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Synthesis of green-engineered silver nanoparticles using *Cymbopogon citratus* (lemongrass) and its antibacterial activity against clinical *Pseudomonas aeruginosa*

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Abstract

Background and Aim: The use of bioengineered nanocomposites as antimicrobials has increased in recent years, but very few investigations have been conducted to test their effectiveness against *Pseudomonas aeruginosa*, a pathogen presenting public health risks that can impact both humans and animals. The aim of this study was to assess the antimicrobial potential of phytofabricated silver nanoparticles synthesized using lemongrass extract against clinical isolates of *P. aeruginosa.*

Materials and Methods*:* The extraction of active compounds from the leaves of *Cymbopogon citratus* was performed using ethanol (80%) as a solvent, high-performance liquid chromatography–mass spectrometry was used to analyze the chemical composition of the extract, the synthesis of silver nanoparticles (AgNPs) was done using silver nitrate ($AgNO_3$) as a precursor, and the antimicrobial and antibiofilm activity of the extract and the AgNPs phytofabricated was assessed against 10 clinical strains of *P. aeruginosa*.

Results: Lemongrass extract was found to consist of the following main compounds: Caffeic acid (445.21 ± 32.77 µg/g), p-coumaric acid (393.32 ± 39.56 µg/g), chlorogenic acid (377.65 ± 4.26 µg/g), quinic acid (161.52 ± 17.62 µg/g), and quercetin-3-glucoside (151.35 ± 11.34 µg/g). AgNPs were successfully phytofabricated using 2.5 mM $AgNO_3$. The ultraviolet (UV)–visible absorption spectra of the AgNPs showed a localized surface plasmon resonance at 464 nm with an absorbance of 0.32 A. The 50× hydrodynamic diameter was 50.29 nm with a surface area value of 120.10 m^2/cm^3, and the volume mean diameter and Sauter mean diameter were 50.63 nm and 49.96 nm, respectively. Despite the compound found in lemongrass extract, no antimicrobial activity was observed with the extract, while AgNPs exhibited noteworthy dose-dependent antimicrobial activity with inhibition diameters up to 24 mm and minimum inhibitory concentration (MIC) and minimum bactericidal concentration ranging from 2 to 16 and 4–64 µg/mL, respectively. AgNPs also demonstrated significant antibiofilm activity by inhibiting biofilms up to 99% between MIC/2 and 2MIC.

Conclusion: The present study suggests that lemongrass is a good candidate for the synthesis of AgNPs with good physicochemical characteristics and having a strong anti-pseudomonas activity. Further research is needed to assess the stability and safety of these AgNPs.

Keywords: antibiofilm capacity, antimicrobial activity, green synthesis, lemongrass, *Pseudomonas aeruginosa,* silver nanoparticles.

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Introduction

The treatment of bacterial infections is becoming increasingly complicated due to the growth of antibiotic resistance [1, 2]. In recent years, various studies have highlighted the mechanisms of antibiotic resistance [3, 4], while others have suggested ways to circumvent this problem, which has major consequences

on human and animal health [5–7]. Antibiotic resistance is defined as the ability of a microorganism to resist an antimicrobial to which it was once susceptible [2, 8]. Resistance can be attributed to four main mechanisms: The degradation of antibiotics by bacteria using different enzymes [9], the ability of the bacteria to modify the antibiotic's target [10], changes that may occur in membrane permeability [11], and the ability of the bacteria to use alternative or unconventional metabolic pathways [12]. Horizontal gene transfer (conjugation, transduction, and transformation), which allows interbacterial transmission of antimicrobial resistance, has worsened the situation worldwide [13]. Antibiotic resistance is particularly observed in Gram-negative bacteria because of the ease of accumulating resistance genes and the availability of efflux pumps in their membranes, which enable them to expel antimicrobials from the cells, thereby making them unresponsive or resistant to various types of antibiotics [1].

Pseudomonas aeruginosa, a ubiquitous non-fermenting aerobic rod-shaped gram-negative bacillus belonging to the *Pseudomonadacea* family, is a frequent causative pathogen in nosocomial infections [14]. *P. aeruginosa* is a ubiquitous non-fermenting aerobic rod-shaped gram-negative bacillus belonging to the *Pseudomonadacea* family and is recognized as a frequent cause of nosocomi Barbier and Wolff [15] reported that this bacterium is responsible for 10%–15% of all nosocomial infections, with a higher frequency being reported in certain categories of high-risk patients: Chronic bronchopulmonary pathologies (cystic fibrosis), immunosuppression (neutropenia and acquired immunodeficiency syndrome), severe burns, and patients hospitalized in intensive care. Pachori *et al*. [16] reported that every year the rate of antibiotic resistance in *P. aeruginosa* increases, making infections in which they are implicated more difficult to treat. In addition to intrinsic resistance to various antimicrobial agents (β-lactam and penem group of antibiotics), *P. aeruginosa* can acquire resistance to other antibiotics using the mechanisms depicted in Figure-1 [16]. Although several antibiotics continue to work well against *P. aeruginosa*, more and more researchers recommend alternative solutions that can counteract antibiotic resistance in *P. aeruginosa*, effectively fight sensitive strains, and reduce the risks of acquiring antibiotic resistance [17–21]. Recent studies [19–21] have suggested that nanoparticles (NPs) and nanocomplexes constitute a suitable alternative for combating microorganisms in general and *Pseudomonas*, in particular, by limiting the possibility for these strains to develop resistance.

NPs are particles with diameters ranging from 0 to 100 nm [22, 23]. In recent years, there has been a considerable increase in research devoted to the antibacterial activity of NPs, with silver NPs (AgNPs) being one of the most investigated [22, 24, 25]. The

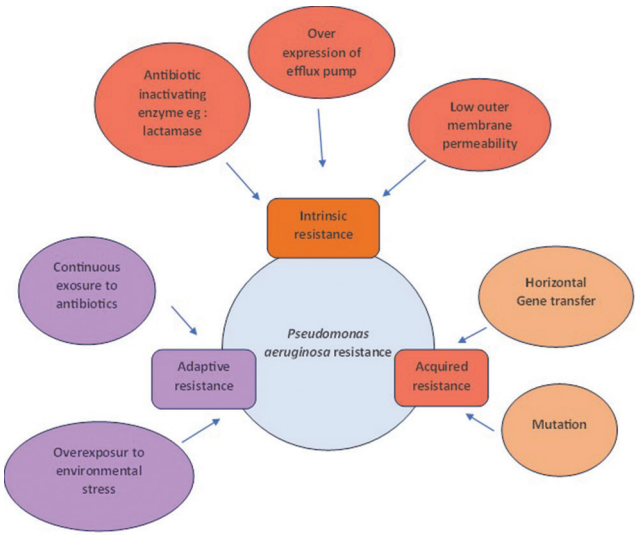


Figure-1: Proposed mechanism of antibiotic resistance in Pseudomonas aeruginosa ([16] with permission from Elsevier; license number: 5433491396764).

synthesis of NPs is very often carried out by chemical or physical means, but unfortunately, these methods are of ecological concern, and therefore, more eco-friendly routes are needed [26, 27]. Biogenic synthesis of NPs is defined as a simple and direct route leading to the formation of NPs using biological materials such as metabolites from animals, enzymes, microorganisms, and plant extracts [23, 28]. The use of plant extracts, in particular, is highly recommended as a replacement for chemical methods because the resulting NPs are more stable and cost-effective [23, 29, 30]. Various medicinal or non-medicinal plants, such as *Panax ginseng* [28], *Euphorbia wallichii* [31], *Salvia verticillata* and *Filipendula ulmaria* [32], *Phoenix dactylifera* [33], *Debregeasia salicifolia* [34], *Moringa oleifera* [35], and *Datura metel* [36], have already been utilized in the phytofabrication and stabilization of AgNPs. To date, very few studies have been conducted to synthesize AgNPs using *Cymbopogon citratus* (lemongrass).

C. citratus is a fast-growing, tall perennial grass belonging to the *Poaceae* family with tuft-shaped leaves arising from the rhizomes, which have a lemon scent (hence its name "lemongrass"). Lemongrass is commonly cultivated in humid subtropical and tropical regions and can reach a height of 3–5 ft. and a width of up to 2 ft. [37]. Depending on the application, lemongrass may be used as fresh leaves, dried powdered concentrated extract, or essential oil [37]. Conventionally, lemongrass has been used to treat coughs, flu, headache, elephantiasis, leprosy, gingivitis, ophthalmology, pneumonia, arthritis, malaria, and vascular disorders [38–45]. Gaba *et al*. [38] recently conducted an extensive review using experience-based sources and reported that the plant can be used to detoxify numerous organs (digestive tract, liver, kidney, bladder, and pancreas), regulate some physiological imbalances (high cholesterol, uric acid, various

toxins, and excess fats), stimulate physiological functions (lactation, digestion, and blood circulation). The same study highlighted several other pharmacological properties of lemongrass, such as anti-inflammatory, antiprotozoal, antidiarrheal, antimutagenic, antinociceptive, larvicidal, antifilarial, antidiabetic, and insecticidal activities. All these properties can be ascribed to its composition, which is rich in minerals, vitamins, macronutrients (including carbohydrates, proteins, and small amounts of fat), and various bioactive compounds such as flavonoids, alkaloids, terpenoids, phenols, saponins, and tannins [37]. All of these compounds can play a significant role not only in reducing silver ions (Ag+) to form Ag, but also as a capping and stabilizing element for AgNPs.

The present study aimed to perform the ethanolic extraction of active compounds from lemongrass, assess its phytochemical composition, perform the green synthesis of AgNPs, and investigate their antibacterial activity against *P. aeruginosa.* We also investigated the antibiofilm activity of the synthesized AgNPs and their extracts.

Materials and Methods

Ethical approval

Ethical approval was not required for this study. All the experiments were performed *in vitro.*

Study period and location

This study was conducted from December 2021 to August 2023 at the Laboratory of Microbiology and Virology of the Medical Institute of the People's Friendship University of Russia, Moscow, Russia.

Plant collection

This plant collection (lemongrass leaves) was implemented during a vacation trip in December 2021 in the city of Nlobison II (Cameroon). We used the mobile professional version of PictureThis-Plant Identifier (Glority LLC, 2021; Hong Kong, China) to identify the plant. After air drying, samples were packed and sent to the Microbiology Laboratory of RUDN University.

Extract preparation

Phytochemical compounds were extracted with 80% hydroethanolic solution (270 mL) and 30 g (30 g) of the vegetal material. The mixture was shaken for 24 h at 11 × *g* at 25°C. Filtration was then performed using Whatman filter paper No. 1(Kent, England) The filtered extract was further concentrated at 40°C in a rotary evaporator (IKA RV8, Staufen, Germany) equipped with a water bath (IKA HB10; IKA Werke) and a vacuum pumping unit (IKA). To avoid significant losses, the extract was recovered when the remaining volume was sufficiently small (approximately 10 mL) and placed in a small petri dish. The Petri dishes were placed in a shaker incubator (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Schwabach, Germany) at 40°C/0 × *g* for complete drying.

Analysis of the extract by high-performance liquid chromatography–mass spectrometry (HPLC-MS/MS)

The HPLC-MS/MS procedure was exactly the same as that described in our previous studies [22, 46, 47]. The 6030 series HPLC-MS/MS (Agilent, Santa Clara, CA, USA) was used, and any modifications were made to the operating conditions [22, 46, 47].

Phytofabrication, purification, and characterization of AgNPs

silver nitrate ($AgNO_3$) (PanReac AppliChem) at different concentrations (1 mM, 2.5 mM, and 5 mM) was prepared, and 9 mL of the solution was mixed with 1 mL of lemongrass water extract. For the bioreduction process, the reaction mixture was incubated in the dark at 42°C for 24 h. After the first visual observation, UV-vis, and size analysis, the optimal mixture was selected, and a reaction mixture with a final volume of 200 mL was prepared in the same proportion (1:10; 180 mL of $AgNO_3$ + 20 mL of extract) under the same conditions (42°C for 24 h). The AgNPs were then recovered by centrifuging the reaction mixture for 1 h at 21428 g, and the pellet was washed 3 times with ethanol (99%) and distilled water (4 times) [26]. The pellets were finally dried at 40°C until complete drying. The AgNPs were characterized by UV-vis spectrophotometry (PerkinElmer Lambda 950 spectrophotometer from 350 to 800 nm), photon cross-correlation spectroscopy (PCCS) (Sympatec GmbH, Clausthal-Zellerfeld, Germany), and Fourier transform infrared spectroscopy (FTIR) (Agilent Technologies, Palo Alto, CA, USA). All operational conditions were identical to those used in our previous article [22].

Antimicrobial activity testing

Preparation of inoculum and susceptibility testing to common antibiotics

Ten strains of *P. aeruginosa* were used throughout the study. strains were cultured in brain heart infusion broth (BHIB) (10 mL) for 18–24 h. Subsequently, 1.5 mL of the culture medium was introduced into Eppendorf tubes (Moscow, Russia) and centrifuged at 6000× *g* at 4°C for 15 min. The supernatant was removed, and the cells were washed thrice with phosphate-buffered saline (PBS). Bacterial cells were finally suspended in PBS to achieve a final concentration equivalent to the 0.5 McFarland scale, which was measured using a DEN1 McFarland densitometer (Grant-bio, Grant instruments Ltd., Cambridge, UK). Antimicrobial susceptibility testing was performed using the modified Kirby–Bauer disk diffusion technique described in our previous study [22]. Amoxiclav (AMC), 30 μg/disk; ampicillin (AMP), 25 μg/disk; ceftazidime (CAZ), 30 μg/disk; ciprofloxacin (CIP), 30 μg/disk; imipenem (IMP), 10 μg/disk; nitrofurantoin (NIT), tetracycline (TE), 30 μg/disk; and trimethoprim (TR), 30 μg/disc.

Preparation of antimicrobial solution

Distilled water was used to dilute the AgNPs to obtain a stock solution concentration of 1024 μg/mL.

Dimethyl sulfoxide (DMSO), purchased from BDH Laboratories, VWR International Ltd., USA, was used to prepare the lemongrass ethanolic extract. The stock solutions were sterilized by microfiltration (0.22 μm), and the resulting solutions were used to create various concentrations required for the antimicrobial testing procedure.

Diffusion method

The antimicrobial activity of lemongrass extract and AgNPs was assessed using well diffusion method. *Pseudomonas* strains were prepared in BHIB following the steps described in the previous section. After incubation at 37°C for 24 h, the strains were centrifuged (6000× *g* for 15 min) and redissolved in sterile PBS at 0.5 McFarland scale (approximatively 1.5×10^8 colony-forming unit/mL). Strains were plated in Petri dishes containing previously poured sterile Muller–Hinton agar. Wells with a capacity of 20 μL were then perforated in the agars and 20 μL of each solution was added. DMSO and distilled water used to prepare the antimicrobial solutions were used as negative controls. The inhibition diameter was observed after 24 h of incubation at 37°C.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

MIC and MBC were determined in a 96-well microplate by the micro broth dilution method the method used was the same as that used in our previous study without any modification [22]. In brief, 100 L of BHIB was introduced into all wells of the microplates, and 100 μL of the test solution was introduced into the first line according to the labeling previously performed. A two-fold dilution followed, and the excess of the last line was discarded so that each well contained the same volume (100 μL). Each microorganism was added to a specific column. The plates were covered and incubated at 37°C for 24 h. The last dilution at which no visible growth was defined was the MIC for each test solution. Subsequently, the lines on which no growth was observed were further inoculated in a solid culture medium after the Petri dishes were labeled accordingly. After further incubation of these Petri dishes at 37°C for 24 h, the last concentration at which no growth was observed was considered to be MBC.

Antibiofilm activity

The antibiofilm potential of AgNPs against *P. aeruginosa* was investigated using the crystal violet attachment assay described in Arsene *et al*. [22] with slight modifications. All antibiofilm activity tests were conducted in 96-well microtiter plates. The first step consisted of preparing three concentrations of AgNPs (MIC/2, MIC, and 2MIC) using BHIB and a stock solution of AgNPs. Subsequently, 190 μL of each mixture was introduced into six different wells (3 for the test and 3 for the specific negative control). BHIB free of AgNPs was used as the negative control. *P. aeruginosa* inoculum (10 μL, 1.5×10^8 CFI/mL) prepared as described in section 3.6.3 was introduced in the test wells. The plate was incubated at 37°C for 48 h. After this incubation, the medium was removed from the wells, and 200 μL of 1% (w/v) crystal violet solution was added to each well. The crystal violet treatment lasted 90 s, and the wells were rinsed 3 times with distilled water and then dried at 37°C. To solubilize the bound crystal violet, 200 μL of 99% ethanol was added, and OD570 was measured using an Allsheng multimode microplate reader (Feyond-A300; Alll Sheng Co. Ltd., Hangzhou, China). OD570 values were used to calculate the inhibition percentage as follows:

$$\text{Inhibition } (\%) = \frac{\text{OD in control-OD in treatment}}{\text{OD in control}} \times 100$$

Results and Discussion

Phytochemical composition of lemongrass extract

In this study, lemongrass extract was prepared using a hydroethanolic solution (80%) as the solvent. The extracts were rotavapor-dried and analyzed by HPLC-MS/MS. Figure-2 shows the chromatogram obtained following the analysis. As depicted in Figure-2, HPLC-MS/MS recorded four peaks corresponding to the bioactive compounds that were recognized by relating their peak retention time (RT), peak area (%), height (%), and mass spectral fragmentation (m/z, MS and m/z, MS/MS) patterns to those of the known compounds (Table-1). The most abundant compound (56.1%) showed a RT of 5.49 min and a molecular mass of 354 Da. This compound has been identified as chlorogenic acid (3-O-Caffeoylquinic acid) due to the m/z, MS and m/z, MS/MS ratios coupled with data available on lemongrass in the literature [42]. The second and third compounds, luteolin 8-C-glucoside (orientin) and orientin 2''-O-rhamnoside, had the same RT (12.83 min) and were identified. The last compound, luteolin 6-C-glucoside (isoorientin), was found in 13.0% of the extract. All the compounds in the hydroethanolic extract of lemongrass are phenolic compounds and flavonoids.

Compounds identified were among those expected in the extract because several studies have already been conducted on this plant, often with other solvents or essential oils [42]. However, the results obtained here differ from those obtained by Ali *et al*. [43], who reported different phenolic compound compositions (in terms of proportions and nature of the identified compounds), including caffeic acid (445.21 ± 32.77 μg/g), p-coumaric acid (393.32 ± 39.56 μg/g), chlorogenic acid (377.65 ± 4.26 μg/g), quinic acid (161.52 ± 17.62 μg/g), and quercetin-3-glucoside (151.35 ± 11.34 μg/g). This difference can be attributed not only to differences in the origins of the plants (Cameroon and Australia), but also, in particular, to the methods of extraction and analytical methods used. In their work, the authors directly extracted

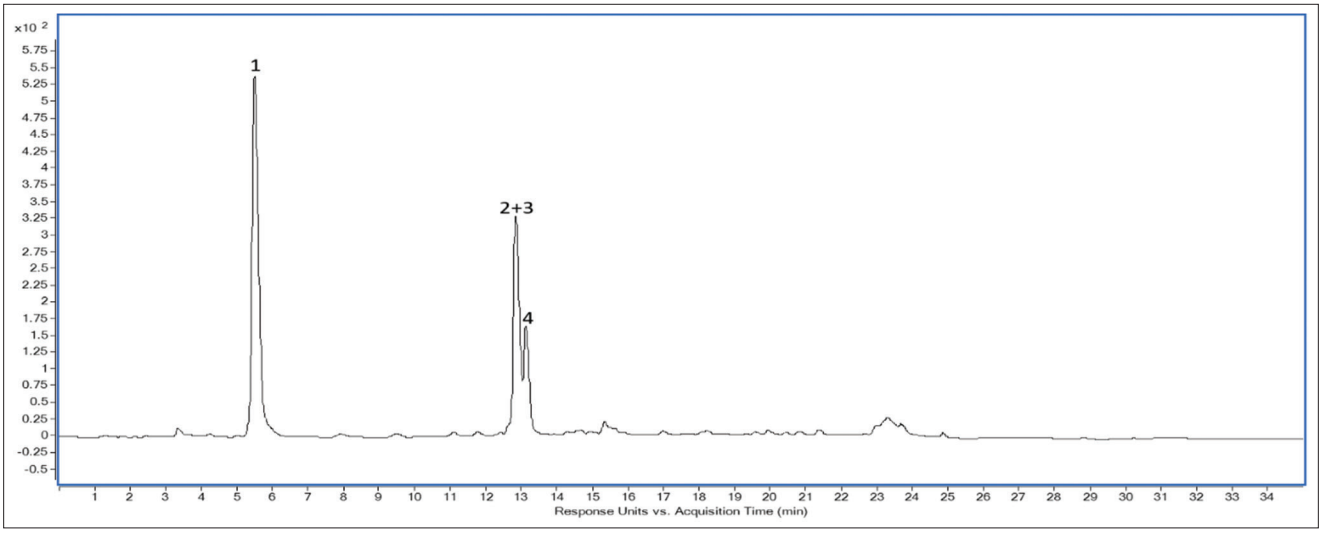


Figure-2: High-performance liquid chromatography - ultraviolet chromatogram of ethanolic extract of *Cymbopogon citratus* extract (λ = 340.0 nm).

and analyzed phenolic compounds [43], whereas in the present study, only the dried hydroethanolic fraction was investigated. Notwithstanding this, because hydroethanolic extraction is relatively simple and easy to carry out, the presence of a significant proportion of chlorogenic acid and the three other phenolic compounds are not negligible and can be exploited for their antioxidant, antimicrobial, anti-inflammatory, anti-hypertensive, antidiabetic, antimutagenic, anxiolytic, and hypoglycemic and hypolipidemic activities [42–44]. In addition, these compounds can play a capping and stabilizing role during the biogenic synthesis of different NPs. Therefore, lemongrass extract was further used in the synthesis of AgNPs before investigating the antimicrobial potential of both hydroethanolic extract and AgNPs.

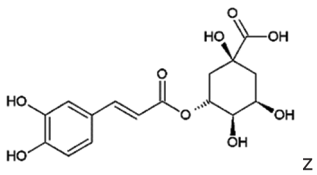
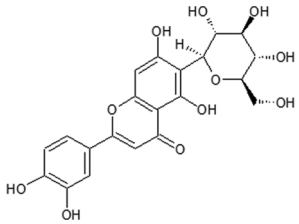
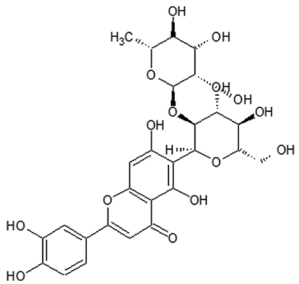
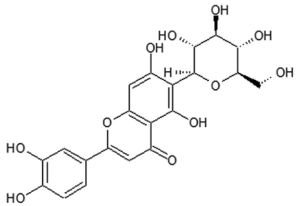
Characteristics of AgNPs synthesized

AgNPs were synthesized with lemongrass extract using $AgNO_3$ at different concentrations (0 mM, 1 mM, 2.5 mM, and 5 mM) as precursor. The phytofabrication process was based on the ability of the reducing agents present in lemongrass extract to convert silver ions (Ag^+) into silver atoms (Ag^0) because silver ions can change an electron from a positive valence to a zero-valent state [45]. After 24 h of incubation of the reaction mixtures at 37°C and avoiding any contact with light, the color change was observed visually. As shown in Figure-3, a color change from colorless to yellowish-brown was observed in all the tubes containing $AgNO_3$+ extract, with an accentuation of color as the concentration of $AgNO_3$ increased. This result agrees well with the findings reported in other investigations [46–50]. Several researchers have attributed this color change characteristic of AgNP formation to the excitation of surface plasmon vibration in AgNPs [45, 51, 52]. On the basis of visual observations, the reaction mixture containing NPs synthesized with 1 mM $AgNO_3$ was very lightly colored (possibly due to a low concentration of NPs), whereas the reaction mixture containing NPs synthesized with 5 mM $AgNO_3$ was highly colored and showed deposits at the bottom of the tube, which indicated the presence of a high concentration of large particles [22].

As depicted in Figure-4, the UV–vis absorption spectra of AgNPs synthesized with 1 mM, 2.5 mM, and 5 mM exhibited localized surface plasmon resonance at 416, 464, and 485 nm. The previous visual observation was confirmed because the absorbance increased with the concentration of $AgNO_3$. 0.1A for 1 mM, 0.32 A for 2.5 mM, and 0.65 A for 5 mM. The tuning fork of the optimal absorptions of the different solutions confirmed the formation of AgNPs because most researchers agree that the absorption spectra of AgNPs have an absorbance peak between 400 and 500 nm [53–59]. Moreover, due to the deposit observed at the bottom of the tube containing the AgNPs synthesized with 5 mM $AgNO_3$ and the weak color of the AgNPs synthesized with 1 mM $AgNO_3$, we decided to continue the study only with the AgNPs synthesized with 2.5 mM $AgNO_3$. Further characterization was performed using photon cross- PCCS and FTIR.

In addition to the particle size, the PCCS provided other important information such as Sauter mean diameter (SMD), volume mean diameter (VMD), and specific surface area (Sv). As shown in Figure-5, the average hydrodynamic diameter of the NPs was 50.29 nm. This diameter highlighted the effective formation of AgNPs according to the definition of NPs, which stipulates that NPs are small particles ranging from 1 to 100 nm [60]. This finding was further confirmed by the Sv value (120.10 m^2/cm^3), since the European commission proposes to define a material as a nanostructure when it has an Sv >60 m^2/cm^3, excluding materials consisting of particles smaller than 1 nm [61, 62]. VMD and SMD were 50.63 nm and 49.96 nm, respectively, and these values were close enough, indicating that these two parameters were closer to each other and that the synthesized particles

Table-1: Chemical composition of ethanolic extract of *Cymbopogon citratus* extract.

Compound	Retention time, min	Molecular formula	Structural formula	Molecular mass, Da	m/z, registration of positive ion	m/z, registration of negative ion	Content, %
Chlorogenic acid (3-O-Caffeoylquinic acid)	5.49	$C_{16}H_{18}O_9$		354	355 $[M+H]^+$, 731 $[2M+Na]^+$	191 $[C_7H_{11}O_6]^-$, 353 $[M-H]^-$, 707 $[2M-H]^-$	56.1 ± 0.1
Luteolin 8-C-glucoside (Orientin)	12.83	$C_{21}H_{20}O_{11}$		448	449 [M+H]+, 919 [2M+Na]+	447 [M-H]-, 895 [2M-H]-	30.9 ± 0.2
Orientin 2''-O-rhamnoside		$C_{27}H_{30}O_{15}$		594	595 [M+H]+, 1211 [2M+Na]+	593 [M-H]-	
Luteolin 6-C-glucoside (Isoorientin)	13.21	$C_{21}H_{20}O_{11}$		448	449 [M+H]+, 919 [2M+Na]+	447 [M-H]-, 895 [2M-H]-	13.0 ± 0.1

were perfectly spherical, as suggested by Kharabi *et al*. [63]. Unfortunately, due to the limitations of our laboratory, we were not able to confirm the real shape of AgNPs by TEM or SEM analysis.

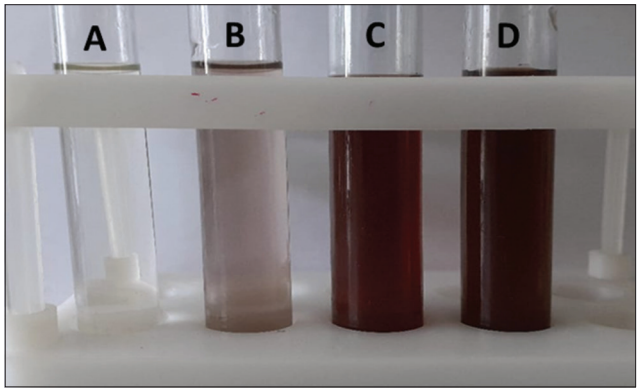


Figure-3: Color of the reaction mixtures after the phytofabrication process of silver nanoparticles with various concentrations of silver nitrate (A: 0 mM, B:1 mM, C: 2.5 mM, and D: 5 mM).

Furthermore, FTIR analysis of the extract and phytofabricated AgNPs was performed to determine whether the molecules present in the extract (Table-1) persist on the surface of the AgNPs. The FTIR spectrum of lemongrass extract is shown in Figure-1 – black line. As expected, the functional groups identified in the extracts (chlorogenic acid, rientin, rientin 2''-O-rhamnoside, and isoorientin) were present (Table-1). We observed a wide band of stretching vibrations of the polymeric hydrogen bond at 3300 cm^{-1} and bending vibrations of phenolic hydroxyl at 1268 and 1374 cm^{-1}. The absorption band of the C-H group was highly pronounced at 2856 and 2916 cm^{-1}, whereas the bending vibrations of the methyl group were detected at 1453 cm^{-1}. In addition, the absorption bands at 709, 765, and

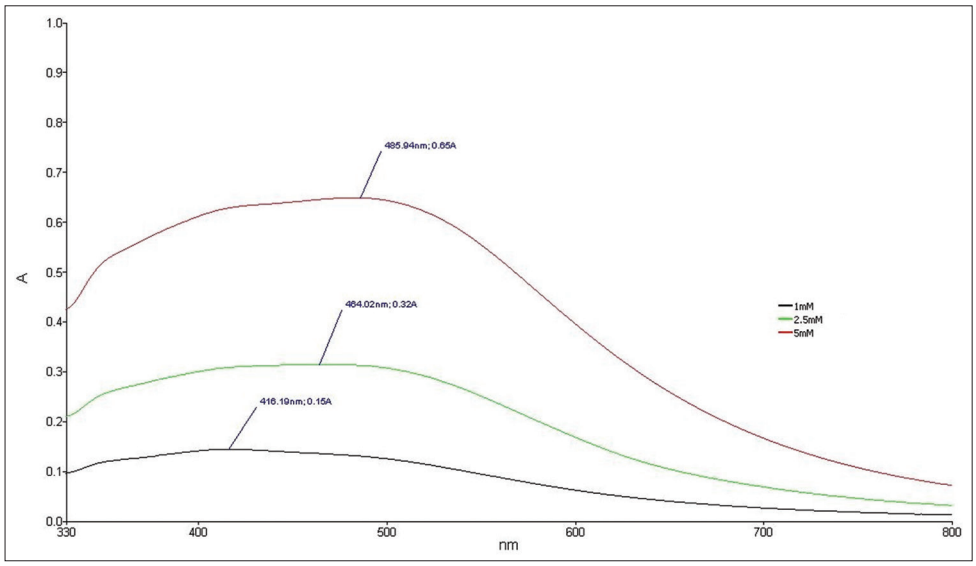


Figure-4: Ultraviolet–visible spectrum of solutions with phytofabricated silver nanoparticles.

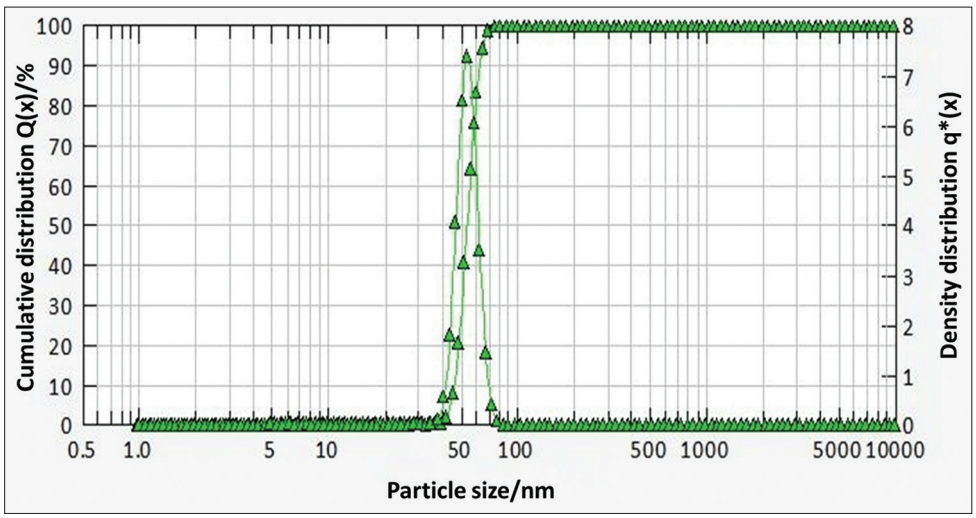


Figure-5: Particle size of AgNPs phytofabricated with lemongrass extract and 2.5 mM silver nitrate. The AgNPs displayed a ×10 = 43.11nm, ×50 = 50.29 nm, ×90 = 58.65 nm, Sauter mean diameter = 49.96 nm, volume mean diameter = 50.63 nm and surface area = 120.10 m^2/cm^3. AgNPs: Silver nanoparticles.

816 cm^{-1} characterize ring torsion. A strong absorption band at 1717 and 1602 cm^{-1} in the spectrum of the extract corresponds to the vibrations of the carbonyl group and most likely indicates the presence of aryl and unsaturated acids in the mixture. The intensive band at 1017 cm^{-1} corresponds to the -O vibration of the alcohols.

In general, the IR spectra of the phytofabricated AgNPs were similar to those of the lemongrass extract spectra with some differences in the position and intensity of some absorption bands. The spectral analysis of the obtained AgNPs indicated the presence of stabilizing organic fragments containing C = O, C-H, and O-H groups on the surface of the NPs (Figure-6, red line), thus revealing that the biogenic synthesis of AgNPs allowed preservation of the stabilizing layer of organic components from the extract on the surface of the NPs.

Susceptibility of the *P. aeruginosa* strains to antibiotics

In general, the assessment of the susceptibility profile of bacteria to antibiotics makes it possible to determine, in which antibiotics should be prescribed for the effective treatment of a specific bacterial infection. In the present study, the antibiotic susceptibility test was performed to evaluate the differences in the efficacy of AgNPs and lemongrass extract against common antibiotic-resistant and unresistant bacteria. It should be noted that the 10 strains of *P. aeruginosa* used in this study were kept and provided by the microbiology laboratory of the RUDN University but had been isolated and identified a few months earlier in children suffering from UTIs in the Russian children's clinical hospital in Moscow, Russia. The resistance profiles of these strains were not available. Thus, the inhibition diameters were measured and interpreted, and the multi-drug resistance index (MDR) was calculated after applying the slightly modified Kirby–Bauer disk diffusion method. Antibiotics tested were AMP, AMC, CAZ, CIP, IMP, NIT, TE, and TR. Table-2 presents the susceptibility profile of the 10 *P. aeruginosa* (PA) strains to antibiotics. All bacteria were resistant to at least three antibiotics (MDR = 0.375), and the least susceptible bacterium (PA5) was simultaneously resistant to six out of eight antibiotics (MDR = 0.750). CIP and imipenem (IPM) were the only antibiotics to which this PA5 strain was not resistant. Figure-7 shows the resistance levels to each antibiotic (in percentage, %). Figure-7 shows that the resistance level ranged from 0% to 100%. All bacteria were resistant to AMP (100%, n = 10), 90% to NIT and TR, 70% to TE, 40%, and 20% to AMC and CAZ, 10% (n = 1) to IPM, and no strain was resistant to CIP.

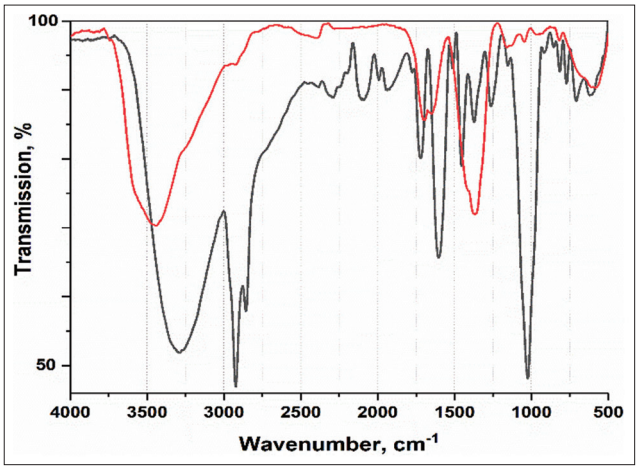


Figure-6: Fourier transform infrared spectroscopy spectra of lemongrass extract (black) and phytofabricated silver nanoparticles (red).

The resistance of *P. aeruginosa* observed in the present study is similar to that reported by other researchers who tested a larger number of strains [64]. For example, Javiya *et al*. [64] tested 56 strains of *P. aeruginosa* against several antibiotics, including AMP, in a tertiary care hospital in Gujarat. It was found that AMP was the antibiotic to which *P. aeruginosa* showed the highest l-resistance with 98.21% (55/56 bacteria). The high resistance of *Pseudomonas* strains to AMP can be explained by intrinsic and natural resistance. It is well known that *P. aeruginosa* is resistant to a variety of antibiotics, including aminoglycosides, quinolones, and β-lactams [65–68]. Pang *et al*. [68] reported that *P. aeruginosa* possesses an *ampC* gene, encoding the hydrolytic enzyme β-lactamase, which can break the amide bond of the β-lactam ring, leading to the inactivation of β-lactam antibiotics, a class of antibiotics to which AMP belongs. Conversely, AMC showed a lower resistance rate (20%). AMC is a combination of amoxicillin and clavulanic acid (amoxicillin/clavulanic acid), while amoxicillin is almost similar to AMP [69]. The higher efficiency of AMC compared to that of AMP can be ascribed to the role of clavulanic acid, an inhibitor of β-lactamase enzymes. Clavulanic acid contains a β-lactam ring that binds to the active site of β-lactamase and inactivates the enzyme, thereby enhancing the antibacterial effect of β-lactam antibiotics, such as amoxicillin [70]. These results suggest that the intrinsic resistance of *Pseudomonas* to β-lactams can be suppressed by combination therapy using these antibiotics with β-lactamase inhibitors.

In addition, the high resistance pattern observed in NIT (70%) and TR (70%) has also been observed by Chavan *et al.* [71]. A recent study by Chavan *et al*. [71] on 445 strains of *P. aeruginosa* showed a resistance rate of 97.6% to NIT. Cunha *et al*. [72], after several years of experience with urinary tract infections, suggested that NIT is active against most common uropathogens, but most *Proteus* species, *Serratia marcescens*, and *P. aeruginosa* are naturally resistant. Regarding resistance to TR, Köhler *et al.* [73] argued that resistance to *Pseudomonas* is due to the overexpression of OprM and OprJ proteins, which play a role in the multiple drug efflux system.

Table-2: Susceptibility to antibiotics of the 10 *Pseudomonas aeruginosa* used in the study.

Pseudomonas aeruginosa strains	CAZ	AMP	NIT	TE	TR	AMC	IPM	CIP	MDR Index
PA1	S	R	R	R	R	S	R	S	0.625
PA2	S	R	R	R	R	S	S	S	0.500
PA3	S	R	R	R	R	R	S	S	0.625
PA4	S	R	R	R	R	S	S	S	0.500
PA5	R	R	R	R	R	R	S	S	0.750
PA6	S	R	R	R	R	R	S	S	0.625
PA7	S	R	R	R	R	S	S	S	0.500
PA8	S	R	R	S	R	R	S	S	0.500
PA9	S	R	R	S	R	S	S	S	0.375
PA10	R	R	S	S	S	S	S	S	0.250

AMP=Ampicillin, AMC=Amoxyclav, CAZ=Ceftazidime, CIP=Ciprofloxacin, IMP=Imipenem, NIT=Nitrofurantoin, TE=Tetracycline, and TR=Trimethoprim, R=Resistant, S=Susceptible.

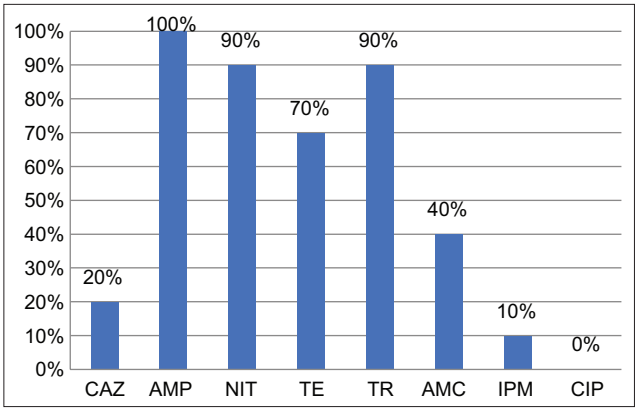


Figure-7: Resistance level of the *Pseudomonas aeruginosa* used (n = 10) to the different antibiotics tested. AMP=Ampicillin, AMC=Amoxyclav, CAZ=Ceftazidime, CIP=Ciprofloxacin, IMP=Imipenem, NIT=Nitrofurantoin, TE=Tetracycline, and TR=Trimethoprim.

Overall, the susceptibility of *Pseudomonas* strains to the tested antibiotics in this study suggests that CIP and IMP can be effective against *P. aeruginosa* infections. Moreover, the results regarding AMP and AMC suggest that it would be beneficial to pay more attention to antibiotics resistant to *P. aeruginosa* by combining them with other molecules in combination therapies. In addition, in view of the MDR indexes observed here, which vary from 0.25 to 0.75, and the observations made in the literature, it seems clear (despite the limited number of strains tested in this study) that resistance to antibiotics in *Pseudomonas* grows very quickly, similar to other gram-negative bacteria. Therefore, in addition to the above-mentioned suggestions, it is necessary to seek better alternative solutions to address the major public health issue of antibiotic resistance.

Antimicrobial activity of AgNPs and lemongrass ethanolic extract

The antimicrobial activity of the hydroethanolic extract of lemongrass and AgNPs was investigated using the disk diffusion method. The MIC and MBC were determined using the microbroth dilution assay, and the antibiofilm effect was assessed against PA3 and PA5, which were the most biofilm producers.

Inhibition zone (IZ)

We investigated the antagonistic activity of AgNPs and lemongrass ethanolic extract against 10 *P. aeruginosa* strains. No IZ was observed irrespective of the concentration of the lemongrass extract used, despite all the compounds found in the extract (Table-1). This result disagrees with the findings of Subramaniam *et al*. [74], who found inhibition diameters ranging from 10 to 15 mm during their study of the antibacterial activity of *C. citratus* against a panoply of gram-negative bacteria, including *P. aeruginosa*. The authors concluded that *P. aeruginosa* was highly susceptible to *C. citratus* [74]. The absence of antimicrobial activity of the ethanolic extract of *C. citratus* observed in this study is also in disagreement with several other studies that have also investigated the antibacterial effect of lemongrass [75–78]. This difference between the results of the present study and those observed in others could be explained by several factors, such as the geographical area of origin of the extracted plants [79], the extraction procedure [80–82], and the phenotype and genotypic differences of the strains tested [83]. De Sá Filho *et al*. [79] recently reported that factors such as the physicochemical characteristics of soils, different dynamics of the action of climatic factors, and the consequent diversity of biological interactions in biomes greatly affect the chemical composition of plants. In addition, the previous studies have revealed that the types of extraction, the extraction parameters such as the nature of solvents used and their concentration, the m/v ratio, the temperature, and the extraction time can lead to very significant differences in the chemical composition of the extracts and therefore of the antimicrobial activity [80, 81]. In a recent study, genotypic and phenotypic differences between microorganisms of the same species can induce changes in susceptibility to existing antibiotics or antimicrobials [81]. Consequently, considering the chemical compounds (chlorogenic acid, orientin, and isoorientin) found in lemongrass extract and considering that these compounds have already demonstrated their antimicrobial activities [84–87], it can be concluded, subject to further research, that the *Pseudomonas* strains tested in

this study are not susceptible to lemongrass extract due to their resistance as observed with the results of their susceptibility to conventional antibiotics (Table-2).

A noteworthy antimicrobial activity was observed against the 10 *P. aeruginosa* strains tested with phytofabricated AgNPs. As shown in Figure-8, the inhibition diameter ranged from 0 to 24 mm, independent of *Pseudomonas* strain and AgNPs concentration. As expected, no IZ (IZ = 0 mm) was observed in the negative control (distilled water used to prepare the solutions of AgNPs). Despite the presence of *P. aeruginosa* strains, IZs decreased with decreasing AgNP concentrations, suggesting that the phytofabricated AgNPs are dose-dependent. Similar results have been reported in most studies investigating the antibacterial properties of antimicrobial compounds [22, 26, 46, 47]. In addition, at the same concentration of AgNPs, there was no significant difference in the IZ between the different *Pseudomonas* strains. This information demonstrates the non-selective effectiveness of the antimicrobial activity of AgNPs compared with our previous study, where it was observed that AgNPs synthesized using phytocompounds from papaya seeds and roots exhibited weak antibacterial activity against most Gram-negative bacteria, whereas bacteria that were more susceptible to common antibiotics were more sensitive to these same AgNPs [46].

MIC and MBC

As previously suggested by Oda *et al*. [88], in addition to the well diffusion method, we further applied the microbroth dilution assay to determine the MIC and MBC values of AgNPs and lemongrass ethanolic extract to better understand their antibacterial potential against *Pseudomonas* strains tested. MIC is the lowest concentration of AgNPs or extracts capable of inhibiting bacterial growth during the serial dilution process. As shown in Table-3, the MIC values of AgNPs against the tested *Pseudomonas* strains ranged from 2 to 16 µg/mL. The highest MIC was observed against PA5, which was the most resistant bacteria to common antibiotics, with an MDR index of 0.75 (Table-1). These results confirm the above hypothesis that the intrinsic phenotype of the bacterium plays an essential role in its susceptibility to antimicrobials. In addition, lemongrass extract was completely ineffective against all the *Pseudomonas* strains, and the MIC could not be determined. These results suggest that the initial concentration of lemongrass used is very low or that the ethanolic extract of lemongrass has no antimicrobial activity against *P. aeruginosa*.

Moreover, MBC was defined as the lowest concentration of AgNPs on the agar plate that showed no growth of *Pseudomonas* strains. In the present investigation, MBC ranged from 4 µg/mL to 64 µg/mL. Interestingly, the MBC of AgNPs was equal to or <4 MIC in all of the bacteria tested, and it became obvious that AgNPs had bactericidal activity because the MBC/MIC ratio was 4 (MBC/MIC 4) [22].

Antibiofilm ability

Biofilm formation is a phenomenon that allows bacteria to regroup inside a matrix and to better resist external conditions, sometimes resulting in increased resistance to antibiotics and the complication of

Table-3: MIC and MBC of phytofabricated silver nanoparticles using lemongrass extract.

Pseudomonas aeruginosa strains	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
PA1	2	8	4
PA2	4	4	1
PA3	2	8	4
PA4	4	8	2
PA5	16	64	4
PA6	4	4	1
PA7	8	16	2
PA8	4	8	2
PA9	4	8	2
PA10	2	8	4

MIC=Minimum inhibitory concentration; MBC=Minimum bactericidal concentration

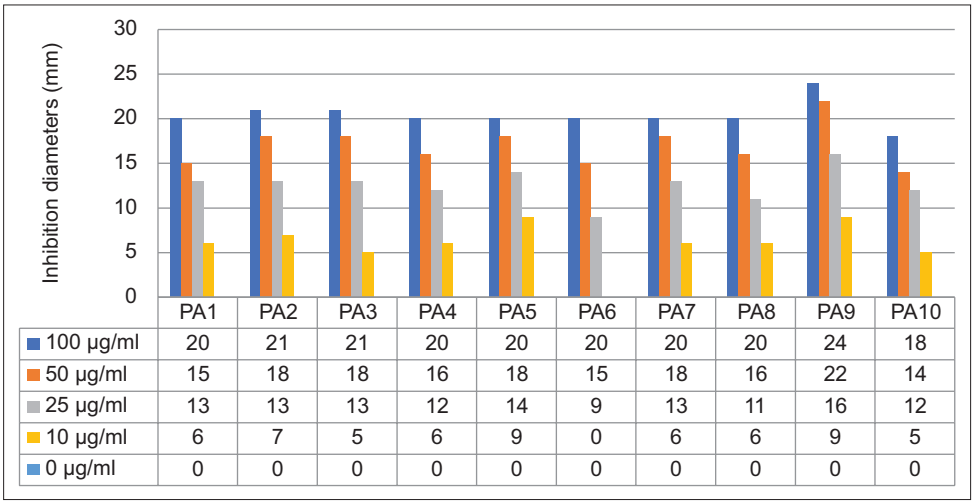


Figure-8: Inhibition diameter of different concentrations (0, 10, 25, 50, and 100 µg/mL) of phytofabricated silver nanoparticles against the 10 *Pseudomonas aeruginosa* strains.

infections [22, 46, 47, 89–92]. Before studying the antibiofilm activity of AgNPs in this study, it was necessary to identify *Pseudomonas* strains capable of forming biofilms. PA3, PA5, and PA6 were the only *P. aeruginosa* strains to produce biofilms. In addition, the crystal violet attachment assay revealed that the phytofabricated AgNPs very strongly inhibited biofilm formation in the three strains tested, including at subinhibitory concentrations (below the MIC). Overall, biofilm formation was inhibited at percentages ranging from 95 to more than 99% in all bacteria, and the lowest inhibition percentage (95%) was observed at MIC/2 (8 μg/mL) in PA5. These results suggest that relatively low concentrations of AgNPs could be sufficient to inhibit biofilm formation even in *Pseudomonas* strains exhibiting antibiotic resistance, similar to PA5 (MDR index = 0.750; Table-1). Although quorum-sensing (QS) investigations were not performed in the present study, the inhibition of biofilm formation might indicate that AgNPs generated using lemongrass may have anti-QS activity. Several studies have recently revealed that biofilm formation and other phenotypes, such as bioluminescence, swarming motility, sporulation, production of degrading enzymes, and other virulence factors, are QS-controlled phenotypes; QS is the ability of microbial strains to communicate with each other through a cell-density-dependent mechanism [91–96]. Anti-QS activity (also called quorum quenching) is increasingly suggested as a promising strategy in the search for new antimicrobials [91]. The findings of this study on a strong biofilm inhibition at MIC/2 present perspectives for future research directed toward the application of phytofabricated AgNPs for quorum quenching.

Conclusion

Despite the compounds found in the extract [caffeic acid (445.21 ± 32.77 μg/g), p-coumaric acid (393.32 ± 39.56 μg/g), chlorogenic acid (377.65 ± 4.26 μg/g), quinic acid (161.52 ± 17.62 μg/g), and quercetin-3-glucoside (151.35 ± 11.34 μg/g)], the lemongrass extract did not display any antimicrobial activity. However, the results of the present study indicated that lemongrass is a suitable candidate for green synthesis of AgNPs. These AgNPs had an average size of 50.29 nm. The use of lemongrass extract for the phytofabrication of AgNPs is environmentally friendly, simple, and fast, as demonstrated by PCSS, FTIR, and UV–vis spectrophotometry. The resulting AgNPs exhibited good antimicrobial and antibiofilm activity against the tested *Pseudomonas* strains. Given the noteworthy antimicrobial potential of the synthesized AgNPs, further research is needed to investigate their safety before they can be recommended for use.

Data Availability

All data are incorporated in the manuscript.

Authors' Contributions

MMJA, KAA, and PIV: Conceptualized and designed the research. MMJA, AKLD, KP, VA, BMN, SLA, YNV, VEA, EIZ, KZS, and SG: Conducted the laboratory experiments. MMJA, AKLD, SLA,YNV, VEA, EIZ, and KZS: Wrote the first manuscript draft, edited, and revised the final version of the article. All authors have read, reviewed, and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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