

## SYNERGISTIC EFFECTS OF NEEM OIL AND GENTAMICIN ON *PSEUDOMONAS AERUGINOSA* VIA PHZM GENE DOWNREGULATION: A COMPREHENSIVE REVIEW

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### Review



### ABSTRACT

Exploring the antibacterial potential of neem oil (*Azadirachta indica*) in combination with gentamicin (GEN) against pathogenic molds, especially *Pseudomonas aeruginosa*, has drawn concern due to the quest for natural treatment options against incurable diseases. Prospective research directions include looking for natural cures for many of the currently incurable diseases available now. microbial identification system, were used to identify the isolates. The research utilized a range of methods, such as the diffusion agar well (AWD) assays, TEM (transmission electron microscopy) analysis, minimum inhibitory concentration (MIC) assays, and real-time PCR (RT-qPCR) to analyze bacterial expression and the antibacterial action of neem oil (*Azadirachta indica*) combined with gentamicin (GEN) against the pathogenic bacteria *Pseudomonas aeruginosa* between others. The combined effects of neem oil and GEN on *P. aeruginosa* are extensively investigated in this study, with particular attention paid to the downregulation of the virulent factor gene *phzM*, inhibition of biofilm formation, morphological changes, and gene expression, in that proportion. Results show that neem oil has strong antibacterial activity against *P. aeruginosa* and *S. aureus*, preventing the formation of biofilms and causing morphological alterations. In addition, the synergistic effects of neem oil and GEN are demonstrated, together in order with the downregulation of the *phzM* gene in *P. aeruginosa*. These results highlight the increased effectiveness of neem oil when combined with GEN and point to the potential of the oil as a stand-alone antibacterial agent. They also highlight the need for more study to overcome antibiotic resistance in harmful bacteria. Overall, our results point to a possibility that neem oil extract, either alone or in combination with GEN, can suppress the development of pathogenic microbes. This synergistic impact is related to the downregulation of a virulence factor gene. To address the drug resistance linked to pathogenic microbes, greater study is required. Novelty of study addresses the important issue of antibiotic resistance by exploring the potential synergistic effects of neem oil and gentamicin on pathogenic bacteria, particularly *Pseudomonas aeruginosa*. This topic is highly relevant in the context of the global challenge of antimicrobial resistance. Novelty and relevance the important issue of antibiotic resistance by exploring the potential synergistic effects of neem oil and gentamicin on pathogenic bacteria, particularly *Pseudomonas aeruginosa*. This topic is highly relevant in the context of the global challenge of antimicrobial resistance.

**Keywords:** Neem oil, *phzM* gene, Gentamicin, *Pseudomonas aeruginosa*

### INTRODUCTION

Medicinal properties of neem oil, its antimicrobial potential, and the increasing problem of antibiotic resistance. It is well acknowledged that medicinal plants are a rich source of many potential therapeutic agents (I. J. Abed *et al.*, 2020). Interestingly, numerous bioactive compounds (secondary metabolites) have been extensively investigated for use against various pathological conditions, including infections and proliferation of cancer cells. Natural plant products are generally believed to possess disease prevention and treatment properties by modulating the expression of key cellular pathways. Owing to the rich antioxidant content of the neem plant (*Azadirachta indica*), a member of the Meliaceae family, it has health-promoting potential (Ahmed *et al.*, 2018). These properties were confirmed through HPLC analysis, which revealed that the *A. indica* extracts contained azadirachtin. In addition, a long list of chemical compounds extracted and purified from different *A. indica* plant parts may have therapeutic potential. These include nimbolinin, quercetin,  $\beta$ -sitosterol, polyphenolic flavonoids, gedunin, salannin, quercetin ascorbic acid, 17-hydroxyazadiradione, nimbiol, and many others (Al-Halbosiy *et al.*, 2013). Furthermore, the neem plant extract exhibited antimicrobial potential, affecting the growth of different infectious species, including viruses, bacteria, fungi, and parasites. Studies on the antibacterial properties of neem have revealed that neem oil may be utilized for controlling spoilage organisms and foodborne pathogens. Furthermore, extracts from parks and neem leaves considerably impede the growth of adult buccal-isolated bacteria. (ALI, Edris *et al.*, 2021). Interestingly, in contrast to the higher concentration of antibiotics required to inhibit the growth of multidrug-resistant (MDR) pathogenic bacteria, lower concentrations of neem leaf extracts are needed to achieve the minimum inhibitory concentration (MIC) in several poultry MDR bacteria (Amer and Taie 2010). Antibiotic resistance is a severe health issue, and efforts have been

concentrated on tackling such increasing life-threatening medical challenges using different approaches (Arora and Chapman, 2000). In addition, the use of bacteriocin and vancomycin was shown to have a profound inhibitory effect on biofilms of MRSA isolates (Baby AR *et al.*, 2022). Similarly, rosemary volatile oil, used as a chemical preservative agent, was shown to have antimicrobial effects on *Bacillus* spp (Elmassry *et al.*, 2020). Antimicrobial resistance is one of the most significant threats to public health worldwide. Instead of using conventional antibiotics, which are effective against multidrug-resistant diseases, it is vital to concentrate on the most innovative antibacterial compounds (Ahmed *et al.*, 2023). Bacteria and antibacterial agents performed better together than when essential oils were used alone. Using the well diffusion method, antibiotics such as tetracycline, rosemary essential oil, and niacin A were found to be effective (AL-Shimmery *et al.*, 2020). *Pseudomonas aeruginosa* is an aggressive gram-negative bacterium with numerous mechanisms of resistance to medicines. Its ability to manufacture phenazine poses a serious healthcare concern with a range of impacts on patients (Anwer *et al.*, 2024). Pyocyanin, the distinctive pigment of *P. aeruginosa*, must be present for infection. This study looked into how pyocyanin synthesis can be affected by low ethanol concentrations. The synthesis of pyocyanin was ascertained by both subjective and scientific methods. (Jiang *et al.*, 2023). Considering the scarcity of studies investigating this topic in Iraq, this study was designed to assess the provides a comprehensive overview of the medicinal properties of neem oil, highlighting its antimicrobial potential against a wide range of pathogens. It emphasizes the urgency of combating antibiotic resistance and introduces the specific focus of the study on the synergistic effects of neem oil and GEN against *P. aeruginosa*.

## MATERIALS AND METHODS

### Bacterial isolation and identification

*Pseudomonas aeruginosa* and *Staphylococcus aureus* bacterial isolates were initially obtained from clinical sources (wound specimens). Isolates were identified by culturing the clinical specimens on blood and MacConkey agar for *P. aeruginosa* and on mannitol salt agar for *S. aureus* (cultures were incubated for 24 hrs at 37°C). The isolates were identified based on their morphological features on culture media and the results of biochemical tests (Ahmed et al., 2021).

### Antibiotic Susceptibility (Kirby-Bauer Disk Diffusion Susceptibility Test)

Antimicrobial susceptibility testing was performed using the Kirby–Bauer test (Ahmed, 2018). In brief, 3 to 5 discrete colonies were suspended in 2 ml of normal saline and adjusted to McFarland 0.5. The bacterial suspension was spread on the surface of a Muller-Hinton agar plate using sterile cotton swabs. The inoculated plates were then placed at room temperature for 30 minutes to allow the absorption of excess moisture. Subsequently, the antibiotic disks, Cefotaxime (30 µg), Levofloxacin (5 µg), Gentamicin (10 µg), Imipenem (10 µg), Meropenem (10 µg) and Cefepime (10 µg) (HiMedia, India), were placed on the agar surface with sterile forceps. Ultimately, the plates were incubated at 37°C for 24 hours.

### Determination of the minimum inhibitory concentration (MIC)

The MIC of the potential antimicrobial activity of the neem oil extract was determined using a broth microdilution assay (Ahmed and Seddiq, 2018). This test was accomplished using a 96-well microtiter plate. One hundred microliters of neem oil extract at five different concentrations (4, 8, 16, 32, and 64 µg/ml) was diluted in a similar volume of dimethyl sulfoxide (DMSO) to obtain one-half of the original concentration. The volume of neem oil was 100 µl per well, along with 100 µl of double-strength Muller-Hinton broth. Then, 10 µl of overnight diluted bacterial suspension, adjusted to a 0.5 MacFarland turbidity standard, was added to the wells. Three control wells were assigned for each test: positive control (well with neem oil), negative control (well with DMSO), and sterility control (well containing the broth media alone). After overnight incubation at 37°C, 5 µl of Gram stain was added to all wells, and the plates were incubated at 37°C for another four hours. Changes in color (white to blue) were observed and recorded.

### Determination of the MIC of Gentamicin Against the Studied Pathogenic Bacteria

The antibacterial effects of gentamicin on *P. aeruginosa* and *S. aureus* were evaluated at different concentrations (4, 8, 16, 32, and 64 µg/ml). According to the CLSI guidelines (AL-Shimmary et al., 2020), the MIC of gentamicin for each isolate was calculated.

### Determination of the Potential Synergistic Inhibitory Effect of Neem Oil and Gentamicin on *P. aeruginosa* and *S. aureus*

The antimicrobial effect of neem oil and gentamicin on both isolation bacteria was assessed using an agar well diffusion (AWA) assay (Huang et al., 2021). According to these findings, the concentration with the greatest inhibition zone diameter was chosen for further studies.

### Estimation of biofilm formation

Biofilm formation was quantified by a colorimetric microtiter plate assay (Romi and Ahmed, 2024).

### Effect of neem oil on biofilm formation

A 96-well microtiter plate was used for this assay, in accordance with earlier instructions (Faiq and Ahmed, 2023). The examined isolates underwent a 24-hour incubation period in infusion broth of brain hearts at 37°C. Subinhibitory quantities of neem oil (100 µl) and a 0.5 McFarland plate with 100 µl of bacterial inoculum were added to each well. For 48 hours, the tubes in place were incubated at 37°C. A culture of bacteria without neem oil was used as a positive control, and clear broth served as a negative control. Following incubation, the contents of each well were disposed of; the microplate was then dried for 45 minutes at 60°C and washed three times with sterile saline. After staining a microplate with two hundred milliliters of 0.1% crystal violet and letting it sit at ambient temperature for fifteen minutes, the plate was washed three times with sterile normal saline. Next, 200 cc of 30% acetic acid was then added to each well, and using a microtiter plate reader, the optical density (OD) of each well was determined at 630 nm.

### Transmission electron microscopy (TEM) analysis

TEM imaging was used to assess the morphological alterations of the investigated pathogenic bacteria treated with neem oil. For TEM imaging, neem oil-suspended

bacteria (approximately 7 µl for each sample) were compared to bacteria suspensions only. The samples were deposited on specimen grids and allowed to adsorb for 1 minute. (Seddiq et al., 2023).

### Measurement of *phzM* gene expression

The method used for real-time PCR was previously described (Mohammed et al., 2023). The relative expression was determined using the delta-delta-Ct technique, and normalization to the housekeeping gene (*fbp*) unique to *S. aureus* was performed. The sequences of the primers according (Ahmed, M. E., & Al-Awadi, A. Q., 2024) used were as follows: *phzM* (forward: 5'-ACGGCTGTGGCGGTTTA, reverse: 3'-CCGTGACCGTCGCATT) and *fbp* (forward: 5'-CCTACCTGTTGGTCTTCGACCCG, reverse: 5'-GCTGATGTGTCGTGGGTGAGG).

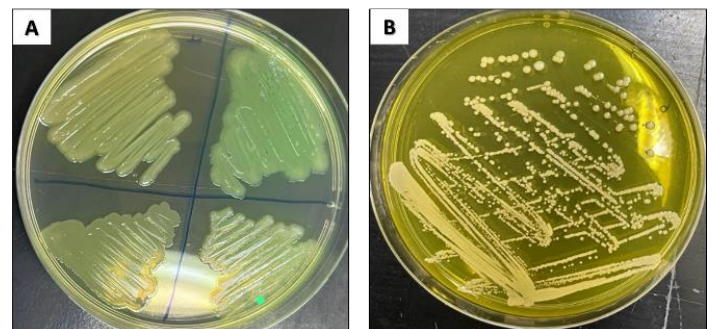
### Statistical analysis

GraphPad Prism software was used for the statistical analysis. One-way ANOVA (TESTS) or independent t tests were used to evaluate the variations in the experimental data. The data are shown as the mean ± standard error; *p* values less than 0.05 were considered significant.

## RESULTS

### Isolation and identification of gram-positive/negative pathogenic bacteria

Bacterial isolates cultured on mannitol salt agar were able to ferment mannitol, resulting in a change in the medium color from red to yellow; thus, the bacteria were presumptively categorized as *Staphylococcus aureus* isolates (Figure 1). The gram-positive bacterium *S. aureus* appeared diplococci under microscopic examination. However, examination of the bacterial isolates on blood agar revealed yellow–gray colonies 3–4 mm in diameter on the zones of β-hemolysis. The morphological characteristics of the growing bacteria were also evaluated. The macroscopic examination of presumptive *P. aeruginosa* isolates on blood agar revealed colonies that produced hemolysis zones with odor and pale colonies. The VITEK<sup>®</sup>2 microbiological identification system further verified the identification of the bacterial isolates mentioned above.



**Figure 1** The bacterial cultures on selective media. (A) *P. aeruginosa* of the clinical isolates cultured on cetrimide agar, and (B) Presumptions of *S. aureus* based on the appearance of the clinical isolates cultured on mannitol salt agar.

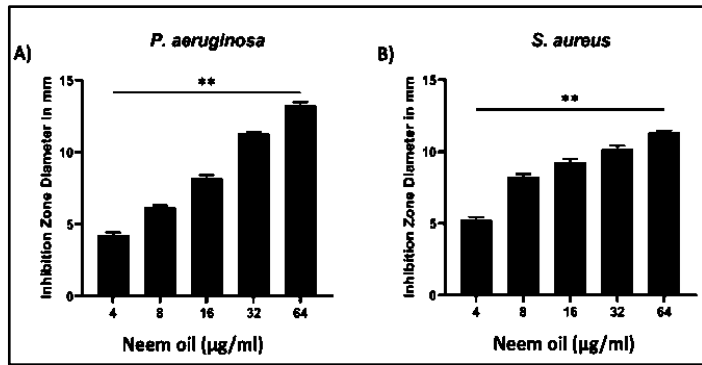
### Antibiotic susceptibility test

Both strains have developed resistance to novel and traditional antibiotics in recent years. As a result, studying the susceptibility pattern helps determine the future problems of effective therapy. To determine the potential resistance of *P. aeruginosa* and *S. aureus*, the isolates were subjected to five antibiotics. In addition, antimicrobial susceptibility tests based on CLSI (2020) were carried out using the widely used Kirby–Bauer disk diffusion technique. Antibiotic susceptibility patterns toward the cephalosporin class showed high resistance compared with other antibiotics, such as gentamicin. This elevated cephalosporin antibiotic resistance in *P. aeruginosa* isolates is due to the increasing incidence of MDR *P. aeruginosa* clinical isolates. Researching and characterizing the bacterial pathogenesis of burn wounds is highly important for accelerating the development of innovative approaches for the prevention and treatment of associated infections. Therefore, *P. aeruginosa* was investigated in this study because it is more resistant than *S. aureus*.

### Minimum inhibitory concentration (MIC)

Both resistant *P. aeruginosa* and *S. aureus* pathogenic isolates were subjected to different concentrations of neem oil extract to determine the MIC. The MIC was determined using Muller Hinton broth via a twofold dilution method, and the lowest concentration at which no growth occurred was chosen as the MIC. The greatest MIC effect on both pathogenic bacterial isolates was obtained with 16 µg/ml of neem oil. As shown in Figure 2, the antibacterial effect of neem oil is

concentration dependent. Interestingly, the diameters of the inhibition zones observed for *P. aeruginosa* (Figure 2, A) exposed to high concentrations (32 and 64 µg/ml) were greater than those observed for *S. aureus* incubated with similar doses of neem oil (Figure 2, B).



**Figure 2** The inhibitory effects of neem oil extract on pathogenic isolates of (A) *P. aeruginosa* and (B) *S. aureus* exposed to increasing concentrations of neem oil extract for 48 hrs. Asterisks denote significant differences ( $p < 0.01$ ).

**Minimum Inhibitory Concentration of GEN by the Well Diffusion Assay Method (WDA)**

For the inhibition zone diameter assay, *P. aeruginosa* and *S. aureus* bacteria were exposed to different concentrations of GEN (4, 8, 16, 32, and 64 µg/ml). A relatively lower concentration (8 µg/ml) significantly inhibited both *P. aeruginosa* and *S. aureus*, and the inhibition zone area increased with increasing concentration. A lower concentration that showed apparent inhibition efficacy reached 6.7 and 5.4 mm, while the highest concentration (64 µg/ml) showed a more pronounced effect, with an inhibition zone reaching 13 and 10 mm against *P. aeruginosa* and *S. aureus*, respectively. The statistical analysis revealed a significant difference ( $p < 0.05$ ) in the mean extent of bacterial growth after treatment with different concentrations of GEN in Table 1.

**Table 1** Zone of Bacterial Inhibition in mm Treated with GEN (8 µg/mL).

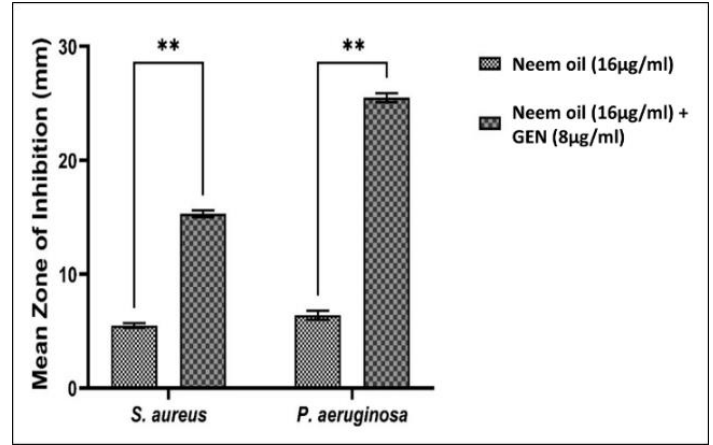
GEN	<i>S. aureus</i>	<i>P. aeruginosa</i>
4 µg/ml	2.5	3.2
8 µg/ml	5.4	6.7
16 µg/ml	8.3	7.2
32 µg/ml	9.2	8.5
64 µg/ml	10	13

**Synergistic Inhibitory Effect of Neem Oil and Gentamicin on *P. aeruginosa* and *S. aureus***

Additionally, the MICs of GEN and the neem oil extract for the multidrug-resistant bacterial isolates *P. aeruginosa* and *S. aureus* were investigated. Interestingly, a greater synergistic effect was observed against gram-negative bacteria (*P. aeruginosa*) than against gram-positive bacteria (*S. aureus*) (mean inhibition diameters: 25.5 and 15.3 mm, respectively) Table 2 and Figure 3. This synergistic antibacterial effect of neem oil plus GEN was much greater ( $p < 0.0001$ ) than that observed for both pathogenic bacterial isolates (*P. aeruginosa* and *S. aureus*) treated with only the neem oil extract. In addition, significant differences between the effects of neem oil extract and GEN at different concentrations (4-64 µg/mL) on *S. aureus* and *P. aeruginosa* were observed.

**Table 2** Mean ± SD of the bacterial growth inhibition zone in mm treated with the neem oil extract (16 µg/mL) and gentamicin GNT (8 µg/mL).

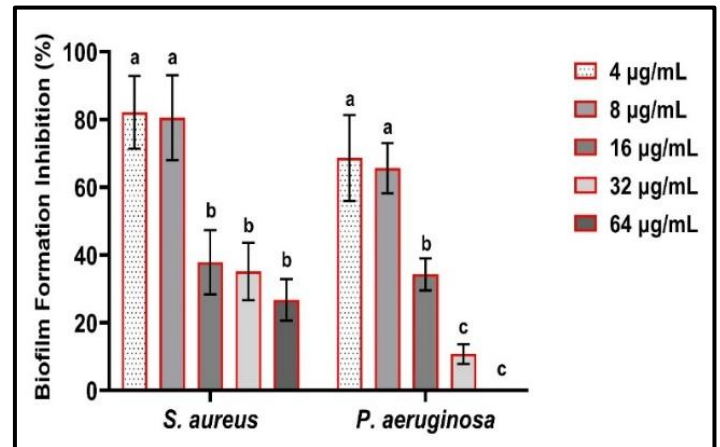
Bacterial Isolate	Mean ± SD Zone of Bacterial Inhibition in mm		p Value
	neem oil extract 16 µg/mL	neem oil extract 16 + Gentamicin GNT 8 µg/mL	
<i>S. aureus</i>	5.5 ± 0.2	15.3 ± 0.3	<0.0001 **
<i>P. aeruginosa</i>	6.4 ± 0.4	25.5 ± 0.4	<0.0001 **



**Figure 3** The mean inhibition zone diameter of *S. aureus* and *P. aeruginosa* cocultured with the neem oil extract alone (16 µg/ml) or combined with 8 µg/ml GEN for 24 hours. The data are presented as the means ± SDs; \*\* $p < 0.0001$ .

**Neem Oil and Effect on Bacterial Biofilm Formation**

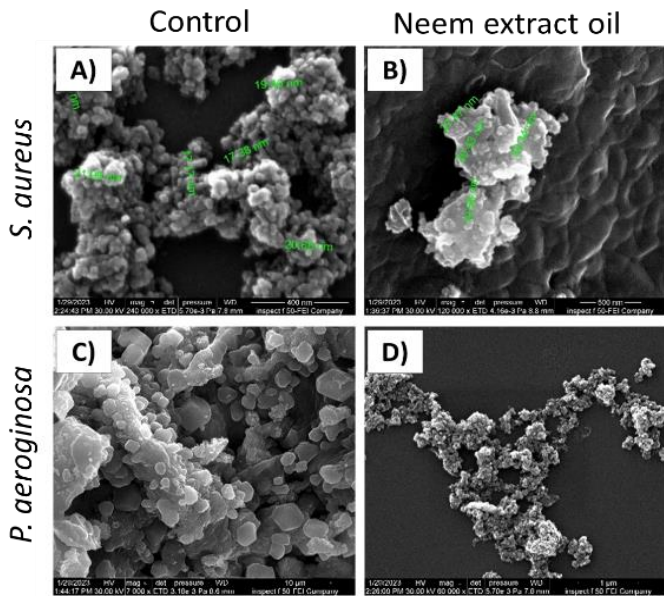
The effect of neem oil on biofilm formation ability was investigated in this study; the results showed that both *S. aureus* and *P. aeruginosa* can form biofilms. This ability was classified as moderate when tested for 24 hours and strong when tested for 48 hours. Our study revealed that biofilm formation was significantly reduced after 48 hours of incubation at 37°C with sub-MICs of neem oil. It was found that neem oil has antibacterial activity against premature biofilms (attachment) of *S. aureus* and *P. aeruginosa*. Premature biofilms of *S. aureus* were inhibited by all concentrations of neem oil at different concentrations. In addition, a concentration of 16 µg/mL of neem oil for each bacterial isolate achieved the highest inhibition rate (Figure 4). Therefore, the antibiofilm activity of both *S. aureus* and *P. aeruginosa* significantly decreased in a concentration-dependent manner after treatment with neem oil Figure 4.



**Figure 4** Biofilm intensity before treatment of *S. aureus* and *P. aeruginosa* with sub-MICs of the neem oil extract. Different letters (a, b, c) indicate significant differences ( $p < 0.05$ ).

**TEM Analysis of the Potential Effect of Neem Oil Extract on the Investigated Pathogenic Bacterial Isolates**

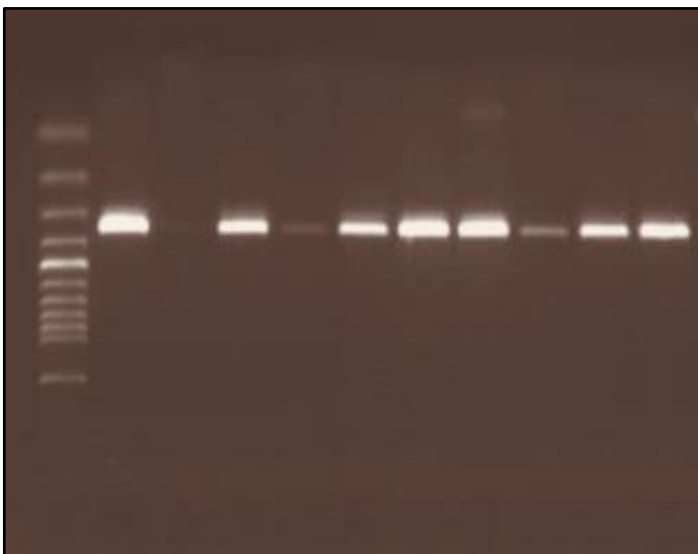
SEM imaging was utilized to analyze the potential effect of treatment with neem oil on the fracture morphology of the investigated bacterial isolates using gamma-irradiated films. Figure 5 shows the morphological characterization before and after treatment with the neem extract oil. After treatment, the spherical morphology of *S. aureus* Figure 5A changed to irregular shapes Figure 5B. Treatment with neem oil containing rod-shaped, sporogenous, and monoflagellated *P. aeruginosa* resulted in irregular and ruptured bacterial cell walls. The results exhibited a far superior surface morphology, with definite indications of a smoother textural surface and an apparent absence of pores, in the cell wall of the treated bacteria Figure 5 C, D.



**Figure 5** TEM images of the tested bacterial isolates before and after treatment with neem oil. The upper panel (A, B) is for *S. aureus*, while the lower panel (C, D) is for *P. aeruginosa*.

**Polymerase chain reaction (PCR)**

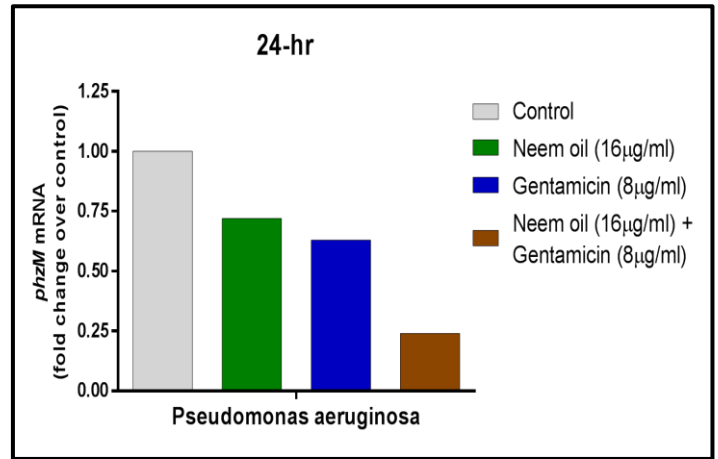
Nine *P. aeruginosa* isolates getting the *phzM* gene were found by the PCR outcomes, and these specimens were chosen according to their multidrug resistance (MDR) status. This procedure on a 1.5% agarose gel stained with ethidium bromide, electrophoresed at 75 volts for 50 minutes, and seen under an ultraviolet (UV) transilluminator was the subsequent procedure used to validate the positive gene result. The results of the study showed that the 180 bp *phzM* gene band was distinct from the DNA pyramid and was crisp, distinctive, and nondispersed. Figure 6.



**Figure 6** Results of the amplification of the *phzM* gene in *Pseudomonas aeruginosa* bacterial samples after separation via 1.5% agarose gel electrophoresis and staining with EthBr. M: 100 bp ladder marker. Lanes Z1-79 resemble 180 bp PCR products

**Effect of neem oil extract, Gen, or their combination on *phzM* gene expression**

The molecular mechanisms underlying the observed antibacterial effects, particularly the downregulation of the virulence factor gene *phzM*. This molecular insight adds depth to the study and provides a basis for further research. To further understand the mechanism underlying the effect of dual exposure to neem oil extract (16 µg/ml) and gentamicin (8 µg/ml) on *P. aeruginosa*, the mRNA level of the virulence gene *phzM* was investigated by RT-qPCR. The relative expression data analysis (Figure 6) revealed the downregulation of the *phzM* gene in bacteria treated with neem oil (0.25-fold) and Gen (0.3-fold). Interestingly, further downregulation was observed in the bacteria exposed to the combination (0.75-fold) Figure 7.



**Figure 7** Mean fold change in *phzM* gene expression in *P. aeruginosa* treated with oil (16 µg/ml), GNT (8 µg/ml), or a combination of both for 24 hours. Following incubation with the given treatments, the fold change over the control is shown by RT-qPCR bars; *fbp* served as the reference gene in this work.

**DISCUSSION**

Antibiotic resistance has emerged as a severe health issue, creating an urgent need to develop novel therapeutic agents (Tang et al., 2023). Identifying natural products that have the potential to cure many of today's intractable afflictions represents a promising research venue (Faiq and Ahmed, 2024). Within this context, the present study investigated antibacterial agents, namely, gram-positive/negative pathogenic bacteria isolated from clinical sources. The results of the present study highlighted the clear antibacterial impact of neem oil on the examined pathogenic bacteria (*S. aureus* and *P. aeruginosa*). Interestingly, both tested bacterial isolates exhibited considerable resistance to several commonly used antibiotics (ceftazidime, levofloxacin, gentamicin, and meropenem). However, the bacterial growth and biofilm formation of both *P. aeruginosa* and *S. aureus* were largely inhibited when they were subjected to all examined concentrations of neem oil. Since antibiotics target essential bacterial processes such as transcription, translation, and cell wall formation, resistance frequently comes with fitness costs when there is no selective pressure (Emad and Salama, 2020). Several lines of evidence indicate that *P. aeruginosa* is a nosocomial pathogen with a high prevalence in burn infections (Nazir S et al., 2023), (Rodrigues et al., 2019) and (Faiq and Ahmed, 2024).

In line with the growing concern about antibiotic resistance, the promising antibacterial potential presented in this study suggests that neem oil extract could be investigated further for its utility in improving the sensitivity of in-use antibiotics to inhibit the growth of poorly eradicated pathogenic bacteria. It is believed that the MDR phenotype in *P. aeruginosa* develops through resistance to the multi-DR phenomenon type, which develops through a variety of pathways, including the loss of outer membrane proteins (porins), beta-lactamase synthesis, multidrug efflux systems, and target changes (Reynolds and Kollef, 2021). In addition, neem oil has been found to have numerous antimicrobial effects against a wide range of microbiological pathogens, including certain pathogenic viral infections as COVID-19 (Sithisarn P et al., 2005), the virus that causes herpes simplex (Shrirangasami et al., 2020), and poliovirus (SaiRam et al., 2000). Neem oil has also been shown to exhibit antifungal effects in a variety of species, such as strains of *Candida albicans* and *Aspergillus flavus* in peanuts (Spernovasilis et al., 2021). (Tian Z et al., 2019). Also observed was the antiparasitic potential (Wayne P., 2019). Currently, extensive research on the antimicrobial activity of *A. indica* (neem tree) is being conducted in various fields, including dentistry, food safety, bacteriology, mycology, virology, and parasitology (Wylie and Merrell, 2022). In addition, combinations of antibiotics and peptidomimetic agents can slow the emergence of antibiotic resistance in *P. aeruginosa* and *S. aureus* upon treatment with ciprofloxacin and Gen separately at subinhibitory concentrations (Yerima et al., 2012). In our recent study, we explored the effect of neem extract oil in vitro and in vivo. We found that neem oil extract has the ability to suppress the development of many rapidly proliferating biological models, such as *A. cepa* root tips and breast cancer cell lines (MCF7 and MDA-MB231), supported by low harm to the exposed mice's essential organs (Mais and Zahraa, 2024).

The results of the present study agree with those of a previous study (Lafta et al., 2023), which showed that *P. aeruginosa* isolates were multiresistant to gentamicin according to the MIC. The aminoglycoside-resistant strains were also identified via *GyrA* gene sequencing. The results obtained in this study (El-Far and Abukhatwah, 2023) show the enhanced antibacterial activity of neem oil after combination with antibiotics such as gentamicin. Thus, the purpose of this study was to determine how certain bacterial strains behaved to the combination of the gentamicin and lavender essential oils extracted from two cultivars of lavender (*L. angustifolia*) and their varied morphological components (flowers and leafy stalks). Another study investigated the Cinnamon oil has antiseptic and anti-efflux properties against XDR *P. aeruginosa* strains for the first time. (Skwirzyńska,

2023). These findings demonstrate the possibility of essential oils as an effective replacement for dosage techniques used in the management of *P. aeruginosa* infections in the future. The present study also revealed that neem oil has antibiofilm activity against multidrug-resistant *P. aeruginosa* and *S. aureus*, which is consistent with the findings of (Abdelatti et al., 2023) who reported that *Piper nigrum* essential oil has antimicrobial effects and antibiofilm activity.

Initially, the minimal inhibitory concentration method was used to evaluate the antimicrobial effects on selected gram-positive and gram-negative bacteria. In this study, two to four samples of five commercially available essential oils—Oregano, Eucalyptus, Rosemary, Clove, and Peppermint—produced by autochthonous enterprises were examined for their antibacterial and antibiofilm properties. (Vuković et al., 2024). Pyocyanin is a chloroform-soluble blue green phenazine pigment made by active cultures of *P. aeruginosa*. Approximately 90 to 95% of *P. aeruginosa* strains produce pyocyanin, the primary phenazine pigment associated with the organism, which has powerful antimicrobial, antioxidant, and anticancer activities (Fawzi and Ahmed, 2024). As pyocyanin can raise the amount of reactive oxygen species (ROS) levels, it can partially cause oxidative stress in humans. Additionally, it has been proposed that pyocyanin facilitates cell-to-cell contacts within cells, helping in the creation of biofilms. (Kothari et al., 2022). Two phenazine-modifying genes (*phzS* and *phzM*) encoding enzymes convert phenazine-1-carboxylic acid to pyocyanin (Martemucci et al., 2023) Interestingly, *phzM* was downregulated in *P. aeruginosa* after incubation with the neem oil extract, Gen, or a combination of both (oil extract and Gen). Both the transcriptome analysis and the qRT-PCR results revealed comparable transcriptional patterns. Moreover, identical reducing patterns were observed in the physiologic and biochemical data after exposure. to the combination, with a decrease in pyocyanin yields (Thi MTT et al., 2020). The latter results agree with (Schmitz and Rosenbaum, 2020) and (Ahmed et al., 2024) which showed that geraniol, a chemical component of nanoparticle same activity essential oil, has antimicrobial and anti-inflammatory activities and low toxicity. However, the effect and mechanism of geraniol against *P. aeruginosa* were linked to the virulence factor *phzM*. In addition, similar findings were observed when EO was used against *P. aeruginosa*, which reduced pyocyanin production (Li, W. R et al., 2023). These three QS quorum sensing systems regulate virulence factors, including pyocyanin, elastase, lectin A, and rhamnolipids. Consequently, chemicals that impede cell-cell contact by blocking one of these three QS systems ultimately decrease the virulence of *P. aeruginosa* (Sepahi, E et al., 2015) Pyocyanin is a dominant phenazine pigment of *P. aeruginosa* that participates in the bacterial pathogenesis of various pigment gene mutants, and its tolerance to nonthermal plasma, which is also called cold atmospheric plasma CAP treatment, was evaluated. Both the transcriptome analysis and the qRT-PCR results revealed comparable transcriptional patterns. Moreover, identical reducing patterns were observed in the physiologic and biochemical data after exposure. (Mahdi et al., 2024). interprets the results in the context of previous literature, highlighting the significance of the findings. It effectively discusses the potential of neem oil as an antimicrobial agent, its synergistic effect with gentamicin, and the downregulation of the virulence factor gene *phzM*. However, it could further elaborate on the underlying mechanisms and implications of the findings.

## CONCLUSION

Emphasizing the potential of neem oil as an antibacterial agent and its synergistic effects with GEN. It urges additional research into the health benefits of neem oil and tackles the problem of antibiotic resistance. Overall, the study provides a thorough analysis of the complementary impacts of neem oil and GEN on *P. aeruginosa* and provides insightful information on multiple approaches for addressing antibiotic resistance in harmful bacteria. When considered together, the study's findings point to the possibility that neem oil extract can prevent harmful bacteria from forming biofilms. Furthermore, our results suggest that neem oil extract, either by itself or in conjunction with GEN, may inhibit the proliferation of pathogenic bacteria. This cooperative effect is linked to a pathogenicity factor gene downregulation.

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**Conflict of interest:** The authors have no competing interests to declare that are relevant to the content of this article

**Author Contribution:** Mais Emad. Ahmed and Fadhel M. Lafta carried out the experiment and wrote the manuscript with input from another authors. Ali H. Alhammer and Rasha K. Mohammed supervise the project and conceived the original idea designed the model and the computational framework and analysed the data

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