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Comparative Evaluation of Pine Seed Oil and Green-Synthesized Silver Nanoparticles on Quorum-Sensing Genes (*LuxS* and *lsrR*) in *Escherichia coli*

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Abstract

Autoinducer-2 (AI-2)–mediated quorum sensing (QS) is a key regulatory mechanism controlling biofilm formation and virulence in *Escherichia coli*. This study aimed to compare the effects of pine seed oil and green-synthesised silver nanoparticles (AgNPs) on the expression of *LuxS* and *lsrR* genes involved in QS regulation. A total of 155 clinical samples were collected from patients in Babylon hospitals, from which 75 *E. coli* isolates were obtained; 25 were selected for molecular analyses. AgNPs were synthesised using pine seed oil extract and characterised by UV-Vis spectroscopy, FTIR, SEM, AFM, EDX, and XRD. Sub-minimum inhibitory concentrations (sub-MICs) of pine oil (1 mg/ml) and AgNPs (32 µg/ml) were used for gene expression studies by qRT-PCR, using *16S rRNA* as a housekeeping gene. The results revealed significant down-regulation of *LuxS* and *lsrR* genes in isolates treated with AgNPs compared to pine oil (mean fold-change for *LuxS*: 0.84 ± 0.05 vs 0.98 ± 0.07; for *lsrR*: 0.57±0.03 vs 0.89 ± 0.04; $p \leq 0.05$). The findings indicate that green-synthesised AgNPs more effectively suppress QS gene expression in *E. coli* than pine seed oil, suggesting their potential as alternative anti-virulence agents, pending further in vivo and toxicity studies.

Keywords: *E. coli*, QS, AgNPs, *LuxS, lsrR*.

تقييم مقارن لزيت بذور الصنوبر وجسيمات الفضة النانوية على جينات استشعار النصاب *LuxS* و*lsrR* في الإشريكية القولونية

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الخلاصة

يُعد نظام استشعار النصاب المعتمد على الجزيء Autoinducer−2 (AI−2) من أهم الآليات التنظيمية التي تتحكم في تكوين الأغشية الحيوية وإظهار عوامل الضراوة في بكتريا *Escherichia coli*. هدفت هذه الدراسة إلى مقارنة تأثير زيت بذور الصنوبر والجسيمات النانوية الفضية المحضّرة بطريقة التخليق الأخضر في

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التعبير الجيني لكل من *LuxS* و *lsrR* المرتبطين بتنظيم هذه الآلية. جُمعت 155 عينة سريرية من مستشفيات محافظة بابل، وتم الحصول على 75 عزلة من *E. coli*، اختير منها 25 عزلة للتحليل الجزيئي. تم تحضير الجسيمات النانوية الفضية باستخدام مستخلص زيت بذور الصنوبر، وتم توصيفها باستخدام تقنيات UV–Vis و FTIRو SEM و AFM و EDX و XRD. استُخدمت تراكيز دون الحد الأدنى المثبط (sub–MIC) من زيت الصنوبر (1 ملغم/مل) ومن الجسيمات النانوية (32 ميكروغرام/مل) لدراسة التعبير الجيني بواسطة تقنية qRT–PCR مع استخدام الجين 16S rRNA كجين مرجعي. أظهرت النتائج انخفاضًا معنويًا في مستوى التعبير الجيني لكل من *LuxS* و*lsrR* في العزلات المعاملة بالجسيمات النانوية مقارنة بزيت الصنوبر (قيمة التغير النسبي للتعبير الجيني *LuxS*: 0.84 ± 0.05 مقابل 0.98 ± 0.07، و *lsrR*: 0.57 ± 0.03 مقابل 0.89 ± 0.04، $p \leq 0.05$) تشير هذه النتائج إلى أن الجسيمات النانوية الفضية المحضّرة بالتخليق الأخضر كانت أكثر فاعلية في تثبيط التعبير الجيني لجينات الاستشعار النصاب في بكتريا *E. coli* مقارنة بزيت بذور الصنوبر، مما يبرز إمكان استخدامها كعوامل مضادة للضراوة بعد إجراء المزيد من الدراسات الحيوية ودراسات السمية.

1. Introduction

Escherichia coli is a common opportunistic pathogen responsible for a broad spectrum of infections, including urinary tract infections, gastroenteritis, and wound infections [1,2]. Its ability to form biofilms and regulate virulence factors is primarily governed by quorum sensing (QS), a communication system mediated by small signalling molecules called autoinducers [3]. Among these, autoinducer-2 (AI-2) synthesized by the enzyme S-ribosylhomocysteine lyase encoded by the *LuxS* gene, plays a key role in interspecies signalling and biofilm development [4], while lsrR acts as a transcriptional repressor within the AI-2 uptake and processing operon [5].

Targeting the QS system has recently emerged as a promising anti-virulence strategy to control bacterial pathogenicity without exerting the selective pressure associated with conventional antibiotics [6]. Natural plant-derived products, such as essential oils, have attracted increasing attention for their potential quorum-sensing inhibitory (QSI) activity [7]. Essential oils, including pine seed oil rich in terpenes, flavonoids, and polyphenols, have been explored for their potential to interfere with QS-regulated pathways [8, 9].

Recently, there has been an increased focus on the green synthesis of metal nanoparticles using plant extracts, due to their ecological, low-cost, and biocompatible nature [10]. Green-synthesised AgNPs are highly effective antimicrobial and antibiofilm agents that damage membranes, cause oxidative damage, and interfere with QS gene expression [11–13]. However, there is limited research directly comparing pine seed oil to green-synthesised AgNPs regarding their ability to regulate QS genes in *E. coli*, especially *LuxS* and *lsrR* [14].

Therefore, the present study aimed to compare the effects of pine seed oil and its green-synthesised silver nanoparticles (AgNPs) on the expression of quorum-sensing (*LuxS* and *lsrR*).

2. Materials and Methods

2.1 Sample collection and bacterial identification

A total of 155 clinical samples were collected from patients attending various hospitals in Babylon between January and March 2024. The samples included urinary tract infections (urine) and diarrhea (stool) . Each specimen was cultured on MacConkey agar and Eosin Methylene Blue (EMB) agar, then incubated at 37°C for 24 hours. Presumptive *E. coli* colonies displaying a metallic green sheen on EMB were subjected to Gram staining and standard biochemical tests, followed by confirmation using the VITEK-2 Compact system (bioMérieux, France) [15,16].

2.2 Antimicrobial susceptibility testing

Antibiotic susceptibility was determined using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar in accordance with CLSI guidelines (2025). The antibiotic panel included ampicillin, ciprofloxacin, ceftazidime, cefotaxime, gentamicin, meropenem, and imipenem. Results were recorded as susceptible, intermediate, or resistant based on inhibition zone diameters [17].

2.3 DNA extraction and molecular detection of QS genes

Genomic DNA was extracted from confirmed *E. coli* isolates using a commercial DNA extraction kit (Zymo Research, USA) according to the manufacturer's protocol. The purity and concentration of the DNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The presence of *LuxS* and *lsrR* genes was detected by conventional PCR using gene-specific primers listed in Table 1 [18].

Table 1: The sequences and reference of the gene primers used in the study.

Primer	(5′–3′)	Length of primer	Product size (bp)	Reference
LuxS	F:5-CATACCCTGGAG CACCTG TT -3 R:5-TTCTTCGTTGCTGTTGATGC-3	20	315	Abbas *et al*., [19]
lsrR	F:5- TTAACTACGTAAAATCGCCGCTG-3 R:5- ATGACAATCAACGATTCGGCAAT-3	23	954	Zuo *et al*., [20]

2.4 Primer design and PCR amplification

Primers for *LuxS* and *lsrR* were synthesised by Macrogen (Korea). Each PCR reaction (25 µL) contained 12.5 µL of 2× PCR Master Mix (Bioneer Corporation, South Korea), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA (approximately 50–100 ng/µL), and nuclease-free water. Amplification was performed in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, annealing at 55–58°C for 30 s, and extension at 72°C for 45 s; followed by a final extension at 72°C for 5 min [21]. PCR products were analysed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

2.5 Green synthesis of silver nanoparticles (AgNPs)

AgNPs were synthesised using pine seed oil extract following the modified green synthesis method described by Minarchenko *et al*., [22]. Briefly, 10 mL of pine seed oil extract (pine seed oil was extracted using a Soxhlet apparatus with n-hexane as solvent for 6-8 hours, and the solvent was evaporated to obtain the crude oil) was mixed with 90 mL of 1 mM aqueous $AgNO_3$ solution and sonicated (100 W, 42 kHz) for 20 min. The mixture was incubated in the dark at room temperature until a colour change from pale yellow to brown indicated nanoparticle formation. The colloidal solution was centrifuged at 10,000 rpm for 15 min, and the pellet was washed three times with distilled water and ethanol, then dried at 40°C and stored at 4°C until use [23].

2.6 Characterization of AgNPs

The synthesised AgNPs were characterised using several techniques.

- UV–Visible spectroscopy: measured absorbance spectra at 300–700 nm to confirm surface plasmon resonance.
- Fourier-transform infrared spectroscopy (FTIR): identified functional groups involved in nanoparticle stabilisation.

- Scanning electron microscopy (SEM) and atomic force microscopy (AFM): determined shape and particle size distribution.
- Energy-dispersive X-ray spectroscopy (EDX): confirmed elemental composition.
- X-ray diffraction (XRD): assessed the crystalline structure of AgNPs [24–26].

2.7 Determination of minimum inhibitory concentration (MIC) and sub-MIC

MIC values for pine seed oil and AgNPs were determined by broth microdilution in 96-well plates, according to CLSI standards. Sub-MIC concentrations (one-quarter MIC) were used in subsequent gene expression assays to avoid bactericidal effects [27].

2.8 RNA extraction and cDNA synthesis

Total RNA was extracted from treated and untreated *E. coli* isolates using the RNeasy Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. The extracted RNA was then evaluated for purity and quality using the A260/A280 ratio; only samples with an A260/A280 ratio of 1.8–3.0 were used. Following this, the RNA concentration was adjusted to 100 ng/µL for downstream applications. For the reverse transcription procedure, 1 µg of total RNA was used in the cDNA reaction mix following the manufacturer's instructions with random hexamer primers and a commercial reverse transcription kit, providing potential high-quality cDNA for downstream PCR amplification [28].

2.9 Quantitative real-time PCR (qRT-PCR)

Gene expression of *LuxS* and *lsrR* was analysed by qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, USA) in a StepOnePlus real-time PCR system. The *16S rRNA* gene served as the internal control. The amplification protocol consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [29].

2.10 Statistical analysis

All experiments were performed in triplicate (n = 3). Data are expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using one-way ANOVA followed by Tukey's post hoc test or the Chi-square test, with $p < 0.05$ considered significant. Statistical analysis was carried out using GraphPad Prism version X (GraphPad Software, USA) [30].

2.11 Ethical approval

This study was conducted in accordance with the ethical standards of the local institutional review board. Ethical approval was obtained from the Local Research Ethics Committee of the College of Science, University of Baghdad (Approval No. 269, dated 2 July 2024). All procedures involving clinical samples were performed in accordance with relevant guidelines and regulations. Verbal consent was obtained from patients before sample collection, and all collected data were kept strictly confidential.

3. Results

3.1 Isolation and identification of E. coli

Of the 155 clinical specimens collected from various sources (urinary tract infections and diarrhea), 75 isolates were identified as *E. coli* based on colony morphology, Gram staining, and biochemical characteristics. Confirmation with the VITEK-2 Compact system verified all isolates as *E. coli*. The distribution of isolates by sample type and patient gender is shown in Table 2.

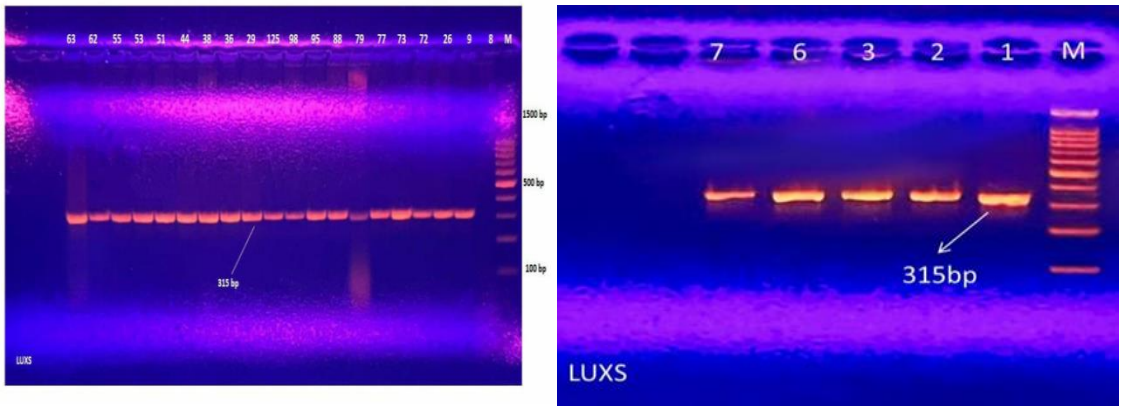
Table 2: Distribution of clinical *Escherichia coli* isolates according to sample type (urine and stool) and patient gender.

Sample Source	No. of Isolates	Male (n, %)	Female (n, %)	Total (%)
Diarrheal cases (Di.)	40	22 (55.0%)	18 (45.0%)	53.3%
Urinary tract infections (UTI)	35	12 (34.3%)	23 (65.7%)	46.7%
Total	75	34 (45.3%)	41 (54.7%)	100%

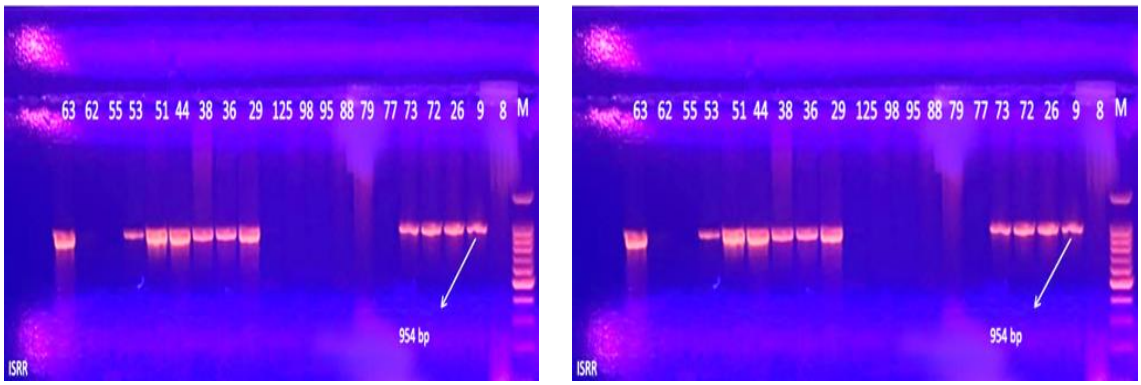
Data were analyzed using the Chi-square test; $p \leq 0.05$ was considered statistically significant

3.2 Detection of quorum-sensing (QS) genes

Molecular screening by conventional PCR revealed that 25 of 75 *E. coli* isolates carried both the *LuxS* and *lsrR* genes, which are associated with the AI-2–mediated quorum-sensing system. The PCR amplification results, showing specific bands for *LuxS* (315 bp) and *lsrR* (420 bp), are illustrated in Figure 1. The frequency of gene detection among isolates is summarised in Table 3.



(a)



(b)

Figure 1: PCR amplification of *LuxS* (a) and *lsrR* (b) genes in selected *E. coli* isolates showed specific bands at 315 bp and 420 bp.

Table 3: Prevalence of quorum-sensing genes (*LuxS* and *lsrR*) among *E. coli* isolates detected by conventional PCR.

Gene	Positive isolates n (%)	Negative isolates n (%)	Total (n = 75)	Statistical analysis (χ^2, p-value)
LuxS	25 (33.3%)	50 (66.7%)	75	$\chi^2 = 10.33$, $p = 0.0013$
lsrR	25 (33.3%)	50 (66.7%)	75	$\chi^2 = 10.33$, $p = 0.0013$
Both genes	25 (33.3%)	50 (66.7%)	75	—

Statistical analysis using the Chi-square test indicated a significant difference ($p \leq 0.05$) between positive and negative isolates

3.3 Green synthesis and characterization of silver nanoparticles (AgNPs)

Successful synthesis of AgNPs using pine seed oil extract was confirmed by a visible colour change from pale yellow to dark brown, indicating surface plasmon resonance (SPR). The UV–Vis absorption spectrum of the AgNPs showed a distinct peak at approximately 420 nm (Figure 2), confirming nanoparticle formation.

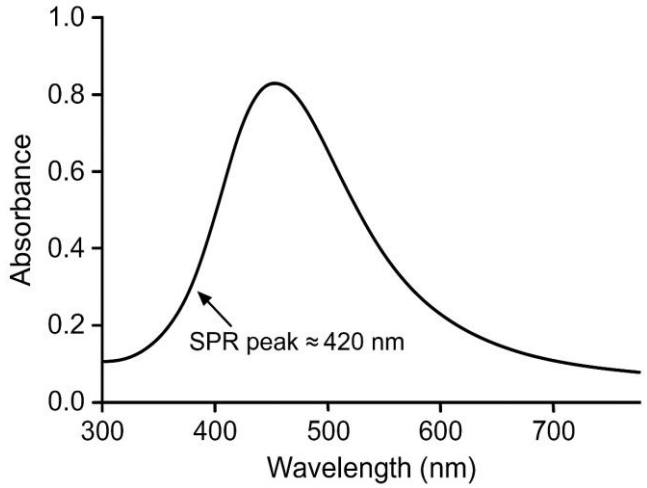


Figure 2: The UV–Vis absorption spectrum of the AgNPs showed a distinct peak at approximately 420 nm

FTIR analysis identified characteristic absorption bands corresponding to O–H, C=O, and C–O functional groups, indicating the involvement of phenolic and carbonyl compounds in nanoparticle reduction and stabilisation (Figure 3). SEM and AFM images showed predominantly spherical nanoparticles with a mean particle size of 35–45 nm (Figure 4). EDX analysis confirmed the presence of elemental silver with strong signals around 3 keV, while XRD patterns displayed distinct diffraction peaks corresponding to the face-centred cubic structure of silver (Figures 5 and 6). The summarised physicochemical characteristics of AgNPs are presented in Table 4.

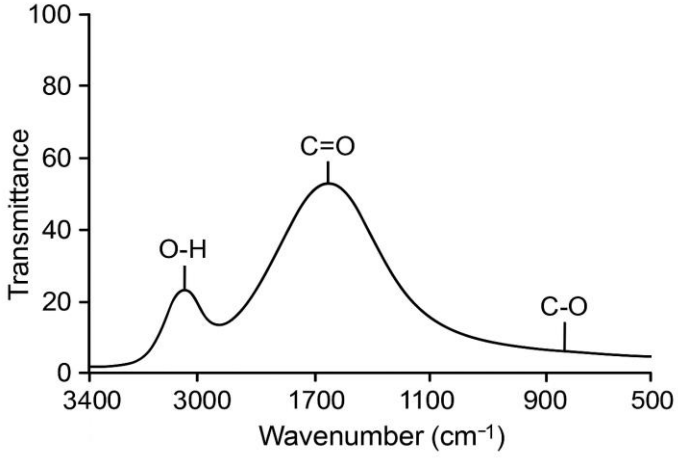
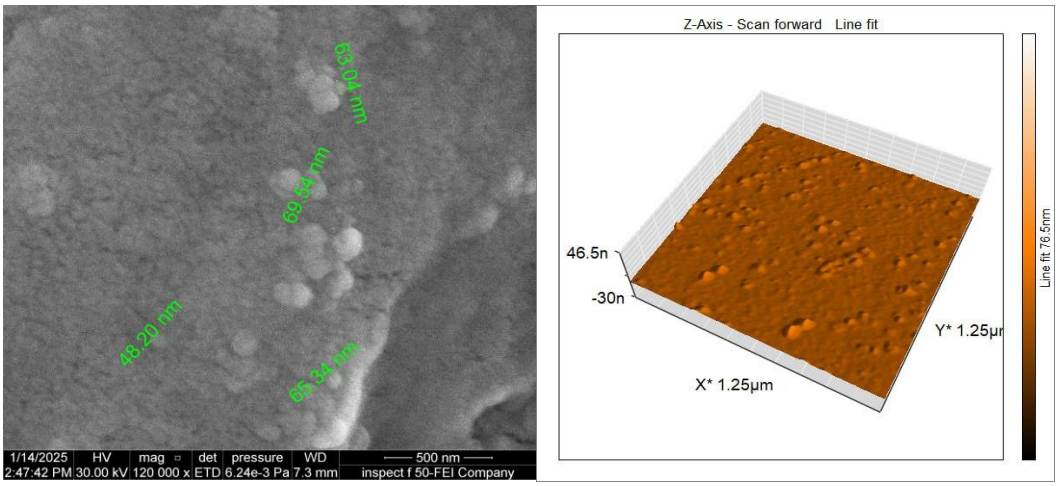


Figure 3: FTIR spectrum of pine seed oil–synthesized AgNPs showing characteristic functional groups responsible for reduction and stabilization**.**



(a) **(b)**

Figure 4: (a) SEM and (b) AFM micrographs of green-synthesized AgNPs demonstrating spherical morphology and size distribution (35–45 nm)**.**

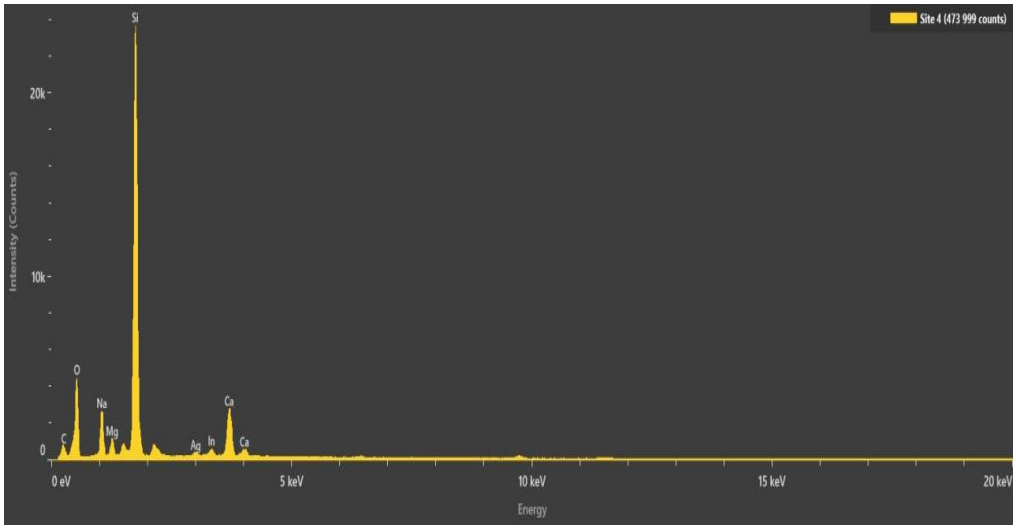


Figure 5: EDX spectrum of AgNPs confirming the presence of elemental silver with a strong signal at 3 keV**.** The average particle diameter was 35–45 nm**.**

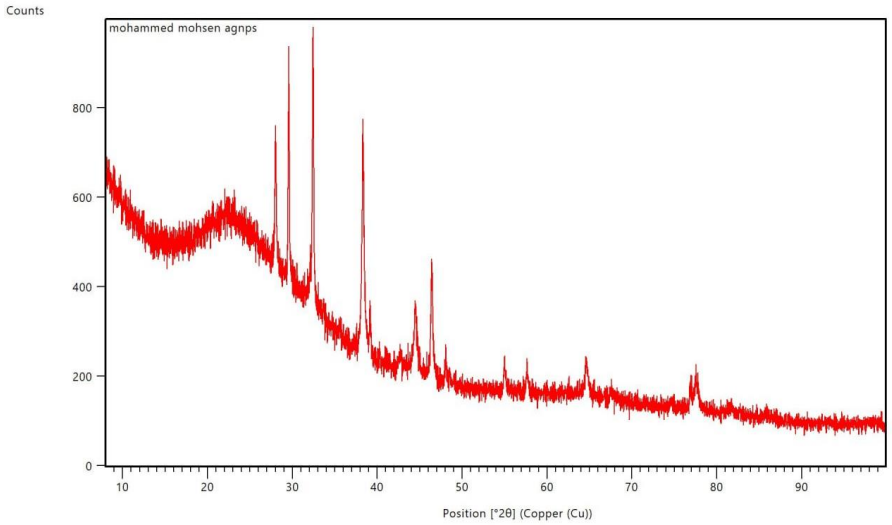


Figure 6: XRD pattern of green-synthesized AgNPs recorded using Cu-Kα radiation, showing characteristic diffraction peaks corresponding to the face-centered cubic (fcc).

Table 4: Physicochemical characterization of green-synthesized silver nanoparticles (AgNPs) using pine seed oil extract.

Characterization technique	Result	Interpretation
Visual observation	Color change from pale yellow to dark brown	Indicates formation of AgNPs due to surface plasmon resonance (SPR)
UV–Visible spectroscopy	Distinct absorption peak at ~420 nm	Confirms the synthesis of silver nanoparticles and the SPR excitation
FTIR spectroscopy	Presence of O–H, C=O, and C–O bands	Suggests involvement of phenolic and carbonyl compounds in the reduction and stabilization of AgNPs
SEM analysis	Predominantly spherical nanoparticles	Reveals particle morphology and uniform shape
AFM analysis	Average particle size 35–45 nm	Confirms nanoscale dimension and surface topology
EDX analysis	Strong signal at ~3 keV	Confirms presence of elemental silver
XRD analysis	Distinct diffraction peaks corresponding to face-centered cubic (fcc) structure of Ag	Confirms crystalline nature and phase purity of AgNPs

3.4 Antimicrobial activity and MIC determination

The antimicrobial activity of both pine seed oil and green-synthesised AgNPs was evaluated using the microdilution method. AgNPs exhibited a lower MIC (32 µg/ml) compared with pine seed oil (1000 µg /ml), indicating greater antibacterial potency . These sub-MIC concentrations were subsequently used for gene expression analysis to ensure non-lethal exposure (Table 5)**.**

Table 5: Minimum inhibitory concentration (MIC) values of pine seed oil and green-synthesized AgNPs against *E. coli* isolates.

Test sample	MIC (µg/ml)	Mean ± SD	Statistical significance
Pine seed oil	1000 mg/ml	1000 ± 25.4	a
AgNPs (green-synthesized)	32	32 ± 2.1	b

Different letters (a, b) indicate statistically significant differences ($p \leq 0.05$) between treatments.

3.5 Quantitative analysis of LuxS and lsrR gene expression

Quantitative real-time PCR (qRT-PCR) was used to assess the effect of sub-MIC concentrations of pine seed oil and AgNPs on the expression levels of *LuxS* and *lsrR* in selected *E. coli* isolates. The *16S rRNA* gene served as an internal control. The ΔCt and ΔΔCt values were calculated, and relative expression levels (fold-change) were determined using the $2^{-\Delta\Delta Ct}$ method (Tables 6-8). Treatment with AgNPs resulted in significant down-regulation of both genes compared to the untreated control and pine oil treatment ($p \leq 0.05$).

Table 6: Relative expression of *LuxS* gene in *E. coli* isolates (control group, untreated).

Sample No.	Housekeeping gene Ct	Target gene Ct (LuxS)	ΔCt	ΔΔCt	Fold change ($2^{-\Delta\Delta Ct}$)
1	20.40	24.17	3.77	–0.447	1.36
2	21.21	25.12	3.91	–0.307	1.24
3	15.29	20.26	4.97	0.753	0.59
Mean ± SD	—	—	4.22 ± 0.61	—	1.00 ± 0.07

Table 7: Relative expression of *LuxS* gene in *E. coli* isolates treated with AgNPs (32 µg/ml).

Sample No.	Housekeeping gene Ct	Target gene Ct (*LuxS*)	ΔCt	ΔΔCt	Fold change ($2^{-\Delta\Delta Ct}$)
3	18.32	22.93	4.61	0.393	0.76
4	20.24	24.72	4.48	0.263	0.83
5	19.79	24.12	4.33	0.113	0.92
Mean ± SD	—	—	4.47 ± 0.14	-	0.84 ± 0.05

Table 8: Relative expression of *LuxS* gene in *E. coli* isolates treated with pine seed oil (1000 µg /ml).

Sample No.	Housekeeping gene Ct	Target gene Ct (*LuxS*)	ΔCt	ΔΔCt	Fold change ($2^{-}\Delta\Delta Ct$)
3	17.41	21.11	3.70	–0.517	1.43
4	18.33	23.01	4.68	0.463	0.73
5	20.88	25.24	4.36	0.143	0.91
Mean ± SD	-	-	4.25 ± 0.41	-	0.98 ± 0.07

Mean fold-change values for *LuxS* were 0.84 ± 0.05 for AgNP-treated isolates and 0.98 ± 0.07 for pine oil–treated isolates, whereas *lsrR* expression decreased to 0.57 ± 0.03 for AgNPs and 0.89 ± 0.04 for pine oil. These results demonstrate that green-synthesised AgNPs more effectively inhibited quorum-sensing gene expression than the natural oil extract (Table 9 and Figure 7).

Table 9: Effect of pine seed oil and green-synthesized AgNPs on *LuxS* and *lsrR* gene.

Treatment	Mean fold change (*LuxS*)	Mean fold change (*lsrR*)	Significance (p≤0.05)
Control	1.00 ± 0.07	1.00 ± 0.06	—
AgNPs (32 µg/ml)	0.84 ± 0.05	0.57 ± 0.03	Significant ↓
Pine seed oil (1 mg/ml)	0.98 ± 0.07	0.89 ± 0.04	Not significant

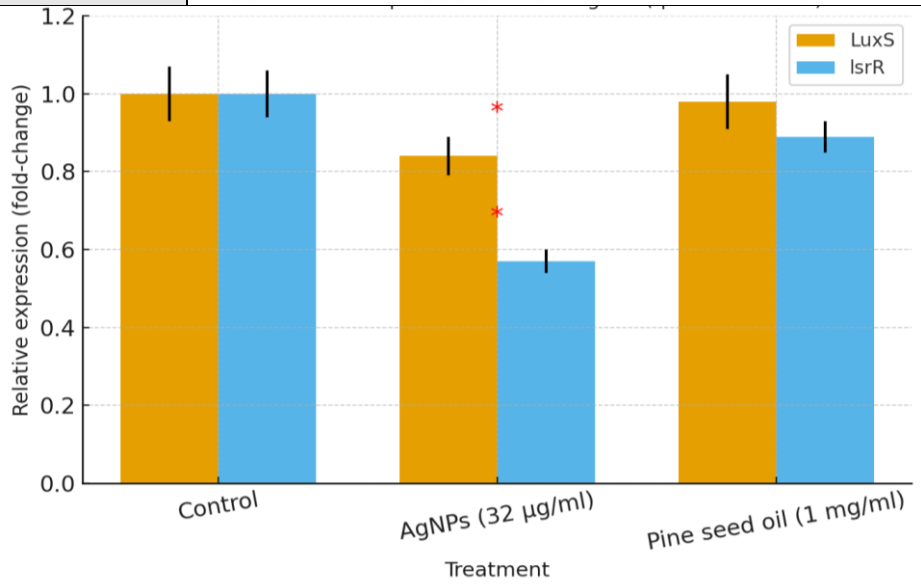


Figure 7: Relative expression levels (fold-change) of *LuxS* and lsrR *genes* in *E. coli* isolates after treatment with pine seed oil and AgNPs (qRT-PCR results), *significant differences (p≤0.05).

4. Discussion

The present study investigated the inhibitory effects of pine seed oil and its green-synthesised silver nanoparticles (AgNPs) on quorum-sensing (QS)-related genes (*LuxS* and *lsrR*) in *E. coli*. The results showed that both treatments down-regulated QS gene expression, with AgNPs having a more pronounced effect. These findings are consistent with previous reports indicating that silver nanoparticles can interfere with AI-2 signalling and attenuate virulence-related traits in Gram-negative bacteria [31–33].

The significant down-regulation of the *LuxS* and *lsrR* genes observed in AgNP-treated isolates may be attributed to multiple mechanisms, including oxidative stress induction, protein–thiol interactions, and membrane damage, which collectively disrupt quorum-sensing pathways [34, 35]. Pine seed oil also reduced QS gene expression, although to a lesser extent. This partial inhibitory effect is likely due to its bioactive compounds, such as terpenes and flavonoids, which have been reported to modulate bacterial signalling and inhibit biofilm formation [36–38].

Our results are consistent with earlier studies that demonstrated natural plant extracts can act as quorum-sensing inhibitors (QSIs) by reducing the synthesis or activity of autoinducers without affecting bacterial viability [39, 40]. For example, essential oils derived from plants such as *Thymus vulgaris* and *Cinnamomum zeylanicum* have been shown to suppress LuxS-regulated quorum sensing in E. coli and other pathogens [41]. Similarly, green-synthesised AgNPs have been reported to inhibit AI-2–mediated communication in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, highlighting the broad-spectrum potential of this approach [42, 43].

Compared to conventional antibiotics, quorum-sensing inhibitors such as AgNPs and natural oils offer the advantage of reducing virulence and biofilm formation without exerting bactericidal pressure, thereby minimising the risk of developing antibiotic resistance [44, 45]. The synergistic combination of phytochemicals and metal nanoparticles could further enhance anti-quorum-sensing activity through multiple molecular interactions [46].

However, despite the promising results, certain limitations must be acknowledged. This study was conducted *in vitro*, and the actual anti-QS efficacy of pine seed oil and AgNPs under *in vivo* conditions remains to be validated. In addition, the potential cytotoxicity and long-term environmental impacts of silver nanoparticles require further investigation [47, 48]. Future research should focus on optimising nanoparticle synthesis, evaluating combinational therapies, and exploring the molecular mechanisms governing QS inhibition at the proteomic and metabolomic levels.

In summary, this study provides evidence that both pine seed oil and its green-synthesised AgNPs can effectively down-regulate *LuxS* and *lsrR* genes in *E. coli*. The enhanced inhibitory potential of AgNPs suggests that they could serve as promising anti-virulence or anti-biofilm agents for controlling QS-regulated bacterial infections, pending further in vivo and safety assessments.

5.Conclusions

The findings of this study show that both pine seed oil and its green-synthesised silver nanoparticles (AgNPs) exhibit quorum-sensing inhibitory activity against *E. coli*. Treatment with AgNPs resulted in significantly greater downregulation of the *LuxS* and *lsrR* genes compared with pine seed oil alone, indicating stronger interference with AI–2–mediated quorum–sensing pathways. The ability of AgNPs to inhibit quorum sensing and potentially reduce virulence and biofilm formation highlights their promise as alternative or

complementary agents to traditional antimicrobials. Although the *in vitro* results are encouraging, further *in vivo* investigations are required to confirm the safety, efficacy, and stability of these biosynthesised nanoparticles. Future studies should also explore their potential synergistic applications with plant-derived compounds and assess their broader impacts on microbial communities and host systems. Overall, this study supports the concept that eco-friendly, green-synthesised nanomaterials combined with natural oils can serve as novel, non-antibiotic approaches to control bacterial communication and pathogenesis.

6. Limitations of the Study

Although this study provides valuable insights into the quorum-sensing inhibitory effects of pine seed oil and green-synthesised silver nanoparticles (AgNPs) against *E. coli*, certain limitations should be acknowledged. First, the study was conducted entirely *in vitro*, which may not fully reflect the complex interactions that occur in host environments. Second, the sample size of isolates analysed for gene expression was relatively small, which could limit the generalisability of the results. Third, the potential cytotoxicity and environmental impact of AgNPs were not assessed in this study. Future work should therefore include *in vivo* investigations using appropriate animal models, evaluation of nanoparticle biosafety, and exploration of the molecular pathways underlying quorum-sensing inhibition at the proteomic and metabolomic levels.

7. Acknowledgments

The authors express their sincere gratitude to the Department of Biology, College of Science, University of Baghdad, for providing laboratory facilities and technical assistance during this research. Special thanks are extended to the postgraduate research unit and the Central Laboratories for their support in conducting the molecular and nanoparticle characterisation experiments. The authors also thank the clinical laboratories of Babylon hospitals for their cooperation in providing the bacterial isolates used in this study.

8. Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this research. The study was conducted solely for academic and scientific purposes, without financial or commercial influence.

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