

ORIGINAL ARTICLE

Antibacterial Efficacy of the Phenyl Lactic Acid-Based Chitosan Nanoparticles against Virulence Protein Regulation in *Acinetobacter baumannii*

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ABSTRACT

Background: The biosynthesis of nanoparticles using the green route is an effective strategy in nanotechnology that provides a cost-effective and environmentally friendly alternative to physical and chemical methods.

Aim: The aim is to prepare chitosan-based silver nanoparticles (AgNPs) conjugated with phenyl lactic acid (PLA) through the green route and test their antibacterial activity. The study included isolation and identification of 20 clinical samples which were collected from patients from laboratories of Al-Nokhba diagnostics center, Baghdad, Iraq, between March 2024 to May 2025 with the diagnosis of *Acinetobacter baumannii*.

Methods: In this study *A. baumannii* isolates were obtained from Medical city hospital. The NPs synthesized were characterised by UV spectrometric analysis, FTIR, and particle shape and size morphology by SEM. The particles were evaluated for their antibacterial efficacy by studying the membrane damage and differential regulation of pili proteins.

Results: The nanoparticles are almost spherical to oval or rod-shaped with smooth surfaces and have a mean particle size in the range of 85 nm with a zeta potential of -1.8 mV. The NPs exhibited significant MIC values of 105.43 µg/mL (positive control 44.45 µg/mL) and are effective in inhibiting *A. baumannii* growth. At 20 and 40 µg/mL of AgNPs, the inhibition zone measured 5 mm and 17 mm, respectively ($p < 0.05$). The percentage inhibition for biofilm inhibition during a 24-hour period was 56, 78, and 94.5% at $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC, respectively. The membrane damage of 86.10% at MIC dose was also confirmed by the cellular protein leakage experiment. When compared to the control, it was discovered that the expression levels of pilA, pilB, pilC and pilF were downregulated. On the other hand, we did not observe any variation in pilM and pilN expression patterns upon treatment.

Conclusions: The current study's findings demonstrated that AgNPs synthesized had encouraging antibacterial qualities and may be viable candidates for other bacterial infection treatment approaches.

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KEYWORDS

Acinetobacter baumannii. chitosan, phenyl lactic acid, PCR, protein leakage, FTIR

LIST OF ABBREVIATIONS

AgNPs - silver nanoparticles
PLA - phenyl lactic acid
SEM - scanning electron microscope
NPs – nanoparticles
MIC - minimal inhibitory concentration

A. baumannii - *Acinetobacter baumannii*
 CS-AgNPs - chitosan-based silver nanoparticles
 Ag⁺ - silver ions
 ROS - reactive oxygen species
 MRS - Man-Rogosa-Sharpe
 Phe - phenyl alanine
 FTIR - Fourier-transform infrared
 EPS - extracellular polymeric substance
 HPLC - high-performance liquid chromatography
 UV-Vis - ultraviolet and visible
 β -lactamases - Beta lactamases
 DLS - dynamic light scattering
 cDNA - complementary DNA
 MDR - multi-drug resistance

INTRODUCTION

Acinetobacter baumannii is a Gram-negative opportunistic zoonotic pathogenic bacteria that can cause nosocomial infections ranging in severity from mild to lethal [1]. Due to the synthesis of various virulence factors and the evolution of multiple resistance mechanisms, the clinical importance of this zoonotic disease is rapidly increasing. Even in the absence of flagellum-mediated motility, it can move by twitching or surface-associated motility [2]. In surface adhesion and biofilm development, type IV pili's extension, attachment, and retraction result in a synchronized multicellular movement called "twitching motility." Surface-associated motility is a form of movement that doesn't require appendages and is probably brought on by the release of extra polymeric molecules. This kind of motility is linked to 1,3-diaminopropane synthesis, lipooligosaccharide formation, efflux pump proteins, and natural competence [3]. Because *A. baumannii*'s motility is strongly related to its virulence traits, it can be used as a specialized prophylactic or therapeutic strategy [4]. By mediating adhesion to host cells, creating biofilms, and promoting motility, *Acinetobacter baumannii* pili are essential virulence factors that enhance its pathogenicity. While other pili, like Csu pili, are necessary for adhesion to host cells, colonization, and the formation of mature biofilms, Type IV pili allow for twitching movement and DNA uptake. For the bacteria to cause hospital-acquired illnesses, several roles are essential. In order to cause infection, pili mediate adhesion to both biotic (like host epithelial cells) and abiotic (like medical devices) surfaces [5]. Additionally, they are crucial parts of biofilms, which are bacterial populations that stick to surfaces and provide a protective matrix. Biofilms contribute to chronic or persistent infections by shielding *Acinetobacter baumannii* from immune cells and drugs. The bacteria can move across surfaces thanks to a type of surface motility known as twitching motility, which is mediated by type IV pili [6]. Surface-associated motility is another kind of motility that is linked to type IV pili and is similarly essential for pathogenicity. Pili aid the bacteria in avoiding the hu-

man immune response by sticking to surfaces and producing biofilms. Additionally, type IV pili contribute to the reception of foreign DNA, which can facilitate horizontal gene transfer and the bacterial acquisition of novel genes, including those that confer resistance to antibiotics [7].

Both Gram-positive and Gram-negative bacteria as well as fungi are susceptible to the broad-spectrum antibacterial action of phenolic acid (PLA) [8]. It functions by breaking down the bacterial cell membrane, which results in intracellular contents leaking out, interfering with metabolic processes, and preventing the formation of biofilms [9]. PLA is regarded as a promising natural antibacterial agent for food preservation because of its efficacy, low odor in comparison to other organic acids, and potential for economical manufacture [10]. Important intracellular components like DNA and ATP may seep out as a result of PLA's ability to harm the cell membrane. Additionally, it reduces the metabolic activity and cell viability of bacteria. By introducing itself into the genomic DNA of bacteria and interfering with their function, PLA is thought to prevent the development of new biofilms as well as harm those that already exist [11].

By fusing the antibacterial activity of silver ions with the characteristics of chitosan, chitosan-based silver nanoparticles (CS-AgNPs) demonstrate strong antimicrobial activity against a variety of bacteria and fungi [12]. The chitosan serves as a transporter and has antibacterial properties of its own, while the silver ions released by the nanoparticles harm bacterial proteins and enzymes, causing cell death [13]. Because of their synergistic impact, they work well in applications such as antibacterial wound dressings, which help hasten healing by preventing infection [14]. Silver ions (Si⁺) are released into the microbial cell by the nanoparticles. By attaching to their sulfhydryl groups and deactivating them, these ions disrupt vital proteins and enzymes. Within the cell, silver ions induce oxidative stress, which raises the generation of reactive oxygen species (ROS) and ultimately results in cell death. Chitosan possesses antibacterial qualities of its own. The synergistic impact of both components is utilized by CS-AgNPs to give increased antibacterial efficacy [15].

In the contemporary healthcare system, *Acinetobacter baumannii* is without a doubt one of the most effective bacteria that cause nosocomial infections acquired in hospitals. Few medications are effective in treating infections caused by multi-drug-resistant *Acinetobacter baumannii* because of the high frequency of illnesses and outbreaks generated by this organism [16]. The acquisition of β -lactamases, up-regulation of multidrug efflux pumps, modification of aminoglycosides, permeability problems, and alteration of target sites are some of the mechanisms of antibiotic resistance of this organism that are now of interest in the search for new antibacterial drugs [17].

Efforts were made to investigate the antibacterial activity of nanoparticles conjugated with PLA in light of the

aforementioned findings and the seriousness of the pathogenicity. We selected silver nanoparticles based on chitosan because of their excellent antibacterial properties. The broad aim was to extract, purify, characterize the phenyl lactic acid (PLA) from fermented LAB and synthesize silver nanoparticles conjugated with PLA and studying their antibacterial efficacy against human pathogen *Acinetobacter baumannii*.

MATERIALS AND METHODS

Samples collection

The study included isolation and identification of 20 clinical samples which were collected from patients from laboratories of Al-Nokhba diagnostics center, Baghdad, Iraq, between March 2024 to May 2025 with the diagnosis of *Acinetobacter baumannii*.

Lactic acid bacteria cultivation and fermentation

The powdered inoculum was allowed to grow in Man-Rogosa-Sharpe (MRS) broth (HiMedia, India) at 30°C for 24 - 48 hours. The pure isolate was further used for fermentation on synthetic MRS media containing phenyl alanine (Phe) at 0.1 gm/L. The fermented flasks were incubated at 30°C for 72 hours. Following incubation, the contents were filtered once and centrifuged for 8000 rpm for 10 minutes at 4°C. The supernatant obtained was filter-sterilized (0.45 µm-pore-size, Sigma Aldrich). The culture filtrates obtained were further screened for the phenyl lactic acid (PLA) using HPLC [18].

Extraction and determination of PhLA

About 50 mL of cell-free supernatant (pH 2.0) was extracted 3 times with equal volume of ethyl acetate. Post extraction, anhydrous Na₂SO₄ was added to the pooled extracts and then filtered using Whatman No. 1 filter paper and concentrated using a vacuum rotary apparatus. An aliquot of the filtrate was used for the synthesis of Nanoparticles. The dried residue was re-dissolved with 10 mL of water containing 0.05% Trifluoroacetic acid (v/v), filtered (0.22 µm) and about 100 µL was injected into the HPLC system (AKTA Basic 10, P-900 series pump, Amersham Bio sciences) fitted with a IRPC C2/C18 column. The mobile phase used was a gradient mixture of mobile phases A (methanol + 0.05% trifluoroacetic acid) and phase B (double-distilled water + 0.05% trifluoroacetic acid) for 15 minutes with a flow rate of 0.5 mL/minute [19].

Green synthesis of chitosan-based AgNPs: About 1 gm of chitosan was added to a conical flask and added with 0.5 mL of acetic acid and 50 mL of distilled water. The contents were stirred for 2 hours and added with 1 mM of silver nitrate. The contents were added with 10 mL of supernatant filtrate from the previous section and stirred using a magnetic stirrer until the colour change. The suspension was now centrifuged at maximum speed and the pellet was dried in hot air oven after washing with

distilled water. The dried AgNPs were further characterized and used for the application work [20].

Dynamic light scattering (DLS) and characterization

To verify the generation of AgNPs, a UV-VIS spectrophotometer (Shimadzu, 1800) with a resolution of 1 nm between 200 and 800 nm was used to perform UV-visible spectrum analysis in an aqueous solution. Using a Perkin Elmer Spectrum One FTIR spectrophotometer (Bomem MB100) with a 3,600 - 400 cm⁻¹ range, Fourier-transform infrared (FTIR) spectra were obtained using the KBr pellet method. Using FESEM-EDX at a 15 KV accelerating voltage (SEM; Philips XL30 ESEM), the surface morphology and atomic ratio of the AgNPs were ascertained [21].

Antimicrobial testing

The antibacterial activity of the AgNPs was assessed using the agar diffusion and minimal inhibitory concentration (MIC) methods along with in conjunction with a positive control (Colistin sulfate 10 mg/mL).

Agar well diffusion method

The agar diffusion method was used to test the antimicrobials nature of synthesized AgNPs on LB agar plates. The bacterial culture was poured over LB agar in an amount of approximately 100 µL. The wells were cut with a sterile cork borer (3 mm). The wells received about 50 µL of AgNPs at different concentrations. Colistin sulphate (10 mg/mL) was used as a positive control during the study. After that, the plates were incubated at 37°C for around 24 to 48 hours. Following incubation, the mean diameter (mm) of the growth inhibition zone was measured and recorded. All tests were carried out using the same experimental settings and in triplicate.

Minimal inhibitory concentration (MIC)

In brief, about 10 mL of LB broth and 10 µL of the overnight bacterial suspension was added with 50 µL of AgNPs of varying concentrations added to their respective tube. Colistin sulphate was used as positive control. The tubes were then incubated for 18 to 24 hours at 37°C. The minimum concentration (MIC) at which no turbidity was observed was determined [22].

Biofilm inhibition assay

Biofilm inhibition was performed as described by [23] but with slight modifications. In a 96-well microtiter plate, about 180 µL of LB broth was added and inoculated with 10 µL of bacterial culture, with the exception of the negative control. Positive control was used with Colistin sulphate (MIC). AgNPs were added at varying concentrations of ¼ MIC, ½ MIC and MIC concentrations. The microtiter plates were incubated for 24 to 48 hours at 37°C. Following incubation, the plates were added with 0.1% crystal violet and detained with 200 µL of ethanol/acetone (80:20). Washing was done with PBS (pH 7.4). The intensity of color developed

was analysed using a Plate reader (Genetix Ltd.) at 595 nm. The percent inhibition was calculated using the formula $\text{OD of control} - \text{OD of sample} / \text{OD of control} \times 100$.

Cellular protein leakage assay

The Bradford assay was used to measure the amount of cellular protein that leaked as a result of bacterial mortality and cell membrane disruption (Bradford, 1976). Bacterial cells (2×10^5 CFU/mL) were inoculated into 10 mL of LB media, and different doses of AgNPs ($\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC) were added. Following a 12-hour incubation period at 37°C, 1 mL of each culture was centrifuged for 30 minutes at 8000 rpm, and the Bradford assay (595 nm) was used to measure the amount of cellular protein.

Production of extracellular polymeric substance (EPS)

The culture that had been cultured in the preceding stage was used to extract EPS. To do that, 5 mL of bacterial culture was centrifuged for 15 minutes at 4°C at 8000 rpm, and the supernatant was gathered. In order to precipitate EPS, two volumes of acetone were added to the supernatant, and the combination was then chilled for the entire night at 4°C. Centrifugation of the mixture at 6000 rpm for 30 minutes (at 4°C) was the final method used to collect the EPS product. After the pellet was dried for 24 hours at 40°C, its wet weight was determined and its dry weight was approximated [24].

RNA extraction

About 5 mL of the bacterial culture treated with MIC concentration of AgNPs was stored from previous section for the RNA extraction. The manual's instructions were followed when performing the RNA extraction procedure. The cultures (1×10^8 bacteria) were centrifuged at 8000 rpm for 5 minutes at 4°C, and the resultant supernatant was disposed of. Following the addition of 500 μ L of Buffer RLT, the pellet was vortexed forcefully for 5 - 10 seconds. The contents were collected in a new tube following 10 seconds of full-speed centrifugation. About 70% ethanol in an equivalent volume was pipetted in and stirred well. A 2 mL collection tube containing 700 μ L of the collected lysate was put inside the RNeasy spin column. The RNeasy spin column was cleaned by centrifuging 500 μ L of Buffer RPE for 15 seconds at 8,000 rpm after discarding the flow-through. To clean the membrane, the earlier method was carried out twice. 30 to 50 μ L of RNase-free water was added before the column was placed in a fresh collection tube.

cDNA synthesis and real-time PCR

The Superscript TMII Reverse Transcriptase, 200 U/L (HiMedia) RT PCR kit was used to create the cDNA. In conclusion, the first reaction was initiated using approximately 1.8 μ g of the RNA that was extracted in the preceding phase. The amount of RNA recovered was

1.85 μ g/mL. The real-time primers (Table 1) were designed using Primer3 software and then acquired from Sigma-Aldrich. The real-time PCR experiment was then carried out using the iQTM SYBR Green Supermix (HiMedia), slightly altering the protocol described by [25]. Primers (600 nM) and 1 μ L of the RT products were utilized in the PCR experiment, and a total of 12.5 μ L was used for the reaction. Each reaction was carried out in triplicate and in tandem with its matching negative control in order to confirm the positive amplification.

Expression of the pili protein members

Real-time quantification of the control and treatment samples was done using the Corbett Research cycler (Bio-Rad). Using the RNA products, about 40 program cycles were executed at 93°C for 55 seconds, 64°C for 50 seconds, and with an elongation at 72°C for 50 seconds. To compare the mRNA expression, the relevant genes of interest were amplified along with the house-keeping gene *recA*. The relative amounts of mRNA in the test samples and the control were compared using the $\Delta\Delta C_t$ technique.

Statistical analysis

Three replicates of each experiment were carried out. One-way analysis of variance (ANOVA) was applied to the data when appropriate, and Tukey's test differences between samples were verified as statistically significant ($p < 0.05$). The statistical software SPSS (faculty version) was used for analysis during the investigation.

RESULTS

Characterization of AgNPs

The purified fraction was found to be phenyl lactic acid (PLA) as evident from the HPLC. The retention time for the fraction was found to be 26 minutes, which is nearly matching to the standard PLA (26 minutes). The formation of red color indicated the formation of nanoparticles. UV spectra of the formed AgNPs showed a distinct peak at 435 nm. From the NTA analysis the particles formed were spherical in shape and with an average of 85 nm (Figure 1 and Figure 2).

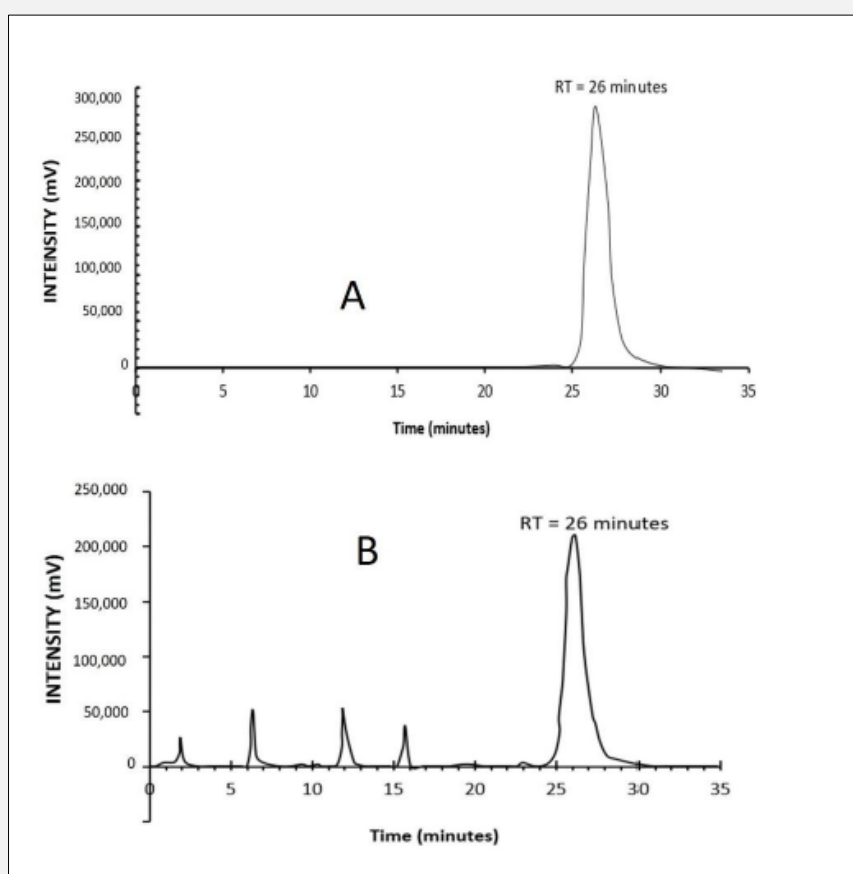
FTIR spectra

The large peak at 3,300 - 3,400 cm^{-1} in the FTIR spectrum of chitosan-based silver nanoparticles (Ag-NPs) represents O-H/N-H stretching, while the acute peak at 2,900 cm^{-1} represents C-H stretching. Amide I band and N-H bending are indicated by bands between 1,600 and 1,650 cm^{-1} , which are associated with C=O stretching and N-H bending, respectively. Peaks in the 1,000 - 1,100 cm^{-1} range indicate C-O extending from the backbone of the polysaccharide.

A FTIR spectra of chitosan-based silver NPs conjugated with PLA showed distinct and peculiar peaks at 1,733, 1,092, 797 and 702 cm^{-1} . These peaks correspond to the

Table 1. Table showing the primer details of the pili gene members used in the study.

Gene		Sequence	Length	Tm	GC %	Product
<i>pilA</i>	FW	ACACTGGTTGAGCTGGTAGTA	20	58.76	50	240
	RV	CCGCCGCGAACTTATCATCTG	20	58.73	50	
<i>pilB</i>	FW	GACACTCGTGGAGCTCGTCA	20	58.99	50	210
	RV	CAGTCTGCTTTATAAGCTTTCCA	20	59.05	55	
<i>pilC</i>	FW	TGCCCCGAGAACAATCCATCC	20	60.1	55	491
	RV	CTTGTCCACCACAAACGCTG	20	60	55	
<i>pilF</i>	FW	AAGCGGTAAAGGTGCGTACA	20	60	50	529
	RV	TTCTGCCCCACTGTACGAAC	20	60	55	
<i>pilM</i>	FW	CACGCGTTGAAGTGCTTGAA	20	60	50	501
	RV	AAAACGGGTTGGCAATCGTG	20	60	50	
<i>pilN</i>	FW	TTACTCCCTTGGCGTGATGG	20	59.7	55	570
	RV	AGTCACCCCCATTTCCTCTA	20	59.97	55	
<i>recA</i>	FW	ATCTCCGTCAATCTCCGCAC	20	59	55	382
	RV	ACGCGCTGAACAAAAGGTTC	20	59.97	50	

**Figure 1.** HPLC chromatogram of PLA produced by the standard (A) and the purified fraction (B).

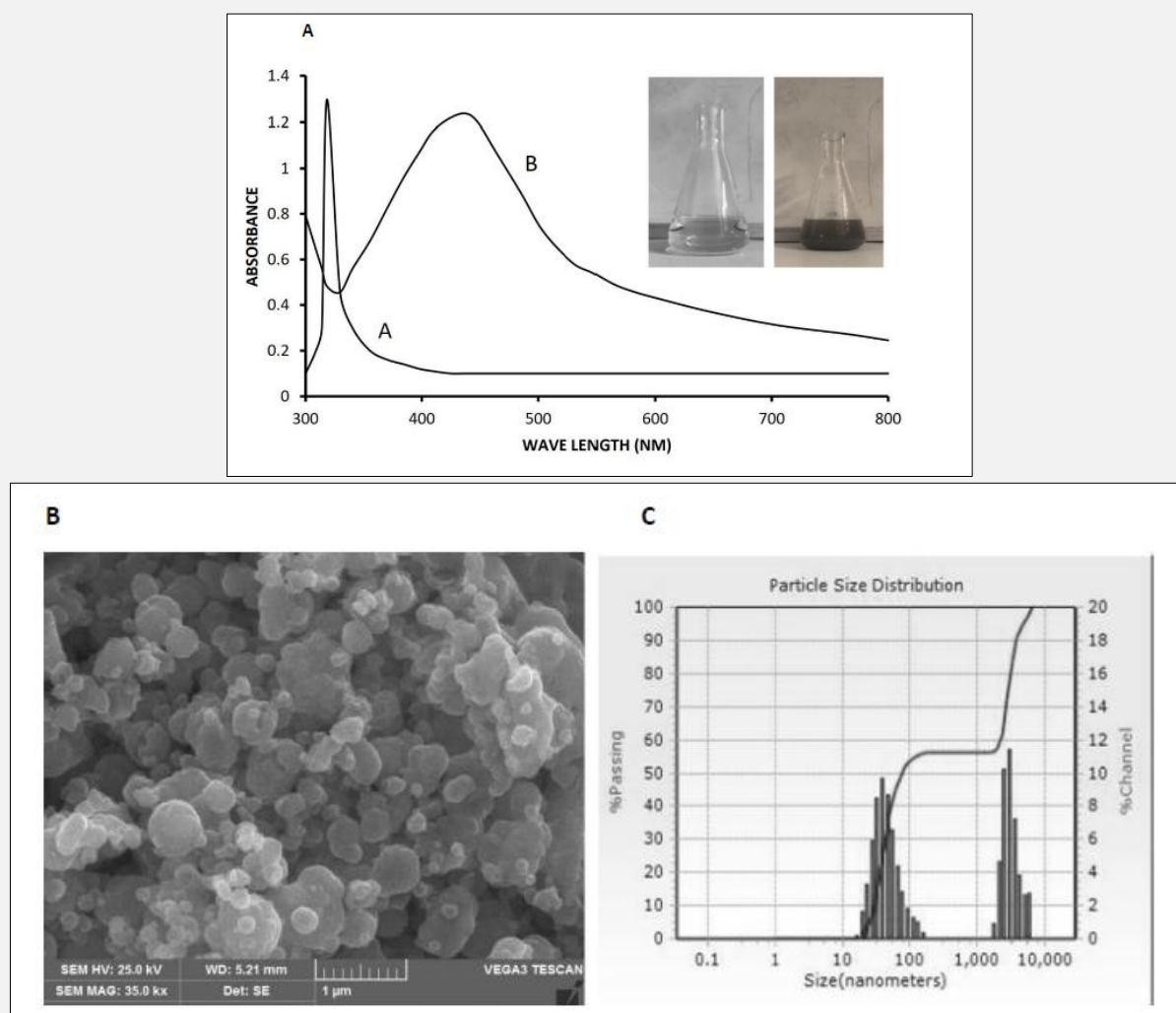


Figure 2. A: UV spectra of the formed AgNPs, B: Picture of the image from the SEM showing spherical shaped NPs, C: Particle size distribution.

phenyl lactic acid (CAS 20312-36-1). Its carboxylic acid functional group's C=O stretching vibration is represented by the peak at $1,733\text{ cm}^{-1}$. The C-O stretching vibration of its carboxylic acid and hydroxyl functional groups is represented by an FTIR signal at about $1,092\text{ cm}^{-1}$. Out-of-plane bending vibrations of the aromatic ring's C-H bonds are probably responsible for the band at 797 cm^{-1} , which is typical of a benzene ring with a certain substitution pattern. The mono-substituted benzene ring's out-of-plane C-H bending is responsible for the FTIR peak at 702 cm^{-1} (Figure 3).

Agar well diffusion and MIC

The antibacterial activity of the formed AgNPs was screened using the agar well diffusion assay. In the fig-

ure below, positive control showed a zone of inhibition of 23 mm, whereas the inhibition was observed to be dose dependent with the case of AgNPs. The inhibition zone was found to be 5 mm and 17 mm at 20 and 40 $\mu\text{g/mL}$ of AgNPs respectively ($p < 0.05$). MIC values were established in order to assess *Acinetobacter baumannii*'s susceptibility to AgNPs. The broth dilution test was employed to assess MICs. Our research showed that the MIC values for positive and AgNPs ($p < 0.05$) were 44.5 and 105.26 $\mu\text{g/mL}$, respectively. At 160 $\mu\text{g/mL}$, the AgNPs' percent inhibition was 83%, and it was determined to be dosage dependent. However, at 44.45 $\mu\text{g/mL}$, the positive control demonstrated 100% inhibition. (Figure 4).

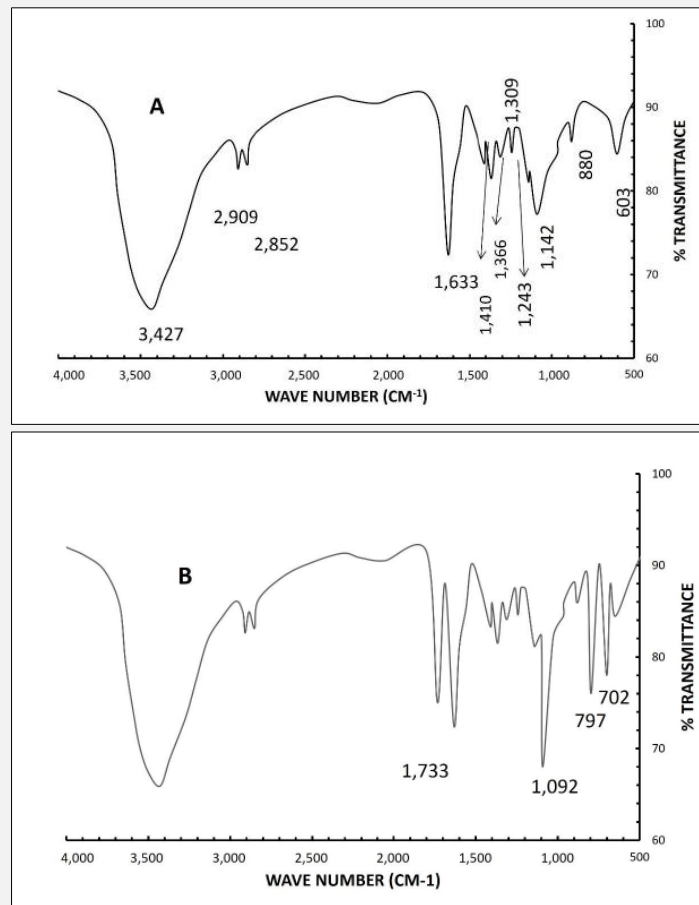


Figure 3. FTIR spectra of chitosan-based A: Silver NPs; B: Silver NPs conjugated with phenyl lactic acid.

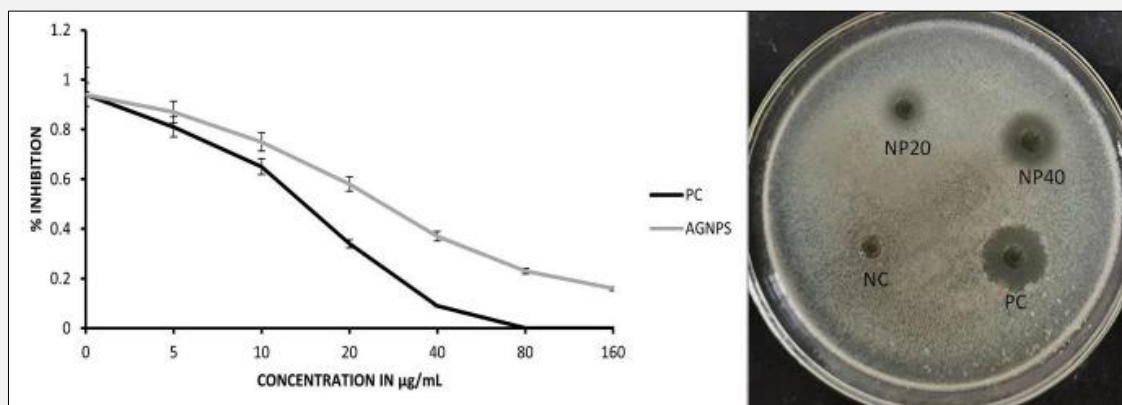


Figure 4. Line showing the % growth inhibition under the treatment. Right: Agar well diffusion showing the inhibition zones. PC: Positive control, NC: Negative control.

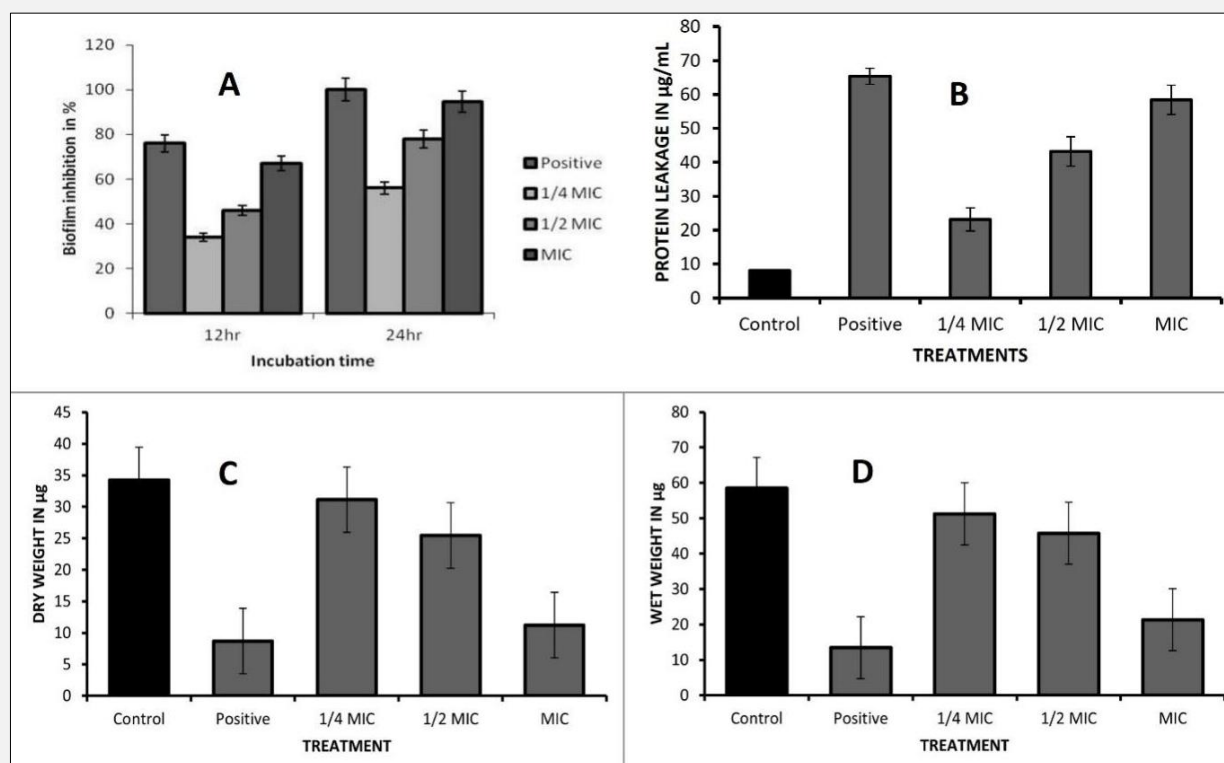


Figure 5. Histogram showing A: the total percent inhibition in biofilm formation under the treatment with NPs, B: the total protein leakage observed under the treatment with NPs, C: the dry weight of EPS under the treatment with NPs, D: the wet weight of EPS under the treatment with NPs.

Biofilm inhibition assay

Following treatment with varying concentrations of NPs ($\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC), the percentage suppression of biofilms in *Acinetobacter baumannii* was also examined. According to the results, biofilm formation was significantly ($p < 0.05$) decreased in the majority of cases when cells were treated with half inhibitory doses of AgNPs in a dose-dependent manner. As the graph below illustrates, at MIC concentrations, the percentage suppression of biofilm development for NPs and the positive control was 94.5 and 100%, respectively ($p < 0.05$). For 24 hours, the percentage inhibition was 56, 78, and 94.5% at $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC, respectively (Figure 5).

Cellular protein leakage assay

The total protein content released into the supernatant was quantified by the Bradford method to estimate the level of membrane damage. The results have indicated that cells treated with AgNPs at $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC showed 64.87, 81.20 and 86.10% increase when compared to the control ($p < 0.001$). On the other hand, positive control showed 87.57% at MIC concentration ($p < 0.05$).

Production of extracellular polymeric substance (EPS)

The results for the wet and dry weights of the extracted EPS showed that the amount of EPS from the cultures treated with NPs was significantly ($p < 0.05$) lower than that of the control cells. At MIC concentrations of AgNPs, the wet weight of EPS decreased by 63.49% and the dry weight of EPS decreased by almost 67.19%, as shown in the figure C&D. Conversely, at MIC values ($p < 0.05$), the positive control demonstrated a 76.98% decrease in the wet weight of EPS and a roughly 74.67% decrease in the dry weight of extracted EPS.

Expression of the pili protein members

RNA extracted from the treated cells was used in qRT-PCR to examine the differential expression of pili gene members. The expression levels of pilA, pilB, pilC and pilF were found to be down regulated to 0.75, 0.54, 0.5 and 0.82 when compared with the control (1 or 100%). But with positive control, there was a minor down regulation of 5 and 9 times respectively. This data demonstrates that the AgNPs can inhibit the bacterial growth. To compare the expression levels at $p < 0.05$, a two-

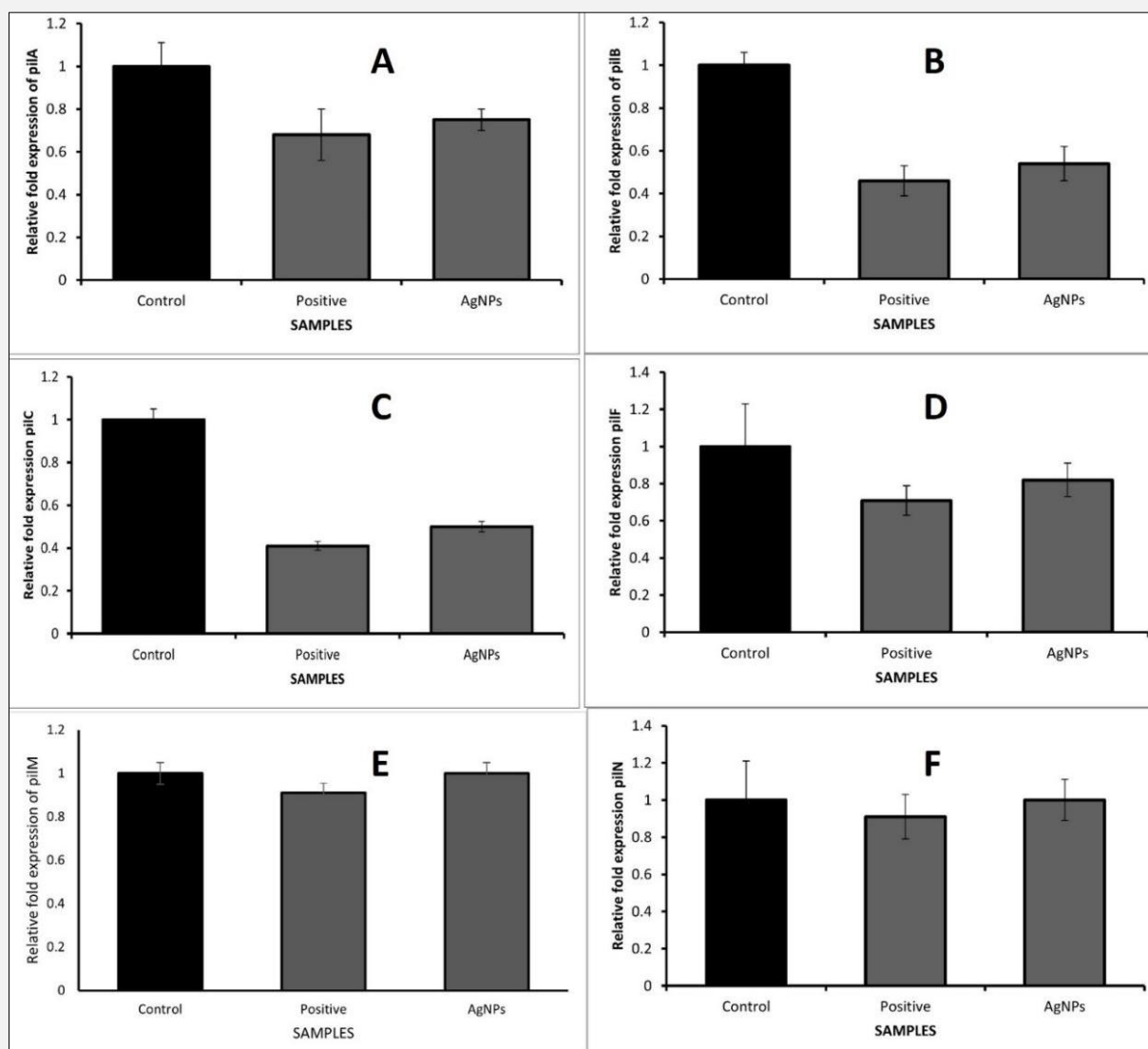


Figure 6. Graph showing the relative fold expression of pili protein members under the treatment.

A: pilA; B: pilB; C: pilC; D: pilF; E: pilM; F: pilN.

way ANOVA was used. The expression of each of the four genes varied significantly between the treatment and control groups [$F(2,10) = 9.819327$; $p = 0.0043$]. Additionally, a significant influence between members was noted [$F(5,10) = 3.97419$; $p = 0.030247$] (Figure 6 and Figure 7).

DISCUSSION

In this study, chitosan-based silver nanoparticles conjugated with PLA were synthesized, characterized, and

their potential as an antibacterial agent against *Acinetobacter baumannii* was further assessed. Our fraction had the same retention period as the standard, according to HPLC analyses. With a peak at 435 nm, we discovered that the NPs' ultraviolet and visible (UV-Vis) absorption spectra verified that silver nanoparticles were formed from silver ions. In the study by [26], similar spectra were also observed. Additionally, SEM analysis of bio-synthesized nanoparticles showed that they have smooth surfaces, are nearly spherical in shape, and had a mean particle size of 85 nm with a zeta potential of -1.8 mV, indicating stable particles.

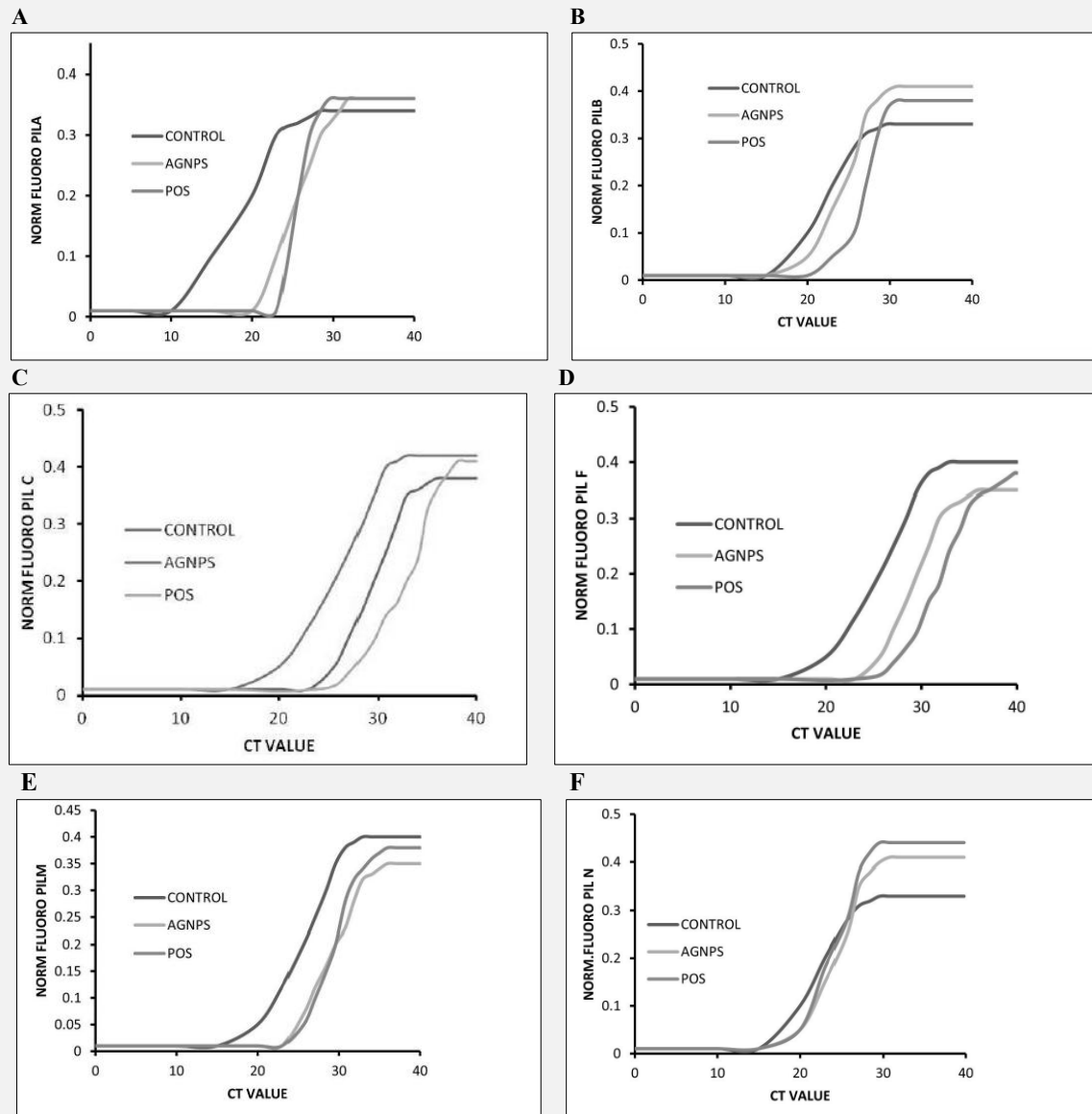


Figure 7. Graphs showing the Ct graphs of pili protein members under the treatment.

A: pilA; B: pilB; C: pilC; D: pilF; E: pilM; F: pilN. All the values are average of triplicates.

According to the current study, the MIC values for AgNPs ($p < 0.05$) and positive were 105.26 $\mu\text{g/mL}$ and 44.5 $\mu\text{g/mL}$, respectively. The percent inhibition of AgNPs was 83% at 160 $\mu\text{g/mL}$ and was found to be dose dependent. Nevertheless, the positive control showed 100% inhibition at 44.45 $\mu\text{g/mL}$.

As found in our study, the wet weight of EPS dropped by 63.49% and the dry weight of EPS dropped by nearly 67.19% at MIC concentrations of AgNPs. On the other hand, the positive control showed a 76.98 decrease in the wet weight of EPS and an approximately

74.67% decrease in the dry weight of extracted EPS at MIC levels ($p < 0.05$). Because of its crucial involvement in antibiotic resistance and biofilm development, extracellular polymeric substance (EPS) is a prospective therapeutic target for *Acinetobacter baumannii*. By attacking EPS, the protective biofilm matrix may be broken, increasing the bacterium's susceptibility to therapy [27]. A promising substitute for conventional antibiotics in the fight against MDR *A. baumannii* is to target EPS. These tactics increase the pathogen's susceptibility to current therapies rather than killing the bacterium di-

rectly, which may restore the effectiveness of traditional medicines [28].

Similar results were also reported by studies by [29], who found that exposure to aluminum oxide nanoparticles disrupted the cellular membrane, as evidenced by an increase in cellular leakage of contents. In the presence of NPs, bacterial adhesion to polystyrene surfaces decreased to 48.8 to 51.9%, whereas biofilm inhibition ranged from 11.64 to 70.2%. Additionally, nanoparticles decreased the biomass of existing biofilms and the synthesis of extracellular polymeric substances.

Research on the use of pilus proteins in *Acinetobacter baumannii* for therapeutic purposes is concentrated on two primary strategies: creating vaccines that use pilus proteins, such as CsuA/B and FimA, to stimulate an immune response, and employing medications that block pilus-dependent processes, such as biofilm formation and motility, like phenothiazine compounds [30].

When compared to the control, it was discovered that the expression levels of pilA, pilB, pilC, pilF, pilM, and pilN were downregulated to 0.75, 0.54, 0.5 and 0.82 when compared to control (1 or 100%). On the other hand, we did not observe any variation in pilM and pilN expression patterns upon treatment. *Acinetobacter baumannii* can be treated by blocking their function, such as by using medications to prevent the production of biofilms, or by creating vaccines that use pilus proteins to trigger a protective immune response. For example, type IV pili are crucial for twitching motility and biofilm production, and some phenothiazine medications can inhibit them, making them possible therapeutic targets. Recombinant pilus proteins, such as CsuA/B and FimA, can also be used to produce an immune response that guards against infection, as demonstrated in animal models [30].

Significantly, synergistic antibacterial and anti-biofilm action against multidrug-resistant (MDR) *Acinetobacter baumannii* has been shown by AgNPs/chitosan nanocomposites. AgNPs and chitosan, a naturally occurring antibacterial biopolymer, work better together at lower concentrations and frequently cause no harm to human cells [31].

AgNPs/chitosan nanocomposites dramatically disrupt existing biofilms (by 22 - 50%) and prevent the formation of new biofilms (by 19 - 21% and, in certain situations, up to 100%) [32].

By suppressing the production of pili and other virulence proteins, silver nanoparticles (AgNPs) have antibacterial action against *Acinetobacter baumannii*. This is a crucial step in halting the formation of biofilms and infection. This method has been validated by numerous investigations, which have found a notable decrease in the genes that produce pilus [33].

Long-term exposure to AgNPs has occasionally been demonstrated to cause gene changes that impair the bacterium's capacity to produce pili and biofilms [34]. We report for the first time, in using the pili proteins as drug targets, as they play a major role in biofilm formation. We found our NPs did inhibit the biofilm formation sig-

nificantly and as confirmed by the down regulation of the pili proteins in the study.

CONCLUSION

Because nanoparticles can directly harm bacterial cell membranes and inhibit efflux pumps, nanoparticles can circumvent or overcome conventional resistance mechanisms, making them important in the fight against multi-drug resistance (MDR). Their special qualities make it difficult for bacteria to become resistant by producing reactive oxygen species (ROS), upsetting cellular structures, and delivering traditional antibiotics in novel ways. The synthesis and characterization of silver nanoparticles based on chitosan and conjugated with phenyl lactic acid that was isolated from fermented LAB were the main focus of our investigation. These environmentally friendly silver nanoparticles were used to combat *Acinetobacter baumannii*, which is resistant to several drugs. Disc diffusion, antibiofilm formation, and differential expression of pili forming gene members were used to investigate the antibacterial activity. At 105 µg/mL (MIC), AgNPs demonstrated strong antibacterial action, and it was discovered that the pili gene members were under-expressed. However, there was no discernible difference between the treatment and control for pilN and pilM members. As a result, the nanoparticles may find application in a number of domains, including drug delivery, pharmaceutical items, and numerous other commercial procedures.

However, we need to mention a few of our study limitations which we are going to address in our continuation work. The NPs were not assessed for their cytotoxicity if any, and also the NPs were not screened for their biocompatibility. We did not perform the release of any silver ions into the surrounding media to confirm the stability of the synthesized NPs.

Explicitly discuss limitations (non-standard susceptibility methods, lack of cytotoxicity/biocompatibility testing, isolate heterogeneity, potential confounding from Ag⁺ release).

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No conflict of interest.

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