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Effect of Green Synthesized Silver Nanoparticles on Virulence Gene Expression and Biofilm Formation in Multidrug Resistant *Proteus Mirabilis*

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Abstract:

This study focuses on the isolation and identification of *Proteus mirabilis* from clinical samples of patients with urinary tract infections at Al-Kindi and Ibn Al-Baladi hospitals between September 2025 and January 2026. Out of 200 samples, 30 isolates were identified using culture media, biochemical tests, Vitek 2 system, and molecular confirmation via 16s rRNA. The results revealed that 100% of the strains harbored virulence genes (*zapA*, *hlyA*, *ureC*). Antibiogram results for 12 antibiotics showed the highest resistance (100%) against (CAZ, F, AMP), while the lowest was 3.3% for (AMC) and 13.3% for (AT). The impact of silver nanoparticles (AgNPs) was evaluated using RT-qPCR on two representatives multidrug- resistant isolates, showing a significant down-regulation in the gene expression of all virulence genes by more than (95%) along with a (87.9%) reduction in biofilm formation. This research suggests that AgNPs could serve as an effective alternative to mitigate the pathogenicity of multidrug-resistant bacteria.

Keyword: (*Proteus mirabilis*, silver nanoparticles, virulence factor, antibiotic, RT-qPCR, gene expression).

Note: The research is based on a master's thesis or a doctoral thesis. (based on a master's thesis).

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Introduction:

Proteus spp. are Gram-negative bacilli belonging to the Enterobacteriaceae family and were first described by Hauser in 1885 (Sellaturay et al., 2012). These bacteria are widely distributed in aquatic environments, soil, and wastewater, where they play an important role in the decomposition of organic matter (Drzewiecka, 2016). *Proteus* species are considered opportunistic pathogens in both humans and animals (Peng,2024). and are associated with a wide range of clinical infections, including respiratory tract infections, soft tissue infections, rheumatoid arthritis, and septicemia in severe cases (Hussein *et al*.,2020). In addition, they are recognized as one of the major causative agents of catheter-associated urinary tract infections (CAUTIs), which may become complicated by urinary stone formation and catheter blockage (Norsworthy and Pearson, 2017).

Proteus species possess several virulence factors that enhance their pathogenicity, including metalloprotease (*zapA*), hemolysin (*hlyA*), and urease (*ureC*) genes, in addition to their strong ability to form biofilms and exhibit swarming motility(Hamilton *et al.*,2018). The increasing clinical concern regarding these bacteria is mainly attributed to their intrinsic and acquired resistance to many conventional antibiotics, such as nitrofurantoin and tetracycline (Girlich *et al*.,2020). Consequently, the search for alternative therapeutic approaches has become essential, and nanoparticles have emerged as promising candidates for combating multidrug-resistant (MDR) bacterial infections (Amin Yavari *et al*.,2016).

Previous studies:

Adeolu *et al.* (2016), in their study "Genome-based phylogeny and taxonomy of the *'Enterobacteriales'*", used comparative genomics to identify Proteus as Gram-negative rods with cell diameters of 0.4 to 0.8 \mum. Drzewiecka (2016) explored the roles of *Proteus* bacteria in natural environments, concluding they are widely distributed in water, soil, and the gastrointestinal systems of humans and animals . Armbruster *et al*. (2018) studied the pathogenesis of *P. mirabilis* in the urinary tract, finding it primarily causes pyelonephritis and contributes significantly to kidney and bladder stone formation. Schaffer *et al*. (2016) examined the mechanisms of urolithiasis, demonstrating that the development of urinary stones requires the combined presence of MR/P fimbriae and the

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urease enzyme. Yuan *et al.* (2021) analyzed crystalline biofilms in clinical samples, reporting that 62% of patients with encrusted catheters also had bladder stones associated with *P. mirabilis*. Liu *et al.* (2015) investigated the role of metalloproteases, showing that the ZapA enzyme in *P. mirabilis* can cleave serum and secretory immunoglobulins, including IgA and IgG. AlQurashi *et al*. (2025) studied the antimicrobial effects of nanoparticles, concluding they interfere with multiple microbial pathways simultaneously, which reduces the probability of resistance development.

Comparison with the current work:

While the previous studies focused on general pathogenesis and traditional resistance, the current study is distinguished by its focus on 30 clinical isolates, specifically demonstrating that AgNPs significantly down-regulate the expression of *zapA*, *hlyA*, and *ureC* genes and reduce biofilm formation, which provides a more targeted molecular solution than general resistance studies.

The main work:

collection, isolation and identification of bacteria

Two hundred mid-stream urine (MSU) samples were collected from patients clinically diagnosed with UTI of both sexes and different ages. The samples were obtained from several hospitals in Baghdad city (Al-Kindi and Ibn Al-Baladi hospitals) through the period from September 2025 to January 2026 using sterile, leak-proof plastic containers. All collected samples were immediately labeled, kept in a cooling box (4°C) and transported to the laboratory within 2 hours for processing. Samples were cultured on blood agar and MacConkey agar. The colonies were identified based on the observed characters, farther identification by microscopic, biochemical, VITEK 2 system and *16srRNA*gene for confirmation diagnosis.

Morphological characteristics

The different media were used to isolate and identify *proteus spp*, when cultured on MacConkey agar proteus appeared as small colonies, convex, circular, smooth edges do not swarm and non-lactose fermenter colonies with special fish-like odor, while *p.mirabilis* exhibits swarming motility and hemolysis on blood agar.

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Microscopical examination

Under the light microscope, *proteus spp* isolates were showed as a Gram -negative bacteria and red color coccobacilli that mostly occurred singly or in short chains.

Biochemical characteristics

The results of biochemical tests, all the isolates tested were positive for citrate, H_2S, urease, methyl red, motility and catalase tests while all tested were negative for indole, voges-proskauer and oxidase teste.

VITEK 2 system

The automated VITEK 2 Compact System (bioMérieux, France) was utilized with GN identification cards. The system evaluated the biochemical and metabolic kinetics of the isolates over an incubation period of 3.85 hours. Key positive reactions included ornithine decarboxylase (ODC), urease (URE), tyrosine arylamidase (TyrA), gamma-glutamyltransferase (GGT), and D-glucose (dGLU). Based on the automated data matrix, all presumptive isolates were successfully confirmed as *P. mirabilis* with an excellent confidence level of 99%.

Identification of *proteus mirabilis* by *16srRNA*

An amplification of the *16srRNA* gene was performed to confirm bacterial identification, primers targeting a conserved region of the *16srRNA* gene were designed and used for amplification of DNA from then separated on agarose gel figure (1). Results showed that all these isolates 30 (100%) were identified as *p.mirabilis*, had *16srRNA* gene band with (1500bp).

Reference Gene

The *16S rRNA* gene was utilized as the internal reference (housekeeping) gene to normalize the expression levels of the target virulence genes (*ureC*, *hlyA*, and *zapA*). The selection of *16S rRNA* is justified based on its established transcriptional stability in Proteus mirabilis under various environmental stresses and antimicrobial exposures. Furthermore, the baseline threshold cycle (Ct) values for *16S rRNA* across all experimental groups in this study the untreated control, Antibiotics-treated, and AgNPs-treated isolates exhibited highly consistent and tight distribution profiles (This confirms that its expression remains constitutional and unaffected by silver nanoparticle-induced oxidative stress.

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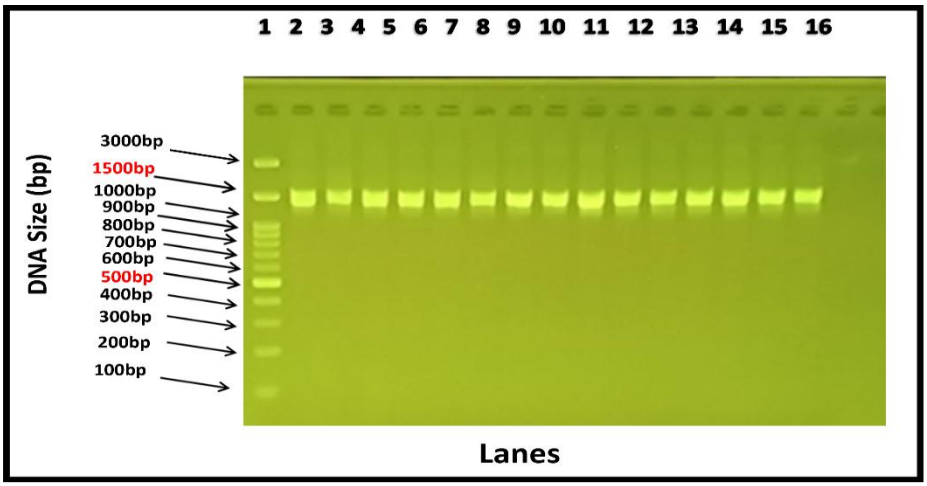

Figure 1: Agarose gel electrophoresis (1.5% agarose,7v/cm2 for 60 min) for *16SrRNA* gene (1500 bp amplicon), lane 100 bp DNA Ladder.

Antibiotics Susceptibility test

Antibiotic susceptibility testing to identify the most successful antibiotic for treating bacterial infection by using Kirby-Bauer method which relies on measuring the diameter of the inhibition zone and comparing it with clinical and laboratory standards institute (CISI,2025). 30 isolates were tested against 12 antibiotics, they showed a varied level of resistance , the highest rate of resistance is seen with Ampicillin (AMP), Ceftazidime(CAZ) and Nitrofurantoin(F)(100%), followed by Tetracycline (TE)(90%), Imipenem (IMI) (73.3%), Trimethoprim-Sulfamethoxazole (TR)(66.6%), Gentamicin (GEN) (40%), Chloramphenicol (C) (56.6%), Cefixime (CFM) (36.6%), a low resistance showed with Levofloxacin (LEV) (20%), Aztreonam (AT) (13.3%) and Amoxicillin-Clavulanate (AMC) (3.3%).

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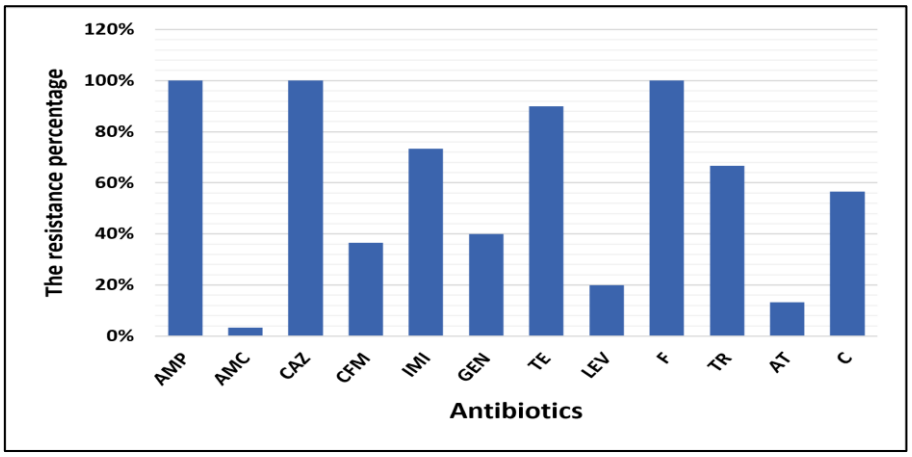

Figure 2: The resistance percentage of *P.mirabilis* isolates to antibiotics.

AMP: Ampicillin TE: Tetracycline LEV: Levofloxacin
AMC: Amoxicillin-clavulanate AT: Aztreonam
GEN: Gentamicin
CAZ: Ceftazidime C: Chloramphenicol
F: Nitrofurantoin
CFM: Cefixime TR: Trimethoprim
IMI: Imipenem

The current results agreed with results (AL-obeidi ,2023) who found that the *P.mirabilis* isolates showed a high resistance to (Ampicillin) (96%), (Ceftazidime) (84%), (Tetracycline) (72%). Our results disagree with results by (Ayoub and Nazir, 2025) and (Kwon *et al*.,2022) results that showed resistance of (Amoxicillin-Clavulanate) resistance was (82%), (Levofloxacin) (67%) and (Aztreonam) (50%) respectively. The resistance of certain bacterial strains to multiple antibiotics is due to the ability of these strains to alter the permeability of the outer membrane as well as the presence of active efflux pump systems (Vergalli *et al*., 2024). Unsuitable or incorrect use of antibiotics can lead to the emergence of MDR bacterial isolates in the public (Al-Zahrani , 2021).

Detection of Virulence Factors

The biofilm-producing ability of the (30) *P. mirabilis* strains was evaluated using the microtiter plate method. The results categorized the isolates into three groups: 47% (14 isolates) were strong biofilm

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producers, 23% (7 isolates) were moderate, and 30% (9 isolates) showed weak biofilm formation.

Phenotypic Expression of Urease, Hemolysin, and Metalloprotease

The phenotypic screening revealed that all 30 clinical strains (100%) were positive to produce urease, hemolysin, and metalloprotease (*ZapA*). These results emphasize the high pathogenic potential of the *P. mirabilis* strains isolated from UTI patient.

Green synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) were synthesized using Apium graveolens extract according to the method of Alhamza *et al*. (2024). Briefly, 10 mL of the plant extract was mixed with 90 mL of 5 mM AgNO_3 solution and incubated in the dark at room temperature for 24 h. The formation of AgNPs was monitored by the visual color change from pale yellow to greenish-brown. The synthesized suspension was then centrifuged at 10,000 rpm for 10 min. The resulting pellet was washed three times with distilled water to remove impurities, transferred to a watch glass, and dried at room temperature for 24 h to obtain the final AgNPs powder for subsequent use.

Characterization of silver nanoparticles

The green synthesized AgNPs using Apium graveolens extract were characterized using analytical techniques to confirm their formation and crystalline nature. UV-Vis spectroscopy successfully verified the biosynthesis of AgNPs, exhibiting a maximum surface plasmon resonance (SPR) absorption peak at 450 nm (Figure 3A). Furthermore, the X-ray diffraction (XRD) pattern demonstrated distinct Bragg reflection peaks at 2\theta angles of 38.1°, 47.0°, 67.9°, and 78.9°. These peaks correspond to the (111), (200), and (220) Miller indices, respectively, confirming the face-centered cubic (fcc) crystalline lattice structure of the synthesized AgNPs (Figure 3B).

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Real-Time PCR (RT-PCR)

Table 1:The sequence and size of the primer were utilized to detect *p.mirabilis* content genes.

Primer	Direction	5 → 3	Size bp	Reference
16SrRNA	F	AGAGTTTGATCCTGGCTCAG	1500	Cobellis *et al* .,2016
	R	5'-TACGGTTACCTTGTTACGACT		
ureC	F	CCG GAA CAG AAG TTG TCG CTG GA	533	AL-obaidi and AL-Hashimy (2023)
	R	GGG CTC TCC TAC CGA CTT GAT C		
hlyA	F	GCGATGGAGATGACACTGCT	324	Designed
	R	CTCCATCAACTACGGCGTGA		
zapA	F	GGTGGTCGTGGACAGGATAC	216	Designed
	R	AGTTGCTTCGCCAGCATTTC		

Reaction Efficiency and Experimental Replicates

To ensure the reliability and reproducibility of the relative quantification, the amplification efficiency for each primer pair was evaluated using a 5-point standard curve generated from 10-fold serial dilutions of cDNA. The amplification efficiencies (E) for all targeted and reference genes fell within the highly acceptable range of 95% to 102%, with linear correlation coefficients (R^2 > 0.99). Furthermore, all steps of the qRT-qPCR workflow were strictly performed using three biological replicates (independent RNA extractions from three different bacterial cultures) and three technical replicates per sample to eliminate intra-assay variations.

Table 2: RT-PCR conditions

Cycle step	Temperature	Time	cycles
Initial denaturation	95ºC	30 seconds	1
Denaturation	95ºC	15 seconds	45-50
Annealing	55,58,63ºC	30 seconds	
Extension	72ºC	30 seconds	
Melting curve	95 ºC	1 minute	1
	60 ºC	30 seconds	
	95 ºC	30 seconds	

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Statistical analysis

The statistical significance of the data was analyzed using SPSS software (version 20). Percentages were calculated, and Fisher's exact test along with the Z-test were performed to evaluate drug resistance levels. Group comparisons were conducted using One-way analysis of variance (ANOVA) followed by the LSD test for multiple comparisons of means. Results were expressed as mean ± standard deviation (SD), and mean values were labeled with letters (A, B, C) in a descending order to indicate significance. A P-value > 0.05 was considered non-statistically significant, while $P \leq 0.05$ was considered statistically significant.

biochemical tests

Table 3: Results of biochemical tests for *P.mirabilis* .

Test	Result	Test	Result
citrate	+	indole	-
H_2S	+	oxidase	-
urease	+	Voges proskauer	-
motility	+	Methyl Red	+
catalase	+		

Synthesis of silver nanoparticles (AgNPs)

The green synthesis of silver nanoparticles (AgNPs) using *Apium graveolens* extract was initially indicated by a gradual phenotypic color change from pale yellow to dark brown due to Surface Plasmon Resonance (SPR) excitation. This visual transformation complies with Fattah *et al.* (2026), who reported similar findings using Pistacia atlantica extract. Biosynthesis confirmation was verified via UV-Vis spectroscopy, exhibiting a sharp, symmetrical SPR peak at 450 nm (Figure 3A), characteristic of stable, spherical AgNPs capped by the plant's active bio-molecules. Furthermore, the XRD pattern (Figure 3B) confirmed the crystalline nature of the nanoparticles, showing distinct Bragg reflection peaks at 2θ angles of 38.1°, 47.0°, 67.9°, and 78.9°. These peaks correspond to the (111), (200), and (220) crystalline planes, verifying a highly pure, face-centered cubic (fcc) silver lattice structure.

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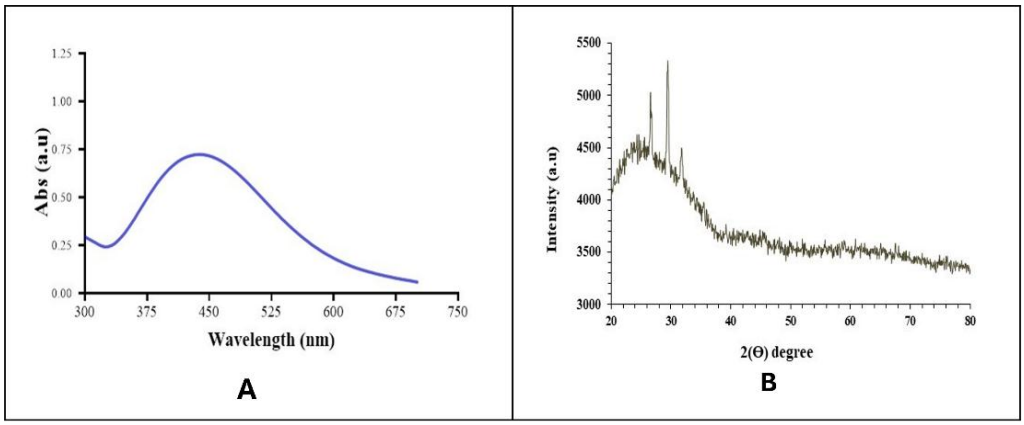

Figure 3: (A) Characterization by UV and (B) XRD Analysis for the AgNPs.

Antibacterial activity of AgNPs

Four multidrug-resistant (MDR) *P. mirabilis* strains were evaluated for silver nanoparticle (AgNPs) susceptibility using a broth microdilution assay with resazurin dye as a viability indicator. AgNPs exhibited potent antibacterial activity against all tested MDR strains. Three strains were completely inhibited at a minimum inhibitory concentration (MIC) of 32 mg/mL, while only one strain demonstrated higher tolerance, requiring an MIC of 64 mg/mL. These relatively low MIC values underscore the potential of AgNPs as alternative antimicrobial agents capable of overcoming conventional antibiotic resistance mechanisms.

Antibiofilm effects of AgNPs

The antibiofilm efficacy of AgNPs was compared to antibiotic against selected *P. mirabilis* strains. Both treatments significantly reduced biofilm formation compared to untreated controls. AgNPs exhibited superior antibiofilm activity, particularly in strains 9 and 28, where optical density (OD) dropped significantly from 1.1±0.1 and 2.22±0.15 to 0.089±0.001 and 0.121±0.001, respectively ($P < 0.005$). antibiotic also showed significant inhibition, notably in strain 38 ($P=0.05$). While strains 50 showed a reduction in biofilm biomass, the change was not statistically significant ($P=0.12$). Overall, AgNPs demonstrated a more potent inhibitory effect on biofilm stability than antibiotic across most tested strains.

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Effect of AgNPs on gene expression

The molecular impact of AgNPs and antibiotics on the expression of key virulence genes (*ureC*, *hlyA*, and *zapA*) was quantified using RT-qPCR and the $2^{-\Delta\Delta Ct}$ method for strains 38 and 50. The results, demonstrated a significant down-regulation across all targeted genes following treatment compared to untreated controls.

•For *ureC*: Both treatments effectively suppressed gene expression, indicating a disruption in urease-mediated pathogenicity.

•For *hlyA*: A marked reduction in fold-change values was observed, confirming the efficacy of AgNPs in inhibiting hemolysin production.

•For *zapA*: The treatment led to a substantial decrease in mRNA levels, suggesting a significant reduction in metalloprotease activity.

Overall, the green-synthesized AgNPs exhibited potent anti-virulence activity by suppressing the genetic pathways essential for *P. mirabilis* colonization and survival, with concentration-dependent inhibition observed in both clinical isolates.

Effect of AgNPs on *UreC* gene expression

Quantitative RT-qPCR analysis demonstrated a profound, statistically significant down-regulation ($P < 0.0001$) in the expression of the urease-encoding gene (*ureC*) following treatment with green-synthesized AgNPs, compared to both untreated controls and antibiotic. Statistical assessment using the Least Significant Difference (LSD) test confirmed that AgNPs reduced the transcription levels (Folding) of the *ureC* mRNA down to 0.063 and 0.366 in the tested resistant isolates (compared to a baseline fold-change of 4.257 in the controls). This multi-fold suppression highlights the anti-virulence superiority of the biogenic AgNPs, which effectively block the primary enzymatic pathways essential for *P. mirabilis* colonization and urinary tract pathogenesis.

Effect of AgNPs on *hlyA* gene gene expression

RT-qPCR analysis of the hemolysin gene (*hlyA*) showed a significant down-regulation ($P < 0.0001$) post-treatment. Based on the LSD test ($P = 0.00001$), the transcription levels (Folding) dropped sharply from control values (0.676 and 1.479) down to 0.419 and 0.019 in AgNPs-treated isolates (N38 and N50 respectively). This marked suppression confirms

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the efficacy of biogenic AgNPs in halting mRNA pathways responsible for cytotoxic hemolysin production during *P. mirabilis* infection.

Effect of AgNPs on *zapA* gene gene expression

For the metalloprotease gene (*zapA*), mRNA expression levels were significantly down-regulated ($P < 0.0001$) post-treatment across both tested isolates compared to their respective untreated controls. Based on the LSD test ($P = 0.00001$), the transcription levels (Folding) dropped markedly from control baselines down to 1.4241 and 0.4175 in the AgNPs-treated strains (N38 and N50, respectively). This consistent suppression demonstrates the high capacity of the biogenic nanoparticles to interfere with and disrupt the primary *P. mirabilis* virulence pathways.

The results of present study were identical to the results of Ibraheem *et al*.,(2020) demonstrating that AgNPs can induce downregulation of virulence-associated gene expression in uropathogenic bacteria. Their study suggested that AgNPs disrupt bacterial membrane integrity, increase membrane permeability, and interfere with nucleic acid stability, thereby contributing to reduced expression of virulence-related genes. These findings highlight the potential of nanoparticle-based approaches to suppress bacterial pathogenicity through modulation of genetic expression.

Conclusion:

This study demonstrates a critical correlation between the high prevalence of multidrug resistance (MDR) and key virulence factors among clinical *P. mirabilis* isolates. To address this therapeutic challenge, silver nanoparticles (AgNPs) were successfully and stably fabricated via an eco-friendly green synthesis approach utilizing *Apium graveolens* extract. Crucially, molecular analysis via RT-qPCR confirmed that these biogenic AgNPs exert a potent anti-virulence effect by significantly down-regulating the expression of essential pathogenicity genes, namely *ureC*, *hlyA*, and *zapA*. Consequently, this study establishes that *Apium graveolens*-derived AgNPs serve as a highly effective, multi-targeted alternative therapeutic agent capable of disrupting both bacterial pathogenicity and biofilm formation in resistant *P. mirabilis* pathogens.

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Recommendations:

Future research should focus on exploring biogenic nanoparticles from diverse microbial sources and evaluating the impact of AgNPs on a broader range of virulence and resistance genes. Additionally, investigating the synergy between AgNPs and conventional antibiotics is crucial for enhancing treatment efficacy. Finally, transitioning to in vivo animal studies is essential to verify the safety and therapeutic potential of these nanoparticles for clinical application.

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تأثير الجسيمات النانوية الفضية المصنعة حيوياً على التعبير عن جينات الفوعة وتكوين الغشاء الحيوي في بكتريا *proteus mirabilis* المقاومة للأدوية المتعددة

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مستخلص البحث:
ركّزت هذه الدراسة على عزل وتشخيص بكتيريا (*Proteus mirabilis*) من العينات السريرية للمرضى المصابين بالتهابات المسالك البولية في مستشفيَي (الكندي وابن البلدي) وذلك خلال الفترة الممتدة من أيلول 2025 إلى كانون الثاني 2026. ومن بين 200 عينة تم فحصها، تم تحديد 30 عزلة بكتيرية باستخدام الأوساط الزرعية، والاختبارات البيوكيميائية، وجهاز(Vitek 2)، بالإضافة إلى التأكيد الجزيئي عبر تحليل الجين *16srRNA*. وأظهرت النتائج أن جميع السلالات المعزولة بنسبة 100% كانت حاملة لجينات الضراوة (*zapA*، *hlyA*، *ureC*). كما كشفت نتائج مخطط الحساسية الدوائية (Antibiogram) ضد 12 مضاداً حيوياً عن تسجيل أعلى معدل مقاومة بنسبة 100% ضد مضادات (CAZ, F, AMP)، في حين سُجلت أدنى نسبة مقاومة بواقع 3.3% لمضاد (AMC) و13.3% لمضاد (AT). عِلاوة على ذلك، تم تقييم تأثير جسيمات الفضة النانوية (AgNPs) باستخدام تقنية التفاعل البوليميرازي المتسلسل الكمي بالوقت الحقيقي (RT-qPCR) على عزلتين ممثلتين متعددة المقاومة للأدوية (MDR)، حيث أظهرت النتائج انخفاضاً جوهرياً في التعبير الجيني لجميع جينات الضراوة بنسبة تجاوزت 95%، متزامناً مع تراجع في تشكيل الغشاء الحيوي (Biofilm) بنسبة 87.9%. وتخلص هذه الدراسة إلى إمكانية استخدام جسيمات الفضة النانوية كبديل علاجي فعال للحد من القدرة الإمراضية للبكتيريا متعددة المقاومة للأدوية.

الكلمات المفتاحية : (*Proteus mirabilis*, AgNPs, جينات الضراوة، مقاومة المضادات الحيوية، تقنية RT-qPCR،التعبير الجيني).

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