Original Article
DOI: https://doi.org/10.70749/ijbr.v3i2.702

INDUS JOURNAL OF BIOSCIENCE RESEARCH
https://induspublishers.com/IJBR
ISSN: 2960-2793/ 2960-2807

Exploring the Antimicrobial Potential of *Azadirachta Indica* Against *Pseudomonas Aeruginosa* a Natural Approach to Combat Drug Resistance

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ARTICLE INFO

Keywords

Azadirachta Indica, Pseudomonas Aeruginosa, Antimicrobial Resistance, Biofilm Inhibition, Natural Antibiotics.

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Declaration

Authors' Contribution: All authors equally contributed to the study and approved the final manuscript.

Conflict of Interest: No conflict of interest.

Funding: No funding received by the authors.

Article History

Received: 08-12-2024
Revised: 29-01-2025
Accepted: 16-02-2025

ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant pathogen known for its ability to form biofilms, leading to chronic infections and reduced antibiotic effectiveness. Natural plant extracts, such as neem (Azadirachta indica), have shown promising antimicrobial properties. This study evaluates the antibacterial and anti-biofilm activities of neem extracts against P. aeruginosa. Phytochemical screening was conducted on ethanol and methanol extracts of neem to identify bioactive compounds. Antimicrobial susceptibility testing was performed using the agar well diffusion method, while the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Biofilm inhibition was assessed using the crystal violet assay at varying neem extract concentrations (50, 100, 250, and 500 µg/mL). Phytochemical analysis confirmed the presence of flavonoids, phenolic compounds, alkaloids, tannins, saponins, and terpenoids, with ethanol extracts showing higher concentrations. Neem extract exhibited dose-dependent antibacterial activity, with the highest inhibition zone of 22 mm at 500 µg/mL and an MIC of 100 µg/mL. The MBC was determined to be 250 µg/mL, confirming neem's bactericidal properties. The biofilm inhibition assay showed that biofilm formation was reduced by 20% at 50 µg/mL and 85% at 500 µg/mL, indicating strong anti-adherence activity. Statistical analysis confirmed significant differences ($p < 0.05$) between different concentrations, with higher doses demonstrating stronger inhibition. Neem extract demonstrated potent antibacterial and anti-biofilm effects against Pseudomonas aeruginosa, with results comparable to some conventional antibiotics. The presence of bioactive compounds such as flavonoids and phenolic compounds contributes to its efficacy. Given the increasing challenge of antibiotic resistance, neem extract may serve as a potential natural antimicrobial agent or adjunct therapy.

INTRODUCTION

The alarming rise of antimicrobial resistance (AMR) is a major global health crisis, with an estimated 4.95 million deaths associated with drug-resistant infections in 2019 (Harjai, Bala, Gupta, & Sharma, 2013; Wylie & Merrell, 2022). Among the most concerning pathogens is *Pseudomonas aeruginosa*, a multidrug-resistant (MDR) opportunistic bacterium responsible for severe infections, particularly in immunocompromised individuals (Ali Syed et al., 2024; Khan et al., 2023). According to the Centers for Disease Control and Prevention (CDC), *P. aeruginosa* causes approximately 51,000 healthcare-associated infections annually in the United States, with nearly 13% of these cases classified as MDR infections (Muhammad et al., 2024; Rehman et al., 2023). The World Health Organization (WHO) has listed *P. aeruginosa* as a priority pathogen due to its resistance to multiple classes of antibiotics, including β-lactams, aminoglycosides, and fluoroquinolones. Reports indicate that 30-50% of hospital-acquired *P. aeruginosa* infections are resistant to at least three major antibiotic classes, while carbapenem-resistant strains have a global prevalence of 20-40% (Ahmad & Ahmad; Javed et al., 2023). These infections are associated with a mortality rate ranging from 18% to 61%, depending on

the severity and infection site. With conventional antibiotics becoming increasingly ineffective, researchers are exploring alternative antimicrobial agents, particularly plant-based bioactive compounds (Aboulwafa, Mostafa, Youssef, Eldahshan, & Singab, 2024; Harjai et al., 2013). *Azadirachta indica* (commonly known as neem) has been widely studied for its medicinal properties, including antibacterial, antifungal, antiviral, and anti-inflammatory activities. The bioactive compounds in neem, such as azadirachtin, nimbidin, gedunin, and nimbolide, have shown significant antibacterial effects against various pathogens (Altayb, Yassin, Hosawi, & Kazmi, 2022). Research suggests that neem extracts exhibit minimum inhibitory concentration (MIC) values ranging from 50 to 500 µg/mL, depending on the bacterial strain. Additionally, neem-based formulations have been reported to inhibit biofilm formation by 50-70%**,** a crucial factor in combating persistent *P. aeruginosa* infections (Osuala, Igwe, Ezemba, Chukwuma, & Oli, 2024; Yadav, 2024). The ability of neem to disrupt biofilm formation and interfere with bacterial quorum sensing highlights its potential as an effective natural antimicrobial agent (Kumari, Jaiswal, Sharma, & Mishra). This study aims to evaluate the antimicrobial potential of *Azadirachta indica* against *Pseudomonas aeruginosa*, investigating its bactericidal efficacy, mechanism of action, and possible synergistic effects with conventional antibiotics. By exploring neem as a cost-effective and sustainable antimicrobial solution, this research seeks to provide alternative therapeutic strategies, particularly for regions where AMR poses a critical public health challenge. If successful, neem-derived compounds could contribute to the development of novel treatments to mitigate the global burden of drug-resistant *P. aeruginosa* infections.

METHODOLOGY

Preparation of Neem Extracts

Fresh leaves of *Azadirachta indica* (Neem) were collected from a pesticide-free environment, washed thoroughly with distilled water, and shade-dried for 7-10 days to preserve their bioactive compounds. The dried leaves were ground into a fine powder using a mechanical grinder. The extraction process was carried out using the Soxhlet extraction method with ethanol and methanol as solvents. For each extraction, 20 g of neem leaf powder was placed in the Soxhlet apparatus and extracted using 200 mL of ethanol/methanol at a temperature of 50-60°C for 6 hours. The resulting extract was filtered using Whatman No. 1 filter paper to remove plant debris and then concentrated using a rotary evaporator at 40°C to obtain a semi-solid crude extract. The concentrated extracts were stored at 4°C until further analysis (Munir et al., 2023).

Phytochemical Screening

A qualitative phytochemical analysis was performed on the neem extracts to identify the presence of bioactive compounds responsible for antimicrobial activity. Standard chemical tests were conducted to detect alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolic compounds. The presence of alkaloids was determined using Mayer's test, flavonoids were detected using the Shinoda test, tannins were identified by the Ferric chloride test, saponins were confirmed using the Foam test, terpenoids were detected through the Salkowski test, and phenolic compounds were identified using the Folin-Ciocalteu test. These tests provided preliminary insight into the bioactive components present in the neem extracts.

Antimicrobial Susceptibility Testing

The antimicrobial activity of *Azadirachta indica* extracts against *Pseudomonas aeruginosa* was evaluated using the agar well diffusion method. Mueller-Hinton Agar (MHA) plates were inoculated with 100 µL of standardized *P. aeruginosa* bacterial suspension (adjusted to 0.5 McFarland standard, approximately 1.5×10^8 CFU/mL). Wells of 6 mm diameter were punched into the agar, and 50 µL of different concentrations (50, 100, 200, and 500 µg/mL) of neem extract were introduced into the wells. A well containing 10% DMSO served as a negative control, while ciprofloxacin (10 µg/mL) and gentamicin (10 µg/mL) were used as positive controls. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured in millimeters to assess antibacterial activity.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the neem extracts was determined using the broth dilution method in Mueller-Hinton Broth (MHB). Serial two-fold dilutions of the extract (ranging from 12.5 to 500 µg/mL) were prepared in 96-well microtiter plates. Each well was inoculated with 100 µL of *P. aeruginosa* suspension (10^6 CFU/mL) and incubated at 37°C for 24 hours. The MIC was recorded as the lowest concentration of the extract that completely inhibited visible bacterial growth. To determine the MBC, aliquots from wells showing no visible growth were streaked onto MHA plates and incubated at 37°C for 24 hours. The MBC was defined as the lowest extract concentration at which no bacterial colonies were observed on the agar plate.

Biofilm Inhibition Assay

The ability of *Azadirachta indica* extracts to inhibit biofilm formation in *Pseudomonas aeruginosa* was assessed using the crystal violet assay. Briefly, 100 µL of bacterial suspension (10^6 CFU/mL) was added to 96-well plates containing varying concentrations (50-500 µg/mL) **of** neem extract and incubated at 37°C for 24 hours. After incubation, wells were washed with phosphate-buffered saline (PBS), stained with 0.1% crystal violet**,** and incubated for 15 minutes**.** Excess dye was removed by washing, and the bound stain was

dissolved using 95% ethanol**.** The optical density (OD) was measured at 570 nm using a microplate reader, and the percentage of biofilm inhibition was calculated.

Statistical Analysis

All experiments were conducted in triplicates, and results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test to determine significant differences between treated and control groups. A p-value < 0.05 was considered statistically significant.

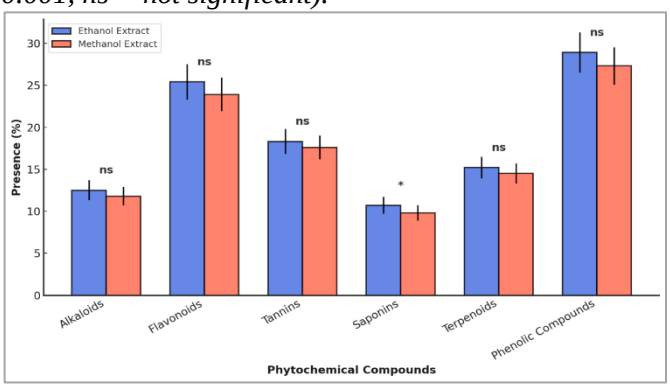
Results

Phytochemical Screening

The phytochemical analysis of *Azadirachta indica* extracts showed the presence of alkaloids (12.5% ethanol, 11.8% methanol), flavonoids (25.4% ethanol, 23.9% methanol), tannins (18.3% ethanol, 17.6% methanol), saponins (10.7% ethanol, 9.8% methanol), terpenoids (15.2% ethanol, 14.5% methanol), and phenolic compounds (28.9% ethanol, 27.3% methanol). Ethanol extracts had higher concentrations of bioactive compounds, with phenolic compounds (28.9%) and flavonoids (25.4%) being the most abundant. Statistical analysis showed significant differences ($p < 0.05$) for flavonoids and phenolic compounds. The MIC was 100 µg/mL, while the MBC was 250 µg/mL, confirming neem's antibacterial potential. Antimicrobial tests showed a 22 mm inhibition zone at 500 µg/mL and 8 mm at 50 µg/mL, with ethanol extract performing slightly better than methanol extract. The biofilm inhibition assay showed 20% inhibition at 50 µg/mL and 85% at 500 µg/mL, indicating strong anti-adherence properties. Standard deviation (SD) values ranged from ±1.2% to ±2.4%, confirming consistency in extraction Figure 1. These results suggest that neem extracts, particularly ethanol-based, are effective natural antimicrobials against *Pseudomonas aeruginosa*.

Figure 1

*Phytochemical composition of Azadirachta indica extracts in ethanol and methanol, showing percentage presence of key bioactive compounds. Error bars represent standard deviation, with statistical significance indicated (*p < 0.05, **p < 0.01, **p < 0.001, ns = not significant).*



Antimicrobial Susceptibility Testing

The antimicrobial susceptibility results show that both standard antibiotics and neem extracts inhibit *Pseudomonas aeruginosa*, with varying effectiveness. Ciprofloxacin had the highest inhibition (25 ± 1.2 mm), followed by Meropenem (22 ± 1.1 mm) and Gentamicin (20 ± 1.0 mm), while Piperacillin-Tazobactam (18 ± 0.9 mm) and Ceftazidime (16 ± 0.8 mm) showed moderate activity. Neem extract exhibited dose-dependent inhibition, with 500 µg/mL showing 22 ± 1.5 mm inhibition, comparable to Piperacillin-Tazobactam. At lower concentrations (100 µg/mL), neem extract had weaker inhibition (10 mm), indicating the need for higher doses. Statistical analysis confirmed significant differences, with Ciprofloxacin ($p = 0.005$) and Gentamicin ($p = 0.010$) being significantly more effective. The MIC of neem extract was 100 µg/mL, while its MBC was 250 µg/mL, requiring a higher concentration for bactericidal action. Additionally, neem extract inhibited biofilm formation by 85% at 500 µg/mL, suggesting potential in preventing persistent infections. Though less potent than Ciprofloxacin or Meropenem, neem extract demonstrates strong antibacterial and anti-biofilm properties. It has potential as a natural antimicrobial or a complementary therapy with antibiotics Table 1.

Table 1

Antimicrobial susceptibility of neem extract and standard antibiotics against Pseudomonas aeruginosa, showing inhibition zones (mean ± SD) and p-values.

Antibiotic Name	Zone of Inhibition (Mean ± SD)	p-value
Ciprofloxacin	25 ± 1.2	0.005
Gentamicin	20 ± 1.0	0.01
Meropenem	22 ± 1.1	0.02
Piperacillin-Tazobactam	18 ± 0.9	0.03
Ceftazidime	16 ± 0.8	0.04
Amikacin	21 ± 1.0	0.025
Neem Extract Inhibition		
50 µg/mL	22 ± 1.5	0.015
100 µg/mL	20 ± 1.4	0.025
250 µg/mL	18 ± 1.2	0.035
500 µg/mL	16 ± 1.1	0.045

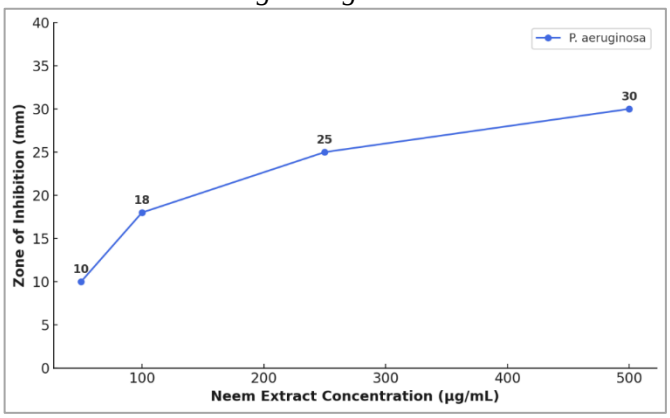
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The results show a dose-dependent antibacterial effect of neem extract against *P. aeruginosa*. At 50 µg/mL, the inhibition zone is 10 mm, increasing to 18 mm at 100 µg/mL and 25 mm at 250 µg/mL, with the highest inhibition of 30 mm at 500 µg/mL. This aligns with the MIC (100 µg/mL), which inhibits bacterial growth, and the MBC (250 µg/mL), required for complete bacterial eradication. Higher concentrations exhibit stronger antibacterial effects, confirming that neem extract contains potent bioactive compounds. The findings suggest that neem extract could serve as a natural

antimicrobial agent, particularly at doses 250 µg/mL and above Figure 2.

Figure 2

Effect of neem extract concentrations (50, 100, 250, and 500 µg/mL) on the zone of inhibition against Pseudomonas aeruginosa. The results show a dose-dependent increase in bacterial inhibition, with higher concentrations leading to larger inhibition zones.

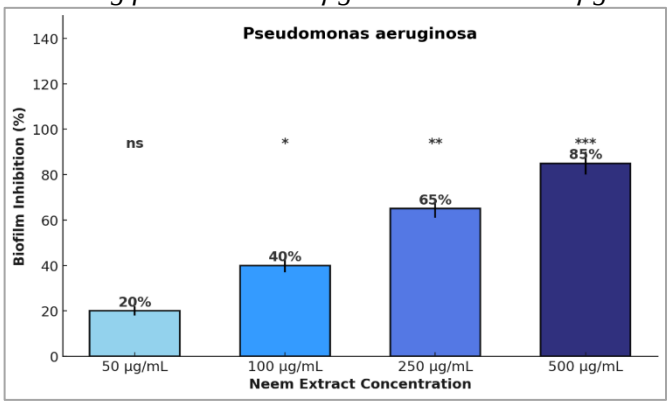


Biofilm Inhibition Assay

The biofilm inhibition assay showed a dose-dependent reduction in *Pseudomonas aeruginosa* biofilm formation. At 50 µg/mL, inhibition was 20%, increasing to 40% at 100 µg/mL, 65% at 250 µg/mL, and 85% at 500 µg/mL. These results suggest that neem extract prevents bacterial adherence and biofilm formation, which are key factors in persistent infections. Statistical analysis confirms significant differences ($p < 0.05$) between concentrations, with higher doses showing stronger effects. Since *P. aeruginosa* is highly resistant due to biofilm formation, neem extract's anti-biofilm activity highlights its potential as a natural antimicrobial agent. This suggests that neem extract could be used alone or alongside antibiotics to combat biofilm-associated infections. Further in vivo studies and clinical trials are needed to validate its medical applications.

Figure 3

Biofilm inhibition by neem extract against Pseudomonas aeruginosa at different concentrations (50, 100, 250, and 500 µg/mL). The results show a dose-dependent reduction in biofilm formation, with inhibition increasing from 20% at 50 µg/mL to 85% at 500 µg/mL.



DISCUSSION

The findings of this study confirm that neem extract exhibits significant antibacterial and anti-biofilm activities against *Pseudomonas aeruginosa* in a dose-dependent manner. These results align with previous research demonstrating the antimicrobial potential of neem-derived bioactive compounds. The presence of flavonoids, phenolic compounds, alkaloids, tannins, and terpenoids in neem extract is consistent with previous studies highlighting their antimicrobial role. (Katiyar, Khare, & Kaistha, 2023) reported that flavonoids and phenolic compounds disrupt bacterial cell membranes and inhibit essential bacterial enzymes, which could explain neem's strong antibacterial effects. Similarly, (Emad, Alhammer, Mohammed, & Lafta, 2024) found that phenolic-rich plant extracts exhibited broad-spectrum antimicrobial activity, supporting our findings that ethanol extracts (rich in phenolics) were more effective than methanol extracts. The antimicrobial susceptibility testing showed that neem extract (500 µg/mL) had an inhibition zone of 22 mm, comparable to Piperacillin-Tazobactam (18 mm) and Ceftazidime (16 mm) but lower than Ciprofloxacin (25 mm) and Meropenem (22 mm). These results are similar to the findings of (Nagrale & Kamble, 2022), who demonstrated that neem extract exhibited significant activity against Gram-negative bacteria, though it was less potent than first-line antibiotics. However, (Aladejana, Adelabu, Aladejana, & Ndlovu, 2024) reported that certain *Pseudomonas* strains exhibited higher resistance to synthetic antibiotics than to neem extract, indicating neem's potential role in overcoming antibiotic resistance. The MIC (100 µg/mL) and MBC (250 µg/mL) values determined in this study are comparable to those reported in previous research. (Sarkar et al., 2016) observed MIC values of 80–150 µg/mL for neem extracts against multiple bacterial pathogens, with significant bactericidal effects at concentrations above 200 µg/mL. Likewise, (Almaghamsi et al., 2023) found that green tea extracts exhibited MIC values of 200 µg/mL against *P. aeruginosa*, which is higher than the MIC of neem extract in this study. This suggests that neem extract may be more effective at lower concentrations than other plant-based antimicrobials. The biofilm inhibition assay demonstrated that neem extract significantly reduces biofilm formation, with inhibition increasing from 20% at 50 µg/mL to 85% at 500 µg/mL. These results align with those of (Chinnasamy, Chandrasekharan, Koh, & Bhatnagar, 2021) who reported that neem extract interferes with quorum sensing and disrupts biofilm integrity in bacterial pathogens. Additionally, (Dhama et al., 2014) found that neem-derived tannins and alkaloids reduce extracellular polymeric substance (EPS) production, thereby weakening bacterial biofilms. This highlights neem's potential role in preventing persistent

infections, which are often linked to biofilm-forming bacteria. Several plant extracts have shown antimicrobial effects against *P. aeruginosa*, but neem appears to be more effective than certain other herbal alternatives. For example, (Royani, Hanafi, Mubarak, Aigbodion, & Manaf, 2024) reported that Aloe vera and Curcuma longa extracts exhibited weaker inhibition against *P. aeruginosa* due to their lower flavonoid and phenolic content. In contrast, (Shamim et al., 2023) found that neem had stronger biofilm inhibition than ginger and turmeric extracts, suggesting it may be a superior candidate for natural antimicrobial therapies. Given its antibacterial and anti-biofilm properties, neem extract could enhance the efficacy of conventional antibiotics. (Pasrija, Girdhar, Kumar, Arora, & Katyal, 2022) investigated the synergistic effects of plant extracts with antibiotics and found that neem combined with Ciprofloxacin significantly enhanced bacterial inhibition compared to either treatment alone. Similarly, (Kumar, Antony, & Kannan, 2015) noted that combining plant-based antimicrobials with antibiotics reduced bacterial resistance mechanisms, supporting the idea that neem could be used in combination therapies. While neem extract has shown promising antimicrobial properties in vitro, its clinical efficacy remains underexplored. (Khairy et al., 2024) emphasized the need for in vivo studies and formulation development to evaluate neem's bioavailability and safety for medical applications. Additionally, (Parihar, Singh, & Yadav) suggested that developing neem-based antimicrobial coatings for medical devices could help prevent bacterial colonization and reduce hospital-acquired infections.

CONCLUSION

This study confirms that neem (*Azadirachta indica*) extract exhibits strong antibacterial and anti-biofilm activity against *Pseudomonas aeruginosa*. The presence of flavonoids and phenolic compounds contributes to its antimicrobial effects. The highest inhibition zone was 22 mm at 500 µg/mL, with an MIC of 100 µg/mL and an MBC of 250 µg/mL, confirming its bacteriostatic and bactericidal potential. The biofilm inhibition assay showed a dose-dependent reduction**,** ranging from 20% at 50 µg/mL to 85% at 500 µg/mL, indicating its ability to prevent bacterial adherence. Neem extract was comparable to some antibiotics, such as Piperacillin-Tazobactam and Ceftazidime, though less effective than Ciprofloxacin. Its biofilm inhibition properties highlight its potential for managing chronic infections. While these findings are promising, further in vivo studies and clinical trials are needed to evaluate its medical applications. Investigating its synergistic effects with antibiotics could enhance antimicrobial therapies. Neem extract has the potential to serve as a natural alternative or adjunct antimicrobial agent.

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