPs on the biofilm-forming ability of *K. pneumoniae* clinical isolates. *p*-values < 0.05 were statistically significant.

Results

The *K. pneumoniae* isolates included in this study were isolated from different clinical samples. Most of the strains were obtained from sputum (18/50, 36%) followed by blood (16/50, 32%), urine (9/50, 18%), and swabs (7/50, 14%). All isolates showed resistance to all used antibiotics and were identified

as MDR. Most *K. pneumoniae* isolates were obtained from male patients.

Biosynthesis and characterization of silver nanoparticles:

Silver nanoparticles have been biologically synthesized by a facile and rapid biological method using aqueous *E camaldulensis* leaf extracts after adding $AgNO_3$ solution; the solution's coloring changed from colorless to yellowish-brown within 24h as an obvious sign that AgNPs were formed. Also, the results of UV-Visible spectroscopy revealed that the synthesized nanomaterial exhibited a well-defined plasmon band at the wavelength of 420 nm corresponding to AgNPs. The TEM analysis of AgNPs indicated that the prepared NPs were spherical with an average size (5.39:23.8 nm) and a mean diameter of 12.22 ± 0.8 nm viewed at (120000x) magnifications. In XRD analysis, the peaks of photosynthesized AgNPs, according to the (111), (200), (220), and (311) planes, respectively, that attributed to the face-centered cubic (fcc) forms of metallic silver (JCPDS 04-0873). Moreover, the FTIR spectrum revealed the involvement of the plant extract during the biosynthesis and stabilization of AgNPs that was confirmed by intensity changes of several peaks; a distinctive strong broad spectral band at 3443.19 cm−1, indicating the presence of the O-H functional group of alcohol, bands at 2076.1 cm−1 attributed to the aliphatic C-H stretching vibration of polyphenols, band at 1635.90 cm−1 corresponds to C═C stretching vibrations of an aromatic alkene and N-H functional groups of primary and secondary amines of amino acids, peptides, and proteins. The bands at about 537.16, 468.41, 452.05, 434.6, and 411.05 cm−1 were attributed to out-of-plane C–H bending vibrations in alkenes and aromatics as displayed in **figure (5A-D)**.

Regarding the antibacterial activity of AgNPs on tested isolates, all isolates were susceptible to AgNPs with MIC ranging from 15.625 µg /ml to 125 µg /ml. Twenty-eight out of 50 *K. pneumoniae* isolates (56%) showed biofilm production. 32% (16/50) were moderate biofilm producers (+2), 24% (12/50) were weak (+1), and 44% (22/50) were non-adherent (0) as shown in **table (1)**. There was a significant reduction in biofilm formation due to the effect of AgNPs on *K. pneumoniae* isolates as 26/28 of isolates became non-biofilm producers (0) and 2/28 became a weak biofilm producer (+1). The mean (±SD) of OD was decreased from 0.5617 (±0.1266) to 0.1190 (±0.0683) as shown in **figure (6)**.

Cytotoxicity activity

The in-vitro cytotoxic effects of phyto-synthesized AgNPs against MRC-5 cells line were evaluated based on cell viability by MTT assay. MRC-5 cells treated with AgNPs at concentrations of 2, and 3.9 g/ml demonstrated 100% and 99.42 percent viability, respectively; the viability of MRC-5 cells declined with increasing AgNPs Levels.

Meanwhile, viability dropped to almost 50% of the initial level, which showed 50 percent, at conc. 45.84 ± 2.43 µg/ml. Therefore, these values were selected as the 50% cytotoxic concentration (CC50). Furthermore, the lowest viability percentages of 6.21 and 3.04 percent, respectively, were reported at maximal concentrations of 250 and 500 µg/ml as indicated in **figure (7)**.

Table 1A. Various values of MIC of all tested *K. pneumoniae* isolates.

Number of samples (%)	MIC
1(2%)	125 µg /ml
47(94%)	62.5 µg /ml
1(2%)	31.25 µg /ml
1(2%)	15.625 µg /ml

Table 1B. Biofilm distribution according to different clinical samples.

Sample type (No)	Non-adherent (0)	Weak (+1)	Moderate (+2)
Fifty samples	22(44%)	12(24%)	16(32%)
Sputum (18)	6(33.3%)	3(16.7%)	9(50%)
Blood (16)	8(50%)	4(25%)	4(25%)
Urine (9)	4(44.44%)	3(33.33%)	2(22.22 %)
Wound (7)	4(57.14%)	2(28.57%)	1(14.29%)

Figure 1. Showed a plate of Muller Hilton agar inoculated by *K. pneumoniae* showing MDR pattern.

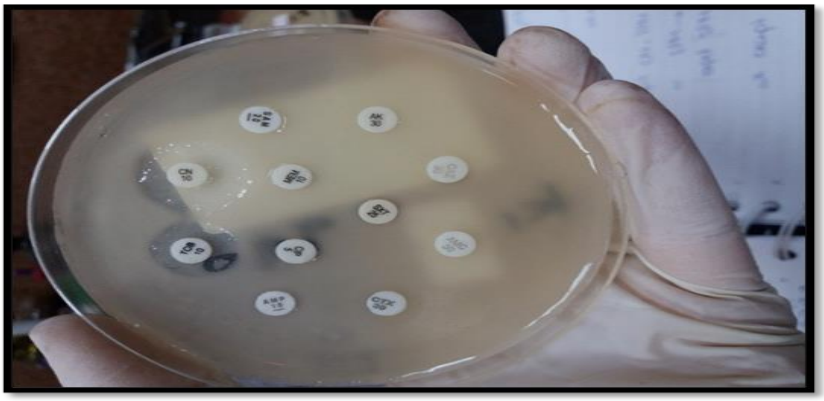


Figure 2. Showed AgNPs antibacterial activities by tubes dilution technique A) Before adding resazurin dye, B) After adding resazurin dye.

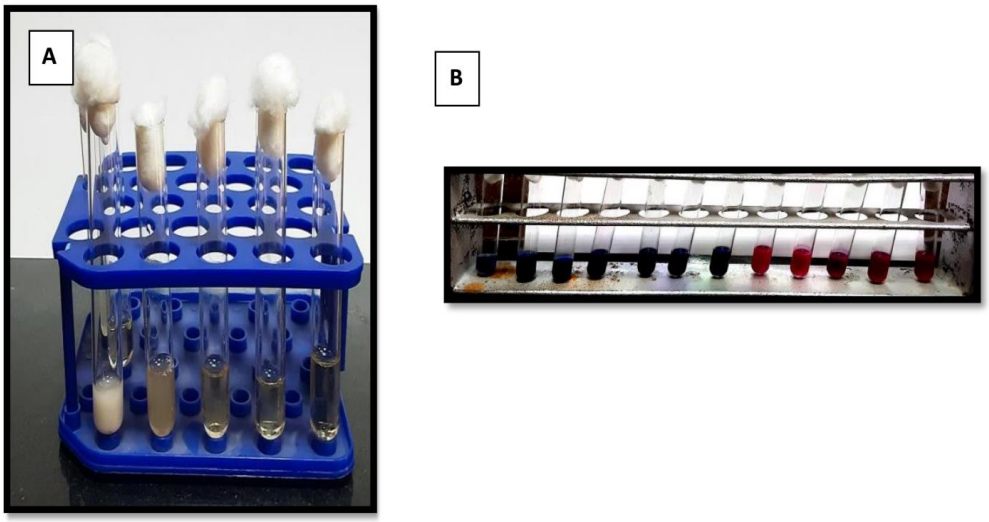
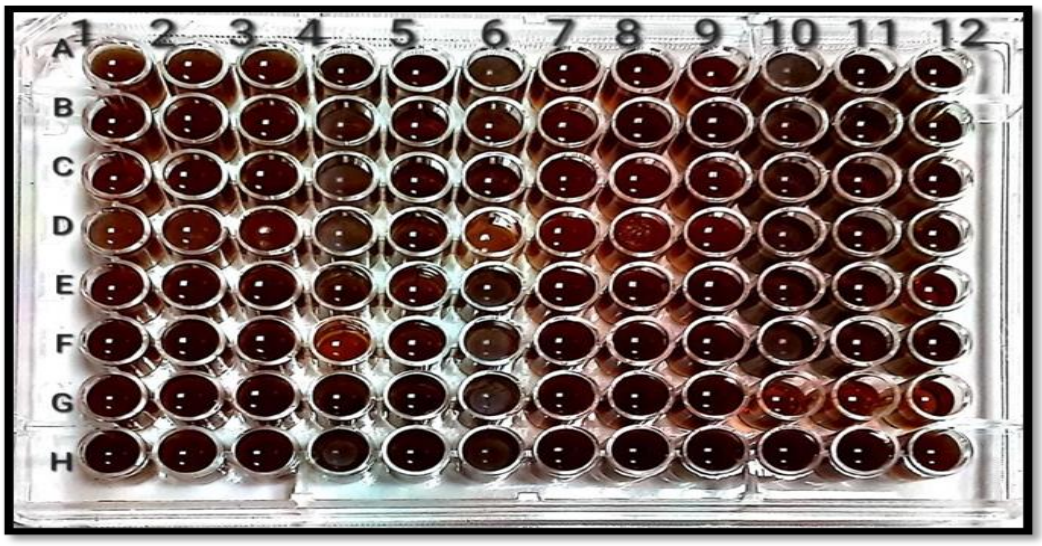


Figure 3A. Microtiter plate assay for assessment of biofilm formation by *K. pneumoniae* isolates.



Row A: showed two isolates, columns 1, 2, and 3 contained isolate no.1 without AgNPs, and columns 4,5,6 contained the same isolate no.1 in addition to AgNPs, and isolate no.2 in columns (7,8,9), and so on till row H (column 6). Row H (columns 7, 8, 9, 10, 11, 12) contained tryptone soya broth only.

Figure 3B. Quantitative crystal violet microtiter plate assay showed the effect of AgNPs on the biofilm formation of *K. pneumoniae* isolates.

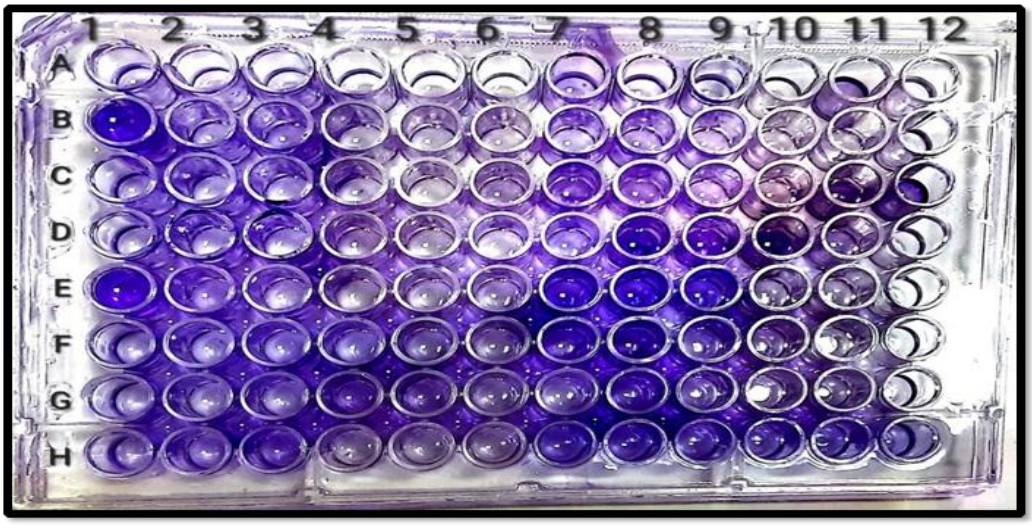


Figure 4. Percentage of *K. pneumoniae* in different clinical samples.

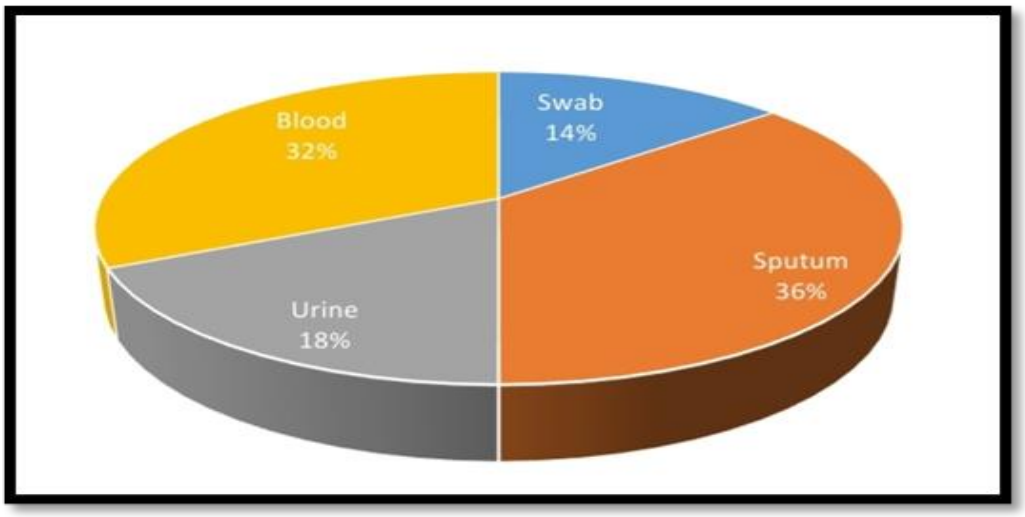
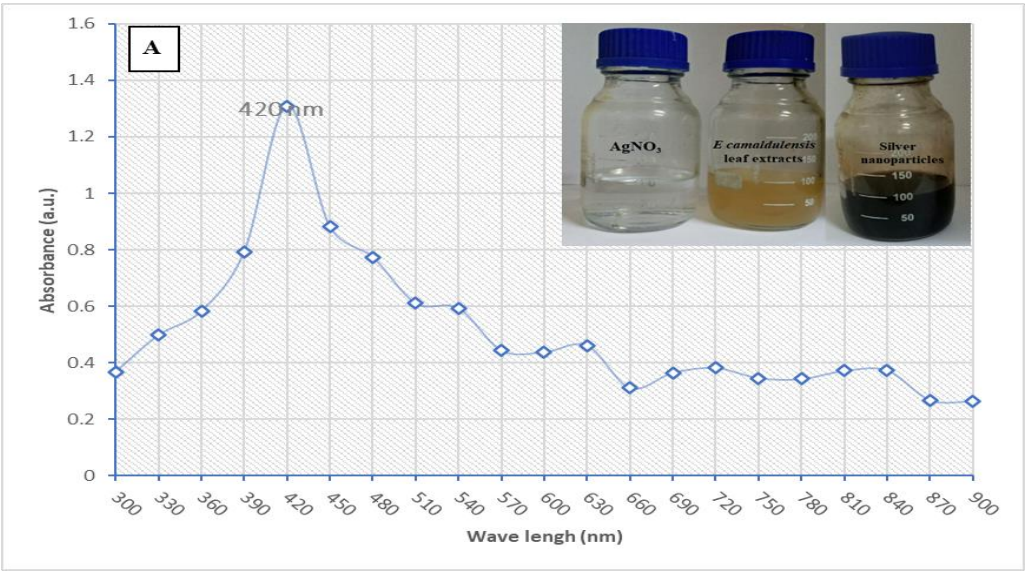
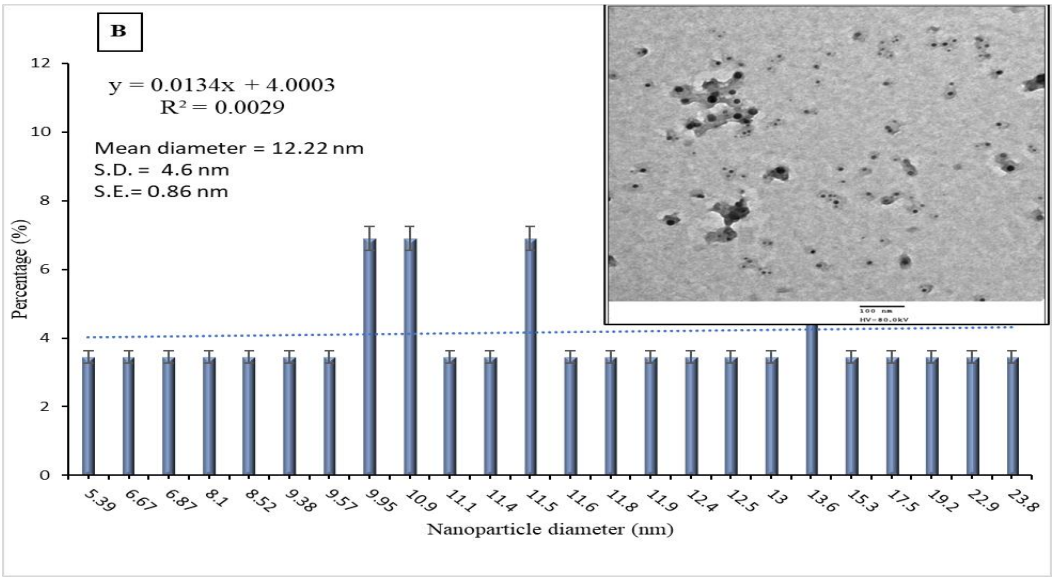
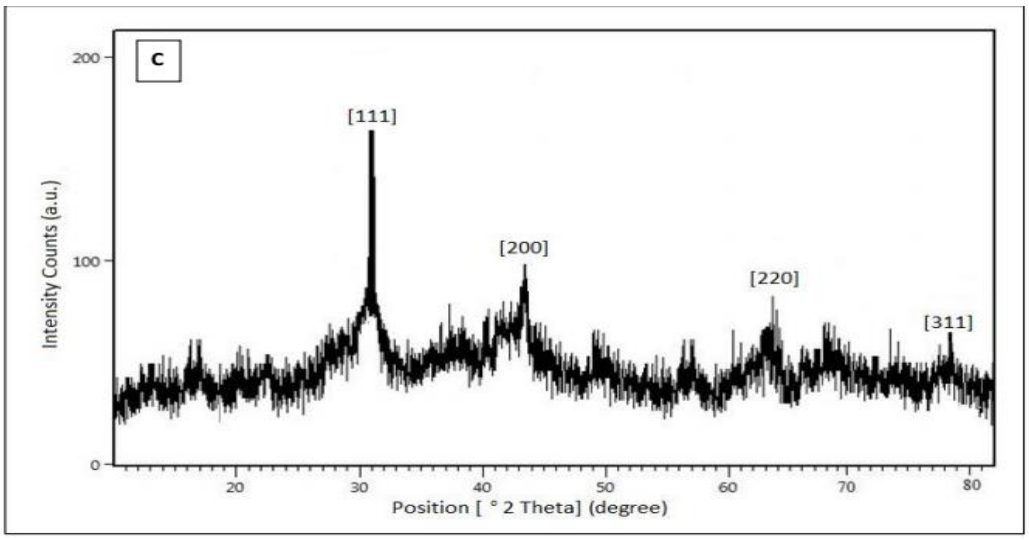
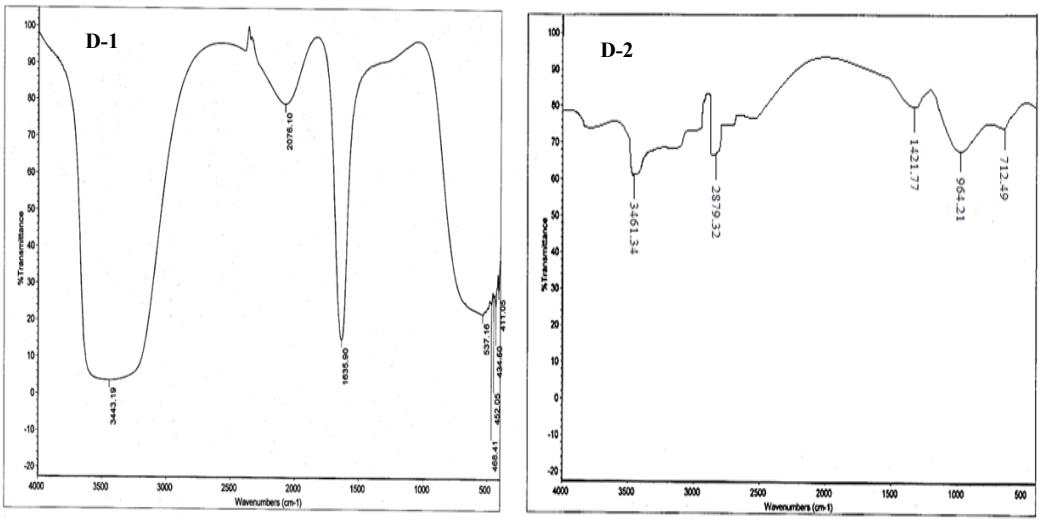


Figure 5. Biosynthesis and characterization of AgNPs by aqueous *E. camaldulensis* leaf extract.









A) UV-visible spectroscopy and visual observation of AgNPs.
B) TEM image of synthesized AgNPs revealing form and particle size distribution.
C) XRD Pattern of AgNPs by aqueous *E. camaldulensis* leaf extract.
D) FTIR spectra of (1) AgNPs in comparison with (2) Control (aqueous *E. camaldulensis* leaf extract).

Figure 6. The effect of AgNPs on the biofilm-forming ability of all *K. pneumoniae* isolates.

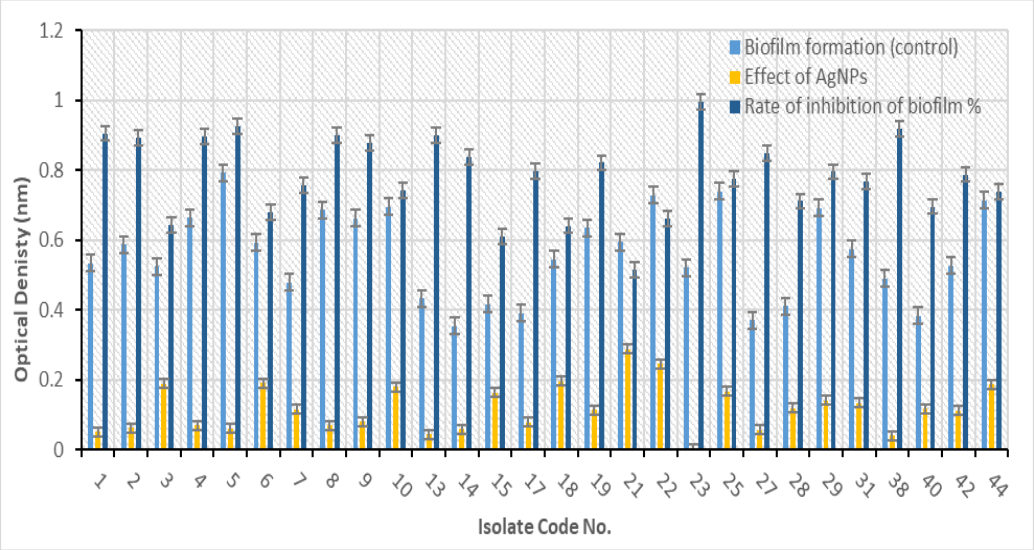
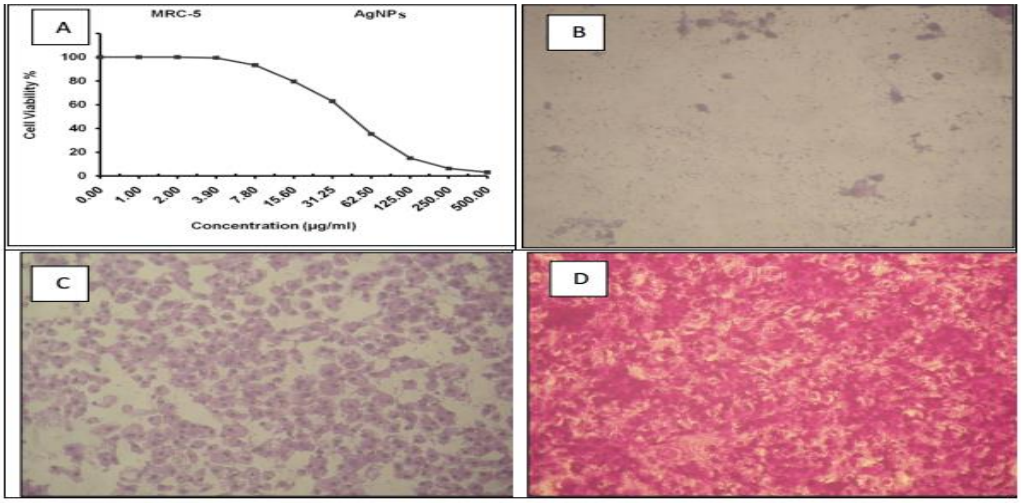


Figure 7. Cytotoxicity and morphological evaluation of cytotoxicity by MTT assay of phyto-synthesized AgNPs treated MRC-5 cells, at different concentrations and overnight incubation.



A) Cytotoxicity by MTT assay.
B) MRC-5 cells treated with AgNPs at 500 µg/ml conc.
C) MRC5 cells treated with AgNPs At 50 µg/ml conc.
D) MRC5 cells non-treated (control).

Discussion

One of the opportunistic nosocomial pathogens is *K. pneumoniae* [19]. It gained international attention due to severity of the diseases caused by it, high rate of antibiotic resistance, and treatment challenges [20].

Results of this study comes in accordance with **Nirwati and colleagues** [2], who found that male patients had a higher tendency to acquire *Klebsiella* infection than female patients (64%). The relationship between sex and the prevalence of *K. pneumoniae* has been linked to unhealthy lifestyle habits like smoking and alcoholism [21]. In a study done by **Jondle et al.** [22], they found that common sites of *K. pneumoniae* colonization is the mucosa of oropharynx and gastrointestinal tract. So, *K. pneumoniae* is considered as one of the most common causes of hospital-acquired infections especially pneumonia. This conclusion is similar to results reported by **Wang et al.** [23], who performed their study in China, they stated that the main site of infection caused by *K. pneumoniae* was the respiratory tract.

Similarly, **Karimi et al.** [21] reported that most of the isolates were obtained from the respiratory samples (61.45%) followed by urine (21.6%) and blood (7.25%), This goes with the

studies of **Yang and Zhang** [24] done in China and **Nirwati et al.** [2] in Indonesia.

On the other hand, *K. pneumoniae* isolates were isolated from urine, surgical wounds, sputum, and blood with percentages of 61.7, 18.1, 11.7, and 8.5%, respectively, according to **Seifi et al.** [25], who performed the study on two hospitals.

All *K. pneumoniae* isolates in the current study were MDR to all tested antibiotics. These results are comparable to those of research by **Shivannavar** [26], which revealed that 37 isolates (90.2% of 41) were MDR. The majority of the MDR *K. pneumoniae* isolates were also found to have high levels of resistance to penicillin, fluoroquinolone, cephalosporin, sulfonamide, and aminoglycoside.

On the other hand, **Nirwati et al.** [2], stated that 54.49% of *K. pneumoniae* were MDR. **Cepas et al.** [27] reported also, that about 38% of tested *K. pneumoniae* isolates were MDR. Also, **Moini et al.** [28] found that about 46.6% of the K. *pneumoniae* isolates *were* MDR and higher resistance rate was reported towards third generation cephalosporins, ampicillin, and aminoglycosides.

The emergence of MDR *K. pneumoniae* is a major challenge worldwide. **Heidary et al.** [29] in a meta-analysis article found that there was a relatively high prevalence of drug-resistant *K. pneumoniae* isolates in Iran. **Shadkam et al.** [30] indicated high resistance to different types of antibiotics. This discrepancy may be attributed to geographic distance, patterns of antibiotic prescription in hospitals, and level of patient hygiene.

Nanoparticles are yet considered as an excellent and applicable alternative to antibiotics, and this may solve the problem of the evolution of MDR among bacteria [31]. Among the different nanosized antibacterial agents, AgNPs have proved to be a highly efficient particle against a wide range of microbes. This effect particularly is important in Gam-negative bacteria especially, *K. pneumonia* [32]. In this regard, the growths of *K. pneumonia* were examined after applying different concentrations of AgNPs and data depicted that most of the assigned concentrations of AgNPs caused growth inhibition of *K. pneumonia* and increasing concentration resulted in more inhibition with a maximum inhibition ranging from 15.625 μg /ml to 125 μg /ml of AgNPs (MIC level). Similarly, **Alotaibi et al.** [33] reported that MIC values of AgNPs ranged from 16–128 μg/mL against Gram-negative bacteria, which is a similar range of previously reported studies. The observed variation in MIC values could be due to the inherent tolerance of the tested strains [34]. Several antibacterial activities were reported for AgNPs, including direct interaction and damage of cell walls, generation of reactive oxygen species, and internalization and release of Ag+ [35,36].

The present study found that 28 (56%) of the 50 isolates were biofilm producers. In a different study, 148 (85.63%) of 167 isolates produced weak or moderate biofilms [2]. Additionally, **Cepas et al.** [27] stated that 37.6% of the examined *K. pneumoniae* isolates produced biofilm. According to **Yang and Zhang** [24], 62.5% of the *K. pneumoniae* that produce biofilm were isolated from urine, sputum, wound swabs, and blood samples. According to **Seifi et al.** [25], the majority of *K. pneumoniae* (93.6%) produced biofilms, and only 6.4% did not produce it.

Also, the level of biofilm production was categorized in the current study as follows: 24% of the strains were weak biofilm producers, 32% were moderate biofilm producers, and 44% were non-biofilm producer strains. The capability of isolates to form biofilms varied because different factors affect this ability, including physicochemical properties, the type of surface to which the biofilm adheres, the physical interactions between different constituents, pH, temperature, etc. [2].

The establishment and recurrence of *K. pneumoniae* infection are both aided by biofilm formation. **Seif et al.** [25] and **Boisvert et al.** [37] reported that strains isolated from sputum samples had higher ability to produce biofilm than other specimens. This proves the role of biofilm in the ability of microorganisms to colonize the lungs [38]. While **Karimi et al.** [39] reported that strong biofilm formation was reported in 20.4% of *K. pneumoniae* isolates, that have a high rate of biofilm formation among all testes isolates. Also, **Yang and Zhang** [24], investigated ability of *K. pneumoniae* strains to produce biofilm these strains were retrieved from blood samples, wounds swabs, sputum samples and urine. Their study indicated that 62.5% of all isolates generated biofilms.

Furthermore, **Karimi et al.** [39] reported that samples taken from the tracheal tube were strong biofilms producer, and sputum specimens formed weaker biofilms in comparison to them, while **Seifi et al.** [25] reported that 93.6% of *K. pneumoniae* isolates in Iran were capable of biofilm formation and 33% of them could produce biofilm strongly.

The ability of *K. pneumoniae* to form biofilms that are also extensively drug-resistant (XDR) was highlighted by **Vuotto and colleagues** [40].

The ability of *Enterobacteriaceae* to generate biofilms was investigated by **Cepas et al.** [27] in order to determine whether there might be a connection between them. While the MDR isolates did not produce a lot of biofilms compared to non-MDR isolates, there was no statistically significant correlation between biofilm development and MDR among *Enterobacteriaceae*.

The present study suggests that AgNPs can be utilized as efficient antimicrobial and antibiofilm agents against MDR *K. pneumoniae.* Nanoparticles can be efficiently used as antibacterial agents against numerous pathogens.

Conflicts of interest : None.

Financial disclosure : None.

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